Tea Tree Oil Derived Plasma Polymer Thin Films:

Biocompatibility, Antibiofilm Effects and Fundamental Properties

Thesis submitted by

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50 WORD SUMMARY

Dr Igras investigated the biocompatibility and anti-biofilm features of tea tree oil derived plasma polymer thin films. She found the novel films have potential as coatings for medical implants. Her work contributes to the knowledge base and development of improved surface coatings for medical devices.

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ABSTRACT

Keywords

biocompatible
biofilm
implanted medical device
plasma polymer
tea tree oil
thin film

Novel plasma polymer thin films derived from tea tree oil have the potential to act as biocompatible, infection resistant surface coatings for medical implants. In order to prove or disprove this hypothesis, variant thin films were manufactured then characterized by determining a set of fundamental properties. Polymers were tested for biocompatibility by murine implantation and semi-quantitative histological analysis of the implant site. Finally polymer infection resistance was assessed by enumeration of bacterial biofilm growth onto polymer housed within a biofilm culture circuit.

Background

Implanted medical devices (IMDs) are ensconced in all areas of modern healthcare and their role is expanding. IMDs encompass a broad spectrum of temporary and permanent devices including catheters, sutures, joint replacements, intraocular lenses and cardiac valves to name a few. Demand for medical implants is fuelled by an aging Western population where devices replace damaged, worn and diseased tissues, aid in administration of supportive therapies or act as diagnostic equipment. Development of increasingly sophisticated implanted medical devices is greatly facilitated by advancing analytic, production, materials and information technologies. In combination, demand for medical implants and the ability to produce them support a large scale, multi-billion dollar medical implant industry.

The two main causes of IMD failure are lack of biocompatibility and biofilm related implant infection. A biocompatible surface treatment that kills biofilm therefore has potential to reduce the massive morbidity and financial burdens associated with failed medical implants. Since the advent of modern implant medicine, a select group of 20 or so synthetic, biocompatible polymers including poly(ethylene), poly(urethane) and poly(vinyl chloride) have dominated implant production. Although touting excellent biocompatibility profiles, none of these well used biopolymers demonstrate active antibiofilm activity. Attempts to confer infection resistance to medical implants based on traditional polymers have proven expensive, contributed to bacterial resistance and have shown little efficacy. The major obstacle to reducing IMD infection is pathological biofilm. Biofilm is a tenacious form of resistant bacteria sequestered in a protective matrix irreversibly attached to an implant surface. The medical device industry's quest for a biocompatible surface polymer with durable antibiofilm effects is ongoing.

Tea tree oil (TTO) is an essential oil harvested from an Australian plant and it kills bacterial biofilm. TTO vapor can be used to create novel surface coatings through plasma polymerization. Plasma polymers are a new and unique class of biocompatible thin films that adhere to almost any surface. Under controlled production parameters, plasma polymers inherit desirable functional groups from parent monomer. Plasma polymer films built from TTO may therefore exhibit the class property of biocompatibility and preserve antibiofilm functions of essential oil precursors. If so, TTO plasma polymer thin films have potential as a new breed of biocompatible, anti-infective medical implant coatings.

Methods

TTO derived plasma polymer thin films were generated in the laboratory under three different power parameters (25, 50 and 75 W). Biocompatibility of novel polymer thin films was determined by implanting polymer coated discs into BALB/c mice. Explanted specimens were scrutinized histo-pathologically and compared to biocompatible controls using a semi-quantitative descriptor.

Antibiofilm effect was gauged by quantifying biofilm growth on polymer films placed into a biofilm culture circuit. Clinically relevant *Staphylococcal* bacterial isolates were sourced to populate the biofilm generating circuit.

The new materials underwent limited and relevant fundamental properties testing as a characterization tool. Properties assessed included film thickness as a function of deposition time, surface topography, hardness, degradation on exposure to ethanol and water, refractive index and dielectric constant.

Results

Results of biocompatibility testing showed little significant difference between TTO derived plasma polymers and control. Skin-implant interactions culminating in sinus formation were the main complication. Microscopically, tissue capsules around TTO polymer implants matured more quickly than control capsules but by 28 days groups were equal in capsular maturity. Small numbers made for weak statistical conclusions.

Novel TTO derived plasma polymer thin films were not born out as antibiofilm coatings. PTFE control surfaces placed within the biofilm culture circuit produced fewer colony forming units than any TTO polymer film ($p<0.01$). All TTO polymer variants behaved similarly. *S.epidermidis* made less biofilm than *S.aureus*.

Production times for plasma polymer thin films were short with films in the micron range deposited in less than an hour. A quadratic relationship between film thickness and deposition time was noted. As a group, the films were extremely smooth and pinhole free with hardness (0.5 to 0.8 GPa) and dielectric constant (2.46 to 2.63) comparable to conventional medical grade polymer coatings. Refractive index of 1.5 was consistent with film transparency similar to standard glass. Films degraded more rapidly in alcohol than in water.

Conclusions and Implications

TTO derived plasma polymer thin films are a new class of biocompatible surface treatments with potential to act as coatings for IMDs. Fundamental property analysis shows polymers can be produced in a time efficient manner and share similar physical properties with traditionally employed medical polymers. The films were not deemed to have antibiofilm effects.

Several avenues of investigation are suggested in order to define the potentials and limitations of TTO plasma polymer thin films as medical coatings. Isolation of the functional antimicrobial moieties from the blend of monomers within TTO would lead to more tailored substrate selection. Expansion and control of polymer fabrication parameters would support discovery of the spectrum of product that can be manufactured and any adjustable polymer properties. Detailed chemical, molecular and structural analysis of films would help better characterize the surfaces. Interaction of films with biofilm and host could then be better understood and optimized leading to improved biocompatibility and antibiofilm performance.

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ABBREVIATIONS

A

AAC	advance analytic center
AFM	atomic force microscope/microscopy
AHL	acylated homoserine lactones
ANOVA	analysis of variance
ALT	antibiotic lock technique
ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
ATTIA	Australian Tea Tree Industry Association

B

C

CFU	colony forming units
CLSM	confocal laser scanning microscopy
CoNS	coagulase negative *Staphylococci*
CSIRO	Commonwealth Scientific and Industrial Research Organization
CVC	central venous catheters

D

DNA	deoxyribonucleic acid
DNase	deoxyribonuclease

E

EDTA	ethylenediamine-tetra acetate
ESEM	environmental scanning electron microscope/microscopy

F

FBR	foreign body reaction

G

GlmU	*N*-acetyl-D-glucosamine-1-phosphate acetyltransferase

H

HIV/AIDS	Human Immunodeficiency Syndrome/ Acquired Immunodeficiency Syndrome

I

IMD	implanted medical device
ISM	industrial, scientific and medical (radio bands)

ISO	International Organization for Standardization
J	
JCU	James Cook University
K	
L	
LCVD	luminous chemical vapor deposition
M	
MBC	minimum bactericidal concentration
MFM	magnetic force microscopy
MHB	Mueller-Hinton broth
MIC	minimum inhibitory concentration
MRSA	methicillin resistant *staphylococcus aureus*
MSSA	methicillin sensitive *staphylococcus aureus*
N	
NCCLS	National Committee for Clinical Laboratory Standards
O	
ONJ	osteonecrosis of the jaw
P	
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDT	photodynamic treatment
PEG	poly(ethylene glycol)
PGA	poly-γ-DL-glutamic acid
PIA	polysaccharide intracellular adhesin
PP	plasma polymer
PMMA	poly(methyl methacrylate)
PTFE	poly(tetra fluoro ethylene)
PVE	prosthetic valve endocarditis
PVC	poly(vinyl chloride)
Q	
R	
RAP	RNAIII activating protein
RF	radiofrequency
RIP	RNAIII inhibiting peptide

RIRDC	Rural Industries Research and Development Corporation
RNA	Ribonucleic acid
S	
SPA	standard plate agar
SPM	scanning probe microscope
STP	standard temperature and pressure
T	
TDMAC	tri-iododecylmethyl ammonium chloride
TEM	transmission electron microscopes
TRAP	target RNAIII activating protein
TSST-1	toxic shock syndrome 1 toxin
TTO	tea tree oil
U	
UHMW	ultra high molecular weight
USA	United States of America
UV	ultraviolet
V	
VCG	Van Oss, Chaudhury and Good
W	
WHO	World Health Organization
X	
Y	
Z	

CHAPTER 1 INTRODUCTION

1.1 Background and Rationale

Implanted medical device (IMD) refers to any object or material inserted or grafted into the body for prosthetic, diagnostic, therapeutic or experimental purposes (Ratner and Bryant, 2004). IMDs are ensconced in all areas of modern medicine and healthcare. Bio-implants may be temporary devices like intravenous catheters, ureteric stents, absorbable sutures and endotracheal tubes or permanent devices such as joint replacements, intraocular lenses, vascular grafts and cardiac valves. Medical implants are common: for example 200,000,000 urinary catheters, 500,000 hip and knee prostheses and over 250,000 vascular grafts are implanted in the United States of America each year (Castner and Ratner, 2002). Demand for medical implants is increasing. Over the past 50 years the bio-implant industry has grown to a value of US$ 100 billion annually, increasing in worth at a rate of 5% per year (Schierholz and Beuth, 2001). Fuelled by the needs of an aging Western population, medical implants support advanced diagnostic techniques, replace damaged, worn and diseased tissues and administer therapies such as fluids and drugs. Development and creation of increasingly sophisticated and novel implanted medical devices such as biosensors and capsular endoscopes is greatly facilitated by advancing computing, production, materials and information technologies. In combination, demand for medical implants and the ability to produce them support the large scale medical implant business and industry that exists today. The implanted device arena is not only an area of medical need but represents a formidable commercial market.

A dominant cause of IMD failure is lack of implant biocompatibility. Biocompatibility is the capacity of a material to perform with an appropriate host response in a specific application (Williams, 1999). A biocompatible implant therefore, is tolerated by host systems and serves its intended function whilst a bio-incompatible material is on some level rejected by host and unable to function. IMDs must be biocompatible to be successful. Unfortunately the probability of finding a single material with physical and bio-chemical bulk properties aligned to both optimal device function and perfect host tolerance is close to zero (Yasuda, 2005). Bio-implants constructed from a single material must therefore trade off material bulk function against host tolerance. This imposed compromise means accepting a medical device with inferior bulk properties, biocompatibility or both in

order to create a viable device. The balance is struck every day in implant medicine: a joint prosthesis for example, must be strong, durable and tolerated by host whereas an intraocular lens must be transparent, flexible and tolerated by host. One alternative to the compromising single material approach to medical device development is a two phase implant. As host tissue reacts only with the outermost atomic layers of bio-implant, the host/implant interface determines degree of implant biocompatibility (Biederman, 2004). In two phase implants, bulk materials are selected for favorable bulk properties and biocompatibility in conferred secondarily by applying a surface treatment or coating to modify and optimize the host/implant interface. Materials used to design two phase bio-implants optimize bulk and bio-tolerance properties independently resulting in improved overall function and biocompatibility.

A second common cause for IMD failure is device infection. Bio-implants are prone to infection because they are foreign bodies exposed to bacteria during implantation, act as microbial havens removed from natural host defenses and are often applied in patients with impaired immunity (Von Eiff et al., 2005). Implant infection causes morbidity for the following reasons: loss of device function deprives the patient of optimal medical care, invasive procedures to remove infected devices put patients at surgical risk and infection itself has detrimental local and systemic effects on host.

Infected medical implants impart high costs to the healthcare system as well as injuring patients. In the United States alone, two million cases of bio-implant infection are reported each year accounting for 45% of all nosocomial infection at a cost of US$ 11 billion (Schierholz and Beuth, 2001). Examining some of the componentry of this data, five million central venous catheters are inserted annually and 2-12% lead to sepsis at a cost of US$ 25 000 per episode (Schierholz et al., 1999, Kojic and Darouiche, 2004, Darouiche, 2001). Urinary catheters are another example of the scope of device related infection. Over 30 million bladder catheters are sited annually in the United States, with a 10 to 30% infection rate (Kojic and Darouiche, 2004). Each case of bladder catheter related infection costs US$ 600 and associated bacteremia increase the outlay to US$ 2800 per episode (Tambyah et al., 2002, Saint, 2000). The cost per episode of an infected joint prosthesis is more than US$ 50 000 (Lentino, 2003). Although the rate of infected joint prostheses is low at 1-3%, the magnitude of this complication is staggering considering hundreds of thousands of joint replacements are performed each year (Kojic

and Darouiche, 2004). Bio-implant associated infections are a large scale morbidity and monetary coast burden to patients and the healthcare system. Because of the magnitude of the problem, far reaching benefits are obtained by preventing device related infections.

Biocompatible and antimicrobial surface treatments or coatings have potential to address the two most common limitations of bio-implants: lack of biocompatibility and implant infection. Synthetic polymers are good candidates for this application because they are versatile, can be mass-produced, function can be engineered according to specifications and they are generally inexpensive. Since the advent of modern implant medicine, a select group of commonly employed polymers with biocompatible profiles have dominated medical implant production. Currently the most prevalent commercial bio-polymers are cellulose derivatives or one of less than 20 major synthetically engineered polymers originally developed for non-medical purposes (Ulbricht, 2006). Examples include poly(ethylene), poly(propylene), poly(urethane), poly(tetra fluoro ethylene), poly(vinyl chloride) and poly(glycolide lactide). Although their clinical biocompatibility reports are excellent, none of these well used bio-polymers actively deter bacterial infection or have formal antibiotic properties (Francolini et al., 2003).

Attempts at producing active antimicrobial surface treatments for medical implants have met with limited success. Strategies have encompassed, simple immersion in antibiotics, antibiotic coatings, electrostatic binding of antibiotics to implant surfaces, drug-polymer conjugates, hydrophilic coatings, coatings of albumin and heparin and matrix loading of polymers with antibiotic substances (Zilberman and Elsner, 2008). Some protection from bio-implant infection is gleaned in the first week with antibiotic impregnated or coated devices but drug release is rapid and poorly controlled, the systems are expensive and may contribute to burgeoning antimicrobial resistance (Hetrick and Schoenfisch, 2006). Effective strategies for durable prevention and control of IMD associated infection are highly desirable but elusive and an area of ongoing and active research.

Plasma polymers are a new class of polymer thin films with unique properties. Whereas conventional polymers comprise an ordered series of intact, repeating monomer units, plasma polymers are highly cross linked, disordered but stable, pinhole-free surface films that promote bio-chemically inert behavior and provide excellent barrier function. This new class of biocompatible polymers tenaciously bind and conform to most surfaces without

altering underlying material bulk properties and act as either occlusive coatings or membranes with selective barrier characteristics (Rosengren et al., 2005, Ulbricht, 2006). Most synthetic bio-surfaces in use today are made from organic polymers manufactured using the classical technique of phase separation (Ulbricht, 2006). In phase separation and other traditional polymerization reactions, only monomers with active double bonds or reactive functional groups will polymerize. Substrate selection is thus limited. Conversely, plasma polymer thin films are specifically engineered from a broad range of organic monomers that fail to polymerize using classical models. Additionally, plasma polymers are manufactured in a dry environment free of solvents and toxic byproduct making production environmentally friendly. Many of the unique characteristics of plasma polymers make them obvious candidates for the design of novel surface treatments for IMDs

Plasma polymers are named after the plasma state of matter that powers their production. There are four states of matter, solid, liquid, gas and plasma. The plasma state of matter consists of positive and negative ions, electrons, free radicals, UV radiation and electrically excited molecules. Plasma is created when an electric discharge occurs in a gas. In plasma polymerization, plasma supplies high levels of energy to activate and fragment gaseous monomer. Stimulated monomer fragments polymerize via a process of statistical recombination as highly stable films on exposure to solid surfaces (Yasuda, 2005). Polymer product is thus a surface associated, dense, irregular, highly cross linked network of strongly bound monomer fragments containing many double and triple bonds. Because of the nature of plasma polymers, they only exist as surface associated films.

Plasma polymers can inherit functional abilities from their monomer precursors. Energy flux is an important determinant of plasma polymer film structure and function (Biederman and Slavinska, 2000). Plasma polymer films produced under low energy flux conditions inherit a higher proportion of intact functional groups from parent monomer. Films produced under high energy flux are made from highly fragmented monomer, are dense, highly cross-linked, impervious and stable but functional moieties of parent monomer are mostly destroyed. Plasma polymer thin film structure and functional moiety preservation are thus titrated by adjusting the energy flux during polymer creation. An optimally titrated film inherits desired functional moieties and still maintains the favorable class

characteristics of plasma polymers. In theory, it is therefore possible to engineer a biocompatible, plasma polymer thin film with inherited antibacterial functional groups.

Bacteria exist naturally in equilibrium between two distinct forms: planktonic bacteria and biofilm. Planktonic bacteria are the more familiar bacterial manifestation of free swimming individuals in solution and are vulnerable to traditional antibiotics and immune attack. Conversely, bacterial biofilm is a sessile, organized community of bacteria sequestered in self-generated, extracellular matrix, irreversibly attached to a surface (Costerton et al., 1978). Biofilm colonies exhibit complex architectures consisting of dynamic towers connected by communicating water channels. Unlike planktonic organisms, biofilm is inherently resistant to traditional antibiotics and immune attack. Physical sequestration of microbes within matrix and a resistant microbial phenotype are designed to protect biofilm organisms from environmental stressors (Cunningham et al., 2011). Biofilm is thus a technique bacteria use to survive in a hostile environment. Unfortunately this effective defense makes bacterial biofilm difficult to eradicate. It is becoming increasingly clear that biofilm is responsible for many refractory, chronic, relapsing and remitting infections in humans (Davey and O'Toole, 2000). Biofilm is also responsible for infection of medical implants (Costerton et al., 2005).

Tea tree oil (TTO) is an essential oil: an aromatic, volatile, oily liquid derived from plant material. It is a complex, composite oil made primarily from a mixture of cyclic hydrocarbons and easily produces a vapor or organic gas (Hammer et al., 2006). TTO is sourced from the uniquely Australian tree *Melaleuca alternifolia* and as such is a renewable and available resource. It has many therapeutic properties but of interest are well described anti-bacterial capabilities (Carson et al., 2002, Hammer et al., 2008). TTO is bactericidal against organisms commonly implicated in medical implant infection such as *S.aureus* and coagulase negative *Staphylococci* (Cox et al., 2000). The oil is also active against antibiotic resistant organisms such as methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE) (Burt, 2004). Significantly, TTO has documented activity against biofilm (Burt, 2004).

Natural TTO is a promising substrate for production of novel, biocompatible, biofilm resistant, plasma polymer thin films for use as anti-infective medical device coatings. The essential oil easily forms organic gas needed for plasma polymerization, provides carbon

molecules for polymeric backbone linkages and has functional moieties active against biofilm organisms responsible for medical implant infections. It is a renewable, inexpensive and available resource unique to Australia. TTO derived plasma polymer thin films may therefore provide a technology that benefits human healthcare through improved biocompatibility and reduced infection of two-phase implanted medical devices.

1.2 Aims and Objectives

The aim of this research was to investigate the hypothesis:

> Tea tree oil derived plasma polymer thin films are biocompatible surface treatments that resist biofilm formation and have potential as novel coatings for implanted medical devices.

The following steps were undertaken (Figure 1.1):

1. Fabrication of variant plasma polymer thin films from tea tree oil
2. Biocompatibility assessment of novel films by implantation in the murine model
3. Assessment of novel film effects on biofilm generation
4. Determination of a subset of fundamental film properties as a characterization tool

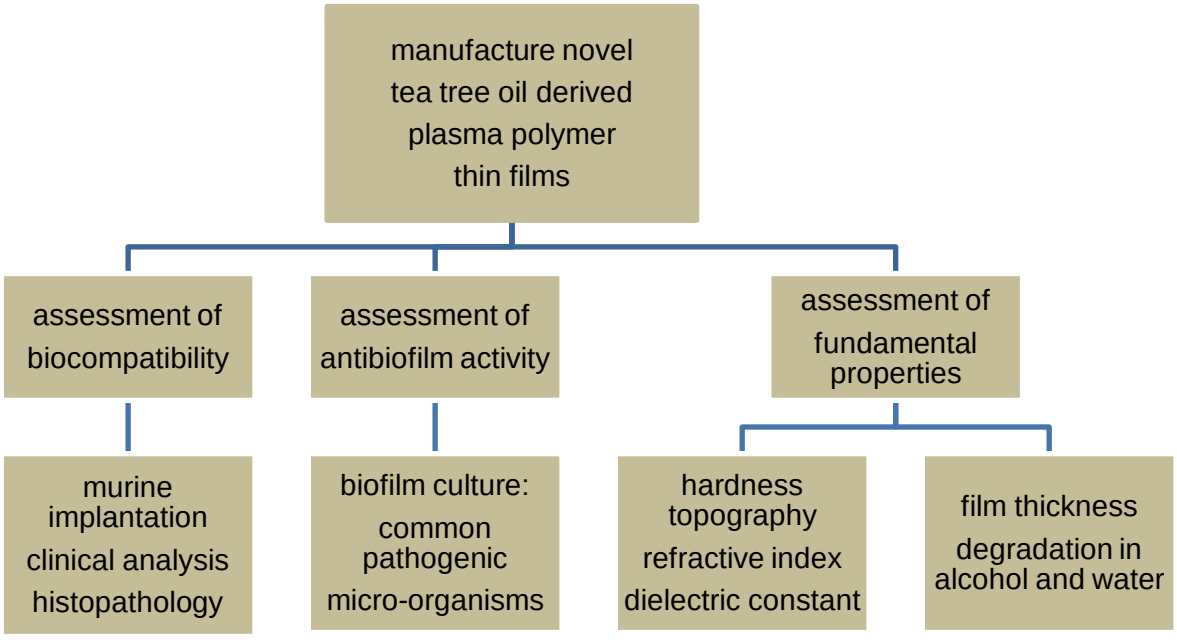


Figure 1.1: Conceptual, high level research plan

The work completed in this thesis is expected to contribute towards the knowledge base and development of more effective biocompatible and biofilm resistant surface coatings for medical implants. In turn to this may lead to more efficient and improved healthcare.

1.3 Thesis Organization

This thesis is presented as a series of chapters, each encompassing a major arm of enquiry as indicated by the conceptual, high level research plan (Figure 1.1).

Chapter 2 is an overarching literature review highlighting relevant knowledge and defining context for research and experimentation. It focuses on medical polymers, medical device surface treatments both novel and commercial and plasma polymer thin films. Implanted medical devices, the definition of biocompatibility and unique and clinically relevant features of pathological biofilm and its relationship to implant infection are also covered.

Chapters 3 to 6 describe experimental methods and results including primary discussion of results. Fabrication, handling, storage and sterilization of tea tree oil derived plasma polymer thin films are described in Chapter 3. Substrate production techniques provide a strong and consistent foundation on which subsequent experiments are based. Biocompatibility, antibiofilm effect and fundamental physical properties of TTO derived plasma polymers are assessed and presented sequentially in chapters 4-6 as follows:

- Chapter 4 deals with polymer biocompatibility testing, explains murine implantation experiments and macroscopic and microscopic results including discussion of a semi-quantitative histology scoring system.
- Chapter 5 represents methodologies and results of biofilm testing and details biofilm culture, detection, enumeration and associated biofilm specific equipment and techniques.
- Chapter 6 concentrates on fundamental properties of novel polymers. A limited spectrum of relevant properties have been selected related directly to medical implant or polymer production requirements and include film thickness as a function of deposition time, surface topography and hardness, refractive index, dielectric constant and degradation on exposure to alcohol and water.

Chapter 7 is a conclusions and recommendations chapter that draws from results to comment on the higher level applicability of tea tree oil derived plasma polymer thin films as biocompatible and anti-infective surface treatments for medical implants. It also includes directions for future work in the field.

1.4 Scope, Limits and Excluded Areas

This research focuses on tea tree oil plasma polymers as novel films to rival established medical surface polymers. Discussion regarding non-polymeric medical materials including metals and ceramics is beyond the scope of this study. Discussion on the burgeoning fields of bio-integration, tissue engineering, nanotechnology, cloning, auto-grafting and tissue transplantation have been excluded. It is acknowledged that tissue recreation will provide a complimentary approach to synthetic medical implants and coatings.

CHAPTER 2 LITERATURE REVIEW

2.1 Spectrum and Scope of Medical Implants and their Cost

A medical implant is any object or material inserted or grafted into the body for prosthetic, therapeutic, diagnostic or experimental purposes (Dzul et al., 2000). The United States Federal Drug and Food Administration defines a device as any instrument, apparatus, implement, machine, contrivance, implant, in vitro reagent or a combination of these elements that is intended for diagnosis, prevention or treatment of a disease (FDA, 2011). The definition of medical implants and devices is broad. An exhaustive list of devices is not presented for this reason however examples will be given to demonstrate that implanted medical devices occupy all medical subspecialties, are common and represent a multi-billion dollar and expanding commercial industry.

The development of biocompatible materials is a relatively new field that draws on the knowledge of physical science, engineering, biology, physiology and clinical science. Over past decades, the number of biomaterials has expanded rapidly. Subsequently there exists a 40-50 year detailed employment log of modern biomedical material use although historical accounts of medical implants date back much further. Synthetic and natural polymers, metals, ceramics and composite materials have been used as components of medical implants. Romans, Chinese and Aztecs used gold in dentistry more than 2000 years ago (Ratner, 1996). Wooden teeth and glass eyes have been in use in much of recorded history. Reports of wooden peg and twisted thread used to repair a brachial artery injury date back to 1759 (Trachtenberg and Ryan, 1994). In 1908 aluminum plates were made to repair skull defects (Sanan and Haines, 1997). In 1939 poly(ethylene) was first employed in plastic surgery and silicone elastomers were used in reconstruction surgery during and after World War II (Love et al., 2011). The first intraocular lens was inserted in 1949 and around the same time the first hip implant, vascular graft and mechanical heart valve were pioneered (Ratner and Bryant, 2004). By the 1950's biocompatible polymers were developed. In 1958 poly(vinyl chloride) was used in heart assist pumps (Litwak et al., 1976). The roles for medical implants have continued to be honed and expanded since that time.

Implanted devices are now used routinely in the practice of medicine. Biomaterials and medical devices have evolved over the last half century into a US$ 100 billion endeavor annually, growing at a rate of five percent per year (Ratner and Bryant, 2004)(Table 2.1). Once the scale and context of medical implants is understood, the value of approaches that improve biocompatibility and reduce infection of implants is better appreciated. Biomaterials have found applications in more than 8000 different kinds of implanted medical devices (Thomas et al., 2006)(Table 2.2). Medical implants can be disposable or reused, temporary or permanent, completely or partially implanted and some degrade *in situ.* Increasingly medical implants are containing active components such as electronics or sensors. The stimuli for implant development include increasing demand from an aging Western population who require medical implants to replace, augment and support damaged, worn and diseased tissues (Castner and Ratner, 2002, Silver, 1994). Implant development has also responded to growing numbers of patients with traumatic and atraumatic conditions amenable to implant derived treatment (Silver, 1994). This demand is cradled by advances in technology that bring design and production of implants into fruition. It is not hard to see how even a small improvement in medical implant biocompatibility or infection resistance can have far reaching beneficial sequelae for patient care.

Table 2.1: Number of medical implants inserted each year in the U.S.A.

Device	Number/year	Biomaterial
intraocular lens	2,700,000	PMMA
contact lens	30,000,000	silicone acrylate
vascular graft	250,000	PTFE, poly(ethylene)
hip and knee prostheses	500,000	titanium
cardiovascular stents	>1,000,000	stainless steel
urinary catheters	200,000,000	silicone, PTFE
breast implants	192, 000	silicone
dental implants	300,000	titanium
pacemaker	130,000	poly(urethane)
renal dialyzer	16,000,000	cellulose
left ventricular assist devices	>100,000	poly(urethane)

(Castner and Ratner, 2002)

Table 2.2: Examples of implanted medical devices

Medical subspecialty	Example of medical implant	
cardiovascular	vascular graft	ventricular assist device
	mechanical cardiac valve	automatic external cardioverter-defibrillator
	pacemaker	coronary artery stent
	cardiac bypass tubing	pulmonary artery catheters
gastroenterology	nasogastric tube	gastrostomy tube
	colonic, esophageal and biliary stents	
maxillofacial	dental prosthesis	titanium plates and screws
neurosurgery	ventriculo-peritoneal shunt	nerve guidance tube
	skull prosthesis	Ommaya reservoirs
	external ventricular drain	ventricular pressure monitor
	brain pacemaker	epidural catheter
ophthalmology	kerato-prosthesis	intra-ocular lens
	contact lens	glaucoma drainage tube
orthopedics	joint prosthesis	bone cement
	fracture fixations devices	
otolaryngology	tympanostomy tube	cochlear implant
	middle ear implant	
plastics & reconstruction	sub-dermal filler	tissue expander
	breast implant	tissue adhesive
reproductive	intrauterine contraceptive	sub-dermal contraceptive
respiratory/thoracic	endotracheal tube	tracheostomy tube
	intercostal catheter	
urology/renal	dialyzer	ureteric stent
	urinary catheter	Tenkhoff catheter
	penile prosthesis	
cross discipline	sutures	staples and clips
	central and peripheral vascular access catheters	introducers
	drains	wound dressings

2.2 Tea Tree Oil (Oil of Melaleuca)

2.2.1 Definition and baseline properties

Oil from the plant *Melaleuca alternifolia* is a complex, composite oil made of cyclic hydrocarbons. It is an aromatic, volatile, oily liquid obtained from plant material and thus part of the essential oils family (Burt, 2004). Primary botanical origin of the essential oil is *Melaleuca alternifolia* of the *Myrtaceae* family, a tree native to central coastal regions of eastern Australia and as such the oil is known as Oil of Melaleuca (Brophy et al., 1989). Oil of Melaleuca is extracted from *Melaleuca alternifolia* by steam distillation of leaves and terminal branches. Six chemo-types of *M. alternifolia* have been described, each producing a unique variant of oil with similar bioactivity (Carson et al., 2006).

Typical constituents of Oil of Melaleuca have been defined using comprehensive gas chromatography and mass spectrometry and consist primarily of terpinoids (monoterpines, sesquiterpenes) and their alcohols (Brophy et al., 1989, Burt, 2004, Shellie et al., 2000)(Table 2.3). Terpinoids are a group of antioxidant substances that occur in natural foods. The basic unit of terpinoids is isopentenyl pyrophosphate (C_5H_8). All terpinoids including those found in Oil of Melaleuca are cyclic unsaturated hydrocarbons where oxygen molecules in constituent groups are attached to basic isoprene units (Amitabha, 2005)(Table 2.4). Major percentage components of Oil of Melaleuca are terpinen-4-ol (>=30%), 1,8-cineole (<=15%) and γ-terpinene (10-28%) however greater than 100 different organic compounds are present within the oil (Hammer, 2006). International Standard for Oil of Melaleuca (ISO 4730) put forward by International Organization for Standardization (ISO) provides a component parameter definition for the essential oil by stipulating range levels of 14 major chemicals (Table 2.5). Plant origin is not specified in ISO 4730 therefore plant species other than *Melaleuca alternifolia* including *M.dissitiflora, M. linariifolia* and *M. uncinata* may be sourced to produce components of ISO standard Oil of Melaleuca or the oil can be created artificially (Carson et al., 2006).

It is important to be specific when discussing “tea tree oil” because the definition varies within the literature. Although international standards to define TTO or Oil of Melaleuca exist, they are not universally applied. The term “tea tree” itself has colloquial and cultural meanings and is an umbrella term for a number of unrelated plant species. Often journal articles do not specify precisely what is meant by “tea tree oil” and thus caution in gathering evidence about “tea tree oil” is prudent. A firm and clear definition for TTO is fundamental to further discussion of the essential oil.

Table 2.3: Common constituents of Oil of Melaleuca (Shellie et al., 2000)

alloaromadendrene	epicubenol	cis-sabinene hydrate
aromadendrene	globulol	trans-sabinene hydrate
bicyclogermacrene	a-gurjunene	spathulenol
cadina-1,4-diene	a-humulene	a-terpinene
d-cadinene	limonene	terpinen-4-ol
b-caryophyllene	cis-p-menth-2-en-1-ol	c-terpinene
1,8-cineole	trans-p-menth-2-en-1-ol	a-terpineol
cubeban-11-ol	a-muurolol	terpinolene
a-cubebene	myrcene	a-thujene
cubebol	b-phellandrene	viridiflorene
a-cupaene	a-pinene	viridiflorol
p-cymene	b-pinene	
epicubebol	sabinene	

Oil of Melaleuca by ISO definition is often referred to as tea tree oil. Conversely, oils from unrelated plants are referred to colloquially as “tea tree oil”. Awareness of terminology is thus vital to avoid confusion. “Ti tree” is Maori and Samoan for plants in the genus *Cordyline*: Asiatic and Pacific trees or shrubs where fragments of the trunk will grow to form whole plants (Weiss, 1997). Essential oils kanuka and manuka, derived from *Kunzea ericoides* and *Leptospermum scoparium* respectively, are referred to as New Zealand tea tree oils (Christoph et al., 2000). In Australia, “paper bark trees” are also known as “tea trees,” and collectively these terms may refer to species in the *Melaleuca* or *Leptospermum* genera, of which there are several hundred varieties (Carson et al., 2006, Lassak and McCarthy, 1983). Even the term “Oil of Melaleuca” can be ambiguous because several chemically distinct oils are distilled from *Melaleuca* species, such as cajuput oil from *M. cajuputi* and niaouli oil from *M.quinquenervia* (Craven, 1999). Despite this, Oil of Melaleuca is the term adopted by both the Australian Standards (AS 2782-1997 - Oil of Melaleuca) and the ISO (ISO 4730 Oil of Melaleuca) when referring to standard composition oil from *M.alternifolia* (Carson et al., 2006). ISO 4730 standard for Oil of Melaleuca is referred to as tea tree oil (TTO) and *M.alternifolia* as “tea tree” within this document.

Table 2.4: Oil of Melaleuca components (Carson et al., 2006)

Component	Type of compound	Chemical formula
terpinen-4-ol	monocyclic terpene alcohol	$C_{10}H_{18}O$
γ-terpinene	monocyclic terpene	$C_{10}H_{16}$
α-terpinene	monocyclic terpene	$C_{10}H_{16}$
1,8-cineole	monocyclic terpene alcohol	$C_{10}H_{18}O$
α-terpinolene	monocyclic terpene	$C_{10}H_{16}$
ρ-cymene	monocyclic terpene	$C_{10}H_{14}$
(+)-α-pinene	dicyclic terpene	$C_{10}H_{16}$
α-terpineol	monocyclic terpene alcohol	$C_{10}H_{18}O$
aromadendrene	sesquiterpene	$C_{15}H_{24}$
δ-cadinene	sesquiterpene	$C_{15}H_{24}$
(+)-limonene	monocyclic terpene	$C_{10}H_{16}$
sabinene	dicyclic monoterpene	$C_{10}H_{16}$
globulol	sesquiterpene alcohol	$C_{15}H_{26}O$

Baseline properties aid definition, allow for comparison with other substances and determine benefits and limitations of biomaterial precursors. TTO is a colorless to pale yellow, mobile liquid (viscosity at 20ºC less than 1 cP) at room and body temperature with a characteristic warm, spicy odor. The essential oil is hydrophobic with poor aqueous solubility, although it is miscible in non-polar solvents. At 20 ºC 1 vol is soluble in 2 vol 85% v/v ethanol. Specific gravity of TTO at STP is 0.885-0.906 making the oil less dense than water. TTO optical rotation at 20 ºC is +5º to +15º and refractive index at 20 ºC is 1.4750-1.4820. Both light based characteristics are routinely employed to assess purity of essential oils. TTO boiling point is 115-270 ºC, freezing point <5 ºC and flash point is 57-60ºC (Pensky Martens Closed Cup). TTO is prone to oxidization if stored incorrectly but is stable for at least 10 years if kept in an airtight, cool and dark environment. Hazardous polymerization of pure TTO does not occur under standard environmental conditions.

Table 2.5: Composition of *M.alternifolia* oil (tea tree oil) (Brophy et al., 1989)

Component	Composition (%)	
	ISO 4730 range	typical composition
1. terpinen-4-ol	>=30	40.1
2. γ-terpinene	10-28	23.0
3. α-terpinene	5-13	10.4
4. 1,8-cineole	>=15	5.1
5. terpinolene	1.5-5	3.1
6. ρ-cymene	0.5-12	2.9
7. α-pinene	1-6	2.6
8. α-terpineole	1.5-8	2.4
9. aromadendrene	trace-7	1.5
10. δ-cadinene	trace-8	1.3
11. limonene	0.5-4	1.0
12. sabinene	trace-3.5	0.2
13. globulol	trace-3	0.2
14. viridiflorol	trace-1.5	0.1

2.2.2 Context of the Australian tea tree oil industry

Medicinal uses of TTO are firmly grounded in Australian history. Indigenous Australians were the first peoples known to exploit the antimicrobial effects of the tea tree, making poultices from its leaves and inhaling oils from the crushed leaves (Low, 1990, Shemesh and Mayo, 1991). The name “tea tree” was coined by Captain Cook of the British Royal Navy at Botany Bay in 1770 (Burdon, 2007). Although Cook's crew first used tea tree leaves for a soothing tea, they later mixed the tea tree leaves with spruce leaves as a beer (Crawford, 2007). Antimicrobial benefits of TTO were first published in 1925 by the leading New South Wales government chemist Arthur Penfold who wrote the paper "Australian Tea Trees of Economic Value" and stimulated a flurry of research into tea tree oil (Penfold and Grant, 1925). During World War II , tea tree oil was standard issue for Australian soldiers who nicknamed the oil “first aid in a bottle” and Australians involved in the tea tree industry were rumored to be exempt from military service (Morris et al., 2003).

In modern times, TTO has a strong consumer appeal as an aromatic home grown Australian product that requires only environmentally friendly processing (distillation) and is perceived as a “natural” and traditional medicine (Penfold and Morrison, 1937, Stephens, 2007). In Australia TTO is conveniently purchased in stores and over the Internet without a prescription and is listed by the Therapeutic Goods Administration as a substance that may be used in medicines (TGA, 1999). TTO is also affordable. Off the shop shelf 30 ml of 100% pure TTO costs around AU$ 25. It is an essential oil routinely recommended by alternative healthcare providers for a wide variety of ailments and as a cosmetic and personal care product. TTO is perceived as a remedy for a number of conditions including dermatitis, respiratory illness, a wide variety of infections and as an aid to strengthen the immune system (Bradley, 2007, Beane, 2011). Enhancement in “green” consumerism has contributed to broad interest in TTO as an alternative to environmentally damaging chemicals and artificial substances (Bradley, 2007). All of these factors contribute to the growing popularity of TTO.

TTO industry is an established, mainstream agricultural venture in Australia cultivating a consistent quantity of high quality product. The TTO industry is one of Australia’s largest native plant industries based on a long term sustainable crop and even features on a new release Australian stamp (ATTIA, 2011). Prior to the 1980’s production of TTO was a cottage industry reliant on harvesting “bush oil” from native stands of *Melaleuca alternifolia* in southern Queensland and northern New South Wales. Annual production from bush harvesting was in the order of 15 to 20 tonnes per annum but since the late 1990’s plantation production has escalated output to around 400 tonnes each year worth AU$ 18 million per annum (Garsden, 1999) . Breeding and improved seed stock have developed plants capable of producing 270 kg of oil per hectare from paddocks that would otherwise yield 148 kg per hectare with unimproved seed manifesting an 80% gain in production per unit area (Stephens, 2007). 90% of Australian TTO is exported to North America and Europe where there is strong demand for the oil (RIRDC, 2006).

In Australia the TTO industry including research and development is overseen by a number of government and professional organizations including Rural Industries Research and Development Corporation (RIRDC), Commonwealth Scientific and Industrial Research Organization (CSIRO) and Australian Tea Tree Industry Association (ATTIA). For example RIRDC’s TTO Research Development and Advisory Committee generated strategic plans on behalf of the TTO industry and the Australian Government with input from both growers and product manufacturers most recently for the period 2006-2011. The Tea Tree Oil Program is part of RIRDC’s New Industries

Portfolio which brings opportunity, diversity and resilience to rural Australia. RIRDC and the other overseeing bodies support continued development of an environmentally sustainable and profitable Australian TTO industry with established international leadership in marketing, value-adding, product reliability and production (RIRDC, 2006). As such the Australian TTO industry is a burgeoning, well run agricultural entity with broad governance supplying both domestic and international markets.

2.2.3 Current tea tree oil applications

TTO is marketed for a wide range of non-medical applications (Morris et al., 2003). Products containing TTO include cosmetics and toiletries such lip balms, soaps, shampoos, toothpaste, mouthwash, shaving cream, depilatories and deodorants. Aromatherapies and massage oils may be based on TTO. Diverse items such as veterinary care products, carpet treatments, industrial disinfectants and agricultural crop protection strategies have taken advantage of the benefits of TTO (ATTOP, 2007). TTO is being investigated as a natural preservative to reduce the incidence of food borne pathogens (Moreira et al., 2005, Cox et al., 2000). It is even a herbalist recommendation to spray the garden with TTO to prevent mold, mildew and fungus from growing on plants and seedlings (Yares, 2007). TTO is common in the non-medical consumer market place.

Health related applications of TTO are also profuse. TTO is a longstanding “cure all” home remedy for a variety of maladies but is finding its feet as an evidence based treatment in conventional medical practice. TTO is used topically to manage burns, skin infections, acne and chronic leg ulcers (Morris et al., 2003, Hammer et al., 2006, Fugh-Berman, 2002). Osteomyelitis associated with chronic wounds has been treated with TTO (Sherry et al., 2001). Sore throat and minor oral infections can be managed with TTO derived lozenges or mouthwash (Martin and Ernst, 2004). Both garden variety cutaneous candidiasis, vaginal thrush and oral candidiasis in the setting of HIV/AIDS have been successfully eradicated with TTO (Evans and Gray, 2003, Van Kessel et al., 2003, Jandourek et al., 1998). Nail bed fungal infections (onychomycosis) remit with application of TTO (Hammer et al., 1996). Some pharmaceuticals for dandruff and head lice treatment employ TTO as their active antifungal or anti-parasitic ingredient (Halcon and Milkus, 2004, Veal, 1996). TTO containing cold sore ointments take advantage of the antiseptic, antiviral and soothing properties of TTO (Carson et al., 2005). Finally TTO in the form of antiseptic hand and body wash is antibacterial against common skin flora including the problematic antibiotic resistant “superbug” methicillin resistant *S.aureus* (Morris et al., 2003, Riley, 2005).

2.2.4 Antimicrobial effects of tea tree oil

2.2.4.1 Introduction

TTO exhibits broad spectrum antimicrobial activity (Cox et al., 2000). TTO has documented antibacterial, antimycotic, antiviral, anti-parasitic and insecticidal effects (Burt, 2004, Hammer et al., 2006). The essential oil is effective against planktonic bacteria and biofilm (Hammer et al., 2005). In particular, the effect of TTO on bacterial biofilm is of interest because biofilm infection is the leading cause of morbidity associated with indwelling devices (Darouiche, 2004). TTO or its derivatives may therefore lead to new treatments for bacterial biofilm associated device infections.

Medical device infections are reduced by targeting common causative pathogens. The skin commensals *Staphylococcus epidermidis* and *Staphylococcus aureus* are by far the most common causes of sepsis related to medical implants (Huebner and Goldmann, 1999, Christensen et al., 1989). Unfortunately, current strategies are often ineffective in preventing or treating pathological biofilms on medical implant surfaces. Evidence is emerging however, to suggest TTO inhibits not only planktonic *Staphylococci* but also staphylococcal biofilm (Al-Shuneigat et al., 2005). On these grounds TTOs antimicrobial profile in not only antibacterial and antibiofilm but targets organisms most frequently associated with medical device infections.

Infection of implanted medical devices with multi resistant organisms such as methicillin resistant *Staphylococcus aureus* (MRSA)*,* vancomycin resistant *Enterococcus, Clostridium difficile,* extended-spectrum ß-lactamase-producing gram-negative bacilli and *Candida* is increasing (Weber et al., 1999). A major common risk factor for infection with multi resistant organisms is exposure to invasive devices of any type (Safdar and Maki, 2002). Because of emerging resistance to standard antibiotics, novel antimicrobial agents and strategies are sought to control medical device infection. TTO has antimicrobial activity against several multi-drug resistant organisms including MRSA, glycopeptide-resistant *Enterococci*, aminoglycoside-resistant *Klebsiellae* and *Stenotrophomonas maltophilia* (May et al., 2000). TTO is thus a promising candidate for development strategies to combat resistant microbial infection of medical implants

2.2.4.2 Testing, measurement and reporting challenges

There are no standard approaches to testing, measurement, interpretation or description of TTO antimicrobial effect. For example antibiotic effect of TTO has been measured with time-kill assays, two-dimensional micro-titer checkerboard, modified breakpoint combination sensitivity test method and multiple combination bactericidal antibiotic testing (Moriarty et al., 2005). Part of the difficulty lies in the fact TTO has poor aqueous solubility making conventional diffusion dependent antimicrobial testing regimes such as agar and broth cultures invalid or difficult to implement (Carson et al., 2006). Testing methodologies are of variable quality and significance and make effects across studies difficult to compare, contrast and quantify.

Even within similar testing methodologies, units employed in essential oil susceptibility testing are inconsistent or difficult to interpret. The most cited measure of antimicrobial performance of essential oils is minimum inhibitory concentration (MIC). In this context, MIC is defined as the lowest concentration of essential oil required for complete inhibition of bacteria for up to 48 hours of incubation or resulting in maintenance or reduction in inoculum viability or that inhibits visible growth (Burt, 2004). Some papers also quote minimum bactericidal concentration (MBC) defined as the essential oil concentration when 99.9% or more of the initial inoculum is killed or the lowest concentration at which no growth is obtained after sub-culturing into fresh broth (Burt, 2004). “MIC” and “MBC” are ambiguous unless exact endpoints are specified and the terms are regularly reported without defining how the measures were interpreted. Of course direct comparison of MIC or MBC with time-kill assays or other testing methodologies is not possible due to incompatible units.

Challenges also arise in misleading interpretation of studies that do not clearly demarcate planktonic cells and biofilm. Biofilm organisms are antibiotic resistant when compared to planktonic embodiments of the same microbial species (Stewart and Costerton, 2001). Biofilm is conceptually recent and biofilm investigation is in evolution (Chapter 5). As a result biofilm testing is not standardized or routine. Studies that do not specify the phase of bacterial lifecycle may present mixed data representing both planktonic and biofilm bacteria and should be viewed with a degree of skepticism.

Inconsistencies in testing methodologies, measurement parameters and reporting techniques do make quantitative and comparative assessment of TTO antimicrobial effect challenging. Despite these difficulties, it is possible with existing approaches to

determine whether organisms are affected by TTO in a qualitative way and to determine the presence or absence of bacterial susceptibility in a broad sense.

2.2.4.3 Tea tree oil effect on planktonic *Staphylococci*

Many studies have shown the susceptibility of Genus *Staphylococci* to TTO. TTO reduces the viability of *S.aureus* and makes bacterial cells sensitive to autolysis with significant loss of tolerance to sodium chloride exposure (Carson et al., 2002). The essential oil is active in liquid or vapor format against *S.aureus* (Edwards-Jones et al., 2004). Coagulase-negative *Staphylococci* which form part of transient and commensal skin flora are also killed by TTO (Brady et al., 2006). In an investigation of skin infections Raman et al. found major TTO components were active against *S.aureus* and *S.epidermidis* (Raman et al., 1995). The essential oil is bactericidal to resistant staphylococcal strains such as MRSA (May et al., 2000). As an example Carson and Riley tested sixty MRSA isolates against TTO and found one hundred percent of isolates were susceptible to TTO in suspension with MIC of 0.25% and MBC of 0.5% (Carson and Riley, 1998). TTO thus has proven antibacterial effect against the primary bacterial cause of implant infections in its free swimming form.

2.2.4.4 Tea tree oil effect on *Staphylococcal* biofilm

TTO has established efficacy again *Staphylococcal* biofilm and other bacterial biofilms as documented in a forty-two page report released by Rural Industries Research and Development Corporation in 2008 (Hammer et al., 2008). Because TTO is considered toxic when administered systemically, antibiofilm testing has focused on cultured biofilm or naturally occurring muco-cutaneous biofilms which can be treated with topical essential oils. Hammer et al. attempted to grow *in vitro* staphylococcal biofilm in the presence of several concentrations of TTO and found no visible biofilm growth at TTO concentrations of 0.5% and above (Hammer et al., 2005). Interestingly, biofilm growth was promoted at concentrations of TTO just below 0.5% perhaps as a protective response to unfavorable environmental conditions insufficient to eradicate the causative microbes (Rachid et al., 2000; Götz, 2002). Bactericidal activity of TTO against established cutaneous biofilms was investigated by Brady et al.. Biofilms formed by both methicillin sensitive *S.aureus* (MSSA) and methicillin resistant *S.aureus* (MRSA) were completely eradicated by 5% TTO after one hour exposure to the essential oil (Brady et al., 2006). Similarly, topical TTO prevented formation of MRSA biofilm on the surface of silicone tympanostomy tubes as verified by Park et al. (Park et

al., 2007). In addition coagulase negative staphylococcal biofilms have been controlled by TTO. Brady et al. showed five of nine biofilms formed by strains of coagulase-negative *Staphylococci* were killed by TTO and a reduction in viable count was apparent in the remaining four isolates (Brady et al., 2006). These results collectively confirmed TTO inhibition of staphylococcal biofilm and bactericidal activity against formed biofilm of MRSA, MSSA and some coagulase negative *Staphylococci* (Brady et al., 2006). Antibiofilm activity of TTO against MRSA and coagulase negative *Staphylococci* was especially significant in a clinical sense as these organisms are common, can be resistant to eradication leading to the carrier state and often show antimicrobial resistance (Halcon and Milkus, 2004).

2.2.4.5 Tea tree oil effect on other bacteria and fungi

Many bacteria other than *Staphylococci* are inhibited or killed when exposed to TTO although to varying degrees (Table 2.6). *Pseudomonas aeruginosa* is less susceptible to antibacterial properties of TTO as compared with many other bacteria. Its tolerance is often attributed to the lipopolysaccharide component of the gram negative outer cellular membrane (Mann et al., 2000). Dental plaques are the prototypical pathogenic medical biofilm and have been studied extensively. Cariogenic and periodontopathic bacteria are present in dental plaques as biofilm. Takarda et al. demonstrated adhesion inhibiting effects of TTO on *P.gingivalis* leading to suppression of dental plaque biofilm (Takarada et al., 2004). Fungi also show susceptibility to TTO (Table 2.8). An increasing proportion of bloodstream and urinary tract infections are caused by *Candida spp.* which can then lead to infection of implanted medical devices (Kojic and Darouiche, 2004). Several fungal isolates including subspecies of *Candida, Saccharomyces, Trichosporon, Rhodotorula, Epidermophyton, Microsporum* and *Aspergillus* have shown susceptibility to TTO or its components (Hammer et al., 2003). TTO therefore has a broad antimicrobial effect.

2.2.4.6 Mechanism of antimicrobial activity of tea tree oil

Broad spectrum antimicrobial properties of tea tree oil have been empirically recognized for decades. Perhaps TTO has evolved antimicrobial properties to prevent microbial infection of the *Melaleuca alternifolia* tree from which it is derived. How TTO works against infection and the active components of the essential oil are however, yet to be fully elucidated. It is likely that multiple mechanisms apply and complex interactions between essential oil components may play a significant role in producing anti-infective properties.

TTO is for the most part bactericidal although it may be bacteriostatic at lower concentrations (Carson et al., 2006). Antibacterial mechanisms are probably multiple, overlapping in their effect and vary according to bacterial species. Proposed mechanisms of bacterial inhibition and killing caused by TTO include:

- Leakage of cellular potassium ions (Cox et al., 2000)
- Leakage of cellular hydrogen ions (May et al., 2000)
- Leakage of other cellular contents (Burt, 2004)
- Cytoplasmic membrane damage and loss of chemi-osmotic control (Cox et al., 2000, Carson et al., 2002)
- Depletion of cellular proton motive force (Burt, 2004)
- Coagulation of the cytoplasm (Burt, 2004)
- Inhibition of cellular respiration (Cox et al., 2000)
- Inhibition of microbial oxygen uptake (May et al., 2000)
- Inhibition of oxidative phosphorylation (May et al., 2000)
- Release of membrane bound cell wall autolytic enzymes (Carson et al., 2002)
- Induced leucocyte differentiation (Morris et al., 2003)

The broad spectrum antimicrobial activity of TTO is often ascribed to monoterpene constituents and in particular to the most abundant component of the essential oil: terpinen-4-ol (Loughlin et al., 2008). Because approximately ninety percent of TTO consists of terpinen-4-ol, 1,8-cineole, α-terpineol, terpinolene and α- and γ-terpinene, these components are generally considered responsible for antibacterial effects of TTO based on their major component status (May et al., 2000). For example, terpinen-4-ol is said to induce leucocyte differentiation (Morris et al., 2003). Alternatively, intact TTO has been reported to have stronger antibiotic effects than the sum of individual components indicating a synergistic interaction between constituents (Takarada et al., 2004). Minor components of essential oils may also be antimicrobial in their own right or may augment the antimicrobial effects of more major TTO components (Burt, 2004) (Table 2.7). Some antimicrobial effects are attributed to the hydrophobic nature of TTO components overall, particularly the hydrocarbon subsets (Carson et al., 2006). Hydrophobic interactions are thought to cause interruption of microbial cellular membranes. Given the diverse antimicrobial activity and many chemical constituents of TTO it is likely more than one antibiotic mechanism are at play. Discovering the anti-infective and specifically antibiofilm mechanism(s) of TTO could greatly contribute to development of novel antimicrobial treatments and prevention approaches to medical device infections.

Table 2.6: Minimum inhibitory concentrations and minimum bactericidal concentrations of tea tree oil

Organism	MIC (% v/v)	MBC (% v/v)	Study
Actinomyces spp.	0.1-1	0.1-2	(May et al., 2000)
Actinomyces spp.	1	1	(Carson et al., 2006)
A.actinomycetemcomitans	0.25-0.5	0.5	(Takarada et al., 2004)
A.actinomycetemcomitans	0.06	0.06	(May et al., 2000)
A.baumanii NCTC 7844	0.25		(Hammer et al., 1999)
A.baumanii	1	1	(Carson et al., 2006)
A.sobria ATCC 9071	0.5		(Hammer et al., 1999)
B.cereus	0.3		(Carson et al., 2006)
Branhamella spp.	0.06	0.06	(May et al., 2000)
E.coli AG100	0.25	0.5	(Cox et al., 2000, Hammer et al., 1999)
E.coli NIHJ JC-2	0.32		(Inouye et al., 2001)
E.faecalis NCTC 8213	2.0		(Hammer et al., 1999)
F.nucleatum	0.13	0.25	(Takarada et al., 2004)
H.influenzae ATCC 33391	0.16		(Inouye et al., 2001)
K.pneumoniae	0.12-0.5	0.12-0.5	(May et al., 2000)
K.pneumoniae	0.5		(Hammer et al., 1999)
Oral bacteria	0.03-0.6		(May et al., 2000)
P.gingivalis	0.25-0.5	0.25-0.5	(Takarada et al., 2004)
P.aeruginosa	>8	>8	(Cox et al., 2001)
S.aureus NCTC 8325	0.25-0.5	0.5	(Cox et al., 2001, Hammer et al., 1999)
S.aureus FDA 209P JC-1	0.32		(Inouye et al., 2001)
S.aureus (MRSA)	0.12-0.5	0.25-4.0	(May et al., 2000, Brady et al., 2006)
S.marcescens	0.5		(Hammer et al., 1999)
S.pneumoniae IP-692	0.32		(Inouye et al., 2001)
S.pneumoniae PRC-53	0.32		(Inouye et al., 2001)
S.pyogenes ATCC 12344	0.32		(Inouye et al., 2001)
S.typhimurium	0.5		(Hammer et al., 1999)
S.sobrinus	1.0	1.0	(Takarada et al., 2004)
S.viridans	0.12-2.0	0.25-2.0	(May et al., 2000)
Vaginal bacteria	0.03-2.0		(May et al., 2000)

Table 2.7: Components of tea tree oil described as antibacterial

Component	Bacteria	MIC (% v/v)	MBC (% v/v)	Study
α-terpineol	*E.coli* 25922		0.45	(Cosentino et al., 1999, Burt, 2004)
	E.coli O157:H7		>0.9	
	E.faecalis 29212		0.9	
	S.typhimurium 14028	0.225	0.225	
	S.aureus 25923	0.9	0.9	
	S.epidermidus 12228		>0.9	
	L.monocytogenes 7644	>0.9	>0.9	
	P.aeruginosa		>0.9	
	Y.enterocolytica 9610		>0.9	
α-pinene	*H.influenzae*	>0.32		(Burt, 2004, Inouye et al., 2001)
	S.pyogenes	>0.32		
	S.pneumoniae IP-692	>0.32		
	S.aureus	>0.32		
	E.coli NIHJ JC-2	>0.32		
1,8-cineole	*E.coli*	1	1	(Cox et al., 2001, Inouye et al., 2001, Burt, 2004)
	E.coli NIHJ JC-2	>0.32		
	S.aureus	0.5	1	
	P.aeruginosa	>8	>8	
	H.influenzae	>0.32		
	S.pyogenes	>0.32		
	S.pneumoniae IP-692	>0.32		
γ-terpinene	*E.coli* O157: H7	>8	>8	(Cosentino et al., 1999, Cox et al., 2001, Burt, 2004, Takarada et al., 2004)
	S.aureus		>0.9	
	S.aureus 25923	0.25	0.5	
	S.epidermidus 12228		>0.9	
	P.aeruginosa 27853		>0.9	
	S.typhimurium 14028	>8	>8	
terpinen-4-ol	*E.coli*	0.125	0.25	(Takarada et al., 2004, Cox et al., 2001, Inouye et al., 2001)
	E.coli NIHJ JC-2	0.32		
	S.aureus	0.125	0.25	
	P.aeruginosa	0.5	>8	
	H.influenzae	0.16		
	S.pyogenes	0.16		
	S.pneumoniae IP-692	0.32		
D-limonene	*S.pyogenes*	>0.32		(Inouye et al., 2001)
	S.aureus	>0.32		
	H.influenzae	>0.32		
	S.pyogenes	>0.32		
	S.pneumoniae IP-692	>0.32		
	S.pneumoniae PRC-53	>0.32		
	S.aureus	>0.32		
	E.coli NIHJ JC-2	>0.32		

Table 2.8: Minimum inhibitory concentrations and minimum fungicidal concentrations of tea tree oil

Organism	MIC (% v/v)	MFC (% v/v)	Study
C.albicans KEM H5	0.125	0.25	(Cox et al., 2001)
non-albicans *Candida*	0.06-0.5	0.5	(Vazquez et al., 2000)
fluconazole resistant *C.albicans*	0.06-0.25	0.5	(Vazquez et al., 2000)
M.furfur	0.12-0.25		(Hammer et al., 1997)
A.fumigatus	>2.0	>2.0	(Vazquez et al., 2000)
A.nidulans	>2.0	>2.0	(Vazquez et al., 2000)

2.2.5 Adverse reactions associated with tea tree oil

World Health Organization (WHO) defines an adverse drug reaction as “an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product” (Edwards and Aronson, 2000). WHO definition excludes therapeutic failures, intentional or accidental poisoning or drug abuse and adverse effects due to errors in administration or compliance (WHO, 1975). Toxic substances or substances that elicit adverse reactions are unsuitable bio-implant materials as they are by definition not biocompatible. If TTO is to be supported as a building block for production of biomaterials then the adverse reaction profile of TTO needs to be understood. Available adverse reaction data for TTO was therefore examined to define its toxicity profile.

It is unknown whether toxic components of a given organic gas or essential oil vapor can be nullified via the process of plasma polymerization. The reverse seems less likely in that plasma polymers are un-reactive and highly stable as a group when compared to parent compounds and therefore should not gain toxicity (Yasuda, 2005). By avoiding parent chemicals known to provoke adverse reactions, some risk mitigation is obtained.

Toxicity, safety and adverse effects of TTO in human beings remains to be assessed within the confines of a randomized, double blind control trial and therefore no level one data on the matter exists. Although a number of lower level trials, reports, case

studies and historical accounts credit a variety of adverse reactions to TTO, these claims should be viewed with some skepticism. Often substances described as TTO within the literature on closer inspection do not meet strict ISO standards for Oil of Melaleuca or are completely unrelated botanical compounds (Carson and Riley, 2001). Variations in percentage concentrations of TTO make comparison between papers and meta-analysis difficult and available studies are generally underpowered due to insufficient participant numbers.

Ironically, a paucity of formal investigation into adverse reactions linked to TTO may stem from historical use of the essential oil. For the past 80 years TTO has remained an over the counter general purpose remedy implemented widely across Australian and international communities. The essential oil is contained within medicines, cosmetics and toiletries and yet there are few reported adverse reactions. Lack of TTO toxicity is therefore implied. Despite quality limitations, available adverse event information relating to TTO was investigated.

Adverse *in vivo* reactions to TTO are grouped according to route of administration and then sub-classified as local or systemic reactions. Data on intravenous, intramuscular, intrathecal, inhalational and subcutaneous administration of TTO is lacking as to date clinical indications for invasive administration of TTO have not existed. Reported adverse reactions reflect common methods of administration of TTO: topical (muco-cutaneous) application and ingestion (Table 2.9).

Table 2.9: Adverse effects of tea tree oil reported in the literature

Adverse reaction	Reference
skin irritation	(Morris et al., 2003)
skin sensitization	(SCCP, 2004)
allergic contact dermatitis	(Van Kessel et al., 2003)
immediate systemic hypersensitivity	(Molzelsio et al., 2003)
erythema multiforme	(Khanna et al., 2000)
altered mental status/ ataxia in children	(Morris et al., 2003)
coma	(Carson and Riley, 1995)
ototoxicity (guinea pig)	(Zhang and Robertson, 2000)
leucocytosis	(Elliot, 1993)
nausea, abdominal pain and anorexia	(SCCP, 2004)
pre-pubertal gynaecomastia	(Henley et al., 2007)

2.2.5.1 Adverse effects from topical application of tea tree oil

Anecdotal evidence dating back 80 years infers that topical use of tea tree oil is safe and any adverse effects are minor, self-limiting and occasional (Hammer et al., 2006). Simple skin irritation and allergic dermatitis as a result of exposure to TTO are uncommon, rarely cause functional impairment and are associated with minor, short lived, local discomfort (Carson et al., 2006). A literature review by Hammer et al. demonstrated that irritant or allergic reactions induced by occlusive patch testing (topical cutaneous application of TTO) occurred in a maximum of 10.7% of patients exposed (3/28 patients) although trials with much larger enrolment numbers have demonstrated significantly lower cutaneous reaction rates as demonstrated in Table 2.10 (Hammer et al., 2006). Incidence of cutaneous reaction to TTO increases with percentage concentration of essential oil but is uncommon even with application of 100% pure tea tree oil (Table 2.10). A single report of erythema multiforme as a result of tea tree oil topical exposure is documented (Khanna et al., 2000). Isolated erythema multiforme is an acute cutaneous eruption characterized by distinctive target lesions on the palms and soles with diagnostic histology and a benign clinical course (Roujeau and Stern, 1994). In rabbits LD50 for topically applied TTO is 2.23 ml/kg which equates in a 70 kg human to 156.42 ml spread over the dermis (Fletcher et al., 2005). 10 ml of TTO is enough to coat an average sized human and intact human skin is less permeable to TTO than rabbit skin (Halcon and Milkus, 2004). A lethal outcome from cutaneous exposure to TTO would therefore be difficult to achieve in man and is not evidenced in the literature. Topical administration of TTO is thus unlikely to cause significant harm.

Aged, oxidized TTO is a three times more potent topical allergen than fresh TTO (Carson and Riley, 2001, Hausen et al., 1999). TTO oxidizes quickly and produces strong allergenic peroxides if the essential oil is exposed to light, heat, oxygen or moisture (Wabner et al., 2006). It is not common practice to comment on degree of oxidization of TTO in adverse reaction papers. Confounding errors may therefore arise due to the unwitting presence of allergenic peroxides in aged TTO. Accounting for peroxides, the incidence of adverse reactions to TTO may be overstated. Oxidation and development of peroxides within TTO is avoided with simple storage methods that ensure a dark, cool, airtight environment (Wabner et al., 2006). Small volume containers reduce the surface area of oil exposed with lid opening. Simple steps such as limiting the number of times a container is opened and accurate expiry date information reduce development of peroxides (Wabner et al., 2006).

Table 2.10: Prevalence of cutaneous reaction to tea tree oil at different essential oil concentrations

Study	TTO (%) applied to skin				
	0.1	1	5	10	100
(Lisi et al., 2000)	0/725	1/725	1/725	-	-
(Coutts et al., 2002)	-	-	-	-	13/550
(Southwell et al., 1997)	-	-	-	-	3/28
(Veien et al., 2004)	-	-	3/217	1/217	-
(Veien et al., 2004)	-	-	0/160		
(Knight and Hausen, 1994)	-	0/20	-	-	-
(Fragrance-Raw-Materials-Monograph, 1988)	-	0/22	-	-	-
(Pirker et al., 2003)	-	-	38/3375	-	-
(Hausen, 2004)	-	-	70/6896	-	-
(Hausen, 2004)	-	-	21/2284	-	-

2.2.5.2 Adverse effects of ingested tea tree oil

Several published case studies report human poisoning with ingestion of modest volumes (10-25 ml) of TTO (Carson et al., 1998). These reports do not specify constituents of TTO or comment on oxidation status. It has been reported that human TTO ingestion has led to transient alteration in mental status and ataxia requiring systemic supportive therapies for less than 24 hours followed by full recovery (Morris et al., 2003). Oral administration of less than or equal to 1.5 g/kg TTO by rats also induces short lived (<72 hours) lethargy and ataxia (Kim et al., 2002). Oral LD50 for TTO in rats is 1.9-2.6 ml/kg and in rabbits 5.0 g/kg (Russel, 1999). For a 70 kg human the lethal oral dose has been extrapolated to 133-182 ml and would be difficult to achieve without intent, persistence and anti-emetics (Wabner et al., 2006). No human deaths due to oral ingestion of TTO are reported in the literature (Hammer et al., 2006). Despite lack of high level evidence relating to ingestion of TTO in humans, existing data suggests potential for toxicity. As safe oral dosing is ill defined, ingestion is not recommended. In fact in Australia TTO is deemed a Schedule 6 poison by the Drugs, Poisons and Controlled Substances Act 1981. Schedule 6 poisons are considered of moderate potential to cause harm and 100% pure TTO must be childproofed and labeled not to be taken internally.

2.2.5.3 *In vitro* adverse effects of tea tree oil

In vitro studies have demonstrated minimal cytotoxicity associated with TTO. Exposure of cultured human umbilical vein endothelial cells to 0.2% TTO has little impact on the cells (Takarada et al., 2004). Increasing TTO concentration to 0.5% increases cell death and decreases activity of surviving cells exposed to TTO (Takarada et al., 2004). Earlier independent cytotoxicity testing on human epithelial cells and fibroblasts deemed TTO to be of low toxicity (Soderberg et al., 1996). Although leaching from a bio implant may achieve similar local concentrations of TTO, achieving comparable systemic concentrations would require parenteral administration or high volume oral ingestion of TTO.

Although not extensively studied, *in vitro* examinations of the mutagenic potential of commercially available TTO products have been performed in a United Kingdom based on a study by Fletcher et al. (Fletcher et al., 2005). The study employed *S.typhi* based Ames testing and determined that neither TTO nor the TTO component terpinen-4-ol had mutagenic effect in *Salmonella* examined with or without metabolic activation.

2.2.5.4 Overview of the adverse effects of tea tree oil

High level evidence supporting clinically significant adverse drug effects due to TTO is lacking but the safety dossier on TTO is incomplete. Existing evidence is insufficient to warrant exclusion of TTO as a monomer source for plasma polymer coatings fit for medical implants. Topical reactions are most relevant for implants with trans-cutaneous components and these reactions are uncommon and minor. Systemic reactions to TTO are poorly documented and rare. Reactions secondary to ingestion are difficult to extrapolate to TTO derived plasma polymer bio-implants as implants are immobilized, contain limited amount of altered TTO derivatives and seldom involve the gastrointestinal tract. TTO is not significantly cytotoxic or mutagenic *in vitro*. Finally TTO has a long standing usage history in the community and a benign historical adverse drug reaction profile.

2.2.5.5 Reduction of tea tree oil adverse effects

Adverse reactions to TTO are minimized by using topical TTO sparingly, diluting topical preparations, preventing ingestion and limiting exposure to aged and oxidized product. The general toxicology profile of *M alternifolia* essential oil suggests that severe reactions are extremely rare in the absence of ingestion (Halcon and Milkus, 2004). An

inner arm cutaneous sensitivity test may help exclude those individuals at increased risk of hypersensitivity reactions (Wabner et al., 2006).

2.2.5.6 Etiology of adverse effects of tea tree oil

TTO, being a heterogeneous substance, has many candidate components linked to adverse reactions although the literature is not in agreement (Table 2.11). It is inaccurate to extrapolate toxicity of an isolated component of TTO such as D-limonene to whole TTO as this strategy ignores possible ameliorating interactions between monomer constituents. No literature exists to describe individual component interactions within TTO probably due to their highly complex and dynamic nature (Hammer et al., 2006). No investigations to date comment on relative absorption of TTO components with topical exposure or ingestion. Absorption is directly related to toxicity (Hammer et al., 2006). It is plausible that different adverse reactions are related not to individual constituents but to component combinations within tea tree oil. Reactions are also influenced by host and environmental factors.

Mechanisms of muco-cutaneous adverse drug reactions may include direct toxicity to cells caused by contact with TTO, stimulation of the inflammatory response or immunological mechanisms such hypersensitivity reactions. Systemic sequelae of ingestion are so rare, inconsistent and poorly described that it is impossible to attempt an explanation of the mechanisms involved.

Table 2.11: Candidate components for tea tree oil adverse reactions

Component	Study
aromadendrene	(Knight and Hausen, 1994)
ascaridole (oxidation product)	(SCCP, 2004)
1,8-cineole	(Patruno et al., 1994)
ρ-cymene	(Carson and Riley, 2001)
D-limonene	(Knight and Hausen, 1994, Van Kessel et al., 2003)
oxidized components	(Hammer et al., 2006)
α-terpinine	(Knight and Hausen, 1994, Hausen et al., 1999)
α-phellandrene	(Knight and Hausen, 1994)
α-pinene	(Cachao et al., 1986)
terpinen-4-ol	(Knight and Hausen, 1994)
terpinolene	(Hausen et al., 1999)
1,2,4-trihydroxy-menthane (oxidation product)	(SCCP, 2004)

2.3 Role of Polymers and Polymer Coatings in Medical Implants

The probability of finding a material that fulfills all requirements in physical and chemical bulk properties for a biomaterial application and whose surface properties are just right for a specific application is very close to zero, if not absolutely zero (Yasuda, 2005). From this point of view all biomaterials should be surface treated to improve biocompatibility. Many different materials have been applied as medical implant coatings with a view to improving their biocompatibility profile including metals, ceramics and polymers. Polymers are favored in many instances because they can be prepared in different compositions with a wide variety of structures and properties. In fact there is enormous potential for the replacement of metals and ceramics by polymers because polymers provide functional improvements at a lower cost whilst still meeting biocompatibility requirements (Rapra Technology Ltd., 2004). Surface modification of biomaterials with polymer coatings is already used to improve the function and lifetime of biomaterials used in medical components, when devices require tailored coatings or when product differentiation is desired (Chu et al., 2002). Medical polymers in common use are natural or synthetic, degradable or non-degradable and some are responsive to their environment.

Medical materials are a big business. Overall the United States medical device market was worth US$ 70.3 billion in 2005 (Toloken, 2003). The value of worldwide sales for all categories of coatings and surface treatment processes used in manufacturing medical devices reached US$ 2.96 billion in 2005 (Piribo, 2006). It's projected that demand for medical polymers will reach US$ 6.55 billion in the United States alone in 2012, based in part on heightened concerns over infection control and increased use of disposable medical products and supplies (Freedonia, 2009).

2.3.1 Medical polymers as an interface

In the 19th Century, the first observations were made that surfaces control biological reactions. In the late 1940s and early 1950s, the first bio-polymers as we know them today were developed (Duke and Plummer, 2002). These were used for intraocular lenses, prosthetic joints and intravascular devices. Over the last 25 years, major advancements in surface science instrumentation have allowed accurate characterization of surface composition and molecular structure of biomaterials. Along with advancements in material

science and molecular biology, these new technologies have instigated the development of the biological model for surface science, where the ultimate goal is to gain a detailed understanding of how surface properties of a material control biological reactivity of a cell interacting with that surface (Castner and Ratner, 2002). This field is the ultimate study of biocompatible materials and has enveloped biomedical polymer science.

Interaction of medical implants with host occurs at the surface of implant. This surface interaction determines biocompatibility of the entire bio-implant. For kinetic and thermodynamic reasons, surfaces are different from corresponding bulk material and contain reactive (unsaturated) bonds, which lead to formation of surface reactive layers and adsorbed contamination layers (Kasemo and Lausmaa, 1994). Encounter with the biological environment triggers specific surface adsorption of water, ions and biomolecules. These moieties are continuously exchanged. Surfaces provide accessibility for reactions and the low energy barrier to mobility in the plane of the surface is used to facilitate complex reactions such as clustering, conformational change and exposure and burial in membranes (Duke and Plummer, 2002). Molecular recognition (a manifestation of both geometry and chemistry) is readily implemented at surfaces and bio-functional surfaces call for advanced design if they are to match the sophisticated recognition ability of biological systems (Kasemo, 2002, Angelova and Hunkeler, 1999). Because cells are inherently sensitive to local mesoscale, microscale, and nanoscale patterns of chemistry, topography and visco-elasticity, the nature of a materials surface determines behaviour of cells approaching the material and in turn the surrounding tissue response to material (Kasemo, 2002, Stevens and George, 2005).

The field of interface science and engineering is highly interdisciplinary and combines knowledge from technical and life sciences disciplines. Although beyond the scope of discussion here, it is vitally important to recognize the key implications of interface interactions and thus the importance of medical implant coatings and surface treatments in the development of bio-implants.

Polymers currently used as biomaterials can be classified in a number of ways: natural or synthetic, absorbable or non-absorbable, according to their chemical grouping, function, properties or applications. All are valid given the appropriate context. Dividing medical polymers into natural and synthetic categories is convenient because it supports some high level comments. Naturally derived polymers are abundant and usually biocompatible

and biodegradable however their disadvantages lie in inconsistent production parameters (the natural environment) and structural complexity which makes modification and purification technically difficult (Angelova and Hunkeler, 1999). Conversely, synthetic polymers are available in a wide variety of modifiable formats and can be manufactured in a highly controlled and consistent fashion. Processing, co-polymerization and blending provide means of optimizing a synthetic polymer's mechanical characteristics and its diffusive and biological properties. Unfortunately synthetic polymers are generally not biocompatible and often elicit unfavorable inflammatory reactions (Angelova and Hunkeler, 1999). There is no perfect medical polymer and as is the case with all industrial materials, medical polymers are employed according to a balance of factors. Subsequently a group of acceptable and well used polymers survive in the commercial marketplace (Table 2.12). Characteristics of the main groups of medical polymeric materials and their broad array of applications are discussed.

Table 2.12: Polymers commonly used in construction of medical devices and coatings

Polymer	Alternate names	Molecular formula
poly carbonate	(PC)	$[C_5H_{12}N_2O]_n$
poly(dimethyl siloxane)	Silastic (SR) (PDMS)	$[R_2SiO]_n$
poly(ethylene)	alkathene (PE)	$[C_2H_4]_n$
poly(ethylene terephtalate)	Mylar, melinex (PET)	$[C_{10}H_8O_4]_n$
poly(hydroxy ethyl) methacrylate	HEMA	$[C_6H_{10}O_3]_n$
poly(methyl methacrylate)	Bone cement (PMMA)	$[C_5O_2H_8]_n$
poly(glycolide lactide)	PGL	$[C_3H_6O_3]_n$-$[C_2H_4O_3]_n$
poly(propylene)	Propathene, prolene (PP)	$[C_3H_6]_n$
poly(tetrafluoro ethylene)	Teflon, fluon (PTFE)	$[C_2F_4]_n$
poly(styrene)	Thermacole	$[C_8H_8]_n$
poly(urethane)	Texin avcothane (PUR)	$[C_{25}H_{42}N_2O_6]_n$
poly(vinylchloride)	PVC	$[C_2H_3Cl]_n$

2.3.2 Natural polymers

Natural substances used as medical polymers include proteins, poly amino acids and polysaccharides. These natural materials come from a variety of animal, plant and microbial sources.

2.3.2.1 Protein and protein based polymers

Proteins and protein based polymers are absorbable, biocompatible, non-toxic, naturally available, typically elastic materials. These materials feature in medical implants and tissue engineering processes because of their favorable properties. The most widely investigated proteins for the use in bio-implants are collagen and albumin.

Collagen is the main supportive protein of bone, cartilage, skin, tendon and other connective tissues. Collagen is also a key component of wound healing. Over the years, collagen has been successfully employed as part of absorbable sutures and sponges, wound dressings and more recently in bio-composite films, nerve cuffs and drug delivery microspheres. Collagen coated non-absorbable sutures have improved tendon to bone integration in rotator cuff surgical repairs by stimulating cellular adhesion and proliferation, alkaline phosphatase and protein synthesis more than uncoated sutures (Mazzocca et al., 2007). Absorbable collagen in the form of spongy sheets permeated with epidermal growth factor have also sped up healing of cutaneous wounds in diabetic mice by promoting granulation tissue influx and re-epithelialization (Kondo et al., 2011). Collagen breakdown products derived from collagen based wound dressings are advantageous because they are chemotactic for cell types required to form granulation tissue, they absorb wound exudates and maintain a moist wound environment (Brett, 2009). Promising results with on lay wound dressings, have also led to development of collagen containing bio-composite films. Coombes et al. produced composite films of collagen and poly(caprolactone) and found film porosity could be tailored by varying processing parameters and that collagen containing films out performed films of plain poly(caprolactone) by encouraging attachment and spreading of human osteoblasts (Coombes et al., 2002). Even nerve healing has been promoted by collagen devices. NeuraGen™ is a collagen based nerve cuff used to encase and support nerve repair sites. In a study by Farole and Jamal , lingual and inferior alveolar nerve injuries sustained following third molar surgery were repaired adding NeuraGen™ (Farole and Jamal, 2008).

Authors reported good preliminary results with sensory improvement, complete bio-resorption of the implant, minimized scar in growth and improved growth factor concentration at the injury site (Farole and Jamal, 2008). Finally the biocompatibility and low immunogenicity of collagen have led to its use in drug delivery micro-particles (Sehgal and Srinivasan, 2009).

The second commonly used protein based natural polymer used in medical implants is albumin. Albumin is the major protein found in plasma, is responsible for colloid osmotic pressure within the circulation and acts as a transport protein for large organic anions and some hormones. Because albumin is a blood component, the protein has been investigated as an anti-thrombotic coating and more recently in cell and drug microencapsulation. Maalej et al. found *in vitro* surfaces coated with modified bovine serum albumin (added nitric oxide functional groups) reduced platelet attachment, adhesion and aggregation (Maalej et al., 1999). This was attributed to anti-platelet actions of nitric oxide combined with anti-adhesive properties of albumin. Similarly, Brynda et al. used cross-linked assemblies of human serum albumin to create albumin based, multi-layered surface coatings (Brynda et al., 2000). On exposure to human citrated plasma the novel coatings displayed no immunoglobulin adherence and favourable platelet interactions suggesting potential as durable, blood contacting medical surfaces (Brynda et al., 2000). Albumin has also been developed as a component of drug delivery devices. Human serum albumin and l-α-dimyristoylphosphatidic acid were applied as a controlled release surface coating to encapsulate ibuprofen microcrystals via layer-by-layer assembly of oppositely charged species in a study by An et al. (An et al., 2004). Release of ibuprofen from microcapsules decreased as capsule wall thickness increased, indicating permeability of these microcapsules can be controlled by simply varying the number of surface layer deposition cycles (An et al., 2004). Ovalbumin has also been used in nano-scale applications. Coombes et al. impregnated knitted vascular grafts with 500 nm microspheres of ovalbumin (stabilized by addition of lactic acid) and found the surface to be resistant to blood platelet adhesion but conducive to endothelialization (Coombes et al., 2000). 60% of ovalbumin was retained by graft up to four weeks suggesting use of this coating technique for intravascular medical devices (Coombes et al., 2000)

2.3.2.2 Poly(amino) acids

Natural poly(amino) acids are a group of biodegradable, poly-ionic molecules with biological functions produced from renewable resources (Obst and Steinbüchel, 2004). Monomers of poly(amino) acids are linked by conventional peptide bonds. Poly(amino) acids used as medical polymers are generally constructed from lysine, glutamic and aspartic acids as they are non-toxic, non-antigenic and biocompatible. Poly-L-lysine has been used in development of nano-tubules for tissue engineering, encapsulation of transplanted pancreatic islet cells and as an enteric coat for glucose bio-sensors (Zhou and Qi, 2011, Liu et al., 2010, Murakami et al., 2011). An interesting medical application for poly(glutamic) acid was an antigen delivery vehicle for dendritic cell based cancer immunotherapy. In a study by Matsuo et al. biodegradable poly(glutamic) acid nanoparticles successfully delivered entrapped antigenic proteins to dendritic cells via major histocompatibility complex class I and II molecules (Matsuo et al., 2010). Dendritic cells normally present tumor associated antigens to cytotoxic T-lymphocytes which in turn act in the immune surveillance system as major effector cells to eliminate malignant cells. Poly(glutamic) acid nanoparticles may therefore function as useful antigen delivery carriers in dendritic cell based cancer immunotherapy (Matsuo et al., 2010). Lastly, poly(aspartic) acid has been used in the manufacture of hydrogels and drug containing micelles as well as biocompatible magnetic nano-particles as contrast enhancers in magnetic resonance imaging (Yang et al., 2011, Torma et al., 2010, Arimura et al., 2005).

Pseudo-poly(amino) acids are a class of polymers based on natural amino acids linked by non-amide bonds. They offer improved mechanical properties, processing, stability, and ease of synthesis over poly(amino) acids joined by traditional peptide bonds (Mallakpour and Zeraatpisheh, 2011). The range of monomers and bonds used to produce pseudo-poly(amino) acids has enabled development of polymers with desired properties such as specific glass transition temperature, crystallinity or hydrophobicity (Mallakpour and Zeraatpisheh, 2011)

2.3.2.3 Polysaccharides and polysaccharide derivatives

Polysaccharides are a class of carbohydrate formed from repeating monosaccharide units linked by glycosidic bonds. Polysaccharides in nature are derived from vegetable, animal, microbial or marine sources such as algae and therefore constitute one of the most abundant renewable polymer resources available today (Habibi et al., 2010).

Vegetable sourced polysaccharides include carboxy methyl cellulose, cellulose sulfate and agarose. Carboxy methyl cellulose easily forms a gel. Cross-linked carboxy methyl cellulose has been instilled as a hydrogel for injectable soft-tissue augmentation in plastic surgery (Leonardis et al., 2010). The substance in gel format has been used to deliver intra-peritoneal drugs in an attempt to reduce post-surgical adhesions and in sub-micron fiber format to deliver anticancer drugs (Helen et al., 2009, Cai et al., 2011). Cellulose sulfate has formed a component of microcapsules as tools for immuno-isolation of allogenic or xenogenic implants (Renken and Hunkeler, 2007, Dautzenberg et al., 1999). Agarose is widely employed in the laboratory as a supporting material and as an immobilization matrix in processes such as gel electrophoresis and chromatography (Angelova and Hunkeler, 1999).

Alginate is an anionic polysaccharide distributed widely in cell walls of brown algae. In extracted form it absorbs 200-300 times its own weight in water quickly forming a viscous gum (Roew, 2009). Alginate is produced commercially from seaweed and from bacteria of Genera *Pseudomonas* and *Azotobacter* (Remminghorst and Rehm, 2009). Because of its excellent gel forming capacity, alginate is widely used in the bio-medical industry for applications such as cell and enzyme immobilization matrices, controlled release of bioactive substances and injectable microcapsules for treating neurodegenerative and hormone deficiency diseases (Angelova and Hunkeler, 1999). Alginate dressings have been widely adopted for the management of postoperative and chronic wounds because of their exudate absorbing and hemostatic properties (Thomas, 2000). Experimentally, alginates have been investigated as a material encapsulating endocrine cells, such as insulin-secreting pancreatic islets (Sabbatini and Zambonin, 1994). Alginates are a versatile and accepted group of natural medical polymers.

Carrageenan is a family of linear, sulfated polysaccharides extracted from red seaweeds that serve as medical polymers. These polysaccharides have excellent thermo-reversible

properties and so have been utilized for microencapsulation and sustained drug release applications (Maolin et al., 2000). They also form foams and are reported as wound management aids (Carp et al., 2004, Lloyd et al., 1998).

Hyaluronic acid, heparin and heparin like glycosaminoglycans are polysaccharides derived from human and animal sources and act as medical polymers. Hyaluronic acid is an anionic, non-sulfated glycosaminoglycan distributed widely throughout connective, epithelial, and neural tissues. It has a very high molecular weight and is an excellent lubricant. Because of its lubricant properties, hyaluronic acid polymer coatings have been applied to improve gliding of extra-synovial tendon graft, to ameliorate the effects of cartilage degradation in osteoarthritic conditions and after joint replacements and in the prevention of postoperative pericardial adhesions (Uebelhart and Williams, 1999, Momose et al., 2002, Mitchell et al., 1994). Heparin is a sulfated glycosaminoglycan of mixed composition with anticoagulant properties. It is released by mast cells and basophils in many tissues, especially the liver and lungs. Heparin coatings have been widely investigated as intravascular and infection resistant surface treatments as means to improving durability of medical devices and in particular intravenous catheters (Jain et al., 2009, Brynda et al., 2000, Mermel et al., 1993).

Dextran is an extracellular polysaccharide produced by beneficial lactic acid bacteria and plaque forming bacteria as a manifestation of biofilm. Dextran has excellent rheological properties and has thus been implemented as a plasma expander and drug carrier (Angelova and Hunkeler, 1999). In hypovolemic patients, dextran infusion improves oxygen flux within the microcirculation (McCahon and Hardman, 2010). Dextran has also formed the basis of bio-medical scaffolds (Yoshikawa et al., 2009)

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (de-acetylated unit) and N-acetyl-D-glucosamine. Commercial production of chitosan is performed by de-acetylation of chitin, the structural element in the exoskeleton of crustaceans and cell walls of fungi. Chitosan and its derivatives are biocompatible, nontoxic, excellent gel and film forming, natural poly-cations widely used in drug delivery systems such as gels membranes and microspheres (Angelova and Hunkeler, 1999). Chitosan microspheres have acted as enteric coatings with controlled release of drugs such as nitrofurantoin into the small intestine (Ribeiro et al., 1999, Hari et al., 1996). More

recently, chitosan coated microspheres have been deployed as an injectable vector for anticancer drug delivery in brain tumor patients (Chandy et al., 2000). In a study by Chandy et al. chitosan coatings delivered 5-fluorouracil in a biphasic manner with an initial peak of drug release followed by a sustained release phase over 30 days (Chandy et al., 2000).

2.3.3 Synthetic polymers

A large group of synthetic polymers are used in medical implant applications including aliphatic poly(esters), poly(amides), poly(anhydrides), thermoplastic poly(urethanes), poly(ethylene), poly(tetra fluoro ethylene) and poly(dimethyl siloxanes) among others. Major benefits of synthetic polymers over natural polymers are the ability to modify and optimize polymer product according to requirement and to ensure a consistent product.

Aliphatic poly(esters) are degradable polymers with a more than 30 year history as suture materials, surgical clips, bone fixation devices, medical implant coatings, drug delivery systems and absorbable meshes and remain one of the most widely used synthetic medical polymers (McIntire, 2002). Polymers derived from lactide, glycolide and caprolactone predominate as medical aliphatic polyesters. All members are insoluble in water but degrade by hydrolytic attack of the ester bonds. Degrading times range from days to years depending on formulation and initial molecular weight (Nair and Laurencin, 2007). Properties of aliphatic poly(esters) are greatly expanded by co-polymerization of monomer units which is one reason they are so versatile.

Poly(amides) are characterized by monomers of amides linked by peptide bonds. Nylon is probably the most well-known synthetic polyamide used as a medical polymer. Poly(amides) have excellent fiber forming capability due to inter-chain hydrogen bonding and a high degree of crystallinity that increases strength in the axis of the fiber (Wong and Bronzino, 2007). Because of its fiber forming ability nylon is widely used as a medical textile, in manufacture of sutures and in creations of membranes for dialysis (Rigby et al., 1997).

Poly(anhydrides) comprise monomers joined by hydrolytically labile anhydride bonds that yield two carboxylic acids upon hydrolysis. The prototype poly(anhydride) for medical

applications is a copolymer of 1,3-bis(p-carboxyphenoxy) propane and sebacic acid (Griffith, 2000). Poly(anhydrides) are biodegradable polymers used primarily in the medical device and pharmaceutical industries and specifically as bio-erodible materials for drug delivery applications (Tamada and Langer, 1992). The anhydride bond of this class of polymers is extremely hydrolytically unstable and thus degradation of poly(anhydrides) is governed by exposure to water and occurs via surface erosion rather than bulk erosion (Griffith, 2000). Surface erosion allows greater control over degradation than bulk erosion and is a favorable feature. Experimental drug delivery systems employing poly(anhydrides) include aerosol particle delivery of etoposide with a view to treatment of small cell lung cancer and oral administration of insulin loaded microspheres in diabetic rodents (Furtado et al., 2008, Tang et al., 2010).

Another group of surface eroding polymers used for sustained drug delivery are poly(ortho esters). Important applications under development are treatment of post-surgical pain, osteoarthritis and ophthalmic diseases as well as delivery of proteins and DNA (Heller et al., 2002). Block co-polymers of poly(ortho ester) and poly(ethylene glycol) have been prepared to investigate biodegradable, injectable *in situ* forming drug delivery systems as an alternative to microspheres and implants as a parenteral drug depots (Packhaeuser et al., 2004). Such systems have a number of advantages including ease of administration, less fabrication complexity and less manufacturing stress on sensitive drug substances.

Poly(cyano acrylates) are solvent free, adhesives that cure rapidly when pressed into a thin film and have found a unique niche as medical glues. As a replacement for classical skin suturing, cyanoacrylate polymer glues provide good cosmetic result, reduced pain, efficient application and are preferred by patients (Pawar et al., 2008). Cyanoacrylates are also widely used by many surgical disciplines as tissue adhesives (Ronis et al., 1984). More recently cyanoacrylates have been employed as nanoparticle delivery systems for nerve growth factors with a view to treating nervous system degenerative diseases such as Alzheimer's Disease (Kurakhmaeva et al., 2009).

Poly(phosphazines) are inorganic, high-molecular-weight, essentially linear polymers with alternating phosphorus and nitrogen atoms in their skeleton. Each phosphorus atom has two functional side groups and these groups can be tailored through macro-molecular substitution to meet the needs of different medical devices making the group of polymers

highly versatile. Other benefits of poly(phosphazines) as medical polymers include potential for hydrolytic degradation and high throughput synthesis (Andrianov, 2009). Phosphazines are highlighted for their potential as potent immunological adjuvants that can dramatically enhance the magnitude, quality and duration of immune responses to a variety of bacterial and viral vaccine antigens (Eng et al., 2010).

Thermoplastic poly(urethanes) are established as medical polymers with widespread applications in medical devices such as joint and vascular prosthetics, catheters and drug delivery systems, pacemaker leads, arterial grafts, semi-occlusive dressings, mammary implants and intra-ocular lenses (Szycher et al., 1991). Poly(urethanes) have desirable bulk properties such as elasticity, tensile strength, durability, tear resistance and ease of fabrication in a variety of compositions which support their use as medical polymers (Sabbatini and Zambonin, 1994). Poly(urethanes) are made by condensation of "hard" diisocyanate and "soft" aliphatic poly(ether) or poly(ester) segments. It is widely accepted that the mechanical properties of segmented poly(urethanes) are due to phase segregation of hard and soft segments.

Poly(ethylene) is a polymerized ethylene resin used in the manufacture of a multitude of medical devices including sutures, catheters, medical membranes and disposable gloves. There are several different kinds of poly(ethylene) that are synthesized with different chain lengths and architecture. Low density poly(ethylene) has branched and linear architecture whereas high density poly(ethylene) is a linear polymer. Poly(ethylene) has fairly good grafting reactivity compared with other polymeric materials and easily forms a coating on medical devices (Sabbatini and Zambonin, 1994).

Poly(vinyl alcohol) is a water soluble polymer made by hydrolysis of a poly(vinyl ester). Depending on the degree of polymerization and hydrolysis of the precursor polymer, the physical properties of poly(vinyl alcohol) can be customized to functional requirements. Poly(vinyl alcohol) has been successfully used in medical device applications for more than 20 years (Alpharetta, 2009). Extensive biocompatibility and functional performance testing have demonstrated the suitability of poly(vinyl alcohol) in applications including gels and blended membranes, contact lenses, ocular wetting solutions, vascular embolic agents, hydrophilic coatings, tissue adhesion barriers, nerve guides, drug delivery and immune-isolation systems (DeMerlis and Schoneker, 2003).

Poly(ethylene oxide) is also known as poly(ethylene glycol) or PEG. Because it is highly biocompatible, PEG is used in a variety of medical devices *in vivo* including drug delivery matrices, wound dressings and soft tissue replacement. In water PEG forms a helical structure and is repulsive of charged molecules but in the cross-linked form its ability to absorb water makes it suitable as a sustained release vector for drug administration (Roedl and Schenk, 2006). Recently PEG shelled micelles have demonstrated rapid intracellular release of doxorubicin, a cancer chemotherapeutic agent (Sun et al., 2009). Amphiphilic networks of PEG have yielded optically transparent, mechanically robust films over a wide range of compositions useful for development of medical lens systems and biosensors (Hu et al., 2008). PEG has even been employed as a hydrogel coatings of urinary catheters to reduce catheter related infection (Roedl and Schenk, 2006).

Poly(hydroxyl ethyl methacrylate) is a polymer that forms a hydrogel on exposure to water. The hydroxyl groups in poly(hydroxyl ethyl methacrylate) take up water and convert the material to a clear, flexible elastomeric gel in which water acts just like a conventional plasticizer. Because of this property, the polymer was initially developed for implementation in soft contact lenses but since that time poly(hydroxyl ethyl methacrylate) and its copolymers have featured widely as materials in medical devices including testicular prostheses, membrane dialyzers, wound dressing, bone tissue substitutes, cell microencapsulation and vocal cord prostheses (Sabbatini and Zambonin, 1994). Poly(hydroxyl ethyl methacrylate) has been implemented as an sustained, antibiotic eluting, hydrogel matrix to prevent blinding infections within the globe of the eye after cataract surgery (Anderson et al., 2009). Fukano et al. demonstrated porous poly(hydroxyl ethyl methacrylate) is biocompatible when implanted into the dermis of mice and in fact allows ingrowth of keratinocytes, fibroblasts and basement membrane proteins consistent with bio-integration (Fukano et al., 2010). Other applications for poly(hydroxyl ethyl methacrylate) include polymeric nanoparticle release of chemotherapy agents, creation of drug coated glaucoma drainage devices and electro-conductive hydrogels as bio-recognition membranes for implantable biosensors, electro-stimulated drug release devices and low interfacial impedance layers on neuronal prosthesis (Anthony, 2010, Chouhan and Bajpai, 2009, Sahiner et al., 2009).

Poly(methyl methacrylate) is a hard, glassy, biocompatible thermoplastic resin of polymerized methyl methacrylate that is used especially in hard contact lenses, prostheses to replace bone, bone cements, dental fillings and maxillofacial/dental prosthetics (Sabbatini and Zambonin, 1994). It is supplied as a powder that when mixed with liquid yields dough-like cement that gradually hardens. A downside to this polymer for medical staff is that the monomer precursor is carcinogenic in the long term although the polymer itself is not a biohazard (Cloft et al., 1999).

Poly(tetra fluoro ethylene)(Section 2.8 Poly(tetra fluoro ethylene))

Poly(dimethyl siloxanes) are silicone polymers consisting of silicon atoms substituted with methyl groups and linked by oxygen atoms. They are physically and chemically stable silicone rubbers with unique flexibility attributed to one of the lowest set of glass transitions of any polymer group (Lötters et al., 1997). The polymers comprise biocompatible materials used as liquids, gels or solids. Poly(dimethyl siloxanes) are implemented as films for artificial membranes, gels for implants (especially in plastic surgery and orthopedic applications), liquids for drug delivery systems, antifoaming agents blood bags and pacemakers (Angelova and Hunkeler, 1999).

Polymer properties can be exploited to make medical devices that respond in a predictable way to their physiological environment. For example poly(ethylene oxide-b-propylene oxide) is a surfactant with amphiphilic and thermo-responsive properties used in temperature dependent delivery of protein and antiseptic skin treatments (Bikram and West, 2008, Angelova and Hunkeler, 1999). Other examples of this theme include poly(vinyl methyl ether) a non-toxic, temperature sensitive polymer with excellent shape memory properties and poly(N-alkylacylamide) a temperature sensitive gel whose lower critical solution temperature can be adjusted via co-monomer incorporation (Angelova and Hunkeler, 1999).

Surface modifications and polymer coatings will continue to play a major role in design and generation of future medical implants. Thin film coatings will not only provide a biocompatible interface but will become biologically proactive by interacting with cells and tissues in a beneficial way. More complex films will exhibit different microarchitectures, chemical patterns, porosities and respond to different inorganic and organic stimuli in a programmed way to meet specific device requirements (Kasemo and Gold, 1999).

2.4 Antimicrobial Medical Implant Coatings

Must research is being undertaken to discover surface treatments or coatings that prevent, reduce or eradicate biofilm from medical implants. The field is a diverse and highly patented area with great clinical and commercial potentials that are yet to be realized. It encompasses traditional approaches such as standard antibiotic therapies presented in surface film format but also introduces some truly novel ant-biofilm approaches with exciting future prospects for anti-biofilm coatings designed with medical implants in mind.

2.4.1 Coatings incorporating antimicrobial drugs and antiseptic moieties

Incorporation of antimicrobial drugs or antiseptic moieties into a device coating is an important technological innovation for the prevention of implant related infection (Francolini et al., 2003). Immersion is the simplest way to load antimicrobial agents onto medical device surfaces. Medical implants can be treated by immersion immediately prior to placement. Successful immersion results in chemical adsorption of the anti-infective agent to implant surface. Dispersion of anti-infective agent through the surface coating (for example a polymer matrix) is uncommon. Furthermore loading by immersion in aqueous antibiotic solutions is more effective for hydrophilic medical devices than hydrophobic devices as better absorption occurs. Immersion of hydrophobic implants in aqueous antibiotic results in weak bonding and early antimicrobial dispersion. Antibiotics such as rifampicin, ciprofloxacin, tobramycin, vancomycin and cephalosporins have all been used for immersion of coated medical implants but effects seldom last more than seven days (Gbureck et al., 2008, Gorman and Jones, 2002). Consequently immersion of medical implants in antimicrobial solutions may reduce early-onset device colonization but is ineffective in preventing biofilm formation long term (Gorman and Jones, 2002).

Because of the limitations of simple immersion techniques, augmented antibiotic coatings have been investigated in order to reduce dilution of antibiotic over time and impede biofilm formation. Surface treatment technologies incorporating anti-infective agents can be classified into four categories: deposited anti-infective thin films, ionically bonded anti-infective coatings, anti-infective agents entrapped within a polymer matrix and modified polymeric surface materials (Shintani, 2004, Tamilvanan et al., 2008).

Devices coated with layers of pure antibiotic such as dicloxacillin, clindamycin, fusidic acid, ciprofloxacin, cefuroxime and cefotaxime inhibit biofilm generation (Sherertz et al., 1989). Unfortunately coating medical implants with antibiotics or antiseptics is short lived due to rapid release, a similar problem as occurs with simple immersion. Polyglactin 910 absorbable suture material coated with triclosan is one example of a medical implant coated with an antiseptic. The suture has an absorption profile of around 63 days but the zone of bacterial inhibition due to triclosan is stated to last a minimum of only seven days (Ethicon, 2011). Barbolt found that polyglactin 910 suture coated with triclosan provided antimicrobial effect sufficient to prevent *in vitro* colonization by *S.aureus* and *S.epidermidis* (Barbolt, 2002). In some temporary or low risk implants, preventing bacterial colonization for less than a week may well be sufficient however moves have been made to stabilize pure antibiotic/antiseptic coatings and extend their duration of action. DeJong et al. stabilized chlorhexidine surface coating by mixing it with lipid and hydroxyapatite (DeJong et al., 2001). In this study, external fixator pins with and without a lipid stabilized hydroxyapatite/chlorhexidine coating were evaluated in a goat model. Pins were deliberately contaminated with *Staphylococcus aureus*. At 14 days, infection developed in 100% of uncoated pins, whereas in coated pins 4.2% became infected, 12.5% colonized and the remainder 83.3% has no bacterial growth ($p < 0.01$) (DeJong et al., 2001). These results demonstrated that lipid stabilized hydroxyapatite/chlorhexidine coating was successful in decreasing infection for up to two weeks, an improvement on simple antiseptic coating alone.

Techniques of binding antibiotic molecules on a medical device surface via ionic bonding have been developed based on the observation many antibiotic molecules possess either positive or negative charge. A surfactant with a positive or negative charge usually serves as an anchor for binding antibiotics with an opposite charge. For example, addition of bonding cationic surfactants such as tri-iododecylmethyl ammonium chloride (TDMAC) in combination with anionic antibiotics is a way of hindering drug dissipation away from bio-implants (Francolini et al., 2003). Negatively charged antibiotics on the surface of a coated implant then exchange with anions in body fluids and release slowly from device surface (Kamal et al., 1991, Darouiche et al., 1999). An *in vivo* rat based comparison study of poly(ethylene) central venous catheters treated by soaking in either penicillin or TDMAC solution or coated with TDMAC-bonded penicillin was performed by Trooskin et al. (Trooskin et al., 1985). Catheters were inoculated with *S.aureus* and harvested on day five

by which time 60% to 80% of catheters untreated or treated only by penicillin or TDMAC soaking were contaminated whereas all TDMAC-bonded penicillin catheters were free of contamination (Trooskin et al., 1985). Another advantage of ionic bonding of antibiotics using anchoring substances such as TDMAC and ethylenediamine tetra-acetate (EDTA) is that although coated devices are manufactured with bound antibiotic they also bind compatible antibiotics after device placement. Systemic administration of antibiotic or local irrigation of bio-implant therefore allows refreshing of antibiotic drug around medical implants (Darouiche et al., 1997, Johnson et al., 1999).

A strategy to combat durability problems of immersion and simple antibiotic coating is matrix loading or entrapping where antimicrobial agents are incorporated into the bulk phase of medical coatings (usually polymers) during synthesis. Polymer matrices can be formed either by *in situ* cross-linking of pre-polymers or non-reactive polymer coatings can be applied to a surface directly (Bach et al., 1993). Although there is no chemical bonding between matrix polymer and anti-infective agent using entrapping method, intermolecular dipole-dipole and Van der Walls inter-actions do occur. The down side of matrix loading is that it inevitably changes bulk polymer properties such as aqueous solubility, transparency or mechanical strength and these material changes must be weighed against antimicrobial benefit (Bach et al., 1993). Examples of coatings that make use of entrapment to resist infection include silver sulfadiazine and chlorhexidine loaded poly(urethane) matrix applied to central venous catheters. These coated catheters have been commercially available in the past and in both *in vitro* and *in vivo* studies the coatings reduce quantitative levels and frequency of bacterial catheter colonization (Bach et al., 1993). The technique has also been used successfully to reduce the incidence of infection within cerebrospinal fluid shunts and endotracheal tubes (Schierholz et al., 1997, Jones et al., 2002). In 1999 Lori and Antoine described an elegant form of surface coat matrix loading using antibiotic containing liposomal hydrogels (Lori and Antoine, 1999). These hydrogels adhered *in vivo* to bio implant surface using a poly(ethylene glycol)-gelatin matrix and inhibited bacterial device colonization and infection for up to seven days (Lori and Antoine, 1999).

Polymer surface coatings can be modified by physical means to become more infection resistant (Tamilvanan et al., 2008). One method is to acquire functional groups at polymer film surfaces using techniques such as gamma irradiation and glow discharge exposure.

Functional groups may convey intrinsic antimicrobial activity or covalently link other antimicrobial substances to a medical implant surface.

Covalent attachment can keep a drug or active moiety present at an implant surface for as long as the device is in place. A common approach is to use quaternary ammonium groups (widely known as disinfectants) as active moieties (Gottenbos et al., 2002, Mermel et al., 1993). Grapski and Cooper showed quaternary amino groups covalently joined to poly(urethane) created a non-leaching, bactericidal surface treatment that acted by disrupting bacterial cytoplasmic membranes (Grapski and Cooper, 2001). In a similar approach, Lewis and Klibanov attached the antimicrobial quaternary ammonium derivative N-alkylpyridinium bromide to a poly(4-vinyl-N-hexylpyridine) via an anchoring polymer onto an inert surface to produce a stable, non-leaching, biofilm killing interface (Lewis and Klibanov, 2005). Impressively Lewis and Klibanov's study showed their antimicrobial-polymer conduit was capable of inactivating 99% of *S.epidermidis, E.coli* and *P.aeruginosa in vitro* and emphasized the action of poly-cationic anchoring polymers in enabling the molecule to "stretch out" and maximize bactericidal activity (Lewis and Klibanov, 2005).

Standard antibiotic agents have also been covalently attached to polymer thin films in order to create antimicrobial coatings for implanted devices. Peng et al. synthesized a polymeric composite containing a 2-aminoimidazole derivative and found it did not leach, was not hemolytic and resisted colonization by *Acinetobacter baumannii* (Peng et al., 2011). This study suggested synthesized polymeric composites may play a role in treating resistant bacteria. Other attempts at this technique have proven less successful. One reason may be the covalent attachment occupies therapeutic sites on the drug rendering it ineffective. For example 6-amino-penicillin-acid based antibiotics bonded covalently to polysaccharides and N-[-2-hydroxypropyl)-methacrylamide copolymer failed as antimicrobial coatings because the amount of active drug was limited by covalent engagement (Ranade, 1990).

2.4.2 Coatings that deter bacterial adhesion

Bacterial adhesion to a surface or interface is the first step in the development of biofilm (Klemm et al., 2007). Surface treatments to reduce bacterial adhesion have therefore

been used to decrease device susceptibility to bacterial infection (Schierholz et al., 1999, Smith, 2005). Alterations in charge, hydrophobicity, polarity and free energy of surface films or addition of anti-adhesins are techniques used to reduce bacterial adhesion to medical implants (Shintani, 2004, Choong and Whitfield, 2000). In particular the effects of polymer coat surface charge and hydrophobicity on the development of biofilm have been investigated in an effort to develop better surface treatments for medical implants.

Charged surfaces, whether positive or negative resist bacterial adhesion. Poly-cationic surface anchored polymers and charged surfaces are anti-microbial in their own right. Gottenbos et al. surface modified silicone rubber with covalently coupled poly-cationic 3 (trimethoxysilyl)propyldimethyloctadecyl ammonium chloride and implanted the modified silicon-rubber into rats to determine the effect on implant infection (Gottenbos et al., 2002). Rats implanted with poly-cationic silicone rubber developed infections one seventh as often as rats implanted with non-modified silicone rubber indicating the independent antimicrobial effect of poly-cationic polymer coatings (Gottenbos et al., 2002). Negatively charged surfaces are also antibacterial. Liu et al. embedded polymers with electro-conductive materials such as carbon particles then imbued them with negative charge and found the charged surfaces inhibited *Staphylococci* (Liu et al., 1993). Presumably the success of negatively charged surfaces was due in part to repulsion between the negatively charged bacterial cell wall and the polymer surface leading to impaired bacterial adhesion.

Increased hydrophilicity of a surface coating decreases adhesion of various bacterial species *in vitro* (Baumgartner et al., 1997, Hogt et al., 1985). As such surface treatments to increase hydrophilicity have been explored. Hydrophilic surface coatings predominantly encompass hydrogels, a class of hydrophilic polymers that can absorb water up to several times their own weight without dissolving (Shintani, 2004). Hyaluronan, poly(vinyl pyrrolidone) and poly(ethylene glycol) are typical examples of hydrogel-polymers used in indwelling medical device surface modifications. In a study by Duran et al. hydrophilic polymer films formed by ultraviolet crosslinking of poly(vinyl pyrrolidone) derivatives showed reduction of uro-pathological bacterial adhesion *in vitro* on materials used to construct urinary catheters (Duran et al., 1995). Hydrophilic coatings constructed from hyaluronan or poly(vinyl pirrolidone) have also reduced adhesion of *S.epidermidis* to poly(urethane) catheters (Bridgett et al., 1993, Morra and Cassineli, 1999). Biomedical device related surfaces coated with the ‘‘superhydrophilic’’ 2-methacryloyloxyethyl

phosphorylcholine displayed highly reduced bacterial retention when compared to hydrophobic surfaces as shown in a study by Hirota et al. (Hirota et al., 2005). Significantly the results were consistent for bacteria associated with biomedical device related infection including *Staphylococcus aureus, Streptococcus mutans* and *Pseudomonas aeruginosa.* In a unique approach, hydrogels containing nitric oxide have been used in an attempt to synergise the abilities of both substances to prevent bacterial surface adhesion. Nablo found sol-gel surfaces capable of nitric oxide release decreased *Pseudomonas* adhesion by 30% to 95% relative to control sol-gel without nitric oxide (Nablo, 2003).

The other group of hydrophilic surface coatings that reduce bacterial adhesion to surfaces describes polymers that incorporate ionic functional groups in the repeating unit (Francolini et al., 2003). Ionic functional groups such as sulphonate, carboxilate and quaternary amino compounds have been added into polymer coatings to this end (Francolini et al., 2003). Biedlingmaier et al. found ionized, processed silicone middle ear ventilation tubes were resistant to *Pseudomonas* and *Staphylococcus* adhesion and biofilm contamination compared with untreated silicone or silver oxide-treated silicone tubes (Biedlingmaier et al., 1998). Ionized surface treatments have modest success against biofilm device contamination *in vivo* (Habash and Reid, 1999).

2.4.3 Metal based coatings

Metal ions have long been investigated for their anti-infective properties (Kim et al., 1998, Heidenau et al., 2005). Localizing metal ions to a device surface is a natural progression when investigating prevention of medical device infection. Subsequently medical implant coatings containing predominantly silver but also other metal ions such as platinum and copper have emerged.

Antimicrobial coatings for medical devices based on silver containing compounds have been extensively investigated (Darouiche et al., 1999). This approach makes sense because of the known broad spectrum bactericidal activity of silver containing clinical treatments such as silver sulfadiazine and Acticoat™ (Stickler, 2000). Silver ions are thought to inhibit bacterial replication by binding to DNA and interfering with vital bacterial metabolic enzymes (Petering, 1976). In more recent work silver based surfaces have been confirmed to kill bacteria and prevent biofilm formation by generating micro-electric fields and electrochemical redox processes (Chiang et al., 2009). Of silver containing coatings

tested for ability to reduce medical implant infections, the most basic is topical silver sulfadiazine cream which although operative has a short lived effect due to dilution and dispersion (Sheng et al., 2000). More sophisticated efforts to maintain silver in the immediate device environment involve more securely linking silver to a surface. A trend towards reduction in nosocomial urinary tract infections was seen with implementation of hydrogel/silver-coated urinary catheters in a study of intensive care unit patients (Bologna et al., 1999). Central venous catheters also have reduced infection when coated with silver derivatives. Samuel and Guggenbichler, showed activated silver nanoparticles imbued into polymer coats of poly(urethane) or silicone resulted in a substantial reduction in catheter incrustation (Samuel and Guggenbichler, 2004). Devices such as the Vita cuff™ have developed from such observations. The Vita cuff™ is a cuff made of silver-protein impregnated collagen intended to provide a barrier to infection along the entry tract of vascular access devices (Schierholz et al., 1998). Finally silver and platinum have been combined in a diamond-like carbon thin film to produce a coating with antimicrobial activity against *Staphylococci* (Narayan et al., 2005).

Copper is another metal used in the creation of novel medical implant coatings. Copper plasma immersion ion implantation has been employed to produce an antibacterial surface on poly(ethylene) effective in preventing *Escherichia coli* and *Staphylococcus aureus* infection (Zhang et al., 2006). Copper-Silver combination coatings also work well together to dissuade biofilm. McLean et al. found that when silver and copper were sputter coated onto several types of catheter as thin films they had significant antimicrobial effects against clinical isolates of *Staphylococcus epidermidis, Staphylococcus aureus* and *Pseudomonas aeruginosa* when compared to catheters lacking the metal thin film (McLean et al., 1993).

2.4.4 Electro-conductive materials and electric fields

Polymer thin films embedded with electro-conductive materials are a novel attempt at forming antimicrobial coatings. Liu et al. found electro-conducting polymers incorporating carbon particles could be connected to an electric current in order to generate electrostatic repulsion between polymer derived cathode and negatively charged bacterial cell wall (Liu et al., 1993). The group went on to suggest an application for the technology for repulsion of *S.aureus* and *S.epidermidis* from bio-implants. Even more complex electro-conductive approaches have been put forward. Poly(pyrrole) is a chemical compound formed from a

number of connected pyrrole ring structures where pyrrole is a heterocyclic, aromatic organic compound. Poly(pyrrole) can store and release drugs upon electrical stimulation. In an *in vitro* study by Sirinrath et al. the antibiotics penicillin and streptomycin were electro-deposited in poly(pyrrole) on titanium (Sirinrath et al., 2011). The research group found 80% of antibiotic stored in poly(pyrrole) was released on demand by applying sweep voltages to the compound (Sirinrath et al., 2011). Electric fields may also augment the effects of antibiotic coatings. Lori and Antoine found antibiotic resistant bacteria became susceptible to previously ineffective concentrations of antibiotics when exposed to low intensity electrical fields (Lori and Antoine, 1999).

2.4.5 Ultrasound activated coatings

Ultrasound activated antimicrobial coatings have been investigated in an attempt to clinically control release or leaching of antimicrobial agents from surfaces of medical implants. Control of antimicrobial delivery offers the promise of reduced antimicrobial resistance, longer lasting antimicrobial effect and targeted antimicrobial action. Kwok et al. developed a ultrasound triggered release system consisting of a drug-containing polymeric monolith coated with an ultrasound-responsive layer based on ordered methylene chains (Kwok et al., 2001). This coating formed an ultrasound-activated switch that confined drug inside the polymer carrier until ultrasound exposure enabled drug release. A novel drug delivery polymer matrix consisting of a poly(2-hydroxy ethyl methacrylate) hydrogel coated with ordered methylene chains that form an ultrasound-responsive coating has also been studied (Norris et al., 2005). This system retained the antibiotic ciprofloxacin inside polymer in the absence of ultrasound but released the drug when low-intensity ultrasound was applied. *In vitro* experimentation showed the successful reduction of *Pseudomonas aeruginosa* biofilms grown on hydrogels using the system in combination with daily ultrasound stimulation (Norris et al., 2005). In a simpler approach, low frequency ultrasound has been shown to markedly enhance the effects of antibiotics such as aminoglycosides against biofilms and in concert with antibiotic coatings for medical implants may improve their antimicrobial effect (Lori and Antoine, 1999).

2.4.6 Enzymes and enzyme activated composite polymer coatings

When any polymer material is inserted in a host, gradual erosion of its surface by host enzymes naturally occurs and this degradation is accentuated by increasing concentrations of enzymes present during an inflammatory response (Woo et al., 1999). Inflammatory response is prominent during times of surgical insertion of a medical implant and during episodes of infection. One concept in development of anti-infective coatings for bio-implants was to create an antibiotic-polymer coating that reacted to inflammatory enzymes by releasing its antibiotic payload. Woo et al. presented work on the feasibility of incorporating antibiotics into the backbone of polymer films as main-chain monomer building blocks (Woo et al., 2002). Other monomers sensitive to enzyme-catalyzed breakdown can also be built into the main polymer backbone and act to release antibiotic when exposed to degrading enzyme. Specifically Woo et al. synthesized polymers of degradable poly(urethanes) combined with fluoroquinolone antibiotics and demonstrated bacterial killing when the antibiotic was released following degradation of the polymer chains by the macrophage-derived enzyme cholesterol esterase (Woo et al., 2002). Taking a slightly different tack, enzymes inhibiting bacterial cell wall synthesis have been employed to manufacture anti-biofilm coatings for silicone urinary catheters. *N*-acetyl-D-glucosamine-1-phosphate acetyltransferase (GlmU) is vitally involved in synthesis of peptidoglycans and lipopolysaccharides in gram-positive and gram-negative bacteria respectively and plays a role in generation of adhesins requisite for biofilm formation in *Escherichia coli* and *Staphylococcus epidermidis* (Burton et al., 2006). Burton et al. developed an antibiofilm coating containing a GlmU inhibitor and found it to be effective against urinary catheter associated bacterial pathogens (Burton et al., 2006). Harnessing enzyme activity to develop antimicrobial coatings holds promise for development of anti-biofilm surface treatments.

2.4.7 Bio-mimetics and bio-mimicry

Another direction in combating device-related infection is to provide coatings that mimic anti-biofilm characteristics or actions of host tissues or naturally occurring systems. The natural world offers the benefits of evolved solutions in action. Anti-biofouling effects of marine organisms in particular provide many instances of natural biofilm resistant coatings. Francolini et al. considered this when they loaded usnic acid into modified poly(urethane) coatings and assessed the effect on biofilm formation by either *S.aureus* or

P.aeruginosa (Francolini et al., 2004). Usnic acid is produced by lichens, naturally occurring surface communities of symbiotic fungus and algae that make antibiotics to protect themselves from colonization by bacteria. Indeed the hybrid surface treatment created by Francolini's team resulted in biofilm inhibition and they postulated the effect was due to interference with biofilm signaling pathways (Francolini et al., 2004). Chitosan is a fiber-like product made from ground shrimp shells that is known to have antibiofilm properties (Carlson et al., 2008). In an experiment to produce novel antibiofilm coatings, Huh et al. grafted chitosan to poly(ethylene terephthalate) and achieved bacterial biofilm inhibition (Huh et al., 2001). Bulwan et al. reiterated the antibiofilm effects of chitosan coatings when they used ionic derivatives of the natural material to form thin films on silicon and glass (Bulwan et al., 2011). The group found chitosan nano-coatings to be resistant to *in vitro* adsorption of proteins including human plasma components and to rebuff the development of *S.aureus* biofilm (Bulwan et al., 2011). Another sea derived material used to create novel antibiofilm coatings is fish muscle α-tropomyosin (derived from fish muscle extract). Preconditioning of inert materials with fish muscle α-tropomyosin, significantly discouraged biofilm formation by uro-pathogenic bacteria in a study by Vejborg and Klemm (Vejborg and Klemm, 2008). In a slightly different approach, Gorman and Jones used high-molecular-weight poly(silanes) to crosslink silicones and form a synthetic exuding surface that was continuously shed and replenished (Gorman and Jones, 2002). The silicones imitated a defense common to mucosal surfaces where surface cells are constantly shed and renewed. In theory, attached bacteria are sloughed along with the shedding surface and infection is avoided. Antibodies and chemicals such as nitric oxide have also been attached to surface coatings in an attempt to reduce biofilm contamination (Hetrick and Schoenfisch, 2006). These studies all borrow from the notion of evolved anti-biofouling mechanisms in nature.

2.4.8 Anticoagulant containing coatings

As part of the endogenous host response, implanted medical devices undergo surface conditioning with proteins such as fibronectin, laminin, fibrin, albumin and components of the coagulation cascade. These proteins interface with bacteria and affect microbial adhesion to and growth on implant. *S.aureus*, for example has a specific fibronectin binding protein that augments adhesion of the bacterium to surfaces (Jeng et al., 2003). Surfaces that reduce surface conditioning may therefore dissuade biofilm. Because many bacteria interact with activated platelets, antiplatelet surface coatings were developed in

an attempt to deter biofilms but with limited success (Francolini et al., 2003). The anticoagulant heparin has been employed as part of an implant surface treatment with modest success in preventing bacteremia and *in vivo* biofilm (Habash and Reid, 1999). Central venous catheter-related bacteraemia occurred less frequently with heparin-coated catheters than with non-coated catheters in a study by Jain et al. (Jain et al., 2009). Tenke et al. showed poly(urethane) ureteric stents coated in heparin prevented biofilm development compared with uncoated stents *in vivo* and the inhibitory effect persisted for six to eight weeks after insertion (Tenke et al., 2004). Similarly Cauda et al. compared deposition of biofilm on double "J" poly(urethane) ureteric stents with and without covalently bonded heparin coating and found heparin coated stents developed less uniform and less compact encrustation than uncoated stents (Cauda et al., 2008). Engineered surfaces containing anticoagulants that limit host conditioning films or produce secondary films that do not support microbial adhesion and/or growth may therefore be effective in preventing or dissuading biofilm (Habash and Reid, 1999).

2.4.9 Phage containing coatings

A bacteriophage or "phage" is a virus capable of infecting and injuring or lysing a bacterial cell. Bacteriophages can be selected for their affinity to invade specific biofilm forming bacteria. Studies suggest a clear potential for phage-impregnated or coated material for the prevention of biofilm infection. PhagoBioDerm™ is a non-toxic, biodegradable polymer film impregnated with lytic bacteriophages against *P.aeruginosa*, *E.coli*, *S.aureus*, *Streptococcus* and *Proteus*. This technology has been applied as a dressing to successfully treat patients with ulcers and infected burns unresponsive to conventional therapies (Kutter and Sulakvelidze, 2005). A further example of the potential of phage coatings was described by Curtin and Donlan who showed *S.epidermidis* biofilm formation on the surface of phage impregnated, hydrogel-coated, silicone catheters was significantly reduced compared with non-phage impregnated catheters (Curtin and Donlan, 2006). In this *in vitro* study not only did bacteriophage reduce the number of viable cells recovered from catheter surfaces but the number of cells adhering to the catheter surface as determined by scanning electron microscopy was also diminished (Curtin and Donlan, 2006). In a similar *in vitro* approach Fu et al. studied the effect of pretreating hydrogel-coated catheters with a *P.aeruginosa* bacteriophage cocktail (containing five different phages) on biofilm formation by *P.aeruginosa* (Fu et al., 2010). Pretreatment with

bacteriophage significantly reduced the 48 hour mean biofilm cell density by 99.9%, a highly significant result (Fu et al., 2010). The prevention of *in vitro* biofilm formation on Foley catheters following impregnation of hydrogel-coated catheter sections with a lytic bacteriophage has also been investigated. A 90% reduction in both *P.mirabilis* and *E.coli* biofilm formation on bacteriophage-treated catheters was demonstrated when compared with untreated controls (Carson et al., 2010). Although phage based antibiofilm surface treatments have promise, safety data for use of bacteriophages in humans is lacking which may hinder development of the technology.

2.5 Luminous Chemical Vapor Deposition and Plasma Polymers

Synonyms for Luminous Chemical Vapor Deposition (LCVD):
plasma polymerization
glow discharge polymerization
plasma enhanced LCVD
plasma enhanced chemical vapor deposition
luminous vapor chemical deposition
plasma chemical vapor deposition

2.5.1 Traditional polymer films

Traditionally polymers are defined as high molecular weight materials composed from chains of orderly, repeating chemical subunits called monomers via a process called polymerization (Williams, 1999). Polymers are also known as macromolecules or giant molecules and may be organic, inorganic, synthetic or natural. Natural polymers include substances such as proteins (polymers of amino acids) and cellulose (polymers of sugar molecules). There are many examples of synthetic polymers for example poly(ethylene) (a polymer of ethene)(Figure 2.1), poly(vinyl chloride)(a polymer of vinyl acetate) and poly(tetra fluoro ethylene)(a polymer of molecules containing fluorine and carbon). Organic polymers consist mainly of hydrogen and carbon but may contain other elements such as nitrogen, oxygen, halogen, phosphorus, silicon and sulfur.

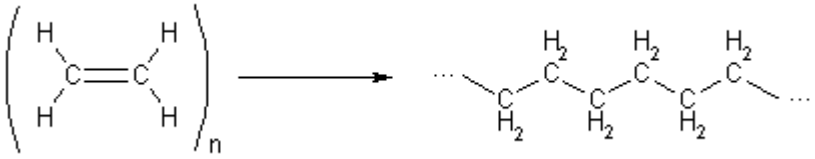


Figure 2.1: Polymerization of ethene to poly(ethylene) through the standard technique of radical chain polymerization. Note the orderly repetitive molecular structure of resultant polymer.

Traditional polymers are employed to manufacture a wide variety of solids and surface coatings in the medical implant industry encompassing fields such as tissue engineering, regenerative medicine and medical devices (Jagur-Grodzinski, 2006)(Section 2.3 Role of Polymers and Polymer Coatings in Medical Implants). About three millions tons of

synthetic polymer are consumed each year in the manufacture of medical devices and usage is increasing at a rate of 10% per year (Rapra Technology Ltd., 2001). There is large scale demand and utilization of polymer coatings for medical implants because polymers meet specific physical, chemical, biological, bio-mechanical and degradation properties required to provide effective therapy whilst still being affordable (Nair and Laurencin, 2007). Use of polymeric materials in medical devices is associated with cost savings in both commodity and high performance implants (Rapra Technology Ltd., 2001). Polymeric materials are cheap enough to make disposable, single use items commercially viable and these one-use items in turn reduce cost of transmissible infection and diminish the need for cleaning and re-packaging (Day, 2004).

The same polymeric materials are used over and over again in the manufacture of medical devices because they work well. From a volume perspective the polymers most often used in bio-medical applications are poly(vinyl chloride), (poly ethylene), poly(styrene) and poly(propylene) but other commonly employed polymers include silicone, poly carbonate, poly(urethane), poly(tetra fluoro ethylene) and acrylics (Rapra Technology Ltd., 2001). It is not surprising to see the polymers in highest usage are also the least expensive of the medical polymers (Rapra Technology Ltd., 2001).

Polymers in the form of thin films acting as medical coatings are increasingly being developed as tailored interface materials for bio-implants. Because optimal bulk properties of existing biomaterials are rarely aligned with optimal surface/interface properties, improvement of medical implants is reliant upon the ability to optimize the material's surface without adversely affecting its bulk properties (Marchant and Yu, 1989). This objective is often achieved through polymer surface treatments. As well as improving interface characteristics of medical devices, advanced medical polymers improve functionality of bio-implants via several routes: “smart” polymers are able to respond to changes in the biological environment associated with infection, inflammation and disease; surface engineering of novel bioactive and bio-inert polymers is generating application specific coatings and incorporation of drug delivery systems is providing pharmacological control over wound healing and tissue regeneration (Rapra Technology Ltd., 2001).

Conventional polymer film and thin film (<100 microns thick) production techniques include solvent casting, painting, thermal spray processing, spin coating, surface adsorption of

monolayers, floating technique, evaporation, sputtering, molecular beam epitaxy, Sol-Gel process and pulsed laser deposition (Krebs, 2007). These techniques are often expensive, time dependent and utilize or create toxic products.

2.5.2 Plasma polymer thin films

2.5.2.1 Introduction

The medical device industry is always looking for ways to improve bio-implants in order to optimize service and care to patients in a resource efficient manner. One way to achieve this goal is to develop better medical polymers and in particular to develop better medical polymer surface treatments.

Plasma polymers are a new class of polymer films (they cannot be formed as bulk materials) with unique properties that make them excellent medical implant coating candidates. In order to understand the benefits plasma polymers offer as medical implant surface treatments, it is helpful to understand how plasma polymers are created and to highlight their benefits when compared to traditional medical polymeric materials.

There are four states of matter: solid, liquid, gas and plasma. Plasma polymers take their name from the “plasma” state of matter and are not related to the blood component called plasma. The plasma state of matter is an ionized gas consisting of a chemical soup of positive and negative ions, electrons, neutrals, atoms, molecules, clusters, free radicals, ultraviolet radiation and other electrically excited molecules but overall is neutrally charged (May, 1998). Plasma is generated when an electrical discharge passes through a gas and is accompanied by dissociation of molecular bonds (Holme, 2007, Sturrock, 1994). In nature, plasmas are present in stars, lightning and the Northern Lights and man has implemented plasma in nuclear reactors and fluorescent lighting.

In the laboratory, plasmas are easily generated by striking a high voltage (normally >1 kV) electrical discharge through a low pressure gas system using a direct current or more usually a high frequency alternating current operating in microwave or radiofrequency ranges (May, 1998). The electrical discharge (electron) originates from photoionization of neutral species by cosmic rays or background radiation or by field emission secondary to electric fields around a sharp point at the electrode surface (May, 1998). The electron is

accelerated by the applied electric field causing it to collide with gaseous molecules leading to a cascade of ionizing collisions within gas. Subsequently plasma is created.

2.5.2.2 Plasma polymer thin film creation: key concepts

How is plasma harnessed to create plasma polymers? Plasma polymerization is not akin to any of the processes that develop traditional polymers. Plasma polymerization occurs in a unique series of steps. After plasma has formed, energy derived from the plasma is transferred to gaseous monomer causing activation and fragmentation of monomer moieties. Chain-producing atoms, such as carbon or sulfur must be present in the initial working gas to serve as a backbone for polymerization. Gaseous monomer interaction with plasma then causes molecular cross-linking, polymer branching, cracking or formation of aromatic rings and creation of double and triple bonds (Yasuda, 2005). Reaction mechanisms proposed for the formation of plasma polymers include monomer fragmentation followed by poly-recombination into randomly structured and cross-linked films; fragmentation accompanied by formation of film-forming intermediates; plasma-initiation of a radical chain-growth polymerization; ion-molecule reactions and ionic chain-growth polymerization although multiple mechanisms are probably at play (Friedrich, 2011). The stimulated radicals and monomer fragments combine, recombine and finally polymerize via a process of statistical recombination to form highly stable films on freely selected solid surfaces (Figure 2.2.).

During the process of plasma polymerization reactive free radical species are trapped within the unpredictable, growing polymer lattice or created within the film as a result of film exposure to plasma derived high energy ultraviolet radiation (Krüger et al., 2006). These radical sites are initiation points of auto-oxidation when the layers are exposed to ambient air, a phenomenon referred to as aging (Gengenbach and Griesser, 1999). Free radicals trapped in polymer films continue to react and change the polymer network over time eventually leading to degradation (Gaur and Vergason, 2000).

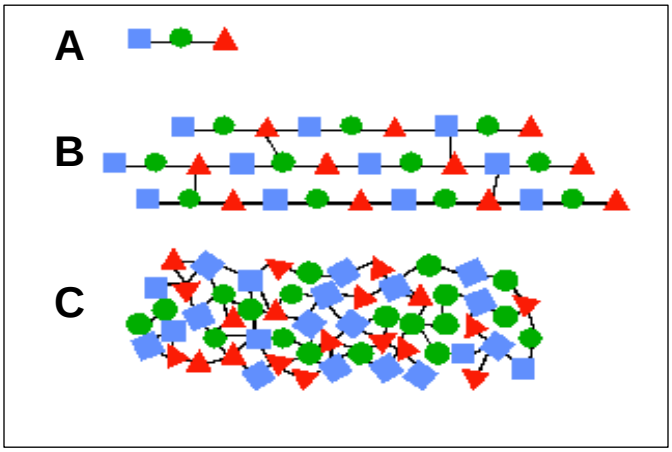


Figure 2.2: Comparison of monomer, conventional polymer and plasma polymer structures: (A) monomer, (B) conventional polymer and (C) plasma polymer. Note how plasma polymers are put together via a process of statistical recombination as opposed to their orderly traditional counterparts (Krebs, 2007).

Using plasma polymerization, a new spectrum of monomers can be polymerized and a new spectrum of previously recalcitrant surfaces can be polymer coated. Energy of plasma polymerization produces polymer films from organic compounds that do not form with traditional chemical polymerization techniques and allows deposition of films onto surfaces that would not usually bind polymer film (Gaur and Vergason, 2000). Although bulk structure of plasma polymers is completely irregular, retention of functional groups tends to be greater than retention of intact monomer structure in the resultant plasma polymer (Friedrich, 2011). This means that although most of a plasma polymer film consists of irregular, stable, highly cross-linked fragments of monomer, the material as a whole maintains active functional groups and therefore active functional potential.

2.5.3 Luminous chemical vapor deposition

2.5.3.1 Description of plasma polymer thin film production in the laboratory

In 1874 the scientific and engineering communities became aware of plasma polymers as contaminant by-product of benzene processing (Easton, 2005). Interest in plasma polymers was rekindled in the 1950s and by the 1960s plasma polymers were employed technically as diaphragms in nuclear batteries (Easton, 2005). Plasma polymerization was used to generate inorganic films (such as silicon oxide or nitride films), organic plasma polymer films, amorphous to polycrystalline films (such as diamond or silicon films) and has been employed in the design of oriented carbon nanotubes (Chen et al., 2000).

Currently, plasma polymer films are applied diversely in the microelectronic, automobile, aeronautic, food packaging and biomaterial industries using methods that were formulated from laboratory based plasma polymerization techniques.

In the laboratory plasma polymerization occurs within a closed system via a process called Luminous Chemical Vapor Deposition (LCVD). Monomer building blocks must be able to form a gas or vapor within a receptacle in order for LCVD to proceed. To create plasma within a reactor (vessel) containing an organic gas, an increasing voltage is applied across the gas between electrodes. An abrupt increase in current indicates breakdown of gases. High-energy electrons collide with gaseous hydrocarbon molecules to produce plasma. The space between cathode and anode containing electrons, ions and radicals, is the electrically neutral plasma or “glow discharge” (Yasuda, 2005). Positive ions are accelerated towards cathode and produce secondary electrons in the process. Excited atoms then emit photons and create a visible glow (Plate 2.1). Power input for plasma polymerization thus serves two functions: it creates plasma and it fragments gaseous monomer. Plasma itself is a direct consequence of ionization of gases within the reactor and fragmentation of organic gas monomer leading to plasma polymerization occurs as a secondary outcome (Gaur and Vergason, 2000).

Plate 2.1: Example of visible glow produced by photons emitted from plasma generated with a glass laboratory tube reactor

Plasma polymer deposition systems are differentiated by geometry of the reactor and frequency of the excitation voltage (radiofrequency/direct current/pulsed)(Easton, 2005). Plasma polymerization is usually performed within the confines of one of three types of reactor:

1. Internal electrode reactor such as the bell jar type reactor with internal, parallel plate metal electrodes
2. External electrode reactors such as glass or silica tubular type reactors fashioned with external coil or ring electrodes

3. Electrode free or high frequency microwave reactors which employ a silica tube that passes through a resonance cavity

The external reactor system is popular as it is easy to implement and overcomes problems of having internal electrodes such as polymer forming on metal electrodes altering discharge characteristics and sputtering of impurities (Figure 2.3) (Easton, 2005). Most plasma polymer is deposited onto a substrate surface made to contact the glow discharge. Plasma polymerization will therefore occur onto a surface that is placed (under favourable conditions) within the glow of a reactor. Not all glow discharges however yield polymer deposition. The plasmas of pure argon and neon gases for example are non-polymer forming and can therefore be utilized to maintain glow within reactor whilst monomer vapor is selectively instilled into the system as a building material for plasma polymerization (Gaur and Vergason, 2000). Under plasma conditions, the instilled monomer molecules fragment and deposit as surface related polymer thin film, and a non-polymer forming by-product like hydrogen gas is evolved (Gaur and Vergason, 2000). Using low pressure, low temperature plasma and weakly ionized gases, the surface of any substrate that can withstand a vacuum and fit within a reactor can be modified or coated (Sakthi and Krishna Pillai, 1999).

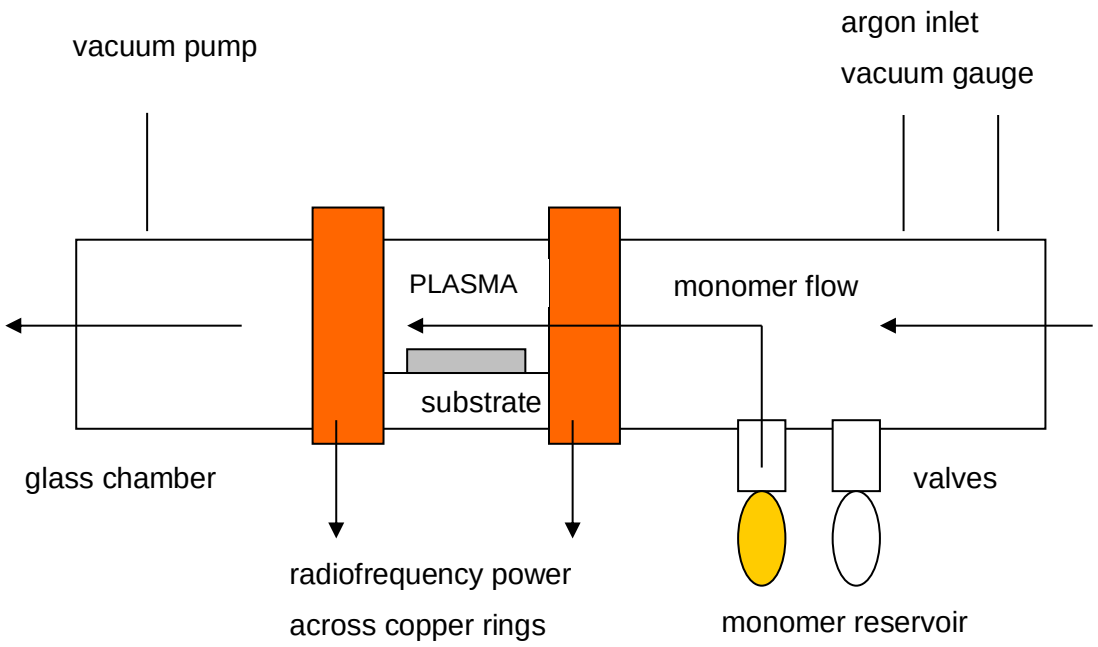


Figure 2.3: Diagram of external electrode type reactor for deposition of plasma polymer on substrate mounted within the reactor chamber and between electrodes (within glow discharge)

The electric field (or potential difference) used to form plasma within a laboratory reactor can be generated using direct-current or alternating-current. Radiofrequency or microwave voltage for the discharge excitation can be applied. A variation to the alternating current discharge is the pulsed glow discharge, which consists of a sequence of short glow discharges (with lengths typically in the microsecond range). The advantage of pulsed glow discharge is that high peak electrical powers can be reached for a low average power, resulting in high peak efficiencies (Bogaerts et al., 2002). Magnetic fields have also been employed to assist plasma polymerization (Biederman and Slavinska, 2000).

2.5.3.2 LCVD and plasma polymer product control

Laboratory based plasma polymerization takes place in a low pressure and low temperature plasma produced by a glow discharge through an organic gas and within the confines of a reactor system. Plasma polymerization is dependent on production parameters including geometry of the reactor system, system pressure, discharge power and frequency of excitation signal as well as the reactivity of the starting monomer, monomer flow rate, substrate temperature, position of substrate within the glow discharge and deposition time (Gaur and Vergason, 2000, Krebs, 2007). It also depends on intensity and energy of the species bombarding the growing film (Biederman, 2004). Any or all of these parameters can be adjusted in order to alter the resulting surface chemistries or physical nature of plasma polymer product (Krebs, 2007). In general, the quality of plasma polymer films can be controlled, precisely and in a reproducible manner by controlling production parameters (Tran, 2004).

Plasma power, monomer flow rate and precursor molecular weight are particularly important parameters in creating films intended to inherit or maintain functionality from parent monomer. By selecting the monomer type and energy density per monomer (known as the Yasuda parameter) the structure and chemical composition of resultant plasma polymer thin film can be greatly varied (Krebs, 2007). Yasuda pointed out that wattage per molar mass of monomer and monomer flow rate (W/MF where W= plasma power; F=monomer flow rate; M=precursor molecular weight) determine the character of continuous-wave plasma polymerization (Yasuda, 2005). Low W/MF values are related to partially undamaged monomer molecules and high W/MF parameters to a nearly complete monomer fragmentation within resultant plasma polymer thin film (Krüger et al., 2006). As

such the number of intact functional moieties derived from monomer building blocks is also proportional to W/MF, with low W/MF systems producing plasma polymer thin films of more intact monomer components and high W/MF systems resulting in plasma polymer thin films constructed of highly fragmented monomer pieces with loss of a greater number of functional moieties.

Energy flux into the growing plasma polymer film is decisive for film structure and final properties. If the energy flux is low, a plasma polymer is created that retains more of the molecular structure of parent monomer and resembles a more conventional, traditional polymer (Biederman and Slavinska, 2000) (Figure 2.4). Increasing energy flux usually causes the film to become harder because it is more disordered, cross-linked and dense. When the energy of bombarding particles is increased even further, the resulting polymer develops unusual properties including high electrical resistivity, optical transparency in the infrared region, hardness higher than sapphire and chemical inertness (Biederman and Slavinska, 2000).

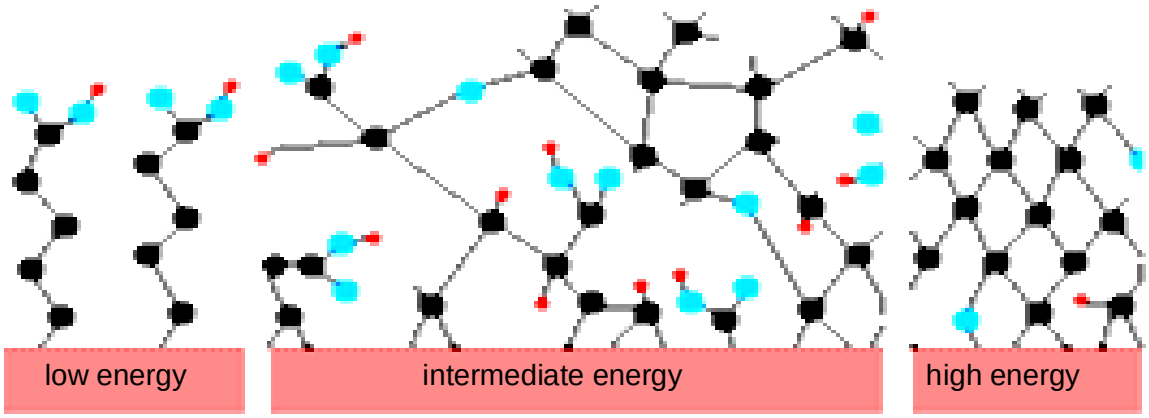


Figure 2.4: Structure of plasma polymers related to energy of production. Low energy flux polymers look more like conventional polymers whereas high energy flux polymers are highly cross-linked and dense.

Pulsed plasma (in the order of thousands of cycles per second) is an added mechanism to control the plasma polymerization process. Plasma pulses cause less monomer fragmentation and allow for chemical chain propagation (which occurs in the absence of plasma) forming more regular polymer structures than the structures seen after continuous-wave plasma polymerization (Krüger et al., 2006). As a consequence pulsed plasma polymers have a more predictable chemical stoichiometry and physical structure.

In addition pulsed plasma polymers do not trap many free radicals within their substance and thus the effects of aging on polymers are diminished (Krüger et al., 2006). Pulsed plasma also results in the highest retention of monomer structure in the deposited polymer film (Krüger et al., 2006).

Magnetically enhanced audio frequency discharge has advantages for producing plasma polymers. This technique employs a shaped, intense magnetic field to entrap electrons, causing them to move in spirals instead of straight lines. Dynamic spiraling of electrons leads to more frequent collision between electrons and gas particles. In turn enhanced fragmentation, higher polymer deposition rates and dense polymer deposits are produced at lower system pressures (Gaur and Vergason, 2000).

There are other practical approaches that may enhance control of plasma polymer film chemistry. Suggestions include use of a Faraday cage around substrate, positioning of deposition substrate downstream from plasma glow discharge, use of monomers with the potential to form double and triple bonds and use of cold substrate (Biederman and Slavinska, 2000).

2.5.3.3 Benefits of plasma polymers and LCVD for medical implants

Plasma polymers differ significantly from traditional polymers. Although plasma polymers tend to be grouped with traditional polymers the only common ground may be monomer substrate. Plasma polymers challenge the very definition of “polymer” as they are not comprised of orderly repeating subunits (as are traditional polymers) but are more a disordered hybrid fusion of irregularly fragmented monomer. Plasma polymers are typically made of short chains that are randomly branched, highly cross linked and highly insulating (Sakthi et al., 2003). Traditional polymers can be made as a bulk material whereas plasma polymers cannot be made as solids independent of a surface and are therefore by definition surface treatments only.

Plasma polymers have unique properties which cannot be obtained by conventional deposition techniques. These include hardness, scratch, abrasion and erosion resistance, long-term chemical, thermal and environmental stability and gas and vapor impermeability making them excellent barrier materials (Ratner, 2007). High energy flux plasma polymers are transparent and have distinctive dielectric properties making them useful as optical

films and coatings (Biederman and Slavinska, 2000). Plasma polymer films can be ultra-thin (minimum 20 nm) and adhere well to nearly all substrates (silicon, steel, glass, other polymers) (Yasuda, 2005). Plasma polymerization is expanding the variety of monomer precursors that can be used to make polymer films because most organic substances can be converted into plasma polymer films so long as they form a gas. In classic polymer chemistry only monomers with reactive functional groups or active double bonds polymerize. In plasma chemistry inactive and saturated monomers are also able to undergo polymerization (Johnson, 1992).

Plasma polymers and the process of LCVD have many specific advantages when considering the production of surface coatings for medical implants. By providing an interface between the bulk phase of a medical implant and its environment, surface coating allows materials within the bulk phase of a medical implant to be chosen for properties independent of surface dependent biocompatibility and anti-infection criteria and therefore allows development of more versatile and effective bio-implants made from a wider range of bulk materials. Biocompatibility and anti-infective features can be imbued secondarily to the medical implant by the plasma polymer surface coating. This is the concept of the two-phase medical implant (Geckeler et al., 1997). Plasma polymers tenaciously bind and conform to almost any surface (glass, other polymers, metal) and easily form over complex geometric objects. Once bound, plasma polymer thin films are highly cross-linked, stable and chemically inert, pin-hole free and impervious giving them good barrier properties and making them biocompatible as a class (Biederman, 2004). Coatings are non-toxic and the production process itself creates a sterile deposition environment (Pan et al., 2000).

Manufacturing benefits of LCVD have been delineated after widespread use of the process by microelectronics industries. The process of plasma polymerization is dissimilar to adsorption of monolayers, floating technique, evaporation, sputtering, spin-coating, painting or any other traditional polymerization technique. The dry processing technique employed to make plasma polymers is clean and efficient, coating times are rapid (often seconds), treatments can be performed on a continuous basis at room temperature, product has good surface uniformity and is relatively easy to procure (Biederman, 2004). Monomer costs are generally negligible and the whole undertaking is environmentally friendly as no solvents, lubricants, accelerators or other toxic chemicals or byproducts are

involved (Tran, 2004, Johnson, 1992). Despite all of these clear advantages, LVCD is yet to be embraced by the commercial medical implant sector suggesting a potential area of future development.

2.6 Plasma Polymer Medical Surface Treatments

Research into plasma polymer surface films in the field of biomaterials is extensive because of the perceived advantages of an imperturbable surface, nano-scale molecular sieve and new surface state of material (Yasuda, 2005) (Section 2.5 Luminous Chemical Vapor Deposition and Plasma Polymers). Using plasma polymer thin film technology, surface properties and biocompatibility can be enhanced selectively while bulk attributes of underlying materials remain unchanged. Research into plasma polymer thin films incorporate development of blood compatible surfaces, non-fouling surfaces, tissue engineering and culture, sterilization of bio-implants, development of biosensors and barrier coatings (Favia and D'Agostino, 1998). Often times the use of plasma polymerization in non-medical industries such as automobile, electronic, and chemical industries have suggested medical applications. Despite the great potential for this technique to develop novel biocompatible surface treatments for medical implants (as inferred by active research), the technology is yet to penetrate the commercial medical implant market on any significant commercial level.

Surface treatment to confer improved biocompatibility to medical implants is highly desirable. Using luminous vapor chemical deposition based plasma polymerization various substances have been investigated to this end. Diamond-like carbon coatings are dense, amorphous, hydrocarbon polymer coatings produced by plasma polymerization of hydrocarbon gas. These thin films have a refractive index and hardness similar to diamond and are smooth, uniformly adherent, abrasion resistant, impervious and thermo-resistant up to 400^{o}C (Kumar et al., 1997). *In vitro* biocompatibility of diamond-like carbon coatings is suggested by mouse fibroblast and macrophage cultures that show no evidence of cellular damage, loss of cellular integrity or significant inflammatory response on exposure to the new material (Thomson et al., 1991). Diamond-like carbon films are already employed to produce biomedical implants such as surgical instruments, implanted infusion pumps, joint prosthetics and cardiac valves (Chu et al., 2002). Non-organic substrates such as glass and silicon have been surface treated with N-vinyl-2-pyrrolidone derived plasma polymer thin films via glow discharge in order to manufacture biocompatible surfaces. Marchant found these films were non-cytotoxic and did not produce significant inflammation (Marchant, 1990). Plasma polymer coatings for metallic implants have also been suggested. Methane, tetra fluoro ethylene and tetra methyl

disiloxane have been used as plasma polymer coatings for orthopedic wire systems in order to decrease the effects of corrosion (Biederman, 2004). Similarly, plasma polymerized hexamethyl disiloxane thin film deposited onto titanium was stable after immersion in phosphate buffer saline solution and encouraged early fibronectin absorption (Hayakawa et al., 2004). Hexamethyl disiloxane coated titanium therefore has potential application as a dental implant material. Finally thin wires have been coated with a flexible fluorocarbon material using plasma enhanced chemical vapor deposition again to prevent alteration of the bulk material *in vivo* (Limb et al., 2009).

A prominent area of interest concerning plasma enhanced chemical vapor deposition is the development of infection resistant coatings for medical implants. Low-pressure plasma processes have been investigated for deposition of poly(ethylene) oxide-like thin films. Poly(ethylene) oxide reduces adsorption of proteins and in turn reduces bacterial adhesion to implanted medical devices (Fabio et al., 2001). Poly(ethylene) oxide-like thin films have thus been suggested as anti-infection coatings. In fact plasma polymerized ethylene films have improved the surface properties of acrylic teeth such *that in vivo* plaque accumulation was much reduced (Kim et al., 2008). Approaching the problem of infection prevention, cotton textiles treated with radio frequency plasma enhanced chemical vapor deposition of titanium oxide films have shown a substantial bactericidal activity on exposure to ultraviolet radiation (Szymanowski et al., 2005). Such plasma polymer treated fabrics may have applications in sterile drapes and theatre clothing.

There are other examples of glow discharge plasma polymer thin films suggested for implementation in bio-implants. For example plasma enhanced chemical vapor deposition is used for fabrication of transparent dielectric optical films to build optical filters, antireflective coatings and optical waveguides that have potential in medical lens systems or biosensors (Martinu and Poitras, 2000). It is clear that the technology holds promise as a means to develop biocompatible and anti-infective surface treatments.

2.7 Existing Data on Essential Oil Based Plasma Polymers

TTO and all essential oils easily form organic gases and so are obvious candidates for plasma polymerization. 100% TTO has not previously been polymerized via luminous chemical vapor deposition or via conventional polymerization techniques however a small number of plasma polymers have been created from other essential oils. Plasma polymer films based on lemongrass oil were created using radiofrequency plasma polymerization by Kumar and Pillai in 1999 (Sakthi and Krishna Pillai, 1999). Reasons stipulated for using lemongrass oil included natural abundance and perceived therapeutic qualities although targeted applications were not given. Conductive and optical properties of eucalyptus oil plasma polymer films have been investigated for use in electronic components (Sakthi et al., 2003). In 2005 a James Cook University thesis described optical and electrical properties of lavender oil plasma polymers with a view to fabricating dielectric thin films (Easton, 2005). Lavender polymer film properties included low dielectric constants and uniform, pinhole free surfaces (Easton, 2005). Cashew shell oil has been polymerized using conventional methods but not via luminous vapor chemical deposition (Chelikani and Dong Shik, 2006). Cashew shells are a byproduct of commercial cashew nut production and are seen as a renewable resource for polymer production.

Rosin is the resinous constituent of oleo-resin exuded by some species of pine and is a naturally occurring film forming polymer. Rosin polymers have excellent biocompatibility and tailored degradability (*in vitro* and *in vivo*) and thus show potential as biomaterials (Satturwar et al., 2003) The pine derived polymer has been pharmaceutically evaluated as a microencapsulating material and as anhydrous binding agents in tablets (Satturwar et al., 2005). Rosin compound is a mixture of diterpene acids, known as resin acids, with a smaller amount of other acidic and neutral bodies.

2.8 Poly(tetra fluoro ethylene)

Poly(tetra fluoro ethylene) (PTFE) is a synthetic polymer of fluorinated ethylene made by free radical polymerization of tetra fluoro ethylene. PTFE has a carbon backbone chain where each carbon atom has two fluorine atoms attached to it.

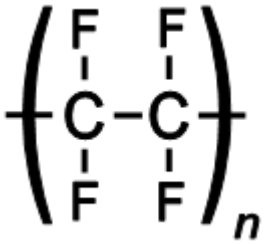


Figure 2.5: Chemical structure of PTFE

PTFE has constant and predicable chemical, thermal and mechanical properties. PTFE is chemically inert and is insoluble in all known solvents. PTFE polymer chains pack well, are crystalline and resist chemical attack due to the packed configuration of fluorine atoms around carbon. The polymer has low and high temperature capability (stable between minus 200^{o}C and 300^{o}C) and is easily autoclaved. It's co-efficient of friction is extremely low (0.1) and the material self-lubricates. PTFE has excellent ultraviolet resistance, is strong (tensile strength of 10-43 MPa) and moderately hard (Rockwell Hardness (R) 58).

Standard PTFE was discovered accidentally in 1938 by Roy J. Plunkett during an attempt to develop gaseous Freon-based coolants (Graffte, 2005). The material was commercialized in 1947 by DuPont as Teflon® (DuPont, 2011, Foundation, 2011). Goretex® is an expanded (stretched and porous) version of PTFE produced by Gore (Gore, 2011). PTFE (both standard and expanded) has a long history of biocompatible and safe *in vivo* applications as part of the medical device material armory dating back to the 1960s (Graffte, 2005). This workhorse of medical implant materials is extensively used to manufacture implanted devices to this day.

PTFE is the best known fluorocarbon polymer used in medical device construction. Flouro-polymers such as PTFE are successful as medical implant materials because of two vital properties: lubricity and biocompatibility (Graffte, 2005). Lubricity in particular has established PTFE as the predominant material used in the construction of medical tubing

as well as other medical devices including synthetic blood vessels and vascular graft and patches, catheters, sutures, surgical clips, prosthetics used in reconstructive surgery, hernia mesh, surfacing for artificial joints, medical guides and stents.

PTFE was an ideal TTO derived plasma polymer deposition substrate for several reasons. PTFE is a well characterized and familiar biomaterial employed in medical implants such as vascular grafts and coronary artery stents dating back 50 years. Because its behavior as an implant is well understood and its role as a successful medical implant material is established, PTFE was also a good implant control material. Beneficial fundamental properties of PTFE included thermal stability, physical robustness and inert biochemical behavior. Standardized PTFE sheets of various thicknesses were freely available, inexpensive, easy to handle and have some flexibility. Implant flexibility reduced trauma and inflammation at the hosted implant sites and in turn reduced confounding factors in biocompatibility analysis. PTFE was straight forward to clean, did not dissolve in alcohol (used in sterilization) or water (physiological solution) and was processed for histological examination using standard equipment. In addition, PTFE had been successfully employed as a deposition surface for essential oil based plasma polymers at the School of Engineering James Cook University, so working with the substrate was familiar.

2.9 Biocompatibility

2.9.1 Definition of biocompatibility

The ideal biomaterial material should not elicit any systemic, immunologic, cytotoxic, mutagenic, carcinogenic or teratogenic reactions when introduced *in vivo* (Ratner, 1975). Rapid resolution or absence of the acute phase inflammatory response and normal wound healing is desired (Johnson, 1992). The ideal biomaterial performs as intended, does not cause harm to the host and integrates seamlessly into host systems or augments host systems. Behavior of autologous grafts or tissues *in vivo* is the gold standard against which biocompatibility of other materials is measured.

“Biocompatibility” is a vague term that implies acceptance of a material on some level within a biological host. The ultimate biocompatible material is autologous host tissue. All other materials will elicit some response that diverges from perfectly compatible self. Biocompatibility thus exists along a continuum. Biocompatibility is also situation specific: it cannot be defined unless the host context is stated. Material acceptance varies according to factors such as type of host, location within host and health of host. Because biocompatibility is reliant on context, only a high level definition is appropriate when speaking in general terms. For the same reason it is not possible to design a single test for biocompatibility. Scientific basis of biocompatibility therefore involves the identification of the causal relationships between material and host tissue such that material can be designed to elicit the most appropriate response (Williams, 1999).

Even at a generic level there is no uniform definition of biocompatibility. The Dorland’s Medical Dictionary says biocompatibility is the quality of not having toxic or injurious effects on a biological system and McCulley simply states biocompatibility is being harmonious with life (McCulley, 2003, Dzul et al., 2000). Williams defines biocompatibility as the ability of a material to perform with an appropriate host response in a specific application (Williams, 1999). A more stringent definition is put forward by the American Society for Testing of Materials who state biocompatibility is the comparison of tissue response produced through close association of implanted candidate material at its implant site within the host animal to that tissue response recognized and established as suitable with control materials (ASTM, 2011). Oshida described biocompatibility of long

term implanted medical devices as the ability of the device to perform intended function with desired degree of incorporation in the host without eliciting undesirable local or systemic host effects (Oshida, 2007). The more complex definitions of ASTM and Oshida are directly applicable to medical implants and emphasize comparison with current alternatives or controls and the importance of device function as well as lack of toxicity and inertness.

Traditional biomaterials are chemically inert and thus passively tolerated through lack of interaction with host systems however materials that stimulate prosthetic incorporation into host tissue offer considerable advantages when compared to inert materials. In addition to the definitions mentioned above, biocompatibility aims at positive promotion of biological processes which further the intended goal of biomaterial application (Kirkpatrick et al., 1998). Active and beneficial participation in host-device interactions and integration with host systems is the ultimate goal of synthetic biocompatible substances.

2.9.2 Generic host response to foreign body

Foreign bodies stimulate a generic cascade of local tissue events leading to encapsulation of foreign body and healing of surrounding tissue. The body reacts similarly to materials identified as biocompatible and walls them off in an avascular, tough, collagenous bag, roughly 50–200 nm thick (Castner and Ratner, 2002). Castner and Ratner made the interesting observation that ten common but very different biomaterials including gold, titanium, alumina, poly(urethane), silicone rubber, poly(tetra fluoro ethylene), poly(ethylene), poly(methyl methacrylate), poly(2-hydroxy ethyl methacrylate) and poly(ethylene terephthalate) after one month implantation into mammals all heal in an identical manner despite the fact they represent materials that are hydrophilic, hydrophobic, hard, soft, polymeric, ceramic and metallic (Castner and Ratner, 2002). What is common about the implanted materials is adsorption of a complex, non-specific layer of host derived proteins. One hypothesis is that the initial host derived protein coating triggers encapsulation of foreign materials and this requisite host interaction emphasizes the relevance of *in vivo* testing of bio-implants (Castner and Ratner, 2002).

Uncontrolled biological encapsulation directly confounds the performance of implanted devices. The presence of extensive capsule seriously degrades implant performance by preventing direct contact between device and host tissue. Inflammation associated with

foreign body response may also inhibit the luminal healing of vascular grafts, trigger capsular opacification of intraocular lenses, lead to the extrusion of percutaneous devices, exacerbate device calcification, induce contact lens discomfort and generally lead to complications that impede optimal device function (Castner and Ratner, 2002).

Biocompatibility is decided by two main factors: the response of host to implant and response of implant to the host (Roach et al., 2005). Host proteins react with outermost atomic layers of the implant and thus the host-implant interface determines biological integration (Roach et al., 2005). Although surface-protein interactions are poorly understood, surface chemistry plays a fundamental role in protein adsorption. Surface properties suggested to confer biocompatibility are low surface energy, negative charge , low surface roughness and low coefficient of friction (Roach et al., 2007). These factors are influenced widely by fundamental material properties, device design, processing and sterilization history, surgical technique on insertion, site of insertion and duration of implantation (Castner and Ratner, 2002). Host health, immune system and individual variations in physiology and anatomy also impact biocompatibility (Castner and Ratner, 2002, Ratner et al., 1996).

2.9.3 Standards for biocompatibility testing

Standards organizations provide guidelines for biocompatibility testing however national and international standards regimes should represent a lowest common denominator and do not ensure optimal biocompatibility results. As standards propagation takes time, all standards bodies lag behind available technologies. Despite this, the global aim is to attain horizontal and harmonious minimal standards for biocompatibility testing of implanted medical devices to allow reproducibility, audit and comparison (Langeland and Langeland, 1965). Although guidelines for biocompatibility testing are available, none are universally adopted.

The most commonly implemented biocompatibility testing standards are provided by the International Organization for Standardization ISO-10993: Biological Evaluation of Medical Devices (Services, 2011). These guidelines aid in selection of tests designed to assess materials that directly or indirectly contact living tissue. ISO standards draw from directives and regulations sanctioned by groups such as the Organization for Economic Cooperation and Development, the American Society for Testing and Materials, the United

States Environmental Protection Agency, the United States and European Pharmacopeias, the American National Standards Institute and the United States Food and Drug Administration. EN 30993 aligns with ISO-10993 and is the standard for biocompatibility testing in Europe. The Therapeutic Goods Administration in Australia has been a contributing member of ISO for more than twenty years and supports ISO standards in biocompatibility testing.

ISO 10993 offers broad guidance in many areas of implant testing. The standards incorporate animal welfare requirements, tests for geno-toxicity, cytotoxicity, carcinogenicity and reproductive toxicity. There are specific tests for interaction of implant with local tissue and with blood as well as for effects of systemic toxicity. ISO 10993 covers clinical investigation of medical implants, material degradation associated with polymers and describes the effects of sterilization techniques on implant materials. Finally the standard characterizes materials using physical and chemical, morphological and topographical parameters and provides methods for immune-toxicology testing of medical devices. ISO 10993 is a good beginning point when planning biocompatibility testing although the standards need to be tailored and adopted depending on the material or implant being tested and the proposed application.

2.9.4 Risk analysis

Prior to formal biocompatibility testing, materials must be free from any know unacceptable risks. Guidelines for performing risk analysis are provided by EN 1441 (Schmalz, 1998). Risk analysis takes into consideration all available data for the device including chemical data (e.g. solubility, corrosion), physical data (e.g. hardness, shrinkage) and historical information about effect of the material on living tissues. Possible threats associated with intended uses of the device or material are identified and assessed. Through careful analysis, some candidate bio-implant materials can be determined unsuitable for implantation at this stage, sparing much time and expense.

2.9.5 Testing biocompatibility

Once aims and context are defined, relevant biocompatibility testing can get underway. In principle the idea is to ensure *in vitro* thresholds are reached prior to implementation of more invasive and costly *in vivo* experiments. Initially the focus is on biosafety: excluding severe deleterious effects on the organism through cytotoxicity, mutagenesis and

carcinogenesis testing. The focus then shifts to bio-functionality and addresses the materials ability to perform an application task with an appropriate host response. In 1970 Autian described a tri-leveled approach to biocompatibility testing consisting initially of nonspecific toxicity testing via cell cultures or small laboratory animals followed by specific toxicity testing using subhuman primates and finishing with clinical testing in humans (Autian, 1970). In a proposal that was adopted by the ISO Technical Report 7405 in 1984, Langeland suggested a sequence of initial tests (cytotoxicity, systemic toxicity and mutagenicity) leading into secondary tests (sensitization, implantation testing and mucosal irritation) and finally usage tests (Langeland and Langeland, 1965). The concept of a graduated approach to biocompatibility testing is still implemented today through ISO-10993.

2.9.6.1 *In vitro* testing

It is generally stated that *in vitro* tests are preferred prior to conducting *in vivo* trials because they are simple, quick to perform, reproducible, inexpensive and avoid harming animals. *In vitro* tests can be used to reject unsatisfactory materials before performing *in vivo* implantation studies if results are known to correlate well with *in vivo* responses. ISO 10993-5 recommends initial *in vitro* cytotoxicity testing as part of biosafety determinations. *In vitro* cytotoxicity tests determine if a device induces sub-lethal or lethal effects at the cellular level. Cell damage can be measured by morphological means, cell growth and metabolism indexes, mitochondrial function, DNA synthesis or membrane integrity testing. *In vitro* mutagenesis and carcinogenesis testing centers on the Ames test which detects mutations in metabolic functioning of bacteria however gene mutations can also be detected in cultured mammalian cells. Bio-functionality testing usually involves transformed permanent cell lines or context specific cell cultures for example endothelial cells for vascular devices or chondrocytes for orthopedic applications. These cell lines are examined for cell adhesion, cell spreading, secretory function, phagocytosis, release of toxins or other context significant biosynthetic functioning. Hybrid organs to simulate *in vivo* conditions have also been developed as biocompatibility models. One example is artificial vascular wall constructed from type 1 collagen gel colonized by smooth muscle cells, placed on a poly(urethane) scaffold and lined with canine endothelial cells to form tunica intima (Kirkpatrick et al., 1998). Despite all of this, the availability of accepted and validated *in vitro* assays is highly limited. *In vivo* testing proceeds if no satisfactory *in vitro* model exists or if initial *in vitro* screening deems a material biocompatible.

2.9.6.2 *In vivo* testing

In vivo testing amounts to implantation of a material or device into a host body cavity or organ depending on intended application. Common sites of implantation include subcutaneous, intramuscular, intra-peritoneal, intra-osseous, within blood circulation or onto cornea/intra ocular. *In vitro* biocompatibility tests are simplified analogies of complex mechanisms and unfortunately do not always correlate with animal implantation (Rosengren et al., 2005). *In vitro* models often contain a single cell type and do not illicit inflammation, immune response, tissue remodeling, inter-cellular reactions, extra cellular matrix interaction, blood interaction or interaction with proteins or hormones. *In vitro* models cannot manifest acute systemic toxicity or pyrogenicity. *In vitro* testing is thus at best a screening test for biocompatibility. Substantial resources have been made available for validation of *in vitro* assays as replacements for animal tests, but it may take years before validated methods can be implemented and any goal of replacing all *in vivo* studies with *in vitro* assays will probably never be met (Fries, 2001). Correct animal models are a better approximation of human response to implant however human implantation is the gold standard in biocompatibility testing (Schiffelers et al., 2005).

The concept that *in vitro* testing of polymers does not accurately reflect *in vivo* results was first described by Wilsnack in 1976 (Black, 1999, Wilsnack, 1976). Wilsnack used ATCC human fibroblast cell line WI-38 to examine *in vitro* cytotoxicity of a large number of polymeric materials and compared *in vitro* results to results obtained after implantation of polymers in rabbits (Wilsnack, 1976). *In vitro* tests were found to be more sensitive than *in vivo* tests and to correlate poorly in general with *in vivo* results (Wilsnack, 1976). Difficulty in extrapolating results was attributed to inherent inadequacies in cell culture that mean cultured cell lines behave differently to native tissue cells, single cell line versus multiple cell types, differences in form of polymer (solid materials versus implanted saline eluted material) and lack of exposure of *in vitro* samples to specific immune system responses (Black, 1999). Another example of the lack of congruency between *in vitro* and *in vivo* polymer testing was demonstrated in a study by Chirila and Zainuddin (Chirila and Zainuddin, 2007). In their study, synthetic polymers with attached carboxyl and oxy-phosphorus groups were postulated to enhance calcification capacity but testing results were contradictory: *in vitro* studies demonstrated an increase in calcification capacity induced by these groups whilst *in vivo* studies demonstrated a decrease of calcification

capacity. It must be appreciated that the complex physiological environment is difficult to model and therefore difficult to predict making *in vivo* testing mandatory prior to formal implementation of medical implants in humans.

Sensitization tests (for allergic contact dermatitis) detect allergy related cutaneous redness and swelling induced by device exposure and are required by ISO 10993-1 standard for all device categories. There are no reliable *in vitro* tests for sensitization. Traditionally the preferred animal model for sensitization testing was the albino guinea pig although more recently the Mouse Ear Swelling Test has been recommended (Gad et al., 1986). Sensitization tests have defined stages: an induction phase, when the potential allergen is presented to the animal, followed by a rest period and a subsequent challenge phase to determine whether or not sensitization has occurred. ISO 10993-1 also requires acute systemic toxicity be assessed for all medical devices requiring blood contact. Acute systemic toxicity occurs when adverse effects are generated rapidly after administration of a single dose of a substance (Anderson, 2001). Acute systemic toxicity is commonly tested by administering extracts of medical devices intravenously or via the intra-peritoneal route in rabbits or mice. ISO 10993-10 covers skin irritation induced by exposure to a medical device and is generally tested by exposing albino rabbits to device extract in polar and non-polar solvent or simple exposure to device itself (Wallin and Upman, 1998).

The purpose of haemocompatibility testing is to detect undesirable changes in blood caused by a medical device or by chemicals leaching from a device. Undesirable effects of device materials on blood include hemolysis, thrombus formation, alterations in coagulation parameters and immunological changes (King and Fries, 2003). Haemocompatibility is an excellent example of the value of *in vivo* biocompatibility testing. Static blood-device interactions are so different to interactions obtained in physiological circulation that a separate ISO standard (10993-4) employing a parallel plate flow chamber system was developed to address inconsistencies.

Material and device implantation can be either surgical or nonsurgical: the surgical method involves dissection of a pouch into host tissues to house implant, while the nonsurgical method uses a cannula and stylet to insert a cylinder-shaped implant into host. After a time delay the implanted sample is harvested and subjected to macroscopic and

microscopic examination to evaluate local tissue reaction as a measure of biocompatibility (Webster, 2007). ISO 10993-6 deals specifically with local tissue response to implant: implants can be subcutaneous, intramuscular, intra-osseous or within the peritoneal or other body cavies. ISO 10993-6 standard recommends four test samples are inserted into the para-spinal muscles on one side of rabbit host and four negative control samples are inserted into the contralateral side of the rabbit. Local responses are assessed histologically at one, four and 12 weeks. A minimum of three animals are recommended for each testing interval and use of a negative control is mandated (Wallin and Upman, 1998).

ISO 10993-11 addresses effects of chemicals that migrate to remote organs and tissues causing systemic toxicity. The rabbit pyrogen test uses fluid extract prepared from test material and administers it intravenously whilst measuring rabbit rectal temp. Over hours a rise in rectal temperature indicates pyrogens and is considered an indication of poor biocompatibility. Test and control groups are also examined and compared looking for signs of systemic illness such as prostration or convulsions. Suggested parameters for systemic toxicity testing include terminal blood routine hematology and chemistry, necropsy results and final body weight (Wallin and Upman, 1998). It is recommended that device testing should use exaggerated doses of test substance to ensure a margin of safety.

More long term *in vivo* exposure and observation is required to determine sub-chronic toxicity and carcinogenicity. Sub-chronic toxicity encompasses adverse effects that occur after repeated daily dosing of a substance to experimental animals over a portion of their life span (Shibamoto and Bjeldanes, 2009). Testing gathers information on target organ effects, toxin accumulation and aids in developing safe dosing levels for human exposure. In sub-chronic or chronic toxicity studies, one or two animal species are dosed daily (with dose escalation), usually for a period of three to six months and monitored for adverse sequelae (Boyd, 1968). The objective of long term carcinogenicity studies is to observe test animals over a major portion of their life span to detect any development of neoplastic lesions after exposure to a test substance (King and Fries, 2003). For medical device extracts, one dose level of the highest practically applicable volume is usually sufficient. At the completion of the dosing period, surviving animals are sacrificed and examined for the

presence of tumors. An increased incidence of tumors in dosed animals indicates the product tested has potential to induce tumors and may be carcinogenic in humans.

2.9.6 Host response to implant: wound healing and foreign body reaction

2.9.6.1 Wound healing in man

Abnormal wound healing around a medical implant can be appreciated and assessed only if the timing and nature of normal wound healing are understood. The healing process in human wounds incorporates a series of complex and coordinated events that begin at injury and can continue for months to years (Baum and Arpey, 2005). Wound healing is divided into three phases: inflammatory phase, proliferative phase and remodeling phase (Mercandetti and Cohen, 2008). Inflammation, epithelialization, formation of granulation tissue, neovascularization, wound contraction and extra cellular matrix reorganization take place in an overlapping fashion during wound healing (Singer and Clark, 1999). Culmination of biological processes results in tissue reconstitution or replacement of normal structures with fibroblastic scar tissue (Figure 2.6).

2.9.6.2 Inflammatory phase of wound healing

The inflammatory phase is the first phase of wound healing, commences immediately post insult and lasts up to six days (Park and Barbul, 2004). Hemostasis is achieved through vasoconstriction, platelet aggregation, platelet activation and activation of the coagulation cascade. Simultaneously the innate immune response to injury is triggered and leads to acute inflammation (Park and Barbul, 2004). Vasodilatation then facilitates inflow of polymorphonuclear cells and tissue fluid to the injured site. Neutrophils predominate over the first three days although macrophages, helper T cells and other immune cells are present in smaller numbers (Amsden, 2006). Neutrophils phagocytose bacteria and debris from damaged tissue and induce bacterial lysis. After three days macrophage numbers at the site of injury increase and replace neutrophils as the dominant immune cell (Amsden, 2006). Macrophages phagocytose non-viable tissue. Granulocytes release chemotactic factors that attract cells involved in the proliferative phase of wound healing and cause them to divide (Park and Barbul, 2004). Inflammation is therefore necessary to remove debris, combat infection and stimulate the proliferative phase of wound healing. As inflammation dies down, fewer inflammatory mediators are secreted, existing ones are

disintegrated and polymorphonuclear and macrophage numbers are reduced at the wound site. These changes indicate the inflammatory phase is ending and the proliferative phase of wound healing is underway (Amsden, 2006).

2.9.6.3 Proliferative phase of wound healing

The proliferative phase of wound healing lasts two days to three weeks (Park and Barbul, 2004). Granulation tissue is produced to fill the defect produced by injury. Granulation tissue consists of new blood vessels, fibroblasts, inflammatory cells, endothelial cells, myo-fibroblasts and immature extra cellular matrix (Biology-online.org, 2011). Proliferative phase commences with the appearance of fibroblasts in the wound (Singer and Clark, 1999). One week post injury fibroblasts are the main cell within the wound and fibroplasia ends two to four weeks after wounding (Amsden, 2006). Fibroblasts deposit ground substance and collagen into the wound bed and thereby increase wound strength. Simultaneous neovascularization occurs as endothelial cells migrate into the wound (Singer and Clark, 1999). Angiogenesis is requisite to wound healing as new blood vessels channel nutrients and remove waste from growing tissue beds. Granulation tissue also underpins re-epithelialization as epithelial cells migrate across new granulation tissue. Epithelial surfacing provides a seal to protect the wound from the environment. About a week after wounding myo-fibroblasts initiate wound contraction (Amsden, 2006). Wound contraction peaks at five to 15 days after injury and continues after re-epithelialization is complete (Amsden, 2006). Wound contraction reduces the surface area of the wound by up to 88% (Berry et al., 1998).

2.9.6.4 Remodeling phase of wound healing

Three weeks to two years after injury, remodeling occurs in an ongoing fashion (Amsden, 2006). Once collagen levels stabilize remodeling or maturation has begun and may continue for years (Baum and Arpey, 2005). Type I collagen is deposited along tension lines with subsequent increases in wound tensile strength (Ehrlich and Krummel, 1996). Ultimately wound strength will reach 80% that of native tissue (Amsden, 2006).

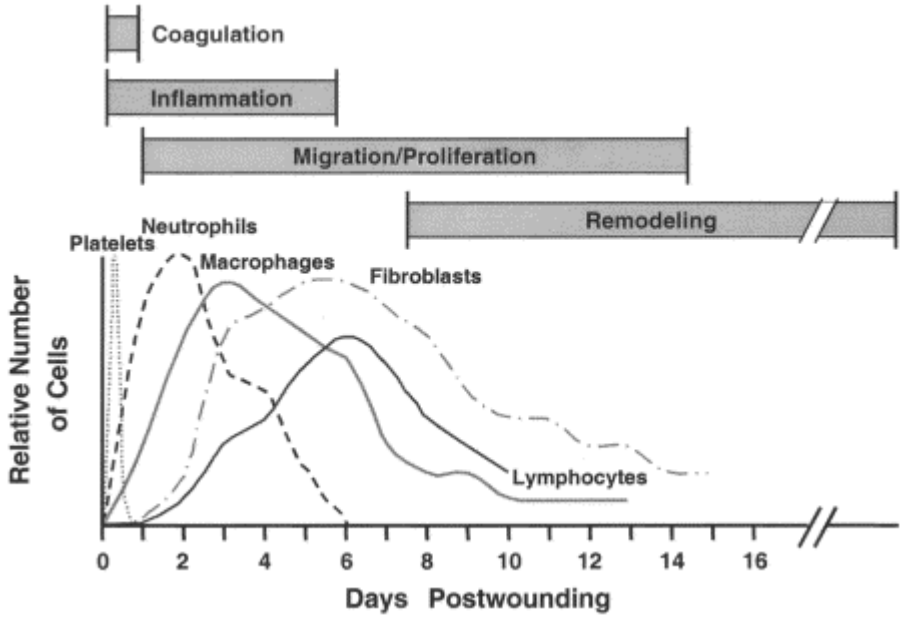


Figure 2.6: Chronology of normal wound healing in adult human skin (Amsden, 2006)

2.9.6.5 Wound healing in the murine model

Chronology and three phase biology of wound healing is similar in all mammals despite anatomical variations (Sullivan et al., 2001, McBrearty et al., 1998). Investigations centered specifically on wound healing in mice illustrate similar inflammatory cellular infiltrates and angiogenic responses to humans (Kameha et al., 2002, Ilse et al., 2000, Drew et al., 2001). Granulation tissue formation assessed by monomorphic analysis of cutaneous wounds in mice peaks on day six as with humans (Gerharz et al., 2007, Park and Barbul, 2004). Four millimeter open punch biopsy wounds in mice are re-epithelialized at 14 days as with human four millimeter punch biopsies left to heal by secondary intention (Mori et al., 2004, Agren et al., 2001). Mouse wound healing thus has correlation with wound healing in humans and is applicable as an early model.

2.9.6.6 Foreign body reaction

Materials alien to host tissues (e.g. foreign bodies, medical implants) may persist within the wound, impair wound resolution and thereby stimulate foreign body reaction (FBR). Definition of FBR is: a granulomatous inflammatory reaction evoked by the presence of exogenous material in the tissues, characterized by foreign body giant cells (Dzul et al., 2000). FBR is the standard reaction of a higher organism to an implanted synthetic material (Castner and Ratner, 2002). FBR associated with medical implants can lead to chronic pain, device rejection and device failure (Hiley and Barber, 2000). Examples of detrimental outcomes due to foreign body reaction include poor wound healing around vascular prostheses, failure of implant electrodes, posterior capsular opacification of intraocular lenses, extrusion of percutaneous devices and implanted biosensor failure (Ratner, 2007).

Three histological criteria must be met to confirm FBR:

1. presence of foreign body giant cells (the hallmark of FBR)
2. encapsulation of foreign material and
3. chronic inflammation (Pernick, 2005).

Foreign body giant cells are multinucleate histiocytes made of fused epithelioid macrophages that display a typical haphazard nuclear arrangement (Anderson et al., 1999)(Plate 2.2). Objects too large for phagocytosis are surrounded by foreign body giant cells and exposed to secreted degrading agents such as super-oxides and free radicals (Plate 2.3). These destructive agents damage the foreign body and may harm nearby healthy tissue. Encapsulation refers to host deposition of a firm, collagenous, avascular barrier around foreign bodies (Vistnes et al., 1978). Capsule formation isolates foreign material from harmful interactions with host tissues but may also impede desired host interactions with therapeutic implants. Chronic inflammation is an inflammatory response of prolonged duration (usually greater than three months) whose protraction is provoked by persistence of the causative stimulus (Hiley and Barber, 2000). Chronic inflammation inevitably causes tissue damage. Cells characteristic of chronic inflammation are macrophages, lymphocytes and plasma cells. The histological definition of chronic inflammation also stipulates the presence of tissue necrosis coexisting with attempts at wound healing and repair (Kumar et al., 2004).

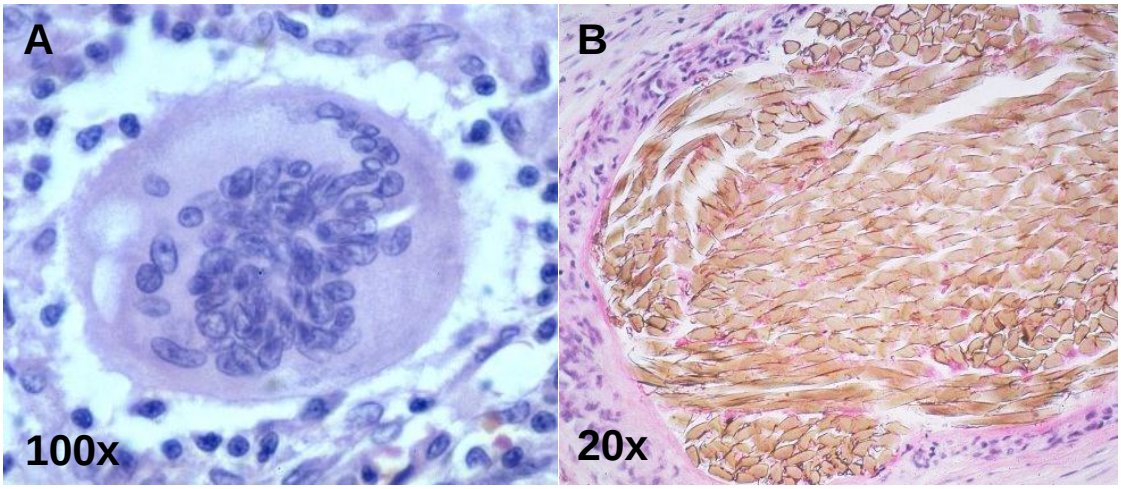


Plate 2.2: Hematoxylin and eosin stained sections of foreign body reaction:
(A) foreign body giant cell (Pernick, 2005). Note typical haphazard, multinucleate arrangement.
(B) foreign body granulomatous reaction to chromic catgut suture material (Rosen, 2007). Foreign body giant cells surround the fibers of the suture material.

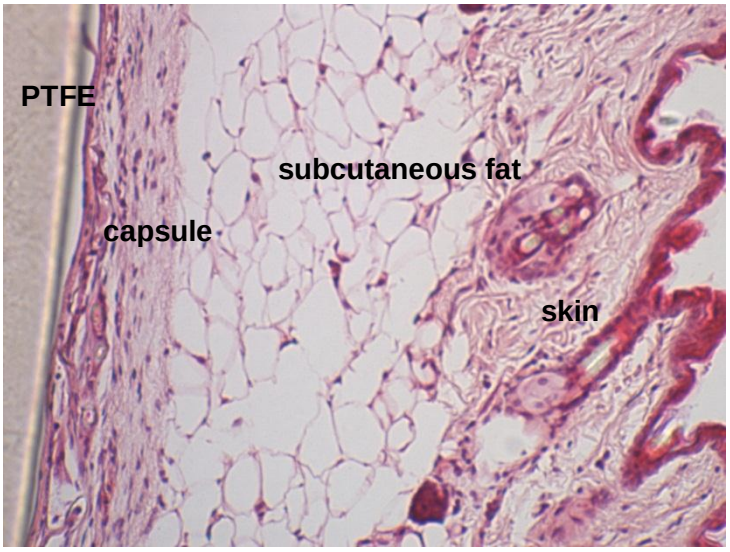


Plate 2.3: Subcutaneous PTFE foreign body and associated capsule (mouse) (Hematoxylin and eosin stain at 40x magnification)

2.9.7 Biocompatibility: choice of animal model

2.9.7.1 Animal species

Animal model biocompatibility tests of implants intended for humans are simulations of human responses to implanted materials. Animal models are utilized in an attempt to predict safety and efficacy of new materials prior to formal human testing. No ideal animal substitute exists. A balance between theoretical and practical experimental needs results is a context specific choice of animal model.

Practical criteria heavily influence selection of animal model in implant tests. Considerations include physical dimensions (animal versus implant), age and gender of the animal as well as its behavioral response to invasive procedures, monitoring and handling. Animal availability is mandatory and as research is schedule driven so procurement time and animal lifecycle play a vital role. Standardized stocks are preferred as they are well characterized and disease free. Healthy animals reduce risk of infection transmission to researchers and other animals and reduce unplanned truncation of experiments due to animal illness or death. Budget and resources contribute to animal model selection. Monies must allow for purchase and maintenance of individuals as well as disposal of biological waste including carcass removal. Animal transport and skilled manpower incur costs and must be factored in. Increased quantities of supplies such as anesthetic agents, cleaning solutions, suture materials and operating equipment are needed in larger animals and raise expenses. Project time constraints, quarantine and regulatory requirements can prohibit the use of animals sourced from outside local areas and therefore limit animal model choice. Personal familiarity with an animal or its use in similar research scenarios within the literature influence animal model choice.

Although necessary practicalities must be addressed, better research results are obtained if animal models closely mimic relevant, context specific human systems so results can be extrapolated more accurately from animal model to man. Mammals are commonly modeled on the basis of their genetic, physiological, pathological and anatomical similarity to man although these similarities exist along a spectrum. As such mammals commonly act as models for medical implant testing. Although non-human primates best replicate human parameters, pragmatism must play a role in choice of animal model and a balance between adequate modeling, experimental function, resource utilization and animal ethics is required. Non-human primates such as baboons have complex social behaviors and many are protected species so there are

ethical concerns about their use (Sykes et al., 2003). In any case non-human primates are not available at James Cook University and their use could not be justified based on the availability of more practical and ethical animal model alternatives. Other mammals ranging from sheep, pigs, cats, dogs and small rodents have served as experimental models to provide critical information during the development of novel polymeric implants (Kameha et al., 2002). Cats and dogs although suitable vectors for experimentation have a companionable and cohabitant relationship with man that generally precludes their experimental use in Australian communities (Davidson, 1998).

The service of pigs in implant testing is considerably less controversial than utilization of non-human primates or domestic pets such as dogs and cats. Pigs are widely bred in captivity, food sources in many societies and are common experimental animal models (Sykes et al., 2003). Pigs have strong advantages as animal models when testing implanted materials intended for humans. Selected pigs are a size, weight and therefore dosing match to adult humans. Proportionate implants can be made to human scale without overburdening the porcine subject or altering the implant. Porcine skin like that of man is firmly attached to underlying structures and is a close structural imitation of human skin although traumatized pig skin bleeds more extensively, has exaggerated fluid loss and a greater susceptibility to infection (Davidson, 1998). Phases of wound healing in pigs correlate well with human responses (Ueberrueck et al., 2005). Pigs have a coagulation system similar to humans (Ueberrueck et al., 2005) and pigs are well matched chronologically and physiologically to humans (Davidson, 1998). Sheep have similar size benefits and in particular have a coagulation system similar to humans (Ueberrueck et al., 2005). Theoretically therefore sheep and particularly pigs are justifiable contenders for testing of novel implant materials.

Rats and mice are the most common models for the investigation of implanted biomaterials but other rodents such as rabbits and hamsters are also extensively sited in the literature (Kameha et al., 2002). The murine research tool set is unparalleled, with at least 40 well-studied inbred strains (Kameha et al., 2002). Genetically modified mice from dedicated laboratories such as the Jackson Laboratory in the U.S.A. allow analysis of regulatory processes associated with tissue response to biomedical implants (Kameha et al., 2002, JAX, 2011). Rat models lack the genetic research repertoire (i.e., transgenic animals, knockouts, SCID, nude) developed in mice however subcutaneous tissue implantation in both rodents provide a high-through-put, relatively low-cost screening technique for testing initial tissue response to implanted materials (Kameha et al., 2002). Rats and mice have a similar angiogenic response in

wound healing, but rats exhibit more extensive inflammation and foreign body response than mice and rats form a thicker capsule around implanted materials (Kameha et al., 2002, Ilse M. S. L. Khouw, 2000). Rats may therefore provide a more appropriate model of inflammatory cell recruitment and differentiation (Kameha et al., 2002). Rats also have advantage of size over mice as they are larger and easier to handle with more body space to implant materials. Both rodents are overall small in size, easy to handle and maintain, have small resource requirements, short generation time and are readily available. Mice and rats are therefore justifiable and commonly employed models for early biocompatibility testing of implant materials.

Hamsters and rabbits act as bio implant test models in a specific subset of experiments. Although hamsters and rabbits are able to tolerate larger implants, they are slower to generate, more difficult and expensive to store and maintain and are less characterized than rats or mice. Because of these disadvantages rabbits and hamsters tend to be utilized in experiments where unique anatomical features are needed. For example, rabbits are employed in intraocular lens testing because leporid ocular size and anatomy allows easy surgery and monitoring in a form similar to the human eye (Han et al., 2003). Another example is the external component of rabbit ears acting as canvass for microscopic examination of *in situ* microvasculature. Rabbit ears have a large, flat surface area, reduced thickness and are easily slide mounted for microscopic analysis.

Rats, mice and rabbits have different cutaneous anatomy to humans or pigs. Mouse epidermis is about 50 µm thick and dermis is less than 1 mm thick and both layers are significantly thinner than human analogs (Sullivan et al., 2001, Davidson, 1998). Loose-skinned animals such as rodents and rabbits have an dense layer of body hair and an added anatomical layer called panniculus carnosus that allows skin to slide and retract over subcutaneous fascia and subcutaneous implants (Aksoy et al., 2002, Sullivan et al., 2001). In rodents and rabbits excision of loose skin results in rapid and marked wound contraction not seen in humans (Davidson, 1998). Mice are also less prone to infection than both humans and pigs (Davidson, 1998). Cutaneous differences can therefore impact choice of animal model in subcutaneous implant testing.

All animal experimentation at James Cook University is subject to ethics approval. The Animal Ethics Committee abides by the Australian Code of Practice for the Care and Use of Animals for Scientific purposes 7th Edition, 2004 and the Queensland Animal Care and Protection Act 2001 to safe keep animal welfare (Langford, 2007). Animal based research at James Cook University is carefully controlled. Appropriate animal

species and animal numbers are chosen to avoid excessive individual burden and yet preserve the likelihood of valid and worthwhile results in a format accepted by the Animal Ethics Committee. Although this committee has the final word on animal selection and implementation at James Cook University, other standards organizations such as American Society for Testing and Materials, International Organization for Standardization (ISO) and the Australian Therapeutic Goods Administration provide information to aid in animal test model selection prior to seeking formal ethics approval. ISO 10993 guidelines recommend the use of mice, rats, hamsters and rabbits for testing implant effects (Wallin and Upman, 1998).

Choice of animal model is multi-factorial, context specific and not an exact science. In most cases a short list of contender species is delivered and after weighted consideration of advantages and disadvantages an animal model is selected. The murine model was chosen for biocompatibility studies of tea tree oil plasma polymer films because mice are proven, standardized and familiar early experimental models for implant assessment. Importantly mice have advantages of easy and inexpensive procurement, maintenance, handling and storage. Ethics approval was more likely with the mouse model than with larger or more companionable animals and mice were recommended by international standards organizations that oversee implant testing. If future expanded studies wish to investigate cellular or genetic response to tea tree oil plasma polymer the mouse model provides established tool sets for genetic and molecular manipulations. Finally baseline comparisons between novel polymer and other implanted polymers are more achievable with a commonly utilized model such as the murine model.

2.9.7.2 Anatomical implant location

Although implantation within any bodily system or structure was possible, at an early stage in testing where scope for implant application was wide, murine subcutaneous implantation was chosen. Subcutaneous insertion was quick, technically simple, minimally invasive and implants were easy to monitor and harvest from the subcutaneous space. In addition many implants such as vascular catheters and ventriculo- peritoneal shunts encounter the subcutaneous space, so this location had clinical relevance. Subcutaneous implantation was recommended for early implantation testing in the literature and by international standards organizations such as ISO (Wallin and Upman, 1998).

2.10 Biofilm

2.10.1 Definition and key concepts

2.10.1.1 Introduction

Bacteria exist in equilibrium between two distinct forms: planktonic bacteria and biofilm. Planktonic bacteria swim as independent cells in liquid medium and are the traditionally familiar manifestation of bacteria. Planktonic microbes group on surfaces where they are known as colonies. A second incarnation of bacteria is *biofilm.* Biofilm is not the same as surface colonies of planktonic bacteria (Table 2.13). Biofilm is a sessile community of microbes irreversibly attached to a substratum, interface or to each other with microbes embedded in self-produced matrix of extracellular polymeric substances (Costerton et al., 1978, Davey and O'Toole, 2000). Biofilm microbes have an altered bacterial phenotype relating to growth and gene transcription and demonstrate nutrient synergy and metabolic co-operation (Costerton et al., 1978). Exchange of genetic traits and complex molecular signaling occurs within biofilm (Davey and O'Toole, 2000). Biofilm is highly differentiated and ubiquitous in the natural aqueous environment (Costerton and Wilson, 2004). Most pathogenic and other bacteria have the ability to exist as biofilm and current concepts on bacterial lifecycles include biofilm.

Biofilms were first identified by the microbiologist Zobell in 1943 and were seen as an industrial nuisance causing marine biofouling through “slime” surface contamination of submerged industrial objects (Lappin-Scott, 1999). The ubiquity of biofilm was not realized until the 1970s (Habash and Reid, 1999). In 1978 the modern definition of biofilm evolved (Costerton et al., 1978). During the 1980s biofilms were studied because they explained anomalies and problems associated with bacterial infection that could not be explained using the concept of planktonic cells (Costerton and Wilson, 2004). The understanding of biofilm continues to evolve.

2.10.1.2 Ultrastructure of biofilm

Biofilm micro-colony is the basic structural unit of biofilm. Living, fully hydrated biofilm micro-colonies are composed of cells (15% by volume) and matrix material (85% by volume) forming heterogeneous, three dimensional, tertiary structures of “towers” and “mushrooms” (Donlan and Costerton, 2002). Micro-colonies combine to create highly organized, complex and interdependent communities. Open water channels are interspersed between the micro-colonies (Lawrence and Korber, 1991). Bulk water

enters these channels to produce convective flow, distribute nutrients and signaling molecules, dispose of waste and aid in environmental sensing (de Beer and Stoodley, 1994). Basic biofilm community structure is conserved among bacteria, with some minor variations (Figure 2.7). The sessile cell fraction of biofilm can be a heterogeneous microorganism milieu or consist of a single bacterial species.

Extracellular matrix excreted by the cellular components of biofilm has specific characteristics. Matrix is predominantly polysaccharide glycol-calyx that contains non-cellular and abiotic components such as proteins and DNA (Sutherland, 2001). Glyco-calyx is highly hydrated and anionic. It mediates adhesion of biofilm to substratum, acts to hold biofilm components together and forms a supporting scaffold for bacteria. Biofilm extracellular matrix provides physical protection to cellular contents of biofilm. Because matrix is an extracellular polymeric substance it is also known as exopolysaccharide.

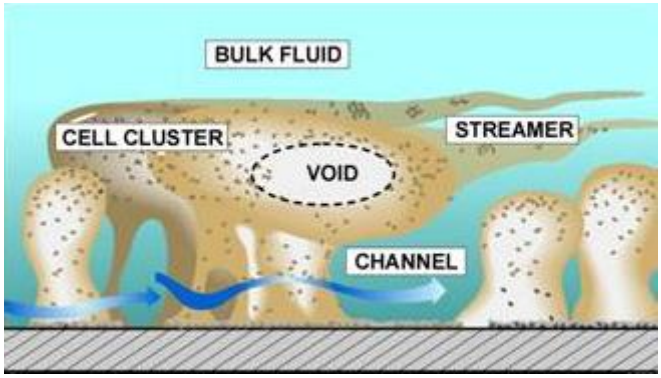


Figure 2.7: Biofilm tertiary structure demonstrating towers and mushrooms interspersed with water channels and voids (Cunningham et al., 2011)

2.10.1.3 Gene expression in biofilm

Gene expression in biofilm is vastly increased compared with planktonic liquid culture and genetic expression changes according to biofilm maturity (Costerton and Wilson, 2004). Approximately 20% of proteins expressed in *Streptococcus mutans* biofilms (found commonly on human teeth) are either up- or down-regulated and glucosyltransferase and fructosyltransferase genes are differentially expressed in mature *S.mutans* biofilm compared with early biofilms (Burne et al., 1997, Svensater et al., 2001). In *Pseudomonas aeruginosa,* a bacteria responsible for biofilm related lung infection, differences in protein expression as large as 50% have been observed between biofilm and planktonic cells and up to 40% between consecutive stages of

biofilm development (Sauer et al., 2002). In addition to increase in the amount of genetic expression, novel gene products are produced in biofilm. For example, cultivation of one biofilm structurally juxtaposed to the biofilm of another species up regulates unique genes that are not expressed in either community alone (Costerton and Wilson, 2004).

2.10.1.4 Induction, formation and maturation of biofilm

Environmental triggers for biofilm formation are ill defined and vary among bacteria. Induction of biofilm may however, be a protective response to environmental stressors such as oxygen limitation, lack of iron or nutrients, temperature changes, osmotic stress, polymorphonuclear cell migration or sub-lethal concentrations of antibiotics. For example sub-inhibitory levels of the protein-synthesis inhibitors tetracycline and quinupristin-dalfopristin strongly induce the formation of biofilm by *S.epidermidis* and nutrient limitation or build-up of toxic metabolites favor formation of biofilm in gram negative bacteria (Donlan and Costerton, 2002, Rachid et al., 2000). *P.aeruginosa* will form biofilm under most conditions that permit growth, but strains of *E.coli* K-12 will not form biofilms in minimal medium unless supplemented with amino acids (Davey and O'Toole, 2000).

Physical factors influence biofilm formation. Biofilm preferentially grows in high shear environments (Reynolds numbers >5000)(Characklis and Marshall, 1990). When biofilms are formed in low-shear environments, they have a low tensile strength and break easily, but biofilms formed under high shear are strong and resistant to mechanical breakage (Donlan and Costerton, 2002). Turbulent flow may enhance bacterial adhesion and biofilm formation by impinging planktonic cells onto surfaces (Donlan and Costerton, 2002). Physical characteristics of a surface such as roughness influence biofilm formation only to a minor extent (Costerton et al., 1995).

Development of biofilm happens in two stages. The first stage involves attachment of planktonic cells to a surface initially via van der Waals forces and then through cell adhesion molecules such as pili (Davies and Geesey, 1995). Microbial cell recruitment follows. The second stage of biofilm development involves microbial multiplication and the formation of exopolysaccharide. Once biofilm is formed it is modified by colonization, co-adhesion, growth, maturation and finally detachment of some micro-organisms (Cunningham et al., 2011, Costerton and Wilson, 2004)(Figure 2.8).

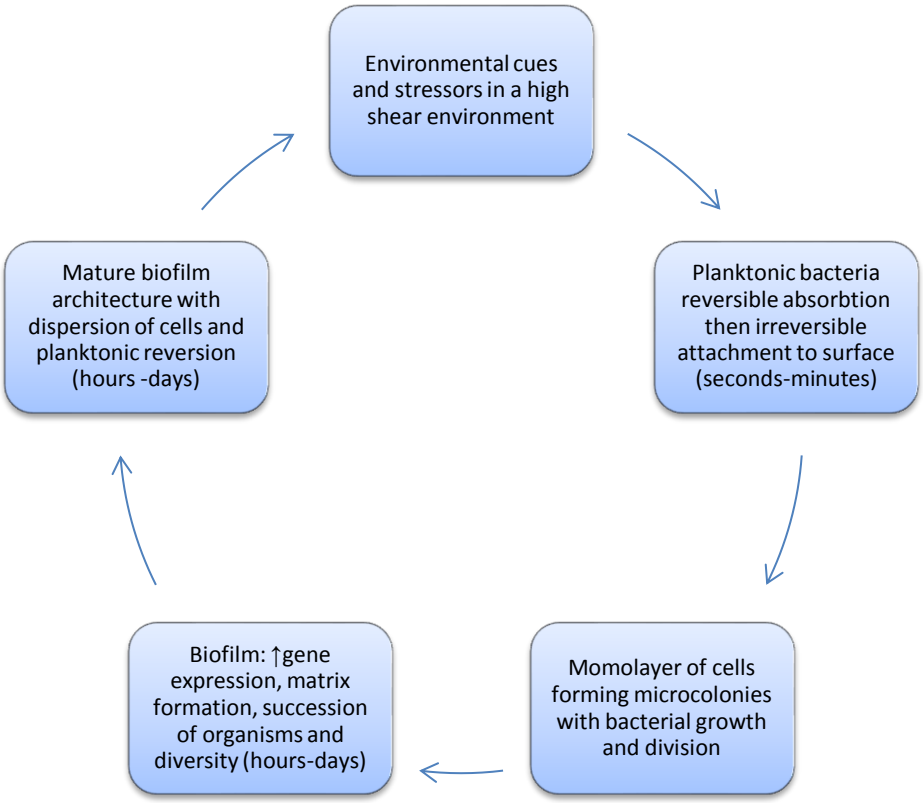


Figure 2.8: Biofilm lifecycle

Expression of genes required to convert planktonic microbes to biofilm is well-regulated once it is triggered. Co-ordinated gene expression is mediated by microbial signal transduction systems (Sauer et al., 2002). Cells undergo a phenotypic shift with large suits of genes being up and down regulated (Parker, 2002). In *S.aureus* and *S.epidermidis,* cell to cell adhesion and biofilm formation is regulated by expression of polysaccharide intracellular adhesin (PIA) the gene product of *icaADBC* (Mathur et al., 2006). In *P.aeruginosa*, flagella bring bacteria into proximity with surface whilst lipopolysaccharide and outer membrane proteins mediate early surface interactions. Once bacteria are on the surface in a monolayer, type IV pilus-mediated twitching is required for cells to aggregate into micro-colonies. Change in gene expression including up regulation of alginate biosynthesis genes and down regulation of flagella synthesis genes herald production of early biofilm (Davey and O'Toole, 2000). Another gram negative bacterium *E.coli* also requires flagellum-mediated swimming to approach and move across surfaces. Type I pili and outer membrane protein Ag43

mediate surface interactions whilst the exopolysaccharide component colanic acid is required for development of *E.coli* biofilm (Davey and O'Toole, 2000). In gram negative bacteria such as *P.aeruginosa* and *E.coli*, signal transduction molecules called acylated homoserine lactones (AHL) play a vital role in biofilm initiation (Davey and O'Toole, 2000). AHL regulated processes inhibit planktonic behavior and induce biofilm. Inactivation of AHL has produced swarming in *Serratia liquefaciens,* biofilm development in *Pseudomonas aeruginosa* and bioluminescence in *Vibrio fischeri* (Bauer and Robinson, 2002). An equivalent two-component peptide signal system is postulated for gram-positive bacteria (Bauer and Robinson, 2002).

Quorum sensing signals may play a role in the initiation and differentiation of biofilm (Fuqua et al., 1994). Quorum-sensing describes a microbial signaling pathway activated as a response to microbial cell density (Miller and Bassler, 2001). Signal molecules called auto-inducers stimulate quorum-sensing systems. Auto-inducers are produced at a consistent basal level by bacteria and auto-inducer concentration is a function of microbial density. The quorum signal is triggered once auto-inducer concentration reaches a threshold and as a consequence biofilm is initiated. Quorum sensing mechanisms may vary between species. It is possible that antimicrobial agents that prevent the formation of biofilm do so by interfering with quorum sensing signaling between cells (Yarwood et al., 2004).

Composition of biofilm is dynamic, evolving and responds to environmental change. Early in its formation biofilm is immature and fragile but over time it matures and becomes increasingly stable. Mature biofilm is characterized by formation of matrix and establishment of organized biofilm architecture (Costerton and Stewart, 1999). Resultant structure is highly viscoelastic and behaves in a rubbery manner although there is a tendency for individual micro-colonies to break off when their tensile strength is exceeded (Stoodley and Lewandowski, 1998).

Table 2.13: Summary of the differences between planktonic bacteria and biofilm

Characteristic	Biofilm	Planktonic bacteria
concept	evolving	traditional
constitutional makeup	heterogeneous	homogeneous
location	surface related	free swimming
matrix	yes	no
exist as	community	individuals
host defenses	protected	exposed
traditional antimicrobial strategies	ineffective	effective
infection	chronic, relapsing, remitting	acute presentation
genetics	biofilm phenotype	planktonic phenotype
metabolism	often inactive	active
grow rate	reduced	standard

2.10.2 Biofilm as a disease vector

2.10.2.1 Introduction

Biofilm is thought to be a microbial protective mechanism evolved to promote bacterial survival on exposure to external stressors or an unfavorable environment (Kojic and Darouiche, 2004). If survival is the premise of biofilm formation then biofilm is expected to resist processes that attempt to eliminate or attenuate it. Indeed, biofilm is much less vulnerable to antibiotics and host immune defenses than planktonic bacteria (Stewart and Costerton, 2001). Planktonic bacteria are metabolically active individuals that rely on their own cellular processes to defend against chemical or host attack. Free swimming bacteria are vulnerable in solution and are cleared by antibodies and phagocytes. They are susceptible to antibiotics that act on active metabolic pathways or exposed structural components. Conversely, biofilm is inherently resistant to phagocytosis, immune processes and antibiotics due to sequestration of cells within matrix and biofilm metabolism may be active or hibernating, anaerobic or aerobic and is in fact often heterogeneous leading to varied antibiotic susceptibility. Once biofilm moves from colonization to a disease causing state it can be difficult to eradicate as it is relatively refractory to traditional medical detection methods, assessment and therapies (Kojic and Darouiche, 2004).

2.10.2.2 Defining pathogenic biofilm

Many potentially pathogenic micro-organisms form biofilms however biofilms are only pathogenic if they confer a negative outcome to host. Like other microbes, biofilm related organisms can form part of benign, healthy, symbiotic or synergistic, commensal micro-flora, such as bacteria lining a healthy gastrointestinal or genitourinary tract. In order to answer the question “is biofilm pathogenic?” negative host outcomes must be defined and then evidence produced that implicates biofilm as the cause of the negative outcomes. Because of the fastidious nature of biofilm detection and culture, such proof is universally difficult. Presence of a biofilm is not enough to make it pathological. The challenge lies in separating the harmless from the harmful.

Typically pathological biofilms present with recurrent and chronic, symptomatic, treatment resistant, culture negative infection or with metastatic infection (Hall-Stoodley et al., 2004).

The diversity of microbe-host interactions is remarkable and ranges from indolent to aggressive (Parsek and Singh, 2003). Indirect clues to implicate biofilm infection include insusceptibility of a disease state to antibiotics, history of long term, low dose antibiotics, a foreign body implant, the presence of a condition known to be associated with biofilm or a past history of biofilm related disease (Costerton and Stewart, 1999). As biofilm infection does not present like acute planktonic infection, a high index of clinical suspicion is warranted in order to consider the diagnosis of biofilm related infection.

Uniformly accepted criteria to determine if biofilm is responsible for any given infection do not exist. Certainly biofilm infection does not conform to Koch's Postulates. Picket proposed an alternate definition of infection to encompass biofilm related infection:

> "Invasion by and multiplication of pathogenic microorganisms in a bodily part or tissue, which may produce subsequent tissue injury and progress to overt disease through a variety of cellular or toxic mechanisms." (Pickett, 2004)

Parsek and Singh went a step further and suggested the following criteria as a starting point to define biofilm infections:

1. The infecting microbes are adherent to some substratum or are surface associated

2. Direct examination of infected tissue shows microbes living in cell clusters or micro-colonies, encased in an extracellular matrix

3. The infection is generally confined to a particular location: although dissemination may occur, it is a secondary phenomenon

4. Infection is difficult or impossible to eradicate with antibiotics despite the fact that the responsible organisms are susceptible to killing as planktonic cells

(Parsek and Singh, 2003)

Although these definitions provide a useful, descriptive starting point, the guidelines supplied by Pickett, Parsek and Singh fail to provide a sound experimental model of cause and effect between biofilm and infection. In reality the diagnosis of biofilm related infection

is often reached through a process of elimination, when symptoms resolve on removal of biofilm from host (often in the context of an implanted medical device) or is based on clinical suspicion alone.

2.10.2.3 Difficulties in detecting biofilm

Biofilm related pathogenic organisms are difficult to detect for a number of reasons. At least a percentage of the organisms in biofilm cannot be cultured or cannot compete with more rapidly growing cells commonly isolated on complex media (Nickel et al., 1989). These culturing difficulties can mislead clinicians, for example in a study by Sanclement et al., specific biofilm cultures taken from patients with chronic sinusitis did not correlate with standard intra operative swab cultures taken from the same patients (Sanclement et al., 2005). Indeed biofilm and planktonic infection may coexist and confound results. In addition, cultured biofilm often grows slowly and requires specialized conditions such as fluid circulation and shear to propagate. Medical tests to detect infection tend to focus on sampling fluid compartments within the body. Shedding of biofilm components into physiological fluid spaces is intermittent so sampling error can make diagnosis elusive. Even when biofilm constituents are shed and cultured they may not be representative of the heterogeneity of underlying biofilm cells. Direct sampling of biofilm is preferable but can be challenging if biofilm is seated on surfaces deep within the body such as on implanted medical devices. Furthermore no definitive physiological marker of the biofilm state exists and as biofilm is an organized mode of bacterial growth, the biofilm phenotype is rapidly lost in *ex vivo* culture (Parsek and Singh, 2003). It's no wonder that until relatively recently, biofilm related morbidity was unrecognized and bizarre viral and/or immunological mechanisms were proposed to explain pathologic outcomes caused by biofilm (Costerton et al., 2003).

2.10.2.4 Biofilm pathogenesis

Exact mechanisms by which biofilm-associated microorganisms elicit disease are poorly understood (Donlan and Costerton, 2002). In all likelihood there are multiple mechanisms that work in concert to produce disease and include:

- Biofilm related direct infection of host tissue (Ramadan et al., 2004)
- Local tissue destruction (Pace, 2006)
- Persistent secretion of toxins (Vincent et al., 1989)

- Reversion to acute planktonic infection (Anderson et al., 2004, Boyd and Chakrabarty, 1994)
- Pathological stimulation of the immune response (Pace, 2006, Chapple and Matthews, 2007)
- Evasion of host immune response (Leid et al., 2002)
- Physical obstruction of organs including embolization (Costerton, 2001, Fux et al., 2004)
- Failure of implanted medical devices with loss of device function and associated surgical morbidity (Khardori and Yassien, 1995)

Whatever the mechanism, persistence and chronicity are features of biofilm pathogenesis linked directly to its problematic detection and eradication (Reid, 1999).

2.10.2.5 Persistence of biofilm

How do biofilms persist or conversely what makes them hard to eradicate? The answer is multifactorial but structural, chemical, nutrient, metabolic and immunological mechanisms are at play. Antibiotic resistance is inherent to biofilm and key to eradication failures.

Structural protection of biofilm organisms exists on several levels. Exopolysaccharide matrix provides physical sequestration of biofilm cells preventing damage from hostile environment, immune systems and antibiotic chemotherapy (Nadar-Macias, 2006). Heterogeneous architecture of biofilm supports development of anoxic and acidic niches (favorable to some bacteria) and zones of nutrient depletion on the interior of biofilm clusters (De Beer and Stoodley, 1995). Nutrient-depleted zones can result in a stationary phase-like dormancy within the biofilm making cells resistant to antibiotics that target metabolic processes (Fux et al., 2004). Community structure of biofilm also provides protection through effects such as the competitive exclusion phenomenon, a principle of theoretical ecology that states no two species can indefinitely occupy the same ecological niche due to limitations in local resources (Bremermann and Thieme, 1989). Established biofilm can thus usurp resources and prevent displacement by commensal and nonpathogenic bacteria. It was recently discovered for example that *Pseudomonas aeruginosa* actively maintains biofilm structure by releasing surfactant molecules that prevent other micro-organisms from clogging biofilm channel systems (Davey and

Caiazza, 2003). Drilling down, the grouping of bacteria in biofilm is associated with increased levels of survival supporting mutations as well as with quorum-sensing-regulated mechanisms (Høiby et al., 2010). Quorum sensing is a cell density dependent form of cell-to-cell signaling that allows bacteria to display a unified response to benefit the bacterial population as a whole (Smith et al., 2004). Protective cellular processes modulated by quorum sensing include symbiosis, transfer of conjugative plasmids, sporulation, antimicrobial peptide synthesis, regulation of virulence and biofilm formation itself (Smith et al., 2004).

Clearly biofilm demonstrates effective strategies to evade the innate and acquired immune systems however specific biofilm defense mechanisms against host immunity are not yet thoroughly characterized (Pace, 2006). Innate immunity is a nonspecific molecular and cellular response to infection and is rapid in onset but does not confer long lasting protection upon host. Innate immunity provides an immediate, generic response to pathogens and includes physical and chemical barriers to infectious agents, recruitment of immune cells, activation of complement cascade and phagocytosis by specialized white blood cells. Biofilm stymies innate immunity in several ways. Cells are less conspicuous to the immune system as antigens are hidden and key ligands repressed within the protected niche of matrix (Donlan and Costerton, 2002). Phagocytosis by macrophages and neutrophils is frustrated by the biomass (Pace, 2006). In addition to ineffective phagocytosis, Leid et al. demonstrated that polymorphonuclear cells have reduced activity due to prolonged stimulation by biofilm leading to a local zone of immunosuppression (Leid et al., 2002). Specifically, leucocytes penetrated channels within *S.aureus* biofilm but demonstrated no evidence of motility or phagocytosis (Leid et al., 2002). Matrix components that confer protection have also been identified. *S. epidermidis* secretes poly-γ-DL-glutamic acid (PGA) as a barrier substance and as a buffer to the bactericidal high salt environment of human skin (Kocianova et al., 2005). The presence of polysaccharide intercellular adhesin (PIA) on the surface of *S.epidermidis* is requisite for matrix formation. In a study by Vuong et al. mutant strains of *S.epidermidis* lacking PIA completely lost extracellular matrix, became vulnerable to phagocytosis by human polymorphonuclear leucocytes and were significantly more susceptible to killing by major antibacterial peptides found naturally on human skin (Vuong et al., 2004).

Acquired (adaptive) immunity is equally vexed by biofilm. As a result, biofilm infections are rarely resolved even in individuals with excellent humoral and cellular immunity (Costerton et al., 1999). Acquired immunity allows host to recognize and remember specific antigens associated with pathogenic bacteria. It provides immunological memory and long lasting immune protection to host through action of effector B and T lymphocytes. B cells orchestrate humoral responses based around antibody production and T cells provide cell mediated adaptive immunity. Biofilm interferes with both arms of adaptive immunity. Even when matrix girt bacterial antigens and toxins are exposed and stimulate antibody production, antibodies are ineffective in killing biofilm bacteria (Costerton and Stewart, 1999). A study by Ward et al. demonstrated this effect nicely. In the study, rabbits vaccinated against biofilm, whose opsonic antibody titers were a thousand times elevated could not mount a phagocytic immune response against biofilm laden peritoneal implants (Ward et al., 1992). Cerca et al. went further to show antibodies can penetrate staphylococcal biofilm but opsonic killing of bacteria is avoided because high concentrations of decoy bacterial antigen shed into matrix prevent binding of antibody to cells (Cerca et al., 2006). Cellular acquired immunity is also thwarted by biofilm defences. Prabhakara et al. used knock-out mice to demonstrate *S.aureus* biofilm elicits inflammatory T helper 1/T helper 17 responses from host instead of host protective T helper 2/ regulatory T cell responses (Prabhakara et al., 2010). Interference in T lymphocyte function by biofilm was also implicated by Taubman et al. in a study of periodontal disease. The study found biofilm interface initiated and perpetuated antigen specific T cell infiltration of gingiva and lead to on-going stimulation of osteoclastogenesis and bony resorption (local tissue destruction) without T cell resolution of biofilm (Taubman et al., 2005). Finally, biofilm associated quorum-sensing signal molecules have been shown to affect cytokine release by stimulated human T cells, reduce T cell proliferation and in turn stunt antibacterial activity of the adaptive immune response (Skindersoe et al., 2009).

Biofilm microbes are generally not as invasive or clinically aggressive as planktonic organisms and reduced virulence may confer survival advantage and biofilm persistence. Biofilm bacteria are adherent to surface and confined within matrix and invasion and motility machinery are down regulated (Parsek and Singh, 2003). Ultimately attenuated virulence may serve the bacteria's interest by increasing the longevity of the host.

Biofilm is inherently resistant to the effects of antibiotic chemotherapy. Depending on the organism, antimicrobial agent and experimental system, biofilm can be up to a thousand times more resistant to antimicrobial stress than free-swimming bacteria of the same species (Parsek and Singh, 2003). It is typical therefore, that standard antibiotics eradicate planktonic bacteria but have little or no effect on biofilm. Resistance occurs due to generic bacterial factors that hamper success of all antibiotics and also via specific biofilm vectors. Focusing on biofilm related factors: matrix may completely inhibit penetration of antibiotic into biofilm, or alternatively it may retard rate of drug penetration long enough to induce expression of antibiotic resistance genes in bacterial cells (Jefferson et al., 2005). Charged polymers and antibiotic degrading enzymes embedded within exopolysaccharide may lead to antibiotic repulsion, binding, deactivation or diffusion gradients of antibiotic making the drugs ineffective (Bagge et al., 2004, Walters et al., 2003). Oxygen and nutrient gradients within biofilm are associated with decreased bacterial metabolic activity and increased doubling times of the bacterial cells leading to dormancy and antibiotic tolerance and some bacteria may differentiate into a protected phenotypic state (Høiby et al., 2010, Stewart and Costerton, 2001, Kojic and Darouiche, 2004). Some biofilm bacteria exposed to antibiotics form inactivated “persister cells” that become reactivated and repopulate the biofilm once antibiotics are discontinued (Kojic and Darouiche, 2004). In zones of nutrient depletion and waste accumulation antibiotic action can also be antagonized (Stewart and Costerton, 2001). Biofilm may accentuate antimicrobial ability seen in planktonic counterparts, for example *Pseudomonas* biofilm cells tolerate the antibiotics tobramycin, gentamicin and ciprofloxacin because of increased expression of a drug efflux pump when compared to planktonic cells (Zhang and Mah, 2008). Other up regulated resistance mechanisms that contribute to survival and persistence of biofilm include chromosomal β-lactamase and mutations in antibiotic target molecules in bacteria (Høiby et al., 2010). The heterogeneity and multicellular, coordinated functioning of biofilm provides a multipronged approach to antibiotic resistance.

2.10.2.6 Human biofilm infections

As time goes by, biofilms are being understood to play a part in a broadening and significant array of human infections (Potera, 1999). Sanclement and Webster predicted 80% of all human infections are associated with biofilm whilst the Center for Disease Control and Prevention in the United States of America estimated the figure to 65% (Fergie et al., 2004, Sanclement et al., 2005). Biofilm infections take on two predominant

manifestations: those that exist independently of medical implants and those associated with medical implants although the difference lies only in the surface onto which biofilm deposits. Refractory device related and chronic bacterial infections now constitute 65-80% of infections treated by physicians in the developed world (Costerton and Wilson, 2004). Overall, it is estimated upwards of 60% of all nosocomial infections are biofilm associated (Archibald and Gaynes, 1997). These biofilm-based hospital acquired infections increase length of hospital stay, morbidity, mortality for patients and cost upwards of US$ 11 billion per year in added costs in the United states alone (Schierholz and Beuth, 2001). There is no dispute that biofilm plays a dominant role in a large proportion of human infections and contributes heavily to the cost of healthcare.

2.10.2.7 Human biofilm infections unrelated to medial implants

Multiple natural surfaces and interfaces occur in the human host. These sites can harbor commensal and pathogenic biofilm in the absence of medical implants. Increasingly biofilm is being linked to multiple and varied human infections related to these natural surfaces. Dental plaque or oral biofilm was first described by Van Leeuwenhoek in the 17th century when he sketched very complex dental biofilms (Hoogenkamp et al., 2011). Oral biofilm is one of the most widely researched biofilm infections in man. In modern dentistry prevention of dental caries and periodontal diseases essentially equals the control of dental bacterial biofilm (Baehni and Takeuchi, 2003). Biofilm is cariogenic on many fronts: oral biofilm damages teeth via enamel surface erosion, subsurface porosity, mineral loss and tubular sclerosis of dentin (Kidd and Fejerskov, 2004). In addition, scanning electron microscopy has demonstrated that bacterial biofilm on the external surface of the root apex in teeth is associated with pulp necrosis (Leonardo et al., 2002). This apical biofilm is postulated to cause failure of endodontic treatment of periodontitis as a consequence of persistent infection (Noiri et al., 2002).

Osteomyelitis, or infection of the bone, is a prevalent issue facing clinicians. There is strong evidence to support biofilm causation of osteomyelitis. The prevalence of *Staphylococcus aureus* in bony infections is likely due to its ability to form a biofilm on the surface of bone (Brady et al., 2008). Transmission and scanning electron microscopy have also imaged biofilm in poly-microbial osteomyelitis (Marrie and Costerton, 1985). An interesting study by Agarwal et al. revealed culture of biofilm from bony sequestrum was the most accurate means to identify the responsible pathogen in chronic osteomyelitis and

instigate effective treatment (Agarwal et al., 2005). Even bony pathology thought to be "sterile" is now being linked to biofilm. Osteonecrosis of the jaw (ONJ) is a known side effect of a group of drugs called bisphosphonates and may occur after radiotherapy. In a study by Sedghizadeh et al. all patients with ONJ requiring surgical bone debridement showed large areas of poly-microbial biofilm within their sequestrum specimens as demonstrated with scanning electron microscopy (Sedghizadeh et al., 2008). These findings suggested a role for microbial biofilms in the disease process of ONJ as well as osteomyelitis.

Biofilm is responsible for several refractory urological bacterial infections. Anderson et al. discovered *Escherichia coli* caused persistent urinary tract infections by invading epithelial cells within the bladder and forming intracellular biofilms (Anderson et al., 2003). The biofilms produced pod-like bulges on the bladder surface surrounded by a protective shell of uroplakin (Anderson et al., 2003). *E.coli* biofilm has been implicated in cystitis, pyelonephritis and prostatitis as well (Soto et al., 2007). In particular prostatitis is associated with biofilm. Using a crystal violet binding assay, Kanamaru et al. demonstrated urine isolated from patients with prostatitis had higher optical density than urine from patients with either cystitis or pyelonephritis (Kanamaru et al., 2006). Increased optical density was associated with more suspended biofilm particles. Similarly strains of *E.coli* commonly associated with prostatitis exhibited significantly higher optical density values than other strains (Kanamaru et al., 2006). These results support an association between bacterial prostatitis and biofilm formation. Finally, Clapham et al. found infection induced urinary calculi consisted of struvite and bacterial biofilm (Clapham et al., 1990).

Native valve endocarditis is associated with biofilm producing strains of *E.faecalis*. Mohamed et al. found that endocarditis producing strains of *E.faecalis* made biofilm significantly more often and also to a greater degree than non-endocarditis isolates (Mohamed et al., 2004). Nallapareddy et al. demonstrated pleomorphic biofilm associated surface pili in *E.faecalis* acted as virulence factors in endocarditis (Nallapareddy et al., 2006). Glycolipid and lipoteichoic acid precursors involved in *E.faecalis* biofilm accumulation were also associated with adherence to host tissue and more virulent endocarditis (Theilacker et al., 2009).

Chronic lung infections in the setting of cystic fibrosis or bronchiectasis are characterized by the presence of biofilm (Fergie et al., 2004). In particular *Pseudomonas aeruginosa*, an opportunistic human pathogen and ubiquitous environmental bacterium, prolifically forms biofilm in the diseased lung and permanently colonizes lung parenchyma within a specialized niche (Wagner and Iglewski, 2008). Microscopy of sputum from cystic fibrosis patients has confirmed *P.aeruginosa* in biofilm configuration and biofilm specific quorum-sensing signals have also been demonstrated in sputum from chronic lung infection patients (Pradeep et al., 2000). Indeed the unique environment of a cystic fibrosis lung selects for a subgroup of auto-aggregative and hyper-piliated *P.aeruginosa* small-colony variants (Häußler et al., 2003). Small colony variants are highly adherent, show increased fitness under stationary growth conditions and augmented capacity for biofilm formation (Häußler et al., 2003). The emergence of small colony variants within the cystic fibrosis lung play a key role in the persistence and pathogenesis of *P.aeruginosa* biofilm lung infection (Häußler et al., 2003).

Chronic wounds including diabetic foot ulcers, pressure ulcers and venous leg ulcers are populated by highly tenacious biofilm communities. In fact 60% of chronic wounds have detectable layers of biofilm displayed on high power imaging or via molecular techniques (James et al., 2008). Bjarnsholt et al. clearly demonstrated biofilm micro-colonies in chronic wounds using fluorescence *in situ* hybridization (Bjarnsholt et al., 2008). Most chronic wound biofilm infections are poly-microbial, contain obligate anaerobes and may enclose populations of bacteria not recognized as wound pathogens, such as *Abiotrophia para-adiacens* and *Rhodopseudomonas* species (Dowd et al., 2008). Although biofilm may simply colonize chronic wounds, delayed healing can also be due to inefficient eradication of pathological biofilm. These studies highlighted the need to target biofilm in chronic, non-healing wounds.

Acute skin infections may also be a pathological manifestation of biofilm. Both staphylococcal cellulitis (Iwatsuki et al., 2006) and streptococcal impetigo (Akiyama et al., 2003) are biofilm related. Necrotising soft tissue infections are rampant bacterial skin and soft tissue infections with a mortality rate of 25% despite optimal care (Cuschieri, 2008). Successful treatment requires early, aggressive surgical debridement of all necrotic tissue, broad spectrum systemic antibiotic therapy and supportive care to maintain oxygenation and tissue perfusion. Grass and colleagues observed structured biofilm communities in

necrotizing skin and soft tissue lesions (Hidalgo-Grass et al., 2004). Biofilm may therefore explain why necrotizing soft tissue infections are so refractory to antibiotics alone.

Gallstone cholecystitis, cholangitis and infected biliary pancreatitis are biofilm diseases (Potera, 1999). In a study of 292 patients with complicated, infected gallstone disease, biofilm on gallstones correlated with colonization and chronic but attenuated disease due to inhibited detachment of bacteria and reduced cholangio-venous reflux (Stewart et al., 2007). Swidsinski et al. used fluorescence *in situ* hybridization with ribosomal ribo-nucleic acid targeted carbocyanine labeled oligonucleotide probes to demonstrate dense multispecies bacterial biofilm in the pancreatic duct of patients with calcific pancreatitis and also in patients with mixed gallstones (Swidsinski et al., 2005). Biofilm associated with gallstones is responsible for *Salmonella* carrier state in humans. Prouty et al. found *Salmonella enterica* efficiently formed biofilm on the surface of gallstones in the presence of bile creating a protected reservoir for bacteria within the gallbladder (Prouty et al., 2002).

Role of biofilm in the human colon and its relationship to colonic disease is poorly understood. It is likely that particle-associated and mucosal biofilm communities in the lower digestive tract, particularly the large bowel are highly evolved assemblages (Macfarlane et al., 2004). Punched fecal cylinders certainly show fecal flora is highly structured and spatially organized (Swidsinski et al., 2008). The most obvious candidate for colonic biofilm pathology is inflammatory bowel disease encompassing ulcerative colitis and Crohn's disease. The etiology of inflammatory bowel disease is unknown, but there is a good case for mucosal bacterial involvement, either through pathogens colonizing the epithelial surface or by inappropriate host immune responses to members of the normal micro flora (Macfarlane et al., 2008). Biofilm may result in higher localized concentrations of bacterial antigens or toxins and precipitate inflammatory bowel disease although this theory remains unproven (Macfarlane et al., 2008).

Ear, nose and throat infections are commonly biofilm related. Biofilms have been implicated in recalcitrant chronic rhino-sinusitis. Using the Calgary Biofilm Detection Assay, Prince et al. determined 28% of patients with sino-nasal muco-purulence had biofilm-forming capacity and that the percentage increased further following interventional sinus surgery (Prince et al., 2008). Bacterial biofilms within the crypts of tonsils removed

for acute infection, chronic disease or hypertrophy have been confirmed using histological and ultra-structural analysis (Chole and Faddis, 2003, Ramirez-Camacho et al., 2008). Similarly, bacterial biofilms are often found overlying adenoid tissue (Winther et al., 2009). A staggering 92% of children with chronic otitis media and effusion have biofilm on the membrane of their middle ear and chronic middle-ear infective disorders are accepted as biofilm-related (Hall-Stoodley et al., 2006). Cholesteatoma is a destructive and expanding growth consisting of keratinizing squamous epithelium in the middle ear or mastoid. Chole and Faddis observed biofilm within sixteen of twenty-four human cholesteatomas studied and postulated biofilm as the cause for recurrence and persistence of infection within these growths (Chole and Faddis, 2002).

Recently obstetric and gynaecological infections have been scrutinised for evidence of biofilm. Romero et al. showed that “amniotic fluid sludge” detected on trans-vaginal ultrasonography and retrieved by trans-vaginal amniotomy was biofilm (Romero et al., 2008). Amniotic fluid samples were examined with scanning electron microscopy and demonstrated bacteria embedded in amorphous matrix and inflammatory cells. Amniotic fluid sludge and chorio-amnionitis are associated with preterm delivery and the attendant risks of premature birth (Bujold et al., 2006, Muglia and Katz, 2011). Bacterial vaginosis, a condition that affects millions of women is also likely a biofilm disease. Using molecular techniques Fredricks et al. compared women with and without clinical bacterial vaginosis and found women with bacterial vaginosis had greater bacterial diversity and included newly recognised bacterial species even when standard cultures were negative (Fredricks et al., 2005). Gonorrhoea is a common sexually transmitted disease caused by the bacterium *Neisseria gonorrhoeae. N.gonorrhoeae* forms a biofilm in flow cells on glass coverslips as well as on primary cervical epithelial cells as demonstrated when infected cervical biopsies were examined with scanning electron microscopy (Steichen et al., 2008). Gonococcal biofilms may be involved in asymptomatic infections, persistence, and increased antibiotic resistance in patients with sexually transmitted diseases.

Some unique biofilm disease vectors have been postulated. One example is plague, an arthropod borne infection. Transmission of plague by fleas depends on dense biofilm infection of the flea’s proventricular valve with *Yersinia pestis* and bacteria released from biofilm are more resistant to human polymorphonuclear leucocytes than *in vitro*-grown *Y.pestis* (Jarrett et al., 2004).

2.10.2.8 Human biofilm infections related to medial implants

Medical implants are vital and ubiquitous in modern-day medical practice. At the same time implanted devices are a leading cause of morbidity and mortality mostly due to nosocomial biofilm infection (Table 2.14). Morbidity of an infected medical implant is due to local, remote and systemic infection, functional implant loss and surgical insult (Vinh and Embil, 2005). In 1957 Elek and Conen demonstrated many fewer bacteria are required to produce infection in the presence of a foreign body (Kojic and Darouiche, 2004). Today, implementation of a medical implant is the greatest exogenous predictor of healthcare associated infections (Manangan et al., 2002). To illustrate the point, between two and four percent of total knee replacements and seven percent of total elbow replacements become infected whilst six percent of vascular grafts, 20% of ventricular assist devices and 100% of the Total Artificial Heart replacements develop infections, all biofilm related (Freitas Jr, 2003).

It is clear device-associated biofilm infections are a major economic and healthcare burden given the widespread use of implants and the cost per episode of implant infection (Lynch and Robertson, 2008). Four types of medical device related infections deserve a special mention. Infection of central venous lines, orthopaedic joint prosthesis, indwelling urinary catheters and mechanical heart valves contribute disproportionately to healthcare costs and poor patient outcomes either due to frequency of biofilm infection, shear number of devices or severity of infection related outcomes (or often a combination of these factors).

Central lines are a major source of biofilm related morbidity because so many are used and a large number become infected. Over five million central venous catheters (CVC) are inserted each year in the U.S.A. (Darouiche, 2001). 500,000 CVC associated bloodstream infections occur in the United States and Western Europe annually (Crump and Collington, 2004). Analysis of the National Nosocomial Infections Surveillance data in the U.S.A. showed 87% of all primary bloodstream infections occurred in patients with a central line (Mermel et al., 2001). Mortality as a result of CVC infection was estimated at 12%-25% per episode and the marginal cost to the healthcare system at US$ 25,000 per infection (Kluger and Maki, 1999). In hemodialysis patients the cost of catheter related infection jumped up to US$ 32,000 per episode (Engemann et al., 2005, Reid, 1999). When

examined by scanning electron microscopy or transmission electron microscopy, mature CVCs almost universally develop biofilm (Raad et al., 1993). This fact makes sense because the equipment is often exposed to extracorporeal environment in some part. Further the intravascular device component becomes coated with platelets, plasma and other tissue proteins that act as conditioning films for adherence for bacteria such as *S.aureus* and *S.epidermidis*. In order of prevalence, the four most common pathogens causing CVC infection are coagulase-negative *Staphylococci, Staphylococcus aureus*, *Candida* species and enteric gram-negative bacilli (Safdar et al., 2004). Based on these statistics, central venous catheters arguably pose a greater risk of device-related infection to patients than do any other indwelling medical devices (Maki, 1994).

Consequence of biofilm orthopedic infection is significant in terms of patient and monetary costs. As the Western population ages and anesthetic and operative techniques support surgery on sicker and older patients, the rate of joint replacements are increasing (Dixon et al., 2004). A current annual estimate of the rate of total hip replacement in the world is approximately one million and knee replacements more than a quarter of a million (Schierholz and Beuth, 2001). Some patients require multiple joint replacements. Rate of infection of joint prostheses is low at between one and three percent however the magnitude of this complication is remarkable considering the number of replacements performed (Kojic and Darouiche, 2004). The cost of prosthetic joint infection is approximately US$ 50,000 per episode and the incidence of recurrent infection approaches 10% (Lentino, 2003). Mortality attendant to intervention for an infected joint prosthesis increases with age and is estimated at 0.4-1.2% for 65 year old patients increasing to 2-7% for 80 year old patients (Fisman et al., 2001). The most common biofilm organism associated with infection at joint replacement sites were found by Arciola et al. to be *Staphylococci* (75.5%) followed by *Enterobacteriaceae* (8%), *Pseudomonas* (7.3%), *Enterococcus* (5.3%) and *Streptococcus* (1.9%) (Aricola et al., 2005). Fracture fixation devices including intramedullary nails, external fixation pins, plates and screws were the other common orthopedic implants affected greatly by the occurrence of biofilm infection. In the United States two million fracture-fixation devices are inserted annually, five per cent of these implants become infected at a cost of US$ 15,000 per episode (Darouiche, 2004). The incidence of infection after internal fixation of closed fractures is generally lower (1-2%), whereas the incidence may exceed 30% after fixation of open

fractures (McGraw and Lim, 1988 , Perren, 2002). The most common infecting organisms of fracture-fixation devices are *S.aureus* (>40%) and *P.aeruginosa* (Aricola et al., 2005).

Greater than a staggering 30 million bladder catheters are inserted annually in the United States (Richards et al., 1999). Zimakoff et al. found 13.2% of hospital patients, 4.9% of nursing home occupants and 3.9% of patients receiving home care undergo urinary catheterization (Zimakoff et al., 1993). The incidence of bacteriuria (and presumably biofilm infection) in these people rises from half to one per cent for a single "in-and-out" bladder catheterization to 10-30% for catheters in place for up to four days and 95% for catheters in place for greater than 30 days (Dickinson and Bisno, 1989). Positive urinary cultures in patients recently catheterized primarily grow *E.coli*, *Klebsiella*, *Proteus* and *Enterococci* whilst organisms associated with long term urinary catheterization include *P.aeruginosa, Enterobacter, Proteus* and *Providencia* (Dickinson and Bisno, 1989). Each catheter associated urinary tract infection adds a mean of US$ 589 to the cost of care (Tambyah et al., 2002).

More than 60,000 cardiac valve replacement operations are performed each year in the United States alone and 60% of these procedures utilize mechanical valves (Vongpatanasin et al., 1996). Prosthetic valve endocarditis (PVE) is not a common phenomenon. PVE has a reported incidence of only two to five percent in valve recipients however among patients who develop this biofilm related infection mortality is as high at 70% (Davey and O'Toole, 2000, Calderwood et al., 1986). Infected cardiac valves cost more than US$ 50,000 to replace and not all patients receiving a replacement survive (Darouiche, 2004). Infected mechanical cardiac valves are a prime example of how biofilm infection can lead to escalating medical costs and life threatening physiological sequelae.

As expressed above, bacteria that infect medical implants are somewhat device dependent. In a more general sense biofilm on bio-implants may be composed of gram-positive or gram-negative bacteria, yeasts or a mixture of organisms. Bacteria commonly isolated from medical implants include gram positives such as *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus viridans* and gram-negatives like *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa* (Donlan, 2001). Biofilm implant infections are due to a single organism or are poly-microbial. Infectious agents originate from the host, healthcare

providers or other environmental sources. Contamination of medical devices occur by direct inoculation with microorganisms, via hematogenous or lymphatic seeding or by trans-coelomic spread (Von Eiff et al., 2005).

2.10.2.9 The link between medical implants and biofilm

Implant infection is a multifaceted interaction between bacteria, device and host. Bacterial factors are probably the most important in pathogenesis of infection, whereas device factors are the most amenable to modification with the objective of preventing infection (Darouiche, 2001). Implanted medical devices are vulnerable to infection for a number of reasons:

- They provide a surface for biofilm formation and a niche for biofilm existence
- Devices may have surface properties favorable to biofilm formation such as higher critical surface tension promoting adherence of red blood cells, platelets and fibrinogen leading to anchoring points for bacteria (Baddour et al., 2003)
- Bio-implants such as intra-vascular devices can be exposed to shear forces that push bacteria toward biofilm formation
- Implants themselves are not vascularized and so are excluded from the anti-infective benefits of circulation including tissue oxygenation and waste disposal
- Devices are sequestered from host immunity and antibiotic chemotherapy
- Implants are surgically inserted and associated with breach in host protective barriers such as skin and mucous membranes leaving them vulnerable to contamination
- Implants are usually associated with a wound when first implanted and are exposed to bacterial contamination if the insertion wound itself becomes infected
- Implants are subject to sterilization and sterile storage and handling techniques which may fail
- Implanted medical devices are more often used in immune-compromised or critically ill patients who are more susceptible to infection or may already harbor infection and seed devices (Darouiche, 2001)
- Implanted medical devices are more common in patients on long term antibiotics and this chemical stimulus may push bacteria into a sessile, protected biofilm state

Development of device-related infections begins with colonization of the foreign material, followed by a complex metamorphosis by the microorganisms with resultant biofilm formation (Vinh and Embil, 2005). Once bacteria contact a foreign body, the cells must adhere to exposed implant surface long enough to become irreversibly attached. Cell attachment is dependent on multiple factors including number and type of bacteria, liquid flow and shear around the device and the physical and chemical qualities of the implant surface (Donlan and Costerton, 2002). Irreversibly attached cells then go on to produce exopolysaccharide matrix and formally evolve into biofilm. Biofilm matures over time and maturation is dependent upon bacterial content and communication within biofilm, fluid flow, nutrient composition, pH and oxygen concentration, antimicrobial drug exposure and ambient temperature (Donlan, 2001).

Table 2.14: Examples of confirmed medical implant biofilm infections

Medical implant	Organism(s)	Reference(s)
gastrointestinal		
PEG feeding tubes	Facultative anaerobes	(O'May et al., 2005)
biliary stents	Poly-microbial	(Speer et al., 1988, Swidsinski et al., 2005)
peritoneal dialysis catheters	*S.aureus and S.epidermidis*	(Finkelstein et al., 2002, Dasgupta, 2002)
hernia mesh	*Staphylococcus spp.* including MRSA, *Streptococcus spp.,* gram negatives and anaerobes	(Taylor and O'Dwyer, 1999, Cobbs et al., 2003)
respiratory		
endotracheal tubes	*Streptococci*, *Prevotella* and *Neisseria*	(Perkins et al., 2010)
tracheostomy tubes	*P.aeruginosa and S.epidermidis*	(Jarrett et al., 2002)

Continued:

Medical implant	Organism(s)	Reference(s)
cardiovascular		
central venous catheters	coagulase negative *Staphylococci*	(Safdar and Maki, 2004)
mechanical cardiac valves	*S.epidermidis, S.aureus, Streptococcus spp.,* gram negatives, diptheroids, *Enterococci*	(Donlan, 2001)
ventricular assist devices	*S.aureus and S.epidermidis*	(Toba et al., 2011)
pacemakers	coagulase negative *Staphylococci*	(Marrie and Costerton, 1984)
defibrillators	coagulase negative *Staphylococci* and *S.aureus*	(Baddour et al., 2003)
intra-aortic balloon pumps	coagulase negative *Staphylococci* and *S.aureus*	(Baddour et al., 2003)
coronary stents	*S.aureus and P.aeruginosa*	(Gray and Baddour, 2002)
vascular prostheses and peripheral vascular stents	*Staphylococci*	(Selan et al., 2002, Antonois and Baddour, 2004)
arterio-venous shunts	*Staphylococci*	(Nassar and Ayus, 2001)
vascular access ports	poly-microbial	(Reed et al., 1986)
neurological		
ventricular shunts	coagulase negative *Staphylococci*, *Corynebacterium spp, Streptococci, Enterococci* and *E.coli*	(Fux et al., 2006)
neurological stimulators	*S.aureus*	(Air et al., 2009)

Continued:

Medical implant	Organism(s)	Reference(s)
musculo-skeletal		
arthro-prostheses	*Staphylococci, Streptococci, Enterococci, Enterobacteriaceae*, non-fermenters, anaerobes and mixed infection	(Trampuz and Zimmerli, 2005)
fracture fixations devices	*Staphylococci*	(Trampuz and Zimmerli, 2006)
urological		
penile implants	*Staphylococci* and gram negative enteric bacteria	(Carson, 1999, Abouassaly et al., 2004)
artificial urinary sphincters	*S.epidermidis*	(Licht et al., 1995)
ureteric stents	*E.coli, P.aeruginosa, E.faecalis, S.aureus*	(Reid et al., 2001)
indwelling urinary catheters	Endogenous gastrointestinal organisms, *P.mirabilis*	(Tenke et al., 2011)
nephrostomy tube	poly-microbial including vancomycin resistant *S.aureus*	(Weigel et al., 2007)
gynecological		
IUCD	poly-microbial and multiple anaerobic and aerobic bacteria implicated	(Pal et al., 2005)
breast and endocrine		
breast prostheses	*S.epidermidis*	(Pajkos et al., 2003)
ear, nose and throat		
tympanostomy tubes	*P.aeruginosa*	(Oxley et al., 2007)
cochlear implants	*S.aureus*	(Pawlowski et al., 2005)
voice prostheses	*Rothia dentocariosa*	(Elving et al., 2002)

Continued:

Medical implant	Organism(s)	Reference(s)
ocular		
intra-ocular lens	*S.epidermidis, E.faecalis*	(Okajima et al., 2006, Kobayakawa et al., 2005)
contact lens	*P.aeruginosa, S.marcescens,* coagulase negative *Staphylococci*	(McLaughlin-Borlace et al., 1998)
scleral buckle	*Staphylococci*	(Zegans et al., 2002)
dental and maxillofacial		
dental implants	poly-microbial	(Elter et al., 2008)
frontal sinus stents	*S.aureus*	(Perloff and Palmer, 2004)
general		
suture material	*S,aureus, E.faecalis*	(Henry-Stanley et al., 2010)

2.10.3 Biofilm culture in the laboratory

Deliberate culture of biofilm poses unique challenges in the laboratory environment and laboratory growth of biofilm is still in its infancy. Artificial biofilm is difficult and laborious to culture and necessitates very different techniques to culturing planktonic bacteria. Tight parameter control is needed to force equilibrium from planktonic toward biofilm formation and ideal culture parameters vary between bacterial species and in many cases are not well elucidated. As well as nutrient, oxygenation and temperature control, quality biofilm requires consistent application of fluid forces such as shear and drag to the culture sample. With such stringent and varied production requirements it is not surprising that standard planktonic techniques fail to culture biofilm and that biofilm is challenging to culture overall.

Once cultured, biofilm is difficult to standardize. A daunting challenge of biofilm research is comparing experimental results produced by multiple laboratories employing different techniques to generate, analyze, and interpret biofilm data (Ramey and Parsek, 2005). Difficulties arise because of the inherently heterogeneous nature of biofilm architecture, limitations in mass transfer and nutrient exchange. Because cells are sequestered within matrix, they are also difficult to measure. As an example, simple concentration based analysis techniques used in planktonic assessment are not valid for biofilm. A lack of standardization and measurement difficulties results in wide variability in biofilm culturing systems and culture output. So far there is only one standard method for growing biofilms (E-2196-02). This standard is sanctioned by ASTM (American Society for Testing and Materials) and uses a rotating disc reactor (Hall-Stoodley and Costerton, 2004). Lack of standardization therefore compounds complexity of biofilm manufacture.

Approaches to laboratory based biofilm cultivation are many and varied (Table 2.15) and include:

- batch-static systems that do not employ shear or continuous flow of bacteria or nutrient medium and simply provide a culture surface and un-refreshed culture medium (O'Toole and R., 1998)
- batch systems with introduced fluid shear without culture medium refreshment (Ceri et al., 1999) and

- flow cells and perfused biofilm fermenters that introduce continuous flow of renewed bacteria and culture medium (Hodgson et al., 1995).

All techniques provide a biofilm culture surface that can be removed and examined once it is colonized to assess biofilm formation.

The effect culture methods have on the diversity of biofilm microbial communities is not well understood (Stach and Burns, 2002). Biofilm culturing techniques therefore are often chosen according to the environment being simulated and the availability of resources and skills. Static models may be preferable to continuous flow models when examining early events in biofilm formation including initial bacterial adherence to surface and micro-colony formation (Merritt et al., 2005). Static generation methods are simple, high through put and can be executed with common laboratory equipment. Their draw backs include limitations in nutrient supply and an inability to reliably generate mature biofilms (Merritt et al., 2005).

Continuous flow systems are frequently utilized because of the advantages fluid forces provide when maturing biofilm. Examples of continuous flow cells include the Modified Robbins Device (Figure 2.10), CDC reactor and the annular reactor (Hall-Stoodley and Costerton, 2004). The drip flow biofilm reactor is a continuous flow device designed for study of biofilm grown under low shear conditions (Figure 2.9). The drip flow reactor is ideal for general biofilm studies, micro-sensor monitoring, biofilm cryo-sectioning samples, high biomass production, medical material evaluations and indwelling medical device testing (Goeres et al., 2009, Schwartz et al., 2010). One example of a drip flow set up is the Kadouri drip fed biofilm system (Merritt et al., 2005)(Figure 2.9). Using the Kadouri system, culture medium is constantly exchanged but shear forces are minimal. The Kadouri system has an added benefit because it can be constructed from standard laboratory equipment. “Off the shelf” drip flow reactors are also available commercially. Commercially available devices have the advantage of know effectiveness in culturing biofilm if implemented according to manufacturer instructions (Plate 2.4).

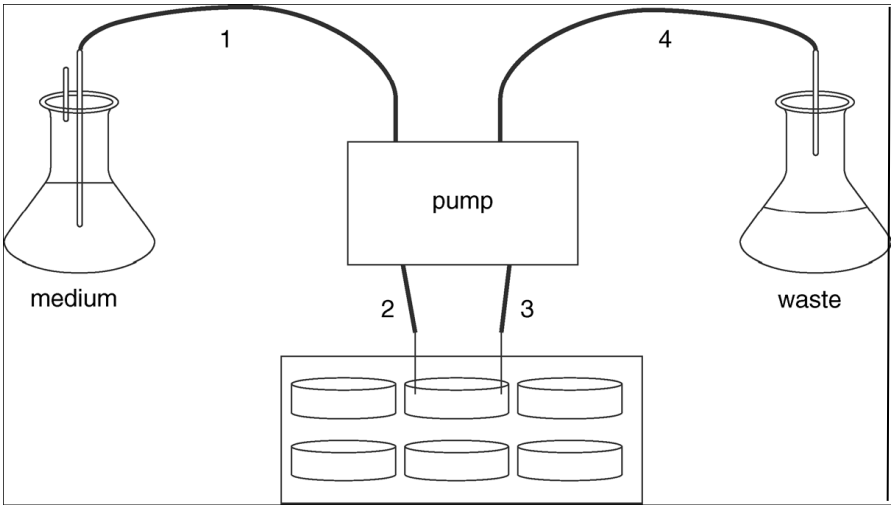


Figure 2.9: Diagram of Kadouri drip fed biofilm system (Merritt et al., 2005)
1 and 2: fresh medium is pumped onto a biofilm grown in a six plate well
3 and 4: planktonic bacteria and spent medium are removed through a needle placed on the other side of the well

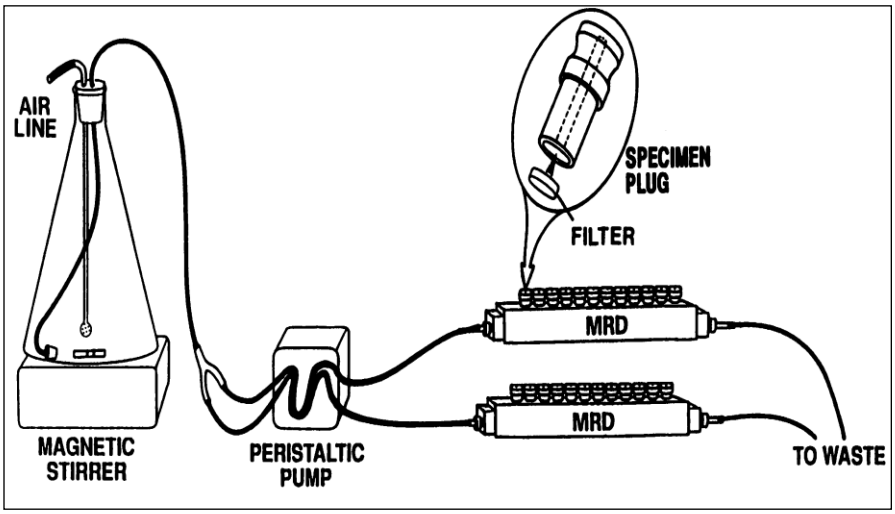


Figure 2.10: Diagram of Modified Robbins Device attached to a continuous flow circuit containing bacteria and culture medium (Ceri et al., 1999)

Plate 2.4: A commercially available drip flow reactor Model DFR 110 from BioSurface™ Technologies Corp. Reactors like this one provide sealed chambers with removable surfaces and use gravity to feed fluid through the chambers providing shear and drag

Table 2.15: Apparatus used for growing and testing biofilms (Donlan and Costerton, 2002)

Apparatus	Organism(s) evaluated	Flow dynamics	Substratum	Method of quantifying biofilm	Study
anaerobic incubator	*Streptococci, F.nucleatum, P.gingivalis, A.actinom.*	static	poly(styrene) wells	air dried biofilm mass absorbance at 492 nm and scraping with viable plate count	(Standar et al., 2010)
Modified Robbins Device	*P.pseudomallei*	batch/mixing	silastic discs	viable plate count	(Vorachit et al., 1993)

Continued:

Apparatus	Organism(s) evaluated	Flow dynamics	Substratum	Method of quantifying biofilm	Study
Calgary Biofilm Device	*P.aeruginosa, S.aureus, E.coli*	batch/mixing	plastic pegs	sonicate peg then viable plate count	(Ceri et al., 1999)
disc reactor	gram negative bacteria	batch/mixing	Teflon coupons	sonicate, vortex, homogenize then viable/direct count	(Donlan et al., 1999)
drip flow and rotating disc reactors	*S.aureus*	continuous flow	Teflon coupons	scraping and microscopic imaging	(Schwartz et al., 2010)
CDC biofilm reactor	gram negative bacteria	continuous flow	needless plastic connectors	sonicate, vortex, homogenize then viable/direct count	(Murga et al., 2001)
perfused biofilm fermentor	*Candida albicans*	continuous flow	cellulose-acetate filters	shake in sterile water then viable plate count	(Ballie and Douglas, 1998)
model bladder	gram negative bacteria	continuous flow	urinary catheters	scanning or transmission electron microscopy	(Stickler et al., 1999)
static incubator, orbital shaker and manual medium renewal every 12 hours	*P. fluorescens*	static, batch/mixing and continuous flow	stainless steel slides	vortex then viable plate count	(Sillankorva et al., 2008)
continuous flow stirred tank reactor	aerobic sewage sludge	continuous flow	tubular reactor	dry weight and epifluorescence	(Turakhia et al., 1983)

2.10.4 Biofilm examination, measurement and imaging

2.10.4.1 Introduction

Today a vast range of tools and equipment exist to support examination, measurement and imaging of biofilm and the information they provide is often complimentary. Some tests aim simply to confirm the presence of biofilm whilst others aim to expose detail of structure and function. Analysis ranges from expensive technology based solutions to simple manual approaches. Regardless, most common modalities for biofilm analysis can be grouped into high resolution imaging and microscopy, molecular studies or direct enumeration techniques. Direct enumeration techniques rely on removal of biofilm organisms from matrix by mechanical force prior to application of traditional bacterial culture methods. A plethora of experimental approaches to biofilm examination, measurement and imaging appear in the literature and a selection of these will be discussed briefly.

2.10.4.2 High resolution imaging and microscopy

High resolution imaging and microscopy provide data on biofilm micro and ultra-structure and include techniques such as scanning and transmission electron microscopy, atomic force microscopy and confocal scanning laser microscopy as well as standard light and epifluorescence microscopy.

Scanning electron microscope (SEM) employs a focused beam of high-energy electrons to generate signals at the surface of solid specimens. The signals provide information on surface topography, chemical composition, crystalline structure and orientation of materials making up the sample. A two dimensional image is generated that displays spatial variations in these properties with magnification ranging from 20x to 30 000x and resolution of 50-100 nm (Swapp, 2011). Conventional SEM techniques have been employed for the examination and characterization of biofilms on medical devices and in human infections (Ferguson et al., 1986, Raad et al., 1993). The main advantage of SEM is its high resolution capability allowing for detailed study of biofilm architecture. Unfortunately SEM distorts biofilm surface configuration (Flemming, 1999). This is due to desiccation of the usually highly hydrated biofilm matrix caused by graded solvents used in SEM specimen preparation. Specimen drying is needed because water of hydration is not compatible with the vacuum through which electron beams must travel in SEM. The result is an artifact where extracellular matrix appears stringy and fibrous instead of thick and gelatinous.

One way around the drying artifact of standard SEM is Environmental SEM (ESEM). ESEM has high resolution capabilities of standard SEM without needing a vacuum. By using specialized electron detectors and a differential pumping system, the electron beam used to create ESEM images can pass from a vacuum to a pressurized specimen chamber. Images are obtained at pressures between 0.1-50 Torr (Little et al., 1991). Vapor pressure can therefore be adjusted to maintain fully hydrated biofilm. Because biofilm architecture is preserved, ESEM is very useful when investigating three dimensional construct of saturated biofilm.

Transmission electron microscopes (TEM) study morphology of bacterial biofilms on an individual cellular or molecular level (Nickel et al., 1989). TEM operates on the same principles as light microscopy but instead of light, a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen on route to create an image. Because of the small de Broglie wavelength of electrons, TEM produces high resolution pictures revealing fine detail within a specimen even as small as a column of atoms. TEM therefore demonstrates items tens of thousands of times smaller than the smallest resolvable object in a light microscope. TEM combined with polysaccharide stains like ruthenium red has played a key role in unravelling the nature and componentry of extracellular biofilm matrix and its relationship to cells (Kokare et al., 2009). This is a major step forward in determining the spatial arrangement of microorganisms in biofilms (Reese and Guggenheim, 2007). TEM has for example differentiated “fibrilar” and “fluffy” appearing exopolysaccharide associated with various *Streptococcal* species and using TEM it is possible to directly visualize spatial patterns of antibiotic action within biofilm (Zahller and Stewart, 2002, Reese and Guggenheim, 2007). TEM is without doubt a useful modality to investigate biofilm however it is a time consuming and complex technique with some prominent limitations. Biological specimens must be held at liquid nitrogen temperatures or be dehydrated and embedded in acrylic resin to withstand a vacuum operating environment and prerequisite ultrathin sectioning (Hannig et al., 2010). In addition specimens cannot be greater that one micrometer thick or electron transparency is lost and staining with toxic heavy element contrast agents such as lead citrate or uranyl acetate is often required (Hannig et al., 2010).

Atomic force microscope (AFM) is an important tool for studying biofilm because it can image surfaces submerged under liquids and it is the only modality that provides biofilm structural, mechanical, and functional information at high resolution (Trache and Merininger, 2008). AFM operates on a different principle than other forms of microscopy, such as optical microscopy or electron microscopy and has the great

advantage of not requiring vacuum conditions. AFM detects deflections of a cantilever tip caused by physical interaction of the tip with molecules on the cell surface. In addition to structural imaging, AFM can therefore determine properties such as cohesive strength within biofilm (Ahimou et al., 2007). Adhesive properties of *S.epidermidis* biofilm have been gleaned via *in situ* AFM and physiochemical characteristic such as hydrophobicity and surface charge in biofilm have also been assessed with AFM (Harimawan et al., 2011, Hu et al., 2011). Forces as small as a few pico-Newtons can be detected and probed with AFM (Lower, 2011). The cantilever tip can image live cells with atomic resolution and probe single molecular events in living cells under physiological conditions (Trache and Merininger, 2008).

Confocal laser scanning microscopy (CLSM) allows thick, hydrated biofilms with structural integrity to be examined at varied depth and without blur, although at lower magnification when compared to SEM (Donlan and Costerton, 2002). Confocal microscopy is an optical imaging technique that builds on epifluorescence microscopy to enhance image contrast. It obtains highly sharpened images by eliminating out of focus light via a spatial pinhole. Fluorescence is detected by a photomultiplier tube and a digital image produced. Three dimensional pictures of biofilm are built by aggregating multiple, thin cross sectional images of the specimen (Semwogerere and Weeks, 2005). Fully hydrated and live biofilm analysis can be performed using this relatively noninvasive and nondestructive technique (Morato et al., 2004).

As with standard epifluorescence microscopy, CLSM requires fluorescent staining of microbes or biofilm components and many different dyes/fluorophores are available for this purpose (Table 2.16). Dyes are chosen based on cost, toxicity, difficulty of application, excitation-emission requirements and according to the processes or structures of interest. Bacterial biofilms can be challenging to dye because they are structurally, metabolically and chemically heterogeneous, exist in various physiological states and may have undefined components (Pitts, 2007). Dyes may have difficulty penetrating matrix thicker than 25 µm although this problem is somewhat overcome by cryo-embedding specimens (Semwogerere and Weeks, 2005). Used in combination with fluorescent molecular probes CSLM can provide information on cell morphology, metabolism and phylogeny in parallel with information on biofilm structure and heterogeneity (Semwogerere and Weeks, 2005). CSLM is a therefore a versatile, applicable and suitable technology for biofilm analysis.

Standard light and epifluorescence microscopy were forerunners to the above modalities and although the techniques are simple, available and inexpensive they

have much less to offer than high resolution imaging solutions described above and will not be discussed further.

Table 2.16 Fluorochromes used for direct staining of biofilms

Stain	Application	Excitation/emission (nm)	Reference
crystal violet	bacterial stain	514/>570	(O'Toole and Kolter, 1998)
FITC	binds to proteins	490/520	(Kokare et al., 2009)
RITC	binds to proteins	575/595	(Kokare et al., 2009)
TRITC	binds to proteins	541/572	(Kokare et al., 2009)
Fluo-3	calcium indicator	506/526	(Kokare et al., 2009)
calcein red-orange AM	esterase activity	400/452	(Pitts, 2007)
calcein violet	esterase activity	400/452	(Pitts, 2007)
CellTrace™ violet	esterase activity	405/450	(Invitrogen, 2011)
fluroscein diacetate	esterase activity	495/520	(Kokare et al., 2009)
rhodamine 123	mitochondria in living cells	505/560	(Johnson et al., 1980)
acridine orange	nucleic acid stain	503/530-640	(Morato et al., 2004)
CTC/dapi	nucleic acid stain	365/602 347/456	(Morato et al., 2004)
hexidium iodide	nucleic acid stain	518/600	(Evans, 2000)
Live/Dead Syto 9 Propidium iodide	DNA and RNA intercalating agents in cells with damaged cell membranes	480/500 490/635	(Morato et al., 2004) (Semwogerere and Weeks, 2005)
Hoescht	stains DNA	362/470	(Kokare et al., 2009)
fluoroscein	pH indicator (negative staining)	490/520	(Kokare et al., 2009)
NCECF	pH indicator	500/530 or 620	(Kokare et al., 2009)

2.10.4.3 Molecular studies

Molecular microbiology affords new insight into biofilm because it greatly enhances detection of microbial components within biofilm or derived from biofilm. Polymerase chain reaction, *in situ* hybridization techniques and various staining methods with or without epifluorescence microscopy assess cellular components, genotypic features, function and viability of cells and identify specific organisms within biofilm. Enzyme linked immune-sorbent assays to detect antibodies against biofilm-specific *Staphylococcal* epitopes have confirmed colonization of vascular grafts where conventional culture techniques have failed (Costerton et al., 2003). Intercellular signaling molecules unique to the biofilm state such as those involved in quorum sensing or genetic assays to differentiate phenotypic states offer the prospect of noninvasive diagnostic tools to confirm the presence of biofilm via remote sampling of physiological fluids. A limulus lysate assay has been used to quantify β-1,3 glucan in the serum of rats to detect intravascular catheter biofilm infection and increased transcription of regulatory RNA molecules has been detected in *Staphylococci* demonstrating extracellular pathogenicity associated with biofilm formation (Shirtliff et al., 2002, Nett et al., 2007). Genetic bio-signatures developed using whole genome analysis of biofilm forming organisms may play a role in characterizing biofilm in the future.

2.10.4.4 Enumeration

Enumeration techniques generally rely on removal of biofilm bacteria from matrix using some type of mechanical force, such as vortexing or sonication, prior to examination and measurement of organisms (Appendix 2A). Such techniques are commonly employed to quantify biofilm because the tests are simple and can be achieved with standard laboratory equipment and little specialized training. Regardless of approach, a determination of the recovery efficiency of the method (i.e., the percentage of cells actually recovered from the biofilm) is needed to authenticate each test. *In situ* procedures that do not require removal of implanted medical devices provide a distinct advantage in the clinical setting.

Frequently described procedures include the following:

- Viable plate count in which suspended and dispersed biofilm cells are plated onto a solid microbiological medium, incubated and counted in the standard way as for planktonic bacteria

- The Maki roll (roll-plate) semi-quantitative culture technique was developed for detection of intravenous catheter biofilm contamination. In this test a segment of involved catheter is rolled across non selective blood agar. Catheter infection is defined as > 15 CFU (Maki et al., 1977)
- Raad et al. attempted to enhance biofilm quantification by using sonication plus vortexing of catheter tips prior to instituting the Maki roll plate method and found a level of 10^4 CFU per tip was predictive of a catheter-related sepsis (Raad et al., 1992)
- Anaissie et al. studied intravenous catheter related biofilms by comparing Maki roll-plate with or without sonication and scanning electron microscope methods. The study defined catheter colonization as ≥15 CFU/tip by the roll-plate technique or ≥100 CFU/tip with the sonication technique. Biofilm surface area was measured by SEM (Anaissie et al., 1995)
- Air Liquid Interface Assays allow live biofilm bacteria existing along a meniscus to be inspected without dyes using phase-contrast or conventional microscopy (Merritt et al., 2005, Zuffrey et al., 1988)
- As part of the Microtitre Plate Biofilm Assay (a tissue culture plate method), biofilm cells are stained with a dye such as crystal violet. The surface associated dye can be solubilized for semi-quantitative assessment of the biofilm using optical density measurements (Merritt et al., 2005, Mathur et al., 2006). Tissue culture plate methods are considered the gold standard basic biofilm enumeration technique.

2.10.4.5 Other biofilm examination, measurement and imaging techniques

As understanding of biofilm grows, the ability to analyse biofilm expands. The literature is replete with novel ideas and techniques. X-ray computed microtomography is a new approach producing three dimensional images of biofilm using X-rays and the X-ray contrast agents barium sulphate and potassium iodide (Davit et al., 2011). Magnetic resonance microscopy has shown spatial assembly and heterogeneity of *Staphylococcus epidermidis* biofilm and the impact on transport dynamics within biofilm (Seymour et al., 2004). Neither of these imaging modalities is widely adopted. Another novel investigation involved miniaturized micro-sensors to characterise physical and chemical microenvironment within biofilm and thus provide data about regional metabolism (Schramm, 2003). Similarly microelectrodes have shown parameters such as pH and dissolved oxygen concentration vary radically between locations as close as 50 µm within a biofilm and may correlate with physiological status and ecology of

microbial communities (Cunningham et al., 2011). Many other approaches to biofilm examination have been proposed including total protein content determination, light absorbance at a fixed wavelength and endotoxin production (Rioufol et al., 1999, Mittelman et al., 1992, Standar et al., 2010). All remain experimental.

2.10.5 Antibiofilm approaches found in nature

Biofilms are ubiquitous on surfaces in the natural environment, for example in the ocean, living surfaces such as seaweeds, corals and fish deal with biofilms originating from millions of microorganisms present in seawater and generally remain unharmed (Kjelleberg and Steinberg, 2001). Natural and traditional medicine advocates have long espoused the anti-infective properties of animal and plant based substances and extracts including herbal remedies and essential oil based therapies but in 1995 Abaruzua and Jakubowski scientifically defined classes of natural substances with potent anti-biofilm activity (Abarzua and Jakubowski, 1995). These included fatty acids, glycolipids, terpenes, terpenoids, phenols, lactones and steroids (Abarzua and Jakubowski, 1995). Natural biofilm resistance strategies make sense as plants and animals must have evolved to defend themselves against the negative consequences of biofilm. These natural combative strategies offer guidance to the development of therapies against human biofilm device infections (Armstrong et al., 2000).

2.10.5.1 Animal derivatives

Animal derived immune proteins have been investigated for anti-biofilm effects. Lactoferrin is a cationic protein found in secretory fluids such as milk, saliva, tears, glandular and nasal secretions, is part of innate immunity and has known bactericidal activity (Shetty, 2006). Such as protein is a good candidate for anti-biofilm properties and indeed Di Mario et al. showed lactoferrin had synergy with antibiotics in eradicating *Helicobacter pylori* bacterial biofilm (Di Mario et al., 2003). Similar synergism was demonstrated when tear derived lactoferrin and the antibiotic vancomycin were employed against *S.epidermidis* intra-ocular and contact lens biofilm infections (Leitch and Willcox, 1999). Another innate immunity protein LL-37 expressed in sino-nasal mucosa had *in vivo* efficacy against preformed *Pseudomonas aeruginosa* biofilms derived from chronic sinusitis specimens in rabbits (Chennupati et al., 2009). As well as known components of the innate immune system, unique proteins found in animals that avoid bio-fouling have been eagerly sought. Distinctin is a heterodimeric antimicrobial compound consisting of two peptide chains linked by a disulfide bond and is made by the skin granular glands of the tree frog *Phyllomedusa distincta*. When

distinctin is used to pretreat central venous catheters, *S,aureus* biofilms become much more vulnerable to standard antibiotic therapy, bacterial load is significantly decreased and bacteremia is avoided (Giacometti et al., 2007). Know components of the immune system and unique animal derived proteins hold promise in developing anti-biofilm treatments.

Honey is a natural, nontoxic, and inexpensive product. Honey and in particular medical grade Manuka honey (produced by bees dining on the nectar of flowers from *Leptospermum scoparium*) is also anti-biofilm. Cooper et al. tested methicillin-resistant *Staphylococcus aureus* and *Enterococci* isolated from infected chronic wound biofilms and found both bacteria were susceptible to Manuka and pasture honey at low doses (Cooper et al., 2002). *In vitro* experiments have also proven the ability of honey to eradicate *Pseudomonas aeruginosa* biofilms cultured from chronic rhino-sinusitis specimens in over 90% of cases, which is a better result than can be obtained by single agent anti-*Pseudomonal* chemotherapy (Alandejani et al., 2009). The active anti-biofilm ingredients in honey are yet to be confirmed but may include phenols, peroxides, antibacterial bee peptides and the antibacterial β-triketone leptospermone (Weston et al., 2000). In addition, honey may possess anti-inflammatory and immune stimulatory properties (Weston et al., 2000).

Marine animals are constantly challenged with bio-fouling because of relentless exposure to bacterial laden aqueous environment. Some animals such as dolphins have evolved structural surfaces that dissuade biofilm. Dolphins have a rough tegument that is smoothed by a shear resistant gel overlying the epidermis and low shear environments are known to deter biofilm (Bienen, 2004). Sponges have enlisted a different approach. Certain families of sponge produce sesquiterpenes, diterpenes and isocyanoterpenoids that resist bio-fouling by inhibiting surface adherence of bacteria at concentrations less than 0.1 µg/ml (Kjelleberg and Steinberg, 2001). Sponges also produce small molecules called 2-amino-imidazoles that are known to disrupt and disperse biofilms and augment the effect of antibiotics against biofilm without being toxic to human cells (Rogers et al., 2010). Japanese soft coral *Dendronephthya spp*. rebuff contaminants with fatty acids and are not fouled (Fusetani, 2003). Looking further into anti-biofilm abilities of marine creatures, the exoskeletons of crabs and shrimp are made of chitin and deter biofilm. Chitosan (produced by the deacetylation of chitin) combined with ammonium salt resisted titanium orthopedic implant infection with *Staphylococcus epidermidis* and *Staphylococcus aureus* biofilms

by inhibiting expression of genes that controlled production of extracellular matrix (Peng et al., 2011). Interestingly, chitosan enhances effects of toxic photodynamic therapies on a wide range of gram positive and gram negative bacteria (Tsai et al., 2011).

2.10.5.2 Plant derivatives

Plant derivatives have been pursued extensively to determine how plant life avoids harmful biofilm related infections. Plants and their derivatives including herbs and spices, essential oils, vegetables, fruit, nuts, grains and other plant stuffs successfully employ anti-biofilm approaches. Many plants use techniques such as natural analogues of bacterial signaling molecules to inhibit or encourage bacterial colonization and biofilm formation on their surfaces (Marks and Neill, 2007, Heurlier et al., 2006). In many cases the observation that a particular plant inhibits biofilm has been made but the method of inhibition remains elusive.

Spices are aromatic or pungent plant substances used to flavor foods or beverages. Spices come from the bark, root, buds, seeds, berry or fruit of tropical plants and trees. Several root derived spices have proven anti-biofilm effects. Baicalein is a flavone isolated from the roots of *Scutellaria baicalensis* and is used widely in traditional Chinese medicine. Zeng et al. found Baicalein interferes with effective quorum sensing in initiation of *E.coli* and *P.aeruginosa* biofilm (Zeng et al., 2008). Another root spice used in traditional Chinese medicine is ginseng. Ginseng has shown *in vitro* and *in vivo* efficacy against *P.aeruginosa*. Wu et al. found ginseng reduces *P.aeruginosa* bacterial swarming and leads to reduced biofilm formation and destruction of mature biofilm (Wu et al., 2011). Subcutaneous administration of aqueous ginseng solution is also associated with attenuated *P.aeruginosa* chronic lung biofilm infections in the rat model probably due to activation of polymorphonuclear immune cells (Song et al., 1998). A third root spice derivative effective against biofilm is curcumin: the principal curcuminoid of the popular Indian spice turmeric, which is a member of the ginger family (*Zingiberaceae*). *Helicobacter pylori* is a bacteria that causes peptic ulcer disease and gastric cancer. Pattiyathanee et al. investigated the effects of curcumin on *H.pylori* biofilm and showed curcumin reduced extracellular matrix production and decreased the ability of *H.pylori* to adhere to HEp-2 cells (Pattiyathanee et al., 2009). The group went on to suggest the use of curcumin as a complementary therapy for treatment of *H.pylori* biofilm related gastric infections.

Spices and chemicals that originate from barks have demonstrated anti-biofilm properties. Topical oak bark ointment successfully prevents methicillin resistant *S.aureus* biofilm infection of burns wounds in the porcine model and aids in hastening wound healing (Davis and Mertz, 2008). This finding is particularly important as infection is a predominant cause of burns associated morbidity and resistant bacterial infections are becoming more common. Salicylate is another bark derived substance. Salicylic acid is a phenolic phytohormone found in willow bark. Salicylate-based poly(anhydride esters) inhibit biofilm formation by *Salmonella enterica serovar Typhimurium* , possibly by affecting surface attachment (Rosenberg et al., 2008).

An essential oil is a plant derived, concentrated, hydrophobic liquid containing volatile, aromatic compounds. A range of essential oils are effective as biofilm therapies. Dental plaque is a good example of biofilm that can be eradicated by an essential oil. The clove oil component eugenol penetrates dental plaque and de-bulks established biofilm more effectively than stannous fluorides or triclosan copolymer antiplaque agents contained in commercial mouthwash (Gera, 2008). In an artificial mouth model, Li et al. also showed the powerful inhibitory effect of eugenol on caries formation associated with *Streptococcus sobrinus* (Li et al., 2011).

Tea tree oil is an essential oil known for its antimicrobial and antibiofilm effects but how it acts is yet to be determined. The essential oil may have evolved to reduce biofilm effect on the higher plant *M. alternifolia*. Constituents of TTO include terpenes and terpinoids, natural chemicals that resist bio-fouling (Abarzua and Jakubowski, 1995). Brady et al. produced a *S.aureus* biofilm from an infected cochlear implant and found a five percent solution of tea tree oil completely eradicated the biofilm following exposure for one hour (Brady et al., 2010). Kwieciński et al. looked further into the effect of tea tree oil on *S.aureus* biofilm and found the minimum biofilm eradication concentration was usually two times the minimum planktonic killing concentration, yet was never higher than one percent v/v (Kwieciński et al., 2009). Tea tree oil is therefore an essential oil that can eliminate biofilm of bacteria responsible for a large proportion of implant infections and is a candidate for clinical anti-biofilm applications.

Cinnamon oil and the organic compound cinnamaldehyde (that gives cinnamon its characteristic flavor and odor) detach and kill existing biofilms commonly associated with *Staphylococcal* medical implant infection. Nuryastuti et al. demonstrated the antibiofilm activity of two percent cinnamon oil on *S.epidermidis* strains and used confocal microscopy to confirm biofilm detachment and cell killing (Nuryastuti et al.,

2009). Methicillin resistant *Staphylococci* are also vulnerable to cinnamon oil. Jia et al. used scanning electron microscopy and confocal microscopy to show cinnamaldehyde detached and killed resistant *Staphylococci* and real time PCR to prove cinamaldehyde also reduced the expression of *sarA,* a biofilm associated gene (Jia et al., 2011). *Staphylococcal* biofilms have also been eradicated by the essential oil of oregano (*Origanum onites*) (Bilge Oral et al., 2010). As cinnamon and oregano are used in food stuffs, their antibiofilm activity may contribute to the anti-spoiling action commonly attributed to many spices and essential oils.

Many edible plants and their produce mount successful anti-biofilm campaigns. Higher plants including pea, soybean, rice, tomato, crown vetch and *Medicago truncatula* (a legume related to alfalfa) secrete substances that manipulate bacterial N-acyl homoserine lactone receptors and prevent biofilm contamination by interfering with quorum sensing (Bauer and Robinson, 2002). Naturally occurring furocoumarins found in grapefruit juice inhibit auto-inducer signaling between a broad variety of bacteria and stymie biofilm production *in vitro* although the anti-biofilm effects of drinking grapefruit juice are unproven (Girennavar et al., 2008). Even cashew shells produce phenolic lipids that resist bacterial cell adhesion and biofilm formation (Chelikani and Dong Shik, 2006). There is much to be gleaned from how vegetables, fruits and nuts avoid biofilm soiling.

Many other examples of plants that fight biofilm exist. *Croton nepetaefolius* is a native plant from Brazil that contains alkaloids, diterpenes and triterpenes effective against biofilm (Carneiro et al., 2010). The constituent triterpene asiatic acid found in the plant *Centella asiatica* (commonly called Gotu Kola) has antibiofilm activity as does corosolic acid found in the plant *Lagerstroemia speciosa* (Garo et al., 2007). The flowering plant *Rhodomyrtus tomentosa* native to southern and southeastern Asia, prevents *Staphylococcal* biofilm formation and kills mature biofilm in a dose dependent manner (Saising et al., 2011). The widespread adoption of antibiofilm strategies in the plant world explains the success of plants in avoiding infection.

2.10.5.3 Combinations of animal and plant derivatives

Compound traditional Chinese herbal medicine has claimed to successfully treat a variety of human infections we now realize are due to biofilm. TanReQing is a Chinese medicine made up of *Scutellaria* (a genus of flowering plants in the mint family *Lamiaceae*), bear gall powder, cornu *caprae hircus* (powdered goat horn), honeysuckle

and fructus. Traditionally TanReQing was used to treat upper respiratory tract infections. Wang et al. used imaging techniques to determine TanReQing not only inhibited formation of *Staphylococcus aureus* biofilm, but also killed viable cells embed in matrix (Wang et al., 2011). The mechanism of TanReQing against biofilm remains unknown.

2.10.5.4 Algal derivatives

Even algae have developed ways to avoid biofilm contamination. Fusetani noticed the Australian red alga *Delisea pulchra* remained free of biofilm surface colonization and went on to show the anti-biofouling effect was due to algal halogenated furanones (Fusetani, 2003). *D.pulchra* algal metabolites are structurally related to the bacterial signaling molecules acylated homoserine lactones that initiate biofilm formation. Furanones may act as specific antagonists of bacterial regulatory systems and thus protect algae from the negative consequences of biofilm contamination (Bauer and Robinson, 2002).

2.10.5.5 Bacterial derivatives

Bacteria in nature have developed ways of eliminating biofilm. Bacteria can disturb quorum sensing in other nearby bacteria, release enzymes that damage biofilm and impede competitive biofilm formation. Lysostaphin is an example of how bacteria sabotage biofilm of competitor bacteria. Lysostaphin is an endopeptidase that cleaves pentaglycin bridges in the cell wall of all *Staphylococci* and is produced by *Staphylococcus simulans*. Surprisingly, lysostaphin kills *S. aureus* and *S. epidermidis* in biofilms and disrupts their matrix at concentrations as low as 1 µg/ml even when high doses of anti-*Staphylococcal* antibiotics have no effect on established biofilm (Wu et al., 2003). Unfortunately lysostaphin only works on sensitive *Staphylococcal* species (Wu et al., 2003). Bacterial antibiofilm products are an area of future research with value in developing new antibiofilm approaches.

2.10.6 Biofilm intervention strategies in the medical implant setting

2.10.6.1 Introduction

Two strategies to intervene against medical implant related biofilm exist: prevention of biofilm formation and elimination of developed biofilm. Interventions aimed at planktonic bacteria are less successful against biofilm because of evolved structural, phenotypic and metabolic tactics biofilm employs to survive adverse environmental pressures (Hammer et al., 2005). Because of inherent resistance to standard antimicrobial approaches, new antibiofilm techniques need to be deployed to address and avoid medical implant infection. Some approaches expand upon standard antibiotic and surgical techniques whilst others are completely novel. Combination attack on biofilm with different antimicrobial vectors is also coming to the fore. As understanding of biofilm triggers and biofilm development improves, steps in these processes will become specific targets in biofilm prevention and eradication. Although the scientific community has many ideas on biofilm attenuation and eradication strategies much work must be done before definitive solutions are reached.

Intervention strategies currently employed for biofilm control will either

(i) optimize host immune response
(ii) prevent initial device contamination
(iii) minimize initial microbial cell attachment to the device
(iv) penetrate the biofilm matrix and kill the biofilm-associated cells, or
(v) remove the infected device (Donlan and Costerton, 2002).

Of course some anti-biofilm attacks act on more than one level. Biofilm prevention is optimal. Failing that, eradication of established biofilm is targeted. Established biofilm is either immature or mature depending on structural complexity and extent of heterogeneous colony formation (Kjelleberg and Givskov, 2009). Immature and mature biofilm have different vulnerabilities. Intervention strategies can involve device modification or target the biofilm directly and can be systemically administered or locally applied (Donlan and Costerton, 2002).

2.10.6.2 General biofilm prevention strategies

General infection reduction strategies apply to biofilm prevention as much as to the prevention of planktonic infection and cannot be overemphasized in importance in biofilm avoidance. Prior to medical device implantation, host can be prepared to reduce infectious implant sequelae. Host immunity and nutrition can be optimized so as to support a more effective host response to infective challenges. Much can be achieved with relatively simple measures that align with overall patient care goals. Adequate intake of calories, proteins and essential fatty acids, micronutrients and vitamins is essential. Good oxygenation and perfusion of implanted tissue discourages infection and may necessitate supplemental oxygen and blood product transfusion. Immunosuppressive medications and interventions such as steroids, cancer chemotherapeutics and radiotherapy can be discontinued or tapered. Control of co-morbidities detrimental to host immunity such as diabetes mellitus should be obtained. Systemic sepsis is ideally resolved prior to device implantation. Finally immunization of host can improve response to some pathological microbes (Von Eiff et al., 2005).

Implantation technique and device handling strongly influence contamination opportunity for bacteria and subsequent biofilm formation. Manufacture specifications and expiry dates should be observed. Standard aseptic surgical technique should be employed with sterile device storage, preparation and insertion to prevent contamination at the outset. Disposable and single use implants reduce infection. Devices should not traverse an infected or contaminated field and perioperative, high-dose, systemic, prophylactic antibiotics should be administered at the time of implantation (Orlando Regional Medical Center, 2006). External device components are best avoided but if required should be handled aseptically and anatomically separated from internal components via tunneling or use of cuffs. Inline filtration of ingress fluids is another anti-infective strategy for implants such as central venous lines and ports. Choice of insertion site can reduce biofilm risk. An example is increased risk of infection of venous catheters sited in the lower extremity compared to upper extremity linked to increased infective phlebitis risk (Von Eiff et al., 2005). Probably the best anti-biofilm strategy is to remove medical implants once they are no longer required (Maki, 1994, Donlan and Costerton, 2002).

Permanence of many bio-implants highlights importance of biofilm prevention. Once an implanted device obtains a biofilm, anti-biofilm strategies are limited by host factors as

many effective *ex vivo* processes such as irradiation or autoclaving are not feasible in the living host (Habash and Reid, 1999). Permanently implanted devices are difficult to remove and removal is often morbid to the patient. It is clear that prevention of biofilm on medical implants will remain a cornerstone of therapy.

2.10.6.3 Specific biofilm prevention and interruption strategies

Assuming an optimized host and uncontaminated medical implant, the next point of intervention is to prevent development or eradicate biofilm on an *in situ* implant. Biofilm production is a sequence of events: a condition favors biofilm deposition, microbes approach and become reversibly then irreversibly attached to a surface, bacterial growth and colonization in initiated and lastly maturation and development of biofilm architecture occurs as genetic and phenol-typical alterations become apparent (Costerton et al., 2003). The whole process is a highly organized and dynamic evolution that requires complex communication between microbial cells. Derailment of any or all of these signals or processes provides opportunity to disrupt the orderly construction of biofilm (Costerton et al., 2003).

Antibiotics at concentrations of 10^2 to 10^4 times the mean inhibitory concentration of free swimming bacteria cause no killing effect on counterpart bacteria in biofilm communities (Tetz et al., 2009). Because biofilm is resistant to antibiotic strategies targeted against planktonic bacteria, different approaches to use of antibiotics are being investigated in the hopes of improving their success against biofilm. Strategies encompass development of new antibiotics or enhancement of old antibiotics to take effect against biofilm, delivery of increased dose or concentration of antibiotics locally at the site of biofilm and antibiotic combination therapies (including both synergistic antibiotic combinations and novel antibiotic-antibiofilm vector combinations). Regardless of approach, the basic tenants of accurate microbial identification and sensitivity testing still apply to biofilm. Accurate microbial identification may require enlistment of biofilm specific culture and imaging techniques (Section 2.9.3 Biofilm culture in the laboratory).

Prolonged, tailored, traditional, systemic antibiotics over several months may eradicate biofilm (especially immature biofilm) but more often suppresses infection as bacterial biofilms usually survive in the presence of systemic antibiotic concentrations (Costerton and Stewart, 1999). Protracted systemic antibiotics can therefore play an adjunct role in

management of biofilm where removal of an implant is unfavorable and attenuation of infection is required or once an infected implant has already been removed. Long term systemic antibiotics and in particular parenteral antibiotics do however have their draw backs because they are expensive, resource intensive, toxic to patients and limited in definitive potential to resolve biofilm implant infections. In addition chronic, sub-lethal doses of traditional antibiotics can precipitate biofilm development as part of the armory of microbial survival techniques. For example in a study by Rachid et al., sub-inhibitory levels of protein-synthesis inhibitors tetracycline and quinupristin-dalfopristin strongly induced the formation of biofilm by *S.epidermidis* (Rachid et al., 2000). Rationalization of antibiotic regimes and avoidance of chronic low dose protocols is therefore recommended in order to reduce the incidence of biofilm on implants.

Some bactericidal agents and antibiotic classes are simply more effective against bacterial biofilm and these agents should be preferentially employed when targeting biofilm on implants. Biocides such as peroxide and chlorhexidine interfere with the attachment and expansion of immature biofilms (Tenke et al., 2004). Wilson et al. compared the effectiveness of quaternary ammonium compounds against one day old biofilm of *Serratia marcescens*, *P.aeruginosa*, *S.epidermidis* and *S.pyogenes* and found three percent hydrogen peroxide was more effective than chlorhexidine-gluconate in inactivating bacterial biofilm organisms (Wilson et al., 1991). Certain subclasses of antibiotics such as transcription inhibitors are also inherently more effective against biofilm. Rifampicin is a transcription inhibitor that is among the most effective molecules for treating biofilm related infections and has led to investigation of other transcription inhibitors in the battle against biofilm (Gualtieri et al., 2006). It makes sense to use and investigate available antibiotic agents already known to work against biofilm.

Increased local concentration of traditional antibiotics may offer a way to counter biofilm resistance and improve antibiotic efficacy. For example the antibiotic moxifloxacin at MIC for planktonic *S.aureus* is ineffective against *S.aureus* biofilm however moxifloxacin at a dose 1000x MIC gives a 2 to 2.5 log reduction in number of viable biofilm bacteria *in vitro* (Desrosiers et al., 2007). Local delivery of antibiotics at the site of biofilm formation can be augmented in several ways. Topical application of antibiotics to medical implants by immersion in antibiotic solution, antibiotic ointment or coatings reduces biofilm occurrence in the short term (Smith, 2005). Veenstra et al. reviewed results of 13 different clinical

studies (2,830 catheters) in which antibiotic-impregnated central venous catheters were compared with untreated catheters and concluded impregnated catheters reduced incidence of catheter colonization and catheter-related blood stream infections (Veenstra et al., 1999). Unfortunately antibiotics or disinfectants applied to implants leach to surrounding tissue over time and not only become ineffective in preventing biofilm but may propagate resistant microbial strains (Lewis and Klibanov, 2005). Higher local antibiotic concentrations can also be attained by way of delivery. In a study by Adair et al. investigating ventilator associated pneumonia, nebulized gentamicin attained higher concentrations within the endotracheal tube lumen and was more effective in preventing biofilm airway infection than parenterally administered gentamicin or cephalosporin (Adair et al., 2002).

Antibiotic combinations are synergistic against biofilm and sometimes work when single agents are completely ineffective. A study by Anwar and Costerton compared the effectiveness of the antibiotics piperacillin and tobramycin alone or in combination against planktonic, immature and mature biofilm variants of *P.aeruginosa* (Anwar and Costerton, 1990). Planktonic cells were eradicated by either antibiotic in isolation. Young sessile bacteria were slightly more resistant to piperacillin or tobramycin than planktonic cells however eradication of immature biofilm was readily achieved with combination antibiotic therapy. Mature biofilm was extremely resistant even to combination antimicrobials but increasing the dose of combined antibiotics resulted in enhanced killing of old sessile bacteria. The data presented in this study not only supported combined antibiotic therapy in treating biofilm infection but suggested early intervention against immature biofilm is more effective than intervention against mature biofilm (Anwar and Costerton, 1990). Disinfection chemicals can also be synergistic against biofilm. Devices coated with combination chlorhexidine and silversulfadiazine showed reduced implant related infection in the first week of implantation compared to either chemical used in isolation (Schierholz et al., 1999).

Antibiotic lock technique (ALT) is static instillation of bactericidal antimicrobial drugs within a chamber or component of a medical device for a sufficient dwell time and at high concentrations in order to prevent and destroy biofilm. Biocide concentrations are 100 to 1000 times greater than the maximal tolerated systemic dose. ALT eradication of biofilm is dependent on type of biofilm cells, biofilm maturity, the antimicrobial or combination of

antimicrobial agents used and the dwell time. ALT is a specific example of how antibiotics delivered locally and at increased concentrations can combat biofilm infection. ALT is particularly successful when applied to combat bacterial biofilms commonly found in central venous catheters because the catheters are used intermittently and can be filled with antimicrobial solution then aspirated prior to use (Donlan, 2008). As very high doses of antimicrobials are utilized, ALT is potentially toxic for the patient and in addition can strongly induce antimicrobial resistance. The technique has been tested with various traditional antibiotics and several other agents, such as chelating chemicals, ethanol and taurolidinecitrate with subsequent reduced biofilm incidence (Donlan, 2008). Barbaric et al. studied ALT with 2M hydrochloric acid in pediatric oncology patients with infected central venous catheters and associated blood culture positive sepsis despite 48 hours of treatment with appropriate intravenous antibiotics (Barbaric et al., 2004). Combination of parenteral antibiotics and ALT with hydrochloric acid eradicated 67% of biofilm associated sepsis and salvaged at least half of central venous catheters where successful treatment was defined as no recurrence of infection and no need for catheter removal at 100 days post treatment (Barbaric et al., 2004). Other novel agents have been tried with ALT. Protegrin peptides are potent antimicrobial agents believed to act against a variety of pathogens by forming nonselective trans-membrane pores in bacterial cell membranes. Synthetic protegrin analog IB-367 and the antibiotic linezolid have been used successfully in combination ALT to treat *in vitro* and *in vivo* biofilm catheter infections with *S.aureus* and *E.faecalis* (Ghiselli et al., 2007). Anticoagulant-antibiotic solutions have also been employed as part of ALT. Heparin and gentamicin or taurolidine solution locks can cure up to two thirds of catheter associated bacteremias without needing catheter removal (Michael, 2004).

As biofilm eradication requires increased local chemical concentrations, interest has developed in polymer and nanoparticle based chemical delivery vectors which focus chemical supply in and around medical implants. Chemicals delivered using these technologies include antibiotics, disinfectants and novel agents. Polymer based carrier systems provide a medium for high concentration anti-biofilm drug delivery. Many polymer-based carrier systems have been proposed including fibrous scaffolds of biodegradable polymers, thermo-reversible hydrogels and cements (Smith, 2005). One example of a biodegradable polymer scaffold for antibiotic delivery to optimize biofilm prophylaxis is poly(D,L-Lactide) loaded with gentamicin. Schmidmaier et al. showed the

polymer-gentamicin combination prevented osteomyelitis in the bones of 90% of rabbit tibiae deliberately contaminated with *S.aureus* and was much more effective in preventing osteomyelitis than parenteral gentamicin prophylaxis alone (Schmidmaier et al., 2006). Similarly polyacrylamide hydrogel matrix loaded with methylene blue was found by Wu et al. to be bactericidal against suspended early biofilm cultures of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Acinetobacter spp*.(Wu et al., 2009). Cement impregnated with antibiotic is commonly used in orthopaedic operations with a view to reduce biofilm infection of bone. Calcium hydroxyapatite cement carrying vancomycin is an effective way of treating localized MRSA osteomyelitis after bony debridement in the rabbit model (Shirtliff et al., 2002). Using antibiotic laden cement in combination with debridement 81.8% of infections were cleared compared to less than 50% clearance with debridement alone (Shirtliff et al., 2002). Polymer based delivery systems have in common the ability to trap and deliver antibiotics at high concentrations in and around medical implants which can lead to improved biofilm prevention and eradication.

Nanoparticles are being investigated as delivery vehicles for antibiofilm substances. Nanoparticles are microscopic particles less than 100 nanometers in diameter and can be used as small scale chemical reservoirs to attack biofilm. Experimentally, nitric oxide releasing silica nanoparticles have been reported to kill greater than 99% of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilm cells (Hetrick et al., 2009). The same study showed nitric oxide silica nanoparticles inhibited fibroblast proliferation to a lesser extent than antiseptics such as chlorhexidine and in turn produced wound healing as well as anti-biofilm benefits (Hetrick et al., 2009). Another advantage of nanoparticles is they can be engineered to release substances in a controlled fashion. Drug release profile has an equally significant influence on biofilm eradication rate as antibiotic dose so manipulation of drug release is an important antibiofilm strategy (Cheow et al., 2010). Cheow et al. designed levofloxacin-loaded polymeric nanoparticles with a favorable, biphasic, extended release profile and effectively inhibited *E.coli* biofilm growth on surfaces (Cheow et al., 2010). Biphasic release ofloxacin microspheres demonstrating initial burst release of antibiotic followed by a slow release phase have also been found to eradicate bone associated bacterial biofilm infections (Habib, 1999). Nanoparticles and microspheres containing antibiotics are thus a

tailored mechanism to control local antimicrobial dose and release profile in the treatment of bacterial biofilm.

A liposome is an artificial microscopic vesicle consisting of an aqueous core enclosed in one or more phospholipid layers, used to convey vaccines, drugs, enzymes, or other substances to target cells or organs (Dictionaries, 2007). In a similar way to nanoparticles and microspheres, liposomes can be utilized as small scale chemical reservoirs for directed delivery of chemicals targeting biofilm (Smith, 2005). Tamilvanan et al. suggested liposomes loaded with antimicrobial agents as an antibiofilm coating for medical devices and as drug delivery carriers to biofilm interfaces (Tamilvanan et al., 2008). Halwani et al. went one step further to demonstrate effectiveness of liposomes containing gentamicin and gallium against clinical isolates of biofilm producing variants of *Pseudomonas aeruginosa* derived from the sputum of cystic fibrosis patients (Halwani et al., 2008). Liposomes are another means of controlled and focussed drug delivery to manage biofilm on implants.

Increase in antibiotic-resistant microorganisms and general antibiotic resistance of biofilm phenotype has prompted interest in the use of metals such as gold, copper and silver as antibiofilm agents. The idea was sparked by the observation that several implants made of metal have reduced biofilm infection rates such as St Jude silver cardiac valves and copper intra-uterine contraceptive devices (Monteiro et al., 2009, Cook et al., 2000). Silver and silver ions in particular are established as bactericidal and used extensively in anti-infection wound applications for example a concentration of 5–10 μg/mL silver sulfadiazine cream is known to eradicate *Pseudomonas* biofilm in chronic wounds (Bjarnsholt et al., 2007, Monteiro et al., 2009). SEM studies of *S.epidermidis* biofilm have also shown silver-treated biofilms become less compact and cells more exposed suggesting silver as an adjunct to standard antibiotics as biofilm therapies (Chaw et al., 2005). Kim et al. confirmed that silver in combination with tobramycin enhances microbial efficiency against *Pseudomonas* biofilm by more than 200% (Kim et al., 2008). Most metal cations however have some anti-biofilm effect. Harrison et al. confirmed the anti-biofilm activity of heavy metal ions when they tested six cations (Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Al^{3+} and Pb^{2}) successfully against *Pseudomonas aeruginosa* biofilm (Harrison et al., 2005). Gold chloride at concentrations of 0.5 mM for 30 minutes at room temperature also kills *P.aeruginosa* biofilm via bio-mineralization (Karthikeyan and Beveridge, 2002). Microscopic examination of mature *P.aeruginosa* biofilm exposed to copper stress has demonstrated biofilm cells

near the exterior are preferentially killed perhaps because internal cells are protected by matrix chelation of metal ions (Teitzel and Parsek, 2003, Karthikeyan and Beveridge, 2002, Chaw et al., 2005). Metal toxicity for biofilm is therefore probably due to bio-mineralization, destabilization of matrix and heavy metal stress.

High affinity, metal-binding chelators such as EDTA and citrate inhibit bacterial growth by disrupting surface adherence and preventing biofilm production (Raad et al., 2008). Time lapse confocal scanning laser microscopy showed that EDTA caused a dispersal of biofilm and killing of biofilm cells within mushroom-like matrix structures (Banin et al., 2006). Gram negative bacteria are also affected if cations that stabilize lipopolysaccharide and the outer membrane are chelated leading to membrane instability and bacterial cell lysis (Mulcahy et al., 2008). EDTA has been widely investigated as an antibiofilm agent because of these characteristics mostly in the context of antibiotic lock technique. Juda et al. ascertained EDTA prevents *S.epidermidis* adherence to thermoplastic poly(vinyl chloride) catheters and also eradicates mature biofilm from the tubes when applied at higher concentrations (Juda et al., 2008). In another catheter based study, EDTA was tested on gram positive, gram negative and mixed biofilms found within hemodialysis catheters harvested from patients moving on to fistula based therapy (Kite et al., 2004). After a 24 hour lock, 40 mg of EDTA per ml was effective at eradicating total biofilm viable count in almost all cases (Kite et al., 2004). EDTA combinations have also been investigated for effectiveness against biofilm. Checkerboard assays have demonstrated a synergism between EDTA and aspirin in activity against *Pseudomonas aeruginosa* and *Escherichia coli* biofilm and EDTA-gentamicin combination is 1000 times more effective in eliminating *P. aeruginosa* biofilm than gentamicin alone (Banin et al., 2006, Al-Bakri et al., 2009). Chelating therapies provide an additional antibiofilm strategy.

Disturbance of biofilm matrix has been postulated to make biofilm cells vulnerable to elimination as disintegration of exopolysaccharide exposes cells to damaging factors residing in aqueous environment such as antibiotics and host immunity. Several strategies have been investigated including muco-lytics, enzymes that degrade matrix components directly and investigation of anti-biofilm proteins and enzymes. Some biofilms make use of mucous and secretions produced by host to hydrate and bulk up their matrix or to further sequester biofilm bacterial cells. N-acetylcysteine breaks disulfide bonds in mucous causing its liquefaction. Because of these mucolytic properties, activity of N-acetylcysteine has been investigated against bacterial biofilms with mucoid components.

Helicobacter pylori biofilm resides in the mucous layer of the stomach and on microscopic examination is often found surrounded by mucous. The organism is linked to gastritis, peptic ulcer disease and gastric cancer and is becoming increasingly resistant to combination antibiotic therapy (Megraud, 2004). Cammorata et al. found pre-treatment with N-acetylcysteine followed by a culture-guided antibiotic regimen obtained *H.pylori* eradication in 65% of samples compared with 20% eradication obtained by culture-guided antibiotics alone (Cammarota et al., 2010). N-acetylcysteine has also been combined synergistically with the antibiotic tigecycline to eradicate biofilm of methicillin resistant *S.aureus* and *S. epidermidis* on catheter segments (Aslam et al., 2007). Similarly, Perez-Giraldo et al. discovered a dose related decrease in *S.epidermidis* biofilm formation when exposed to N-acetylcysteine (Pérez-Giraldo et al., 1997). Mucolytics seem to hold promise as weapons against mucous associated biofilms.

A promising strategy may be the use of enzymes to dissolve biofilm matrix directly (Høiby et al., 2010). Both gram positive and gram negative bacterial biofilms use extracellular DNA to control and maintain biofilm biomass, architecture and morphology (Tetz et al., 2009). Tetz et al. discovered cleavage of extracellular DNA with DNase leads to formation of a weakened biofilm that permits penetration of antibiotics (Tetz et al., 2009). Alipour et al. investigated both DNase and alginate lyase (which dissolves the bacterial anchoring molecule alginate) as adjuncts to aminoglycoside antibiotics in the *in vitro* killing of *P.aeruginosa* biofilm in sputum (Alipour et al., 2009). Activity of aminoglycosides against biofilm was increased with the addition of DNase or alginate lyase and was increasingly evident with concurrent administration of both enzymes (Alipour et al., 2009). Directed enzymatic dissolution of staphylococcal extracellular matrix has also been achieved. Chaignon et al. eradicated a variety of staphylococcal biofilms by exposing them to dispersin B followed by the proteases proteinase K or trypsin (Chaignon et al., 2007). Enzymes that target structural parts of biofilm matrix may aid in biofilm break down and weaken biofilm to the effects of standard antibiotics.

Quorum sensing is a community communication system of stimulus and response correlated to population density and is employed by bacteria to co-ordinate gene expression and microbial behavior including biofilm formation. A variety of molecular signals are used. Common classes of signaling molecules are oligo-peptides in gram

positive bacteria, N-acyl homoserine Lactones (AHL) in gram negative bacteria and auto-inducers in both gram positive and negative bacteria (Miller and Bassler, 2001). Quorum quenching is achieved by degrading or diluting the biofilm signaling molecules or the elements involved in their production or perception (Kok Gan et al., 2009). Control of quorum sensing and quenching pathways and molecules is a keenly pursued target as a means to control and eradicate biofilm (Dror et al., 2009).

Quorum sensing can be manipulated to prevent staphylococcal biofilm formation on implants. A community signaling pathway that leads to *Staphylococcal* biofilm formation involves the release of RNAIII Activating Protein (RAP). RAP diffuses to surrounding cells, is bound to cell surface through Target RNAIII Activating Protein (TRAP) which then activates RNAIII and biofilm formation is initiated (Harraghy et al., 2007). This pathway of communication can be shut down using an inhibitor of TRAP called RNAIII Inhibiting Peptide (RIP) (Balaban et al., 2003). RIP is a hepta-peptide than prevents binding of RAP to TRAP, inhibits production of RNAIII and stymies biofilm formation. RIP stops biofilm formation on implants challenged with *S.aureus* and *S.epidermidis* by disrupting quorum-sensing mechanisms (Costerton, 2001, Harraghy et al., 2007, Balaban et al., 2003). As well as preventing biofilm, RIP has demonstrated synergism with antibiotics in eliminating 100% of drug resistant, graft associated *in vivo S.epidermidis* mature biofilm implant infections (Balaban et al., 2003).

Recently, farnesol was described as a quorum sensing molecule that can inhibit biofilm. With viability assays, biofilm formation assessment and ethidium bromide uptake testing, farnesol was shown to inhibit S*taphylococcal* biofilm formation and compromise cell membrane integrity (Jabra-Rizk et al., 2006). It was also useful in eradicating methicillin resistant *Staphylococci* and acted to sensitize bacterial biofilms to antibiotics (Jabra-Rizk et al., 2006). Dried farnesol on biofilm contaminated titanium alloy discs has the ability to reduce the number of viable biofilm bacteria fiftyfold with the effect lasting at least three days (Unnanuntana et al., 2009). Although the exact mechanism of action is unknown, farnesol is a candidate for quorum sensing manipulation of biofilm.

Gram negative bacteria are susceptible to manipulation of quorum sensing to prevent and treat biofilm. A range of gram-negative bacterial species use N-acyl homoserine lactone (AHL) molecules as quorum-sensing signals to regulate virulence factors including biofilm

formation (Miller and Bassler, 2001). AHL can be inactivated by the enzyme AHL lactonase (Dong et al., 2002). Kiran et al. investigated biofilms of multidrug resistant *Pseudomonas aeruginosa* (a gram negative bacterium) to see if they were susceptible to quorum sensing manipulation and found lactonase inhibited biofilm formation and made established biofilm more susceptible to both ciprofloxacin and gentamicin antibiotics (Kiran et al., 2011). On scanning electron microscopy, lactonase was seen to disrupt biofilm of *P.aeruginosa* and virulence factor production was also reduced (Kiran et al., 2011). Chow et al. extended the concept of lactonase as a quorum quenching protein by enhancing the catalytic efficiency and substrate range of the wild-type enzyme (derived from *Mycobacterium avium ssp. paratuberculosis*) through a single point mutation (Chow et al., 2009). The mutant enzyme exhibited a thirty fold increased inactivation of AHLs and also inactivated N-hexanoyl-l-homoserine lactone and N-butyryl-l-homoserine lactone, quorum sensing molecules that were previously not hydrolyzed by the wild-type enzyme (Chow et al., 2009). This interesting study shows that quorum quenching molecules can be augmented and enhanced in order to fight biofilm more effectively.

A bacteriophage (phage) is a virus capable of infecting bacterial cells and causing cell lysis. Phage therapy is the use of bacterial viruses to treat bacterial infections. Phage therapy has been around for over a century but has remained outside the mainstream of modern medicine presumably because of doubts about its efficacy and the boom of antibiotics (Levin and Bull, 1996). Now that antimicrobial resistance is increasing and biofilms are proving difficult to treat, phage therapy is undergoing a revival. Bacteriophages can reside in bacterial genome as a pro-phages or enter a lytic state by usurping bacterial gene expression machinery to synthesize hundreds of new phage progeny and cause bacterial lysis. Many phages produce destructive enzymes such a peptidoglycan hydrolases that degrade bacterial cell wall and holin that literally creates a hole in the cell wall (Donovan, 2007). Phage mixtures, engineered phages and their gene products are potential antibiofilm weapons (Donlan, 2009).

Several in vitro experiments have shown that phages are able to infect biofilm cells and lyse them, usually with the help of phage encoded polysaccharide lyase that acts specifically against biofilm matrix (Azeredo and Sutherland, 2008). The use of phages requires identification of virus-enzyme combinations effective against a given biofilm. Biofilm organisms must therefore be isolated and screened against a bank of known

phages or genetically engineered with desired characteristics (Azeredo and Sutherland, 2008). Bacteriophage-polysaccharide depolymerase combinations have averted enteric single species *in vitro* biofilm formation by *Enterobacter cloace* (Tait et al., 2002). Phage efficacy against *Klebsiella pneumoniae* biofilm in combination with amoxicillin has also been proven although the effect of phage alone against biofilm in this incidence was not significant (Bedi et al., 2009). Lu and Collins confirmed the better performance of bacteriophages bearing antibiofilm specific enzymes in eradicating and controlling biofilm (Lu and Collins, 2007). They engineered enzymatic phages to target biofilm and reduced bacterial cell counts by 99.997% within biofilm (Lu and Collins, 2007). This is an example of the benefits of engineered phages against biofilm.

Because biofilm is so difficult to eradicate using conventional antimicrobial techniques, physical forces have been tested to determine their effect on biofilm viability. Physical therapies enlisted against biofilm include ultrasonic and sonic energy, extracorporeal shock waves, electric fields, photodynamic and other light therapies.

Ultrasound and sound waves used in combination with antibiotics or other microbial agents have a synergistic effect in eliminating established biofilm. Biofilms on removable components such as ultrafiltration membranes where access is easy and host toxicity is a non-entity can be destroyed using ultrasound alone up to 95% of the time (Zips et al., 1990). At levels that do not harm human tissue, lower frequency ultrasound seems to produce greater biofilm killing compared to higher frequency ultrasound but takes up to six hours to sterilize biofilm incurring a significant time cost (Johnson et al., 1998). Unfortunately not all biofilms can be explanted so combination therapies have arisen to augment the antibiofilm effects of ultrasound and reduce the dose and time required for effective biofilm elimination. The application of low-power ultrasound in concert with antimicrobial agents to enhance antimicrobial effectiveness is known as the bio-acoustic effect (Freitas Jr, 2003). An example of the bio-acoustic effect is that one third of *E.coli* biofilm can be removed from surfaces using 40 kHz of ultrasonic energy alone but the addition of protease or trypsin increases efficacy of biofilm removal to greater than 80% (Oulahal-Lasgsir et al., 2003). Similarly ultrasonic irradiation at 500 kHz and 10 mW cm^{-2} of a 24 hour old biofilm of *P.aeruginosa* enhances the killing of bacteria by gentamicin and ultrasonic and sonic agitation of dental plaque in combination with chlorhexidine has a pronounced synergistic effect in killing biofilm (Shen et al., 2010, Qian et al., 1996). Apart

from its own intrinsic antibiofilm activity and the bio-acoustic effect, ultrasound has been used as a “switch” to release antibiotic from bone cement and from hydrogel coatings. Ensing et al. showed antibiotic loaded cements could be triggered to release drugs when exposed to pulsed ultrasound (Ensing et al., 2006). In the same study ultrasound responsive cements were more effective than antibiotic cement alone against biofilm producing clinical isolates of *E.coli, S.aureus,* coagulase negative *Staphylococci* and *P.aeruginosa* (Ensing et al., 2006). In a similar vein a drug delivery polymer matrix consisting of poly(2-hydroxy ethyl methacrylate) hydrogel coated with ordered methylene chains has been designed to retain antibiotic inside the polymer in the absence of ultrasound and release antibiotic on exposure to low-intensity ultrasound (Norris et al., 2005). Ultimately these techniques may facilitate the development of medical devices that can release bactericidal agents “on-demand” in response to external ultrasonic impulses.

Extracorporeal shock waves utilize a focused acoustic pulse to impact biofilm. In an *in vitro* study by Wanner et al. *S.aureus* and *S.epidermidis* biofilms grown on steel washers were exposed to low energy shock waves at 0.16 mJ/mm^2 for 500 impulses then exposed to antibiotics and examined with scanning electron microscopy (Wanner et al., 2011). Complete eradication of biofilm was achieved using the antibiotic-shock wave combination therapy (Wanner et al., 2011). Shock waves are thus another synergistic, physical antibiofilm strategy.

Electrical fields combined with a low current density have enhanced the efficacy of several commercial biocides against biofilm (Blenkinsopp et al., 1992, Ehrlich et al., 2005). The bioelectric effect refers to electric fields that improve the efficacy of biocides in killing biofilm. In some instances antimicrobial potentiation by electric fields have allowed biofilm killing at antibiotic doses effective against planktonic bacteria (Costerton et al., 1994). Electrical approaches either released antimicrobials from device surfaces or acted to drive antimicrobials through biofilm (Smith, 2005). Biofilm bacteria are readily killed on active electrodes and on conductive elements that lie within electric fields but do not themselves function as electrodes (Costerton et al., 1994).

Photodynamic treatment (PDT) of biofilm involves three components: a photosensitizer taken up by biofilm, a light source and oxygen. Light is employed to excite the photosensitizer and produce reactive oxygen species which then go on to damage biofilm.

Favorable features of PDT include broad spectrum of action, efficient inactivation of antibiotic-resistant bacteria, low mutagenic potential and lack of selection for photo-resistant microbial cells (Jori et al., 2006). Photodynamic therapy has been extensively researched as a tool against biofilm because it is an extrapolation of the established practice of light therapy in dentistry. George and Kishen showed matured endodonic biofilm plaques are eradicated by advanced non-invasive light-activated disinfection in combination with methylene blue (a cationic, phenothiazinium photosensitizer) and perfluorodecahydronaphthalene (an oxygen carrier) (George and Kishen, 2008). *Enterococcus faecalis* biofilms have also been exposed to PDT with promising results. Kishen et al demonstrated methylene blue in combination with the microbial efflux pump inhibitor verapamil supported successful PDT based eradication of *Enterococcal* biofilm (Kishen et al., 2010). Street et al. quantified the effect of PDT on *P.aeruginosa* biofilm and found both power density and illumination duration affected biofilm kill (Street et al., 2009). Finally, Di Poto et al. looked at PDT against mature *S.aureus* biofilm combined with antimicrobial chemotherapy and host immunity (Di Poto et al., 2009). Biofilms exposed to a porphyrin photosensitizer and visible light followed by vancomycin antibiotic or whole blood (containing phagocytes) were completely eradicated (Di Poto et al., 2009). The results suggested that PDT was a useful independent or adjunctive approach to biofilm killing.

Light therapies are effective against biofilm even in the absence of photosensitizing agents. Both ultraviolet light and laser have been employed to kill biofilm. Light administered by portable instruments delivered into catheters is propagated through poly(tetra fluoro ethylene) and silicone, two common polymers used in catheter and other medical device construction. Ultraviolet C light reduced the number of viable biofilm bacteria on urinary catheters harvested from patients in the *ex vivo* setting but as with ultrasonic biofilm therapies, very large doses and protracted treatment times were needed to obtain 99.9% disinfection rates (Bak et al., 2009). Artificial *Pseudomonas aeruginosa* biofilm grown on catheters was exposed to ultraviolet diodes in an attempt to determine biofilm killing effect in a study by Bak et al. (Bak et al., 2009). Killing effect was proportional to ultraviolet exposure time, catheter length and catheter construction material, but the study group managed a disinfection rate of 99.99% for a 10 cm, silicone peritoneal dialysis catheter exposed to ultraviolet light for 300 minutes (Bak et al., 2009). Ultraviolet disinfection may therefore be useful in the treatment of *in situ* catheter

associated biofilm. Q-switched Nd:YAG laser pulses are an alternative light source effective against *Pseudomonas* biofilm. Krespi et al. delivered laser to *Pseudomonas aeruginosa* biofilm grown from clinical otorrhea isolates *in vitro* and showed laser shockwave and plasma production caused almost instantaneous detachment of biofilm from surface (Krespi et al., 2008). Laser technology has the potential to generate a powerful stress wave sufficient to disrupt biofilm without any ill effect to the underlying host structure (Krespi et al., 2008).

2.10.6.4 Device removal

The gold standard and definitive management of biofilm contamination and infection of medically implanted devices is physical removal of the device. Device removal is not infrequently the only effective treatment for bacterial biofilm infection on medical implants.

2.10.6.5 Future targets and other novel anti-biofilm approaches

There has been no shortage of novel suggestions to treat biofilm infection of medical implants. Genes essential for biofilm viability, cell attachment or biofilm formation are potential targets (Scheie and Petersen, 2004, Donlan and Costerton, 2002). It was recently discovered that some bacteria maintain their three- dimensional biofilm structure at least in part by releasing surfactant molecules which prevent clogging of biofilm channel systems making anti-surfactant therapies attractive (Davey and Caiazza, 2003). Conversely bio-surfactants in combination with antibiotics have been employed to successfully reduce biofilms of *E.coli* (Rivardo et al., 2011). Brady et al. have made some progress in developing multicomponent vaccines against *Staphylococcal* biofilm (Brady et al., 2011). Finally planned colonization of implanted devices with genetically modified organisms or organisms of low virulence is another clever, novel concept (Freitas Jr, 2003).

2.11 *Staphylococci*

2.11.1 Definition, taxonomy and epidemiology

Genus *Staphylococci* are gram positive, spherical bacteria and are immobile, non-spore forming, fermentative, facultative anaerobes. Under the microscope these bacteria classically appear as grape-like clusters of cream to yellow cocci (Ahmad et al., 2010). More than 32 species of *Staphylococci* are described and most reside on skin and mucous membranes of mammals as commensal bacteria (Chiller et al., 2001). A small proportion of *Staphylococci* are soil microbial flora (Ahmad et al., 2010).

In addition to characteristic microscopic appearance and gram stain results, biochemical traits identify *Staphylococci. Staphylococci* are usually catalase positive and oxidase negative (Baker, 1984). Coagulase activity, hemolysis, nitrate reduction, and 10 different carbohydrate reactions are routinely implemented to serially classify species of human *Staphylococci* (Kloos and Schleifer, 1975). *Staphylococci* can also be identified by the production of DNase and or thermo-stable nuclease (HPA, 2007). Historically coagulase positive *Staphylococci* are referred to as *S.aureus* and other *Staphylococci* are grouped as coagulase negative *Staphylococci* (CoNS). Recently other (non *S.aureus*) coagulase positive staphylococcal species have been described such as *S. hyicus*, *S. schleiferi* subspecies *coagulans* or *S. intermedius* however these strains are rarely significant in human infection or carriage (HPA, 2007). *S.epidermidis* is the most common CoNS (Kawamura et al., 1998). *S.aureus* and *S.epidermidis* are differentiated on the basis of hemolysis. *S.aureus* shows β hemolysis on sheep blood agar and *S.epidermidis* does not (Todar, 2005). Detection of surface proteins such as clumping factor (slide coagulase test) or protein A (commercial latex tests) allow rapid identification of *S.aureus* (Personne et al., 1997). Virulence factors and production of extracellular toxins aid in classification. *S. aureus* produces virulence factors such as protein A, capsular polysaccharides and α toxin. Some strains of *S.aureus* produce toxic shock syndrome 1 toxin (TSST-1), Panton-Valentine Leucocidin or other toxins (HPA, 2007).

Staphylococci are common human colonizers and often part of commensal bacterial populations. *Staphylococcus aureus* colonizes approximately 30% of the general population and up to 50% of persons who are intravenous drug users, diabetics, or healthcare workers (Boubaker et al., 2004). Methicillin resistant *S.aureus* colonizes 2.7% of the community (Jernigan et al., 2003). *S.epidermidis* is considered part of ubiquitous human commensal flora (Trueba et al., 2006).

2.11.2 Staphylococcal infections

Staphylococci are a major cause of both hospital acquired and community onset infections (Diekema et al., 2001). 28% of all nosocomial infections are bloodstream infections: 40% due to CoNS and 9.3% due to *S.aureus* (Ngyuen, 2007). Surgical site infections make up seven percent of all nosocomial infections of which *Staphylococci* contribute 35% (Ngyuen, 2007). The 10 *Staphylococcus* species most often responsible for human infection are *S.epidermidis* (31.3%), *S.aureus* (23.3%), *S.haemolyticus* (12.2%), *S.caprae* (10.7%), *S.simulans* (4.4%), *S.hominis* (4.0%), *S.capitis* (3.9%), *S.saprophyticus* (3.6%), *S.warneri* (2.2%) and *S.lugdunensis* (1.3%)(Kawamura et al., 1998).

S.aureus produces a wide variety of disease in humans via tissue invasion or toxins. Epidermal infections occur through breaches in the skin and include impetigo and ecthyma. Dermal infections consist of erysipelas, cellulitis and necrotizing soft tissue infections. The pilo-sebaceous unit is infected in folliculitis, furunculosis, and carbunculosis. A pathological hallmark of *S.aureus* infection is abscessation and septicaemia with metastatic haematogenous spread to any organ possible (Chulamokha and Watanakunakorn, 2001). Common end organ infections include pneumonia, endocarditis and osteomyelitis. Food poisoning can occur due to ingested heat stable *S.aureus* enterotoxins. *S.aureus* super-antigens cause widespread activation of T-cells and subsequent massive release of pro-inflammatory cytokines leading to toxic shock. *S.aureus* mediated super-antigen syndromes include scalded skin syndrome, toxic shock syndrome (TSST-1, enterotoxin B and C) and scarlet fever (Chiller et al., 2001).

CoNS produce fewer extracellular products and toxins than do coagulase positive strains and are often opportunistic pathogens. *S.epidermidis* is a common cause of infection in the neonatal population and pediatric oncology, burns and ophthalmic patients are high-risk groups for coagulase negative staphylococcal infections (Weisman, 2004). CoNS are also a principle cause of prosthetic device infections in adults (Vernachio et al., 2002). Medical implant infection is commonly caused by *S.epidermidis* because despite low virulence the bacterium is well adapted to adhere to smooth metal and plastic surfaces as biofilm (Ziebuhr et al., 1999). Biofilm encapsulation of CoNS also contributes to broad spectrum antibiotic resistance including methicillin and penicillin (Balaban et al., 2003). *S.saprophyticus* is part of normal vaginal flora and may cause urogenital tract infection in sexually active young women (Latham et al., 1983).

2.11.3 Treatment of staphylococcal infection

Treatment of staphylococcal infection is by the host immune system and iatrogenic techniques. Host immune cells phagocytose *Staphylococci* and antibodies are produced to neutralize toxins and promote opsonization of bacteria (Todar, 2005). Removal of infected foreign bodies and surgical drainage of pus or debridement of infected, necrotic tissue are mainstays of treatment. Supportive therapies such as fluid management, blood pressure support and artificial ventilation may be required depending on the location and severity of infection.

Antibiotic therapy is instituted in staphylococcal infection. Penicillin remains the drug of choice if the isolate is sensitive to the drug. Alternatives to penicillin include other β-lactam agents (e.g. cefazolin, ceftriaxone, cefepime, amoxicillin-clavulanate, and imipenem)(Diekema et al., 2001). A semisynthetic penicillin (nafcillin, oxacillin, methicillin) is indicated for β-lactamase–producing strains (Lowy, 1998). Approximately 20% of *S.aureus* and 68% of CoNS strains are resistant to methicillin (Marshall, 1998). Glycopeptide antibiotics vancomycin and teicoplanin are generally effective in resistant cases (Smith et al., 1999). Resistance to glycopeptide antibiotics is also emerging in *Staphylococci* (Trueba et al., 2006, Leung and Chow, 2000). Patients unable to be treated with glycopeptides have been treated with fluoroquinolones, trimethoprim–sulfamethoxazole, clindamycin or minocycline. Rifampin is another potent anti-staphylococcal drug, but resistance invariably develops if it is used alone (Lowy, 1998). Drug combinations have been employed in an effort to reduce the development of antibiotic resistance (Lowy, 1998). Complicated resistance patterns encountered in staphylococcal infections require input from infectious disease specialists.

CHAPTER 3 FABRICATION OF PLASMA POLYMER THIN FILMS

3.1 Introduction

Bio-mimetics is where the elegance and brilliance of nature are mimicked or replicated by man. Rather than reinventing the wheel, solutions to scientific problems are sought in nature. Using this approach man can take advantage of evolution and existing successes in the natural environment. Because of the philosophy of bio-mimetics, natural materials and their semisynthetic derivatives are areas of active research. Materials derived from plants are also attractive because they are inexpensive, renewable, readily available, can be chemically modified and are potentially degradable and biocompatible due to their origin (Suniket and Fulzele, 2007).

Tea tree oil (TTO) has proven antimicrobial activity in an essential oil format (Carson and Riley, 1998). In the context of growing antimicrobial resistance, new strategies against pathological microbes are sought to supplement traditional antibiotic approaches. Bio-medical implants are ubiquitous in the practice of modern medicine and the majority of complications associated with bio-medical implants are either infection or rejection related (Christensen et al., 1989). It is postulated that biocompatible, nontoxic, polymeric, surface related thin films can be derived from TTO using the process of plasma polymerization. These TTO based polymer films may preserve the medicinal and antibacterial benefits of their parent compound TTO. As plasma polymers are inherently stable as a class, it is possible TTO derived plasma polymer thin films will be bio-chemically inert, lack systemic toxicity and demonstrate biocompatible traits (Yasuda, 2005). These synthetic derivatives of TTO may provide a novel, biocompatible, antibacterial surface coating for use in the development of two phase medical implants.

3.2 Production of Tea Tree Oil Derived Plasma Polymer Thin Films

Plasma polymers were produced in the Microwave Laboratory at the School of Engineering at James Cook University Townsville Campus. Polymer samples were deposited on standard glass slides, PTFE squares and PTFE discs for fundamental property, antibiofilm and biocompatibility testing respectively. Deposition substrate was clean but not aseptic (Section 3.3.2 Technique for cleaning substrate).

3.2.1 Hardware components and configuration

3.2.1.1 Hardware components

- RF (radiofrequency) plasma generator operated at 13.56 MHz (ISM band) with a maximum power output of 300 W and impedance 50 Ohm (ENI™ ACG-3B RF Generator)
- custom made glass chamber manufactured at the University of Queensland
- single phase JAVAC™ rotary vacuum pump
- vacuum/Pressure gauge (JAVAC™ Acravac Plus™)
- copper ring electrodes (2)
- matching Network
- tea tree oil (100%) monomer source (Appendix 3A)
- argon gas, cylinder and attachments
- connection tubing
- vacuum grease silicon lubricant (to seal glass on glass joins)

3.2.1.2 Hardware configuration

A customized glass chamber supported by metal brackets was attached via airtight tubing to a vacuum pump, vacuum gauge, Argon gas cylinder and Argon flow valve (Plate 3.1 and Figure 3.1). Glass on glass interfaces were lubricated with vacuum grease to reduce gas leaks. The glass chamber was made with two liquid monomer reservoirs attached but separated from the main chamber via adjustable stop-cock valves. The first reservoir was used to house tea tree oil liquid monomer. The second reservoir was initially designed for doping and was not used. Two copper ring electrodes were placed around the glass

chamber 10 and 18 cm from the liquid monomer reservoir. Deposition substrate was placed on a single use, clean, disposable plastic platform within the glass chamber and between copper electrodes. Electrodes were linked to radiofrequency power supply and matching device.

100% pure tea tree oil was stored in one liter air tight aluminum containers in a dark, dry cupboard at room temperature. Aliquots of two milliliters of fresh essential oil were withdrawn for each deposition cycle.

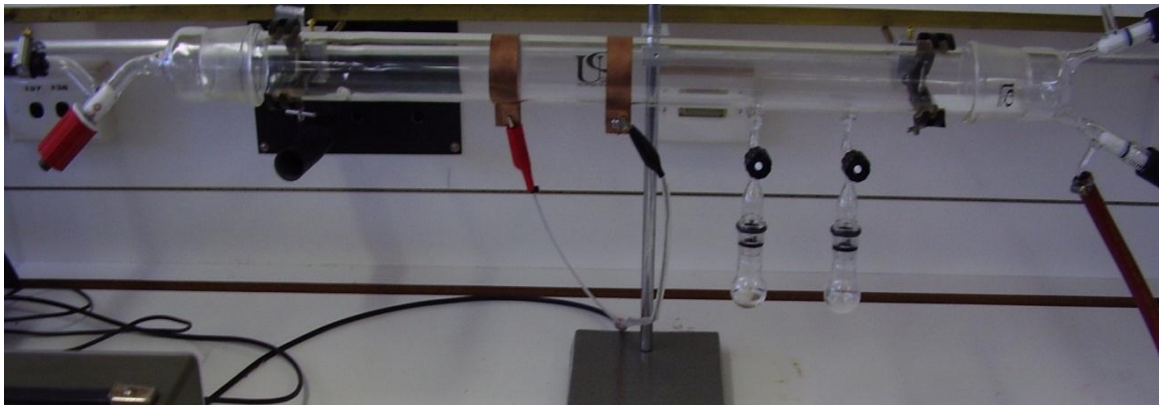


Plate 3.1: Hardware configuration for plasma polymer thin film production

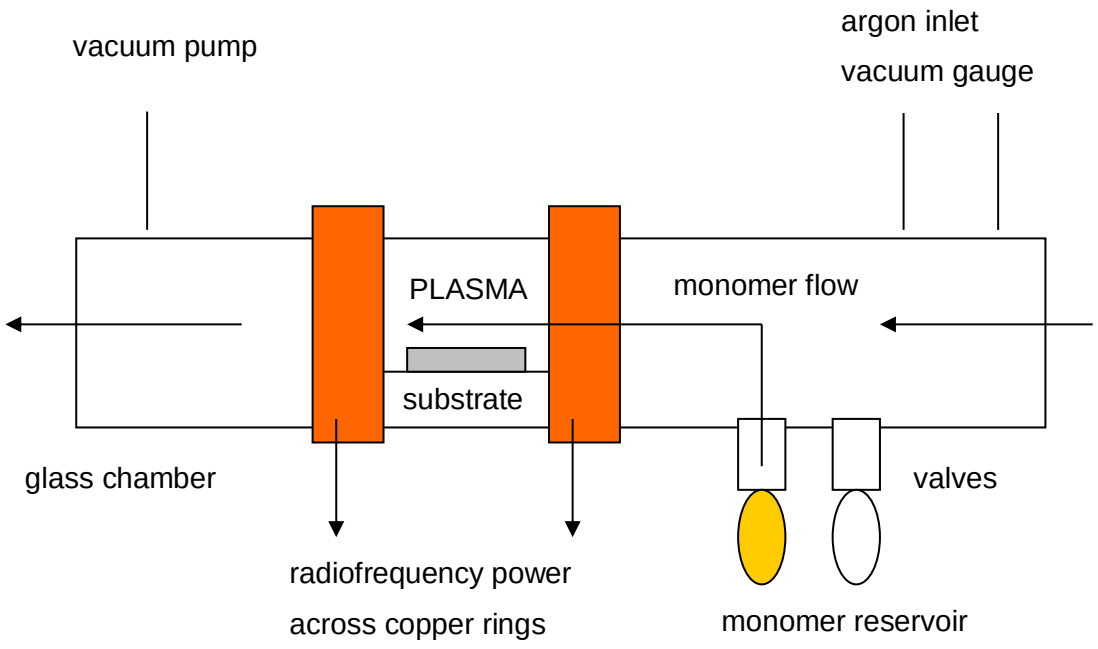


Figure 3.1: Diagram of hardware configuration for plasma polymer thin film production

3.2.2 Plasma polymer thin film production

3.2.2.1 Plasma polymerization on glass substrate

76 x 26 x 1 mm BioSciTech™ glass laboratory slides were employed as plasma polymer deposition substrate for fundamental property studies. Glass slides were inexpensive, available, standardized, fit within the glass vacuum processing chamber and were easy to store, handle and clean. Glass slides were transparent making polymer deposition more easily detectable to the naked eye. Transparency allowed for measurement of polymer film thickness using a spectrophotometer (PTFE leads to erroneous measures with desktop spectrophotometry). In preparation for plasma polymer deposition, slides were processed according to a cleaning protocol (Section 3.3.2 Technique for cleaning substrate), individually labeled with indelible marker and stored at room temperature within clean slide boxes in a dry, sealed and locked cupboard.

For each polymer production run, two milliliters of 100% pure tea tree oil was deposited into monomer reservoir attached to the customized glass chamber with the connecting stop cock firmly closed. Glass chamber was sealed and atmospheric gas within the chamber was evacuated by a vacuum pump to a pressure of 600 microns as detected by the pressure gauge. Monomer within the reservoir was then exposed to the chamber vacuum by opening the connecting stop cock to equalize pressure (600 microns) between the reservoir and glass chamber. Equalizing pressures between the monomer storage reservoir and glass chamber was needed to achieve controlled monomer release into a low pressure environment during the next step of plasma polymer production.

Once pressure between glass chamber and reservoir was equalized, the connecting stop-cock was again closed to preserve 600 microns pressure within the monomer reservoir. The glass chamber was then opened to air to allow insertion of deposition substrate. A single prepared glass slide was mounted on a plastic stage and placed longitudinally within the customized glass chamber between copper ring electrodes. The system was then resealed and the seal tested by generation of a low pressure environment (< 600 micron) using the vacuum pump. Argon gas was then perfused into the glass chamber for one minute to displace any residual air with resultant gradual loss of vacuum.

When the Argon wash was finished, a low pressure environment within the glass chamber was re-established but this time to a pressure of 300 microns. The stop-cock separating TTO reservoir from glass chamber was then opened allowing TTO vapor to permeate the glass chamber as organic gas. A potential difference was generated between the copper electrodes using the radiofrequency generator (13.56 MHz) and plasma formed within the glass chamber. The power on the radiofrequency generator was set at 25, 50 or 75 W (depending on required production parameters) and matching was checked. Plasma polymer deposition reaction was permitted for a predetermined period of time (2, 5, 10, 20, 30 or 60 minutes).

Once the required time point was reached, the stop-cock between the monomer reservoir and glass chamber was firmly closed preventing further release of organic gas into the glass chamber and the radiofrequency generator switched off, stopping the reaction. The glass chamber was opened and substrate with newly deposited plasma polymer thin film was retrieved. Each slide was checked for macroscopic evidence of plasma polymer surface coating, identifying details were confirmed and the samples were stored in a sealed, clean, dry container.

3.2.2.2 Plasma polymerization on PTFE substrate

Plasma polymer films on PTFE substrate were required for biocompatibility studies (insertion into mice) and for biofilm testing (insertion into biofilm circuit and biofilm processing equipment). 0.38 mm and 1 mm thick PTFE sheets were cut to 76 x 26 mm rectangles (dimensions of standard glass slides) making for ease of processing, handling and storage and allowing for standardization of production parameters. PTFE substrate was then plasma polymer coated on both sides using an identical technique to that described for glass slides (Section 3.3.2.1 Plasma polymerization on glass substrate). Radiofrequency power of 25, 50 or 75 W (depending on required production parameters) was applied for ten minutes to develop a polymer film approximating 1000 nm thickness. Films of 1000nm were obvious to the naked eye. Polymer on 0.38 mm PTFE substrate was cut into 6 mm diameter discs in preparation for biocompatibility studies. Polymer on 1 mm PTFE was hand cut into 25 x 25 mm squares ready for antibiofilm testing. PTFE and polymer films were stored in labeled, sealed, sterile and dry containers.

3.3 Cleaning Construction Hardware

3.3.1 General approach

Hardware components and deposition surfaces in direct contact with developing plasma polymer thin films or gaseous precursor monomer must be clean. Surface contamination of deposition substrate with dust and other environmental particulate matter leads to inconsistent polymer deposition, increased bacterial trapping and holes in deposited thin film thereby reducing film quality and sterility. Incidental polymer buildup on hardware after repeated fabrication cycles causes clogging of inlet channels and flaking within the glass vacuum chamber and must also be avoided. Specialized cleaning was therefore required for polymer deposition substrate and glass vacuum chamber components. Standardized cleaning protocols were developed to support efficient, consistent and high quality plasma polymer film generation.

All cleaning took place in the Microwave Laboratory at the School of Electrical and Computer Engineering at James Cook University. Staged cleansing processes addressed different types of surface contamination. A combination of manual mechanical and chemical techniques was adopted. Powder free PVC gloves were worn during the cleaning process to avoid particulate and finger print contamination. Three cleaning chemicals were utilized: acetone to denature and breakdown residual TTO plasma polymer, Extran™ MA03 a commercially manufactured biodegradable, phosphate free, alkaline soap as a general purpose cleaner and isopropyl alcohol (isopropyl-1-ol). Rapid drying was achieved by removing isopropyl alcohol with compressed air. The aim of rapid drying was to dissuade settling of particulate matter. Decontaminated equipment was stored within clean containers in a clean, dry and sealed cupboard.

Plasma polymer deposition substrate (standard glass microscope slide or tailored PTFE sheeting) was ultrasonically cleaned in addition to manual mechanical and chemical cleaning in an attempt to ensure decontamination at the most critical site: substrate-polymer interface. Ultrasonic cleaning works by cavitation whereby microscopic bubbles release sound energy (20-100kHz), provide agitation and dislodge surface debris on a micro scale (Fuchs, 1995).

3.3.2 Technique for cleaning substrate

In preparation for plasma polymer deposition, new, standardized glass slides or tailored pieces of PTFE sheeting were manually cleaned using Extran™, rinsed in distilled water and submerged for 15 minutes in a three liter ultrasonic bath (Soniclean™ 160HT 60 kHz 70 W)(Plate 3.1). Substrate was then immersed in isopropyl-1-ol and rapidly dried using pressurized air. Cleaned substrate was placed in a clean slide box and stored in a sealed cupboard. The protocol for cleaning substrate is presented in a flow diagram below (Figure 3.2).

Plate 3.2: Soniclean™ 160HT ultrasonic cleaning bath used to clean deposition substrate

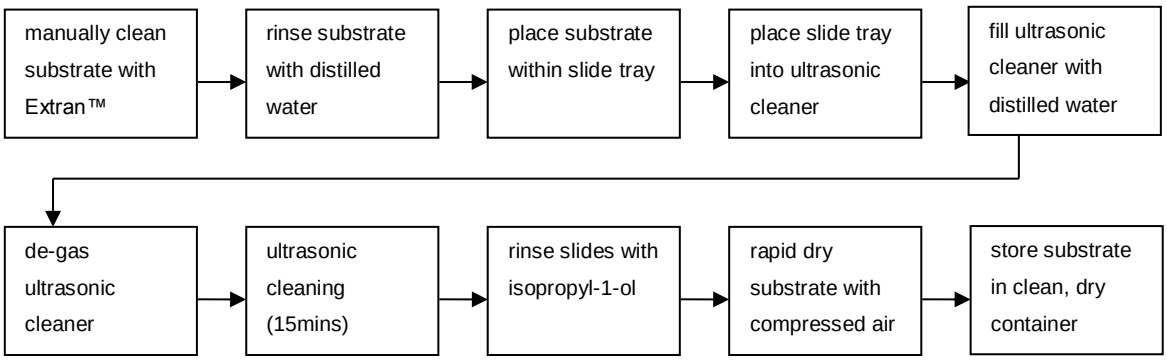


Figure 3.2: Protocol for cleaning substrate

3.3.3 Technique for cleaning glass vacuum chamber

The glass vacuum chamber inside which plasma polymerization occurred was susceptible to incidental plasma polymer contamination. A4 3M™ write-on overhead transparency films made from poly(ethylene terephthalate) were used to line the glass chamber and reduce cleaning requirements caused by incidental polymer deposition on the chamber internal surface. Transparencies were removed and replaced after each deposition cycle. Cleaning of glass chamber itself was undertaken whenever polymer deposits became macroscopically obvious. Chamber was hand cleaned with acetone and a bottle brush to remove all visible plasma polymer followed by an acetone rinse. After hand washing with Extran™, the chamber was rinsed in distilled water and then isopropyl-1-ol. It was too large to clean in the ultrasonic bath. Pressurized air stream was used for rapid drying. The chamber was immediately reconnected to closed production circuit after cleaning to avoid environmental contamination. The protocol for cleaning the glass chamber is presented in a flow diagram below (Figure 3.3).

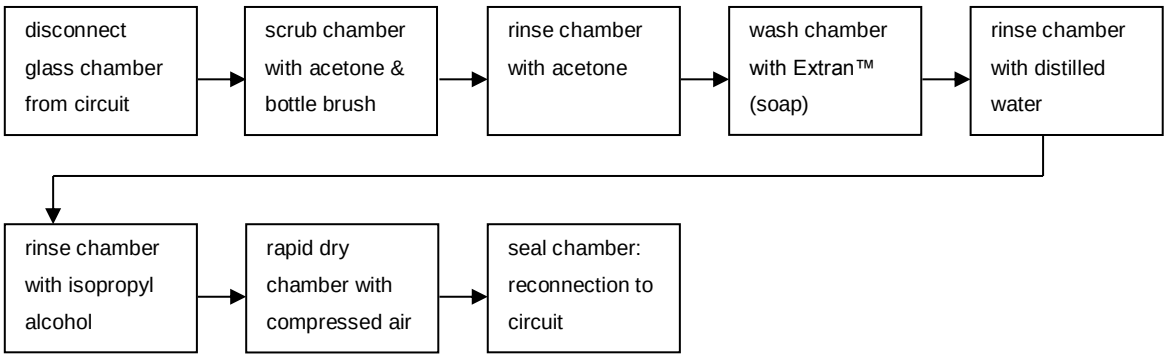


Figure 3.3: Protocol for cleaning glass chamber

3.4 Sterilizing Tea Tree Oil Derived Plasma Polymer Thin Films

Medical device materials need to be sterile on implantation in order to prevent device infection. Sterilization is the elimination of all transmissible agents such as bacteria, fungi, prions and viruses. Bio-materials must therefore be manufactured aseptically or sterilized after production. Post production sterilization is cheaper as it does not need to support a sterile production environment and also reduces contamination due to breaches in sterile production line.

Biocompatibility and antimicrobial testing of tea tree oil derived plasma polymer films required sterile product to avoid microbial contamination of experiments and confounding of results. Although plasma polymerization is a high energy process that in itself is likely to be antimicrobial it is not a standardly applied sterilization technique. Post production decontamination was therefore indicated and sterilization methods were investigated.

Table 3.1: Standard polymer sterilization techniques (Athanasiou et al., 1996)

Sterilization technique	Advantages	Disadvantages
steam sterilization (high pressure, 120-135 ^{o}C)	no toxic residue	degradation due to water attack, may cause melting/softening
dry heat sterilization (160-190 ^{o}C)	no toxic residue	may cause melting/softening
radiation (ionizing or gamma)	high penetration, low chemical reactivity, quick effect	polymer instability, deterioration, cross linking and breakdown of chains toxic
gas sterilization (ethylene oxide)	low temperature range so less likely to cause heat related damage	lengthy process, degassing residues are toxic

The ideal sterilization method is rapid, cheap, nontoxic, available, does not alter the structure, properties, surface or bulk configuration of polymer and does not remove or damage polymer. A sterilization technique was sought with low risk of causing damage, alteration or contamination of tea tree oil derived polymers .The most common standard

polymer sterilization techniques utilize heat, steam, radiation or chemicals such as ethylene oxide (Table: 3.1). Biomedical devices prepared from fragile substances like biodegradable poly(esters) are usually sterilized by ethylene oxide because other sterilization procedures, such as irradiation, heat, steam or acid, cause extensive deformation and accelerated polymer degradation (Gardner and Peel, 1991).

Hospital steam sterilization employs high pressure moisture at temperatures greater than 100 ^{o}C. Such conditions can produce deformation or degradation of materials due to water attack and may exceed melting or thermal transition temperatures. A study investigating the effects of steam sterilization on polymeric suture materials compared seven different steam sterilization techniques. The results showed at least one polymer property changed and molecular weight of the suture material decreased in all cases (Rozema et al., 1991). Specialized equipment (autoclave) is required to perform steam based sterilization. These changes are undesirable when characterizing a new material.

ɣ-Radiation is a rapid sterilization technique with high material penetration and low chemical reactivity. Unfortunately radiation based techniques cause polymer instability, degradation, cross linking and breakdown of polymer side chains (Athanasiou et al., 1996). ɣ-Radiation is known for example, to cause chain scission in poly(lactic acid)-poly(glycolic acid) copolymers (Cheu and Williams, 1983). Under *in vivo* conditions, irradiated poly(glycolic acid) sutures have been shown to degrade faster than samples that were not irradiated (Cheu and D.F., 1983). Administration of radiation requires specialized equipment, trained staff and a protected environment.

Room temperature chemical sterilization with gases such as ethylene oxide is used on heat sensitive polymers. After exposure to ethylene oxide materials require degassing or aerating for at least 24 hours as the gas is toxic. Sterilization via this method is slow because of aerating delays and polymers sterilized with ethylene oxide can retain residues of the gas in harmful quantities (Rickert, 2003). Ethylene oxide does not alter mechanical polymer properties for example ethylene oxide is commonly used to sterilize poly(lactic acid)-poly(glycolic acid) copolymers without detrimental effects on mechanical properties (Athanasiou et al., 1996).

In a novel approach to sterilization, poly(lactic acid)-poly(glycolic acid) copolymer scaffolds were exposed to Argon plasma created at 100 W for four minutes resulting in sterilization (Gardner and Peel, 1991). The scaffolds were studied for morphological changes indicative of degradation as assessed by scanning electron microscopy. Over a period of eight weeks, advanced morphological and volume changes in the polymer scaffold occurred although molecular weight and mass were stable (Gardner and Peel, 1991). Plasma sterilization was also compared to ethylene oxide for reprocessing of poly(urethane) electrophysiology catheters (Lerouge, 2000). Surface analysis techniques showed oxidization was limited to the surface layer in plasma sterilization whereas ethylene oxide induced deeper alkylation (Lerouge, 2000). Argon plasma sterilization used no toxic chemicals and left no toxic residues on treated materials. Plasma exposure is therefore a valid sterilizing technique.

Disinfection in 70% ethanol for thirty minutes affects neither the morphology nor the molecular weight of most organic polymers. While gram-positive, gram-negative, acid-fast bacteria and lipophilic viruses show high susceptibility to concentrations of ethanol in water ranging from 60-80%, hydrophilic viruses and bacterial spores are resistant to the microbial effects of ethanol (Gardner and Peel, 1991). As ethanol does not eliminate hydrophilic viruses and bacterial spores it is a disinfecting technique rather than a sterilization technique.

After examination of the evidence, sterilization of plasma polymer thin films was achieved by two methods: exposure to Argon plasma during plasma polymer production (Section 3.2 Production of tea tree oil derived plasma polymer thin films) followed by 30 minute immersion in 70% ethanol. These techniques were chosen because Argon plasma exposure was inherent to plasma polymer production and ethanol was an effective disinfectant, non-toxic, inexpensive, easy and quick to administer, did not require special equipment or training and did not alter polymer properties with brief exposures. After sterilization, processed samples were stored in sterile vials.

CHAPTER 4 BIOCOMPATIBILITY PROFILE

4.1 Host Response to Implant: Assessment Options

Effects of *in vivo* placement of devices or materials must be interpreted in order to definitively determine device biocompatibility. Biocompatibility results needed to be quantified or categorized to allow for comparison and statistical analysis of data. Although guidelines existed, no universal *in vivo* biocompatibility scoring system was supported and researchers had developed a variety of descriptors and classifications to fit individual experiments. Still, shared themes emerged. Three common techniques assessed host response to implant and device biocompatibility: animal behavior post implantation, gross examination of the implant site and histological examination of implant and surrounding tissue (Athanasiou et al., 1996, Black, 1999, Johnson, 1985, Kirkpatrick et al., 1998). Animal behavior and surgical site assessment were often achieved macroscopically whilst histology was a microscopic study of implant, associated cells and their organization within tissue. Macroscopic information was usually obtained by regular animal observation. Microscopic assessment was achieved post explantation, usually associated with euthanasia of the test animal. Blinded assessment by at least two independent experts was preferred by most researchers as this avoided subjective bias and reduced error (Heinrich, 2003). An applied definition for biocompatible host response post implantation was therefore normal animal behavior, limited wound complications and benign implant histology observed by a minimum of two independent and blinded experts.

4.1.1 Macroscopic assessment of host response to implant

Animal behavior post implantation of test material can be compared to behavior of sham controls as a means of determining biocompatibility. Behaviors such as feeding, socialization, mobility, grooming, alertness and activity may be altered in animals with systemic illness. Sick animals may be restless or lethargic, seizure, develop unhealthy skin or coats, vomit, display altered stool, have stunted growth, lose weight or die. Events such as animal death, disability or disease occurring after material implantation are gross indicators of lack of implant biocompatibility. Similarly surgical wounds and implant sites can be examined macroscopically in a living host or post mortem. Surgical wounds should heal according to known phases of inflammation in the presence of a biocompatible

implant. Signs at the implant site suggestive of impaired wound healing and therefore poor biocompatibility include rash, redness, discharge, abscess or sinus formation, ulceration, florid granulation, dehiscence and implant extrusion. Macroscopic indicators of biocompatibility should be collected at regular intervals with minimal disruption to natural animal behavior and by a consistent observer familiar with the test species (Martin and Bateson, 1993). Macroscopic indicators can often be categorized as present or absent and as such are reasonably easy to record and classify.

4.1.2 Microscopic assessment of host response to implant

Microscopic assessment of host response to implant is more problematic than macroscopic assessment. Microscopic reaction of host tissue to implant is complex, occurs along a spectrum and co-exists with phases of inflammation. Any classification of histological response to implant must be meaningful and correlate with material biocompatibility in order to have worth. As with all biocompatibility tests, histological assessments are context specific and seek out detectable, microscopic changes relevant to the implants desired application.

Histology is widely used to gage host response to implant and implant biocompatibility on a microscopic level (Sumner, 1990, Markovic et al., 2006). In fact ISO 10993-6 “Tests for Local Effects after Implantation” recommends implants are removed with a cuff of surrounding tissue for examination under low power microscopy to determine biocompatibility (Wallin and Upman, 1998). Histological features analyzed include tissue type, architecture and cellular infiltrate around the implant, cell counts, cell morphology, capsule thickness, assessment of neovascularization and extra cellular matrix deposition (Vandevord, 2002, Ruhe, 2005). Histology is a standard technique, cheap, available and capable of high through put. Standard staining with hematoxylin and eosin is sufficient to detect markers of acute and chronic inflammation, determine tissue integrity and define implant capsular features (Cullen et al., 2005, Calnan, 1970). Special stains can augment histology results or provide specific information. Examples of special stains include myeloperoxidase for neutrophils in acute inflammation, Masson’s Trichrome for collagen found in implant capsules, Wright stain for blood components and Gram-Twort stain (Appendix 4D) for bacteria associated with implant infection. Histological assessment is mostly performed manually by trained operators looking down the microscope or

automated through applications such as Image Pro Plus™ Software Package (MediaCybernetics, 2011). Multiple slides per specimen should be examined by board certified veterinary pathologists for adequate inspection and reporting of biocompatibility results (Bellamy and Olexson, 2000).

4.1.2.1 Histological categorization of host response to implant

In order for microscopic examination of implant and surrounding tissue to be meaningful there must be a way of recording, categorizing and comparing results in a discrete fashion. Achieving a meaningful scoring system is probably the most challenging part of assessing implant biocompatibility. Usually some part of the assessment is user dependent or subjective. Either a semi-quantitative or quantitative model can be applied.

Determining microscopic biocompatibility via histological assessment of the inflammatory response and capsule formations around implant by an experienced operator such as a pathologist is a common approach (Black, 1999, Johnson, 1985, Sousa et al., 2004). A semi quantitative inflammation scale or formal quantitative models can then be used to document the host response to implant (Parker et al., 2002, Wallin and Upman, 1998, Lips, 2006). Reactive or non-biocompatible materials generate a capsule of two to four millimeters around bio-implant whereas non-reactive tissues do not generate a capsule (Wallin and Upman, 1998). Severe reactions to implant are hallmarked by increased inflammatory cells and necrotic surrounding tissue (Plate 4.1) or a prominent foreign body reaction.

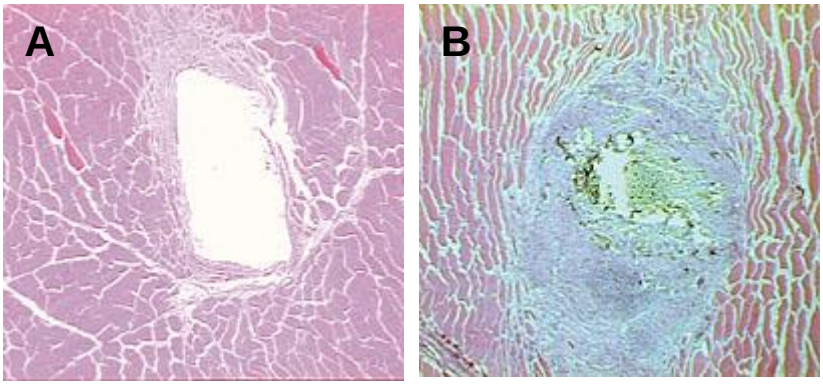


Plate 4.1: Local histological response of tissue to implant:
(A) a negative control demonstrates minimal inflammatory response and
(B) an implant has generated a severe and detrimental host response with necrosis and dense inflammatory cell infiltrate

Lips used a semi-quantitative scoring system to categorize microscopic tissue response to polymer implants (Lips, 2006). Her system divided cellular response around implant into groups with no cellar infiltration, sporadic infiltration or high cellular infiltration. Scores were obtained for macrophages, giant cells, fibroblasts and lymphocytes. Sections were also examined for the presence of fibrin, induction of vascularization and formation of fibrous capsule around polymer implants. Samples were examined at predetermined intervals of between five and 42 days to mirror known phases of inflammation.

A similar approach was taken by Sousa et al. who represented cellular components of endodontic implant response on a star chart (Sousa et al., 2004). Histological evaluation of explanted test materials and surrounding tissue occurred at four and 12 weeks. Criteria scored included: neutrophils, macrophages, lymphocytes, plasma cells, giant cells, dispersed materials, capsule formation, newly formed healthy bone, necrotic tissue, boney resorption and inflammatory response (Sousa et al., 2004). A result was awarded “*” for absent or slight response, “**” for moderate and “***” for severe response.

Both Parker and Royals created semi-quantitative grading scales for histological responses of soft tissue to implant (Parker et al., 2002, Royals et al., 1999). Parker described the reaction zone response around bio-implant in terms of capsule qualities, capsule thickness and interface qualities (Parker et al., 2002). Each subheading was broken down into descriptive categories and designated a score from zero to four to correlate with severity of reaction (Table 4.1). Scores from all three categories were summated to achieve a final biocompatibility assessment for implanted material. In the Royal study on biocompatibility of subcutaneous polymer delivery system implants in rhesus monkeys, presence and level of fibrous connective tissue associated with implant and inflammatory cell infiltrate were graded according to a semi-quantitative scale (Royals et al., 1999). Scores for each category were averaged and represented in a tabular format (Table 4.2).

Table 4.1: Histologic grading scale for soft tissue implants (Parker et al., 2002)

Reaction zone response	Score
Capsule qualities	
capsule tissue is fibrous, mature, not dense, resembling connective of fat tissue in the non-injured regions	4
capsule tissue is fibrous but immature, showing fibroblasts and little collagen	3
capsule tissue is granular and dense, containing both fibroblasts and many inflammatory cells	2
capsule consists of masses of inflammatory cells with little or no signs of connective tissue organization	1
cannot be evaluated because of infection or other factors not necessarily related to the material	0
Capsule thickness rating	
1-4 fibroblasts	4
5-9 fibroblasts	3
10-30 fibroblast	2
>30 fibroblasts	1
not applicable	0
Interface qualities	
fibroblasts contact the implant surface without the presence of macrophages or foreign body giant cells	4
scattered foci of macrophages and foreign body giant cells are present	3
one layer of macrophages and foreign body giant cells are present	2
multiple layers of macrophages and foreign body giant cells are present	1
cannot be evaluated because of infection or other factors not necessarily related to the material	0

Table 4.2: Tissue responses in rhesus monkeys to *in situ* formed implants at 28 days including descriptor and results (Royals et al., 1999)

Reaction zone response	Score
Presence/level of fibrous connective tissue associated with implant	
no difference from normal control tissue, no connective tissue at or around the implant site	0
presence of delicate spindle shaped cells or mild fibroplasia	1
presence of moderate connective tissue	2
disruption of normal tissue architecture and presence of moderately dense fibrous connective tissue	3
severe deposition of dense collagenous/ connective tissue around the implant	4
Qualification of inflammatory cell infiltrate response at implant site	
no difference from normal control tissue, no presence of macrophages, foreign body giant cells, lymphocytes, eosinophils or neutrophils at or around the implant site	0
presence of a few lymphocytes or macrophages, no presence of foreign body giant cells, eosinophils or neutrophils	1
presence of several lymphocytes, macrophages, with a few foreign body giant cells and small foci of neutrophils	2
presence of large numbers of lymphocytes, macrophages and foreign body giant cells, also notable presence of eosinophils and neutrophils	3
severe cellular infiltrate response to implant followed by tissue necrosis at or around the site	4

Test article	Average histology score	
	Fibrous connective tissue	Inflammatory cell infiltrate
Subcutaneous		
polymer delivery system 1	1.43 (7)	1.43 (7)
polymer delivery system 2	1.25 (8)	1.38 (8)
Intramuscular		
polymer delivery system 1	1.25 (4)	1.50 (4)
polymer delivery system 2	0.83 (6)	1.33 (6)

Barker, Framson et al employed a Leica DMR inverted microscope fitted with a SPOT digital camera system to image implant tissue specimens and quantify the result (Barker et al., 2005). Foreign body capsule thickness was calculated on Masson's trichrome stained sections using Image J™ software. Masson's trichrome distinguishes cells from surrounding connective tissue. Average thickness of collagen capsule was measured by integration of the collagen-stained area throughout the entire length of the implant section. This group of researchers analyzed a minimum of 30 sections from each sample. Collagen capsules were further assesses for fiber maturity by staining with PicroSirius Red. The total inflammatory cell response was quantified by determining the percentage of the implant surface lined by inflammatory cells, including foreign body giant cells (Barker et al., 2005). A great advantage of this computerized approach is reduction in subjectivity. Purely quantitative results using direct measurements are more statistically sound as they require no extrapolation and do not employ artificial groupings.

Variations on the above approaches have been employed to indirectly assess biocompatibility of implanted materials. The area of fibrous capsule (seen on histological slides) around implant (mm^2) has been used as a substitute quantitative measure for foreign body reaction (Mang et al., 2005). Bouet used laser doppler flowmetry in non-invasive semi-quantitative assessment of hyperemia due to the inflammatory response associated with implants (Bouet, 1990). Marois et al. quantified enzymatic activity and Ruhe assessed development of heterotopic tissue such as bone at the site of implantation as surrogate markers of biocompatibility (Ruhe, 2005, Marois et al., 1999). Surface changes in polymer implants have also been assessed as biocompatibility measures (Šprincl et al., 1982).

Immune response can be assessed as an indirect measure of biocompatibility. Excessive or aberrant immune reactivity is consistent with poor biocompatibility. *A*ssessment of immune response can be obtained with lymphocyte proliferation assays (Vandevord, 2002). Supplementary immuno-histochemical stains to assess immune cells and proteins associated with implants is a common assessment technique (Kvist et al., 2006). Antibody binding to implant assessed with ELISA assay is a specific example (Vandevord, 2002). Other components of the inflammatory response (white cells, extracellular alkaline and acid phosphatase, the complement component C3, total protein content, haptoglobin) can

be measured from blood specimens and protein bio-fouling of biomaterial surface is a measure of biocompatibility (Kvist et al., 2006, Markovic et al., 2006).

Selection of a categorization or quantification technique for microscopic assessment of bio-medical implants is dependent on resources, experience and the specific hypothesis being tested or research context. Approach is thus individualized.

4.2 Biocompatibility of Plasma Polymer Thin Films

4.2.1 Introduction and perspective

A novel polymer thin film derived from tea tree oil was created. Implantable medical coatings were targeted applications for this new thin film. A surface treatment for medical implants that preserves antimicrobial tea tree oil characteristics has potential to reduce medical implant infection (Chapter 5 Antibiofilm Effects). Currently few antimicrobial implant coatings exist and those in use are predominantly based on antibiotic chemotherapy risking toxicity and development of antimicrobial resistance (Kamal et al., 1991, Sheng et al., 2000).

Tea tree oil has known benefits including antimicrobial, antiseptic, anti-inflammatory and antioxidant properties (Carson and Riley, 1998, Carson et al., 1998, Shemesh and Mayo, 1991). It is locally produced, has a low adverse reaction profile and is used widely and accepted in many forms within the Australian and international consumer communities as a topical therapeutic agent (Web, 2011, Beane, 2011, Garsden, 1999).

Potential benefits of tea tree oil derived polymer are obsolete if polymer is not tolerated by host (is not biocompatible). As there is no current understanding of *in vivo* behavior of novel plasma polymer tea tree oil compounds, biocompatibility investigations are vital to determine the new materials candidacy as a medical device polymer. The complex and dynamic nature of biocompatibility testing means *in vitro* studies are poorly extrapolated to the *in vivo* model. Biocompatibility of novel tea tree oil derived polymer in an animal model is therefore highly preferable. Although human biocompatibility is the gold standard, the chain of clinical testing decries animal models predate human experiments. Mouse model testing provides an available, familiar, disease free, standardized stock that can be thoroughly analyzed in a short time and a controlled environment. In addition mice are relatively easy to procure, maintain and handle and are inexpensive in comparison to other animals. Adequate or improved alternative animal models exist (e.g. ovine, porcine) but these models have many disadvantages including size, procurement, maintenance, storage, handling and a lifecycle that is difficult to assimilate into a 12 month project. At a preliminary stage in materials development it is ethically and practically difficult to justify the use of larger animals. For the stated reasons the murine model was adopted for biocompatibility assessment of tea tree oil derived plasma polymers.

4.2.2 Methodology for biocompatibility testing

4.2.2.1 Overview of methods

Biocompatibility of tea tree oil derived plasma polymers was determined by subcutaneous implantation of polymer film into mice. Subsequent explantation of film and surrounding tissue was performed at predetermined time intervals to correlate with known phases of the inflammatory response. Implant analysis and comparison with controls provided biocompatibility data. In addition daily clinical observation of implanted mice and daily wound examination were utilized as markers of biocompatibility.

4.2.2.2 Experimental design

120 healthy, adult *Mus. Domesticus* BALB/c mice were enrolled into the biocompatibility experiment. Mice were between 10 and 15 weeks of age with a mature immune system and no senescent changes. Weight range of mice was between 20 and 25 grams and all subjects were non-pregnant females. Same sex mice were chosen to reduce confounding due to hormonal differences. Female mice were less aggressive and easier to handle than their male counterparts.

Biocompatibility testing was based on an elemental concept: insertion of a single polymer variant into a single mouse host. A solitary coated implant avoided burdening each mouse with multiple inserted foreign bodies. Operative placement of a single implant was rapid and required low dose anesthetic minimizing surgical risk of mortality and morbidity. Placing multiple implants into one animal can also confound and compromise results. For these reasons single implant/ single host protocol was applied in biocompatibility experiments.

In order to test biocompatibility of three tea tree oil derived plasma polymer variants, four trial arms were required: one arm for each polymer variant (25, 50 and 75 W) and a negative sham arm to act as a control. Polymer variants were generated by changing the radiofrequency power parameter during polymer production. Polymer variants then allowed effects of power production parameter on biocompatibility to be assessed.120 mice were divided into four groups each containing 30 individuals. One group (30 mice) was assigned to each experimental arm (25, 50 or 75 W or control). Host foreign body responses vary according to duration of implantation. To allow correlation with known phases of inflammation, specimen assessment was performed at planned time intervals.

Intervals of three, 14 and 28 days were chosen to represent early and late acute inflammation and the chronic inflammatory state respectively. 10 mice per experimental arm were sacrificed at each predetermined time interval via carbon dioxide asphyxiation as per Animal Ethics Committee at JCU. Implant and surrounding soft tissue were harvested *en bloc* from each mouse immediately post mortem and fixed for 24 hours in formalin. Collected implant specimens were subjected to analysis to assess biocompatibility (described below). 120 mice specimens supported a meaningful statistical experimental result without excessive animal sacrifice. 10 mice per subgroup were recommended for significant statistical interpretation (Pearson's Chi square test). This number was derived after discussion with Dr. Yvette Everingham, statistician at the School of Mathematics James Cook University and condoned by the Animal Ethics Committee at the same university (Table 4.3).

Table 4.3: Grouping of mice for biocompatibility experiments: three polymer variant arms (25, 50 or 75 W) and one control arm were populated. Each arm contained 30 individuals (three groups of 10 mice). 10 mice from each arm were sacrificed at each of three time points (three, 14 and 28 days). A total of 120 individuals were therefore required.

Time to sacrifice	25 W polymer	50 W polymer	75 W polymer	Control (negative sham)	Total
day 3	10	10	10	10	40
day 14	10	10	10	10	40
day 28	10	10	10	10	40
Total	30	30	30	30	120

4.2.2.3 Production of implants

Plasma polymers cannot be produced independently of deposition substrate. Polymer thin film was therefore produced on PTFE substrate to simulate a coated medical implant. Six millimeter diameter, uniform, circular discs were cut from 0.38 mm thick PTFE sheeting. PTFE was used because it is inert, inexpensive, available and readily tailored. More importantly PTFE is employed extensively in production of human medical implants and thus has clinical relevance. A disc with six millimeter diameter fits well within mouse

subcutaneous space but also affords a generous surface for polymer deposition. 0.38 mm thick PTFE also had some flexibility allowing easy insertion. Rounded disc implants were selected because they were less likely to cause local trauma than implants with corners. PTFE discs were coated on both sides with a single polymer variant (25, 50 or 75 W) deposited over 10 minutes (Section 3.2 Production of Tea Tree Oil Derived Plasma Polymer Thin Films). Naked PTFE discs served as controls. A 10 minute time interval for thin film deposition reliably produced a film thickness of greater than 1000 nm. Thicker films reduced risk of uncovered substrate and allowed visual confirmation of the presence of a polymer film coat prior to implantation.

4.2.2.4 Sterilization and storage of implants

Implants were decontaminated by immersion in 100 vol% ethanol for one hour. They were then rinsed for four hours in sterile, physiological phosphate buffered solution (PBS) to remove ethanol and any contaminants. Ethanol does not alter plasma polymer structure during short exposures (although it may do after protracted exposure), has low toxicity and can be removed from implant so as not to compromise results via evaporation. Ethanol is also inexpensive, available and effective as a decontaminant. Discs were stored in sterile PBS prior to implantation (overnight) and were handled aseptically at all times after decontamination.

4.2.2.5 Surgical implantation equipment

All reusable surgical equipment was sterilized by autoclave processing (20 min at 121^{o}C) at the School of Veterinary and Biomedical Sciences at James Cook University prior to use. Non-reusable equipment was purchased sterile. Items for the implantation procedure included:

- dissecting forceps (2)
- artery forceps (2)
- dissecting scissors (1) and suture scissors (1)
- sterile surgical gloves, gown and drapes
- sterile gauze
- sterile saline in sterile pot
- 3/0 Prolene™ suture
- 7.5mg/ml povidone iodine skin preparation solution

4.2.2.6 Technique of implantation

Surgical procedures were carried out in the Animal Surgery (room DB085-008), School of Veterinary and Biomedical Sciences. Participant mice were anaesthetized via intra-peritoneal injection of 0.06 ml ketamine/xylazine/normal saline solution injected with a sterile syringe and 25G needle. Anesthetic mixture was made up as follows:

ketamine (100 mg/ml) 3 ml + xylazine (20 mg/ml) 1 ml + normal saline 6 ml = 10 ml.

Volume of anesthetic was calculated according to recommended weight related dosing schedules for mice (ketamine 0.15 mg/gm, xylazine 0.01 mg/gm) and an assumed individual murine weight of 20 gm. The above regime provided reliable surgical anesthesia for approximately 15 minutes post induction and induction occurred within five minutes of intra-peritoneal anesthetic administration.

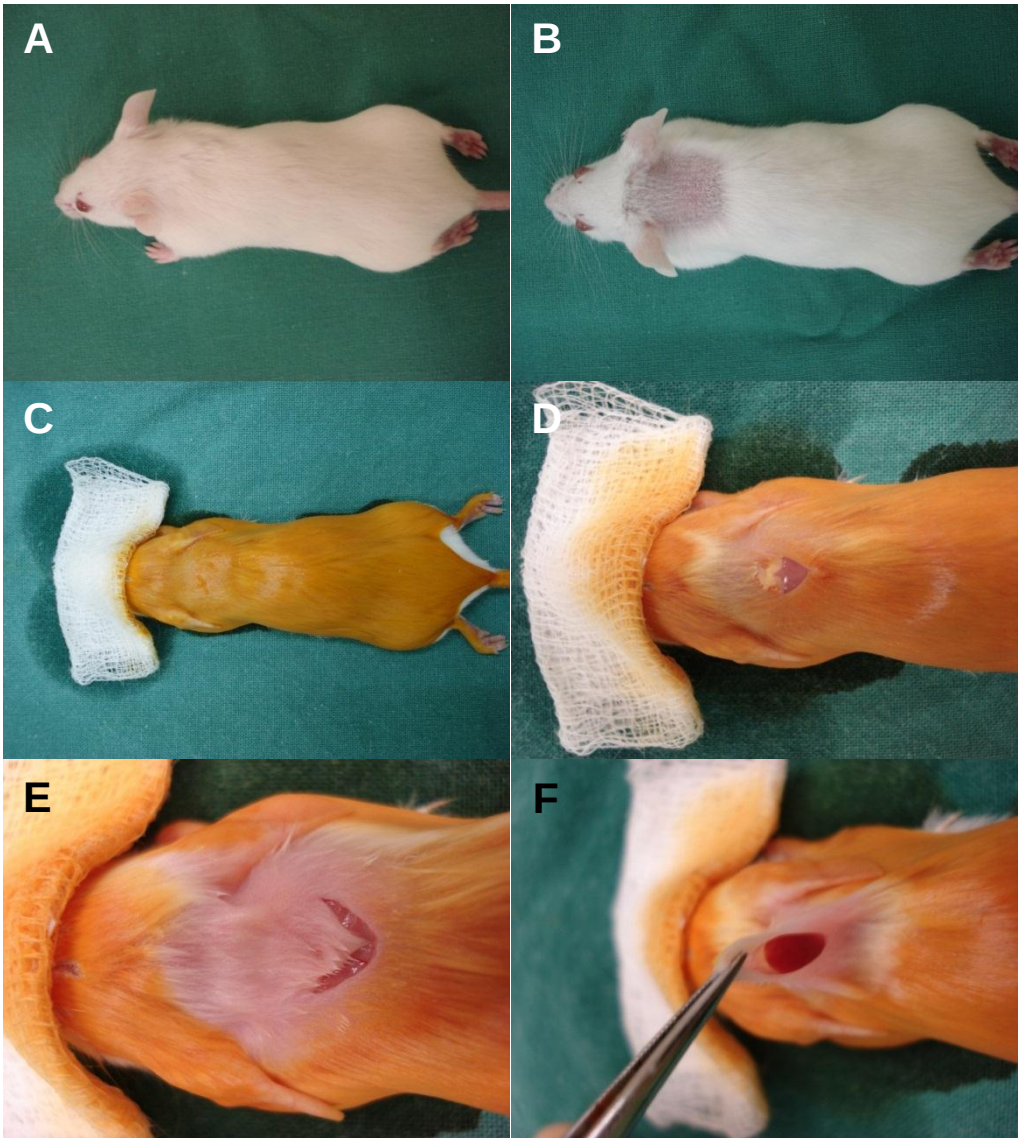
Anaesthetized mice had fur clipped over the dorsal inter-scapular region in preparation for surgical implantation. Mice were transferred onto a sterile surgical sheet overlying a heated pad. 7.5 mg/ml povidone iodine skin preparation solution was applied to the entire dorsum of the anesthetized mouse. Murine ocular desiccation was prevented by intra operative topical application of moist saline gauze.

Under sterile conditions, a small transverse cutaneous incision was made across the prepared inter-scapular region on each mouse. An upper wound flap was raised and subcutaneous pocket fashioned in the longitudinal axis using blunt dissection. Six millimeter diameter PTFE (coated or non-coated) implant was deliberately placed as far from the incision as the subcutaneous pocket would allow. Intention of tunneling was to encourage separation of implant from incision and thus reduce risk of implant infection, sinus and extrusion. Tunneling also separated implant from confounding changes caused by skin incision. The inter-scapular location had advantages of loose skin, allowed reasonably a-traumatic, low risk and quick insertion, made the implant site easy to inspect, was difficult for mice to disturb and was well tolerated by the animals. Finally the skin wound was closed with interrupted, 3/0 Prolene™ monofilament sutures that were minimally inflammatory, have a low infection risk and were easy to remove (Plate 4.2).

The inserted PTFE disc was either:

1. coated with one of three tea tree oil derived plasma polymer variants (25, 50 or 75 W)
2. naked (negative sham)

During recovery from anesthesia, mice were covered with a light cloth and placed under supplemental heat (light bulb). Animals were monitored until they moved on their own accord and then placed back into their housing cage.



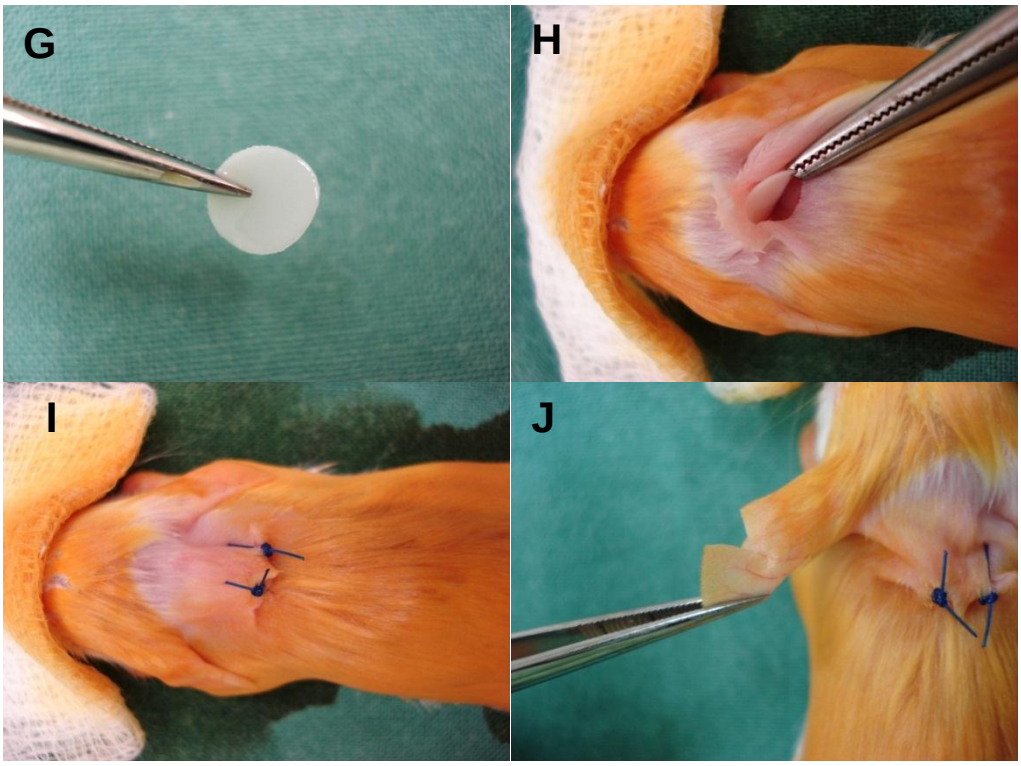


Plate 4.2: Technique of implantation of polymer coated discs into mice:

(A) anesthetized mouse in sterile field
(B) inter-scapular region clipped of fur pre-operatively
(C) operative field prepped with povidone iodine and eyes protected
(D) incision made through inter-scapular skin
(E) position of incision
(F) subcutaneous pocket created by raising superior flap
(G) sterile disc ready for implantation
(H) disc implanted into subcutaneous pocket aseptically
(I) wound closed with interrupted 3/0 Prolene™ sutures
(J) mouse marked with ear notch (Plate 4.3)

4.2.2.7 Inspection of postoperative mice

Inspection of postoperative mice was divided into two phases: the immediate postoperative phase with recovery from anesthesia and then routine daily examinations. Immediate postoperative mice were observed until awake, active, grooming and eating/drinking. Primary postoperative complications were recorded in a logbook. Thereafter mice were housed in the Small Animal Facility (building DB086) of the School of Veterinary and Biomedical Sciences. Each mouse was routinely examined every morning by a single observer up until and including the day of euthanasia. Implants were

not routinely palpated for fear of causing confounding trauma at the implant site. Examinations focused on local wound site inspection and assessment of general behavioral parameters as markers of systemic illness. Wound inspection sought end points of infection, collection, sinus formation, implant migration/exposure/extrusion, wound dehiscence, excessive granulation or scar formation, murine originated disruption of the wound or any other deviation from normal wound healing. General behavior parameters focused on evidence of undue distress including ruffled fur and poor grooming, failure to move, obtundation, emesis, altered stool, failure to eat and drink or weight loss. Focal abnormalities that developed distant from wound and implant site were also noted and considered as possible remote complications. If animals were judged to have undue stress or illness the plan was to euthanize them with carbon dioxide gas and conduct a post-mortem examination. Similarly if animals died independently of being euthanized the plan was for a post-mortem examination to determine the cause of death. Final macroscopic wound descriptions were obtained post mortem prior to implant removal after a thorough inspection of incision, implant site and whole mouse carcass. Results were recorded manually in a logbook and abnormalities digitally photographed.

4.2.2.8 Identification of individuals

Mice were housed in cages in groups of five individuals. As each experimental arm required 10 mice, two cages were required per experimental arm. Mice within a given cage had identical implants, insertion and sacrifice dates. Cages were labeled with type of implant (25, 50 or 75 W or control), date of implantation and planned date of sacrifice. In addition cages were labeled as containing mice No. 1-5 or 6-10. Individual mice were identified by ear notching performed after induction of anesthesia for implantation surgery (Plate 4.3).

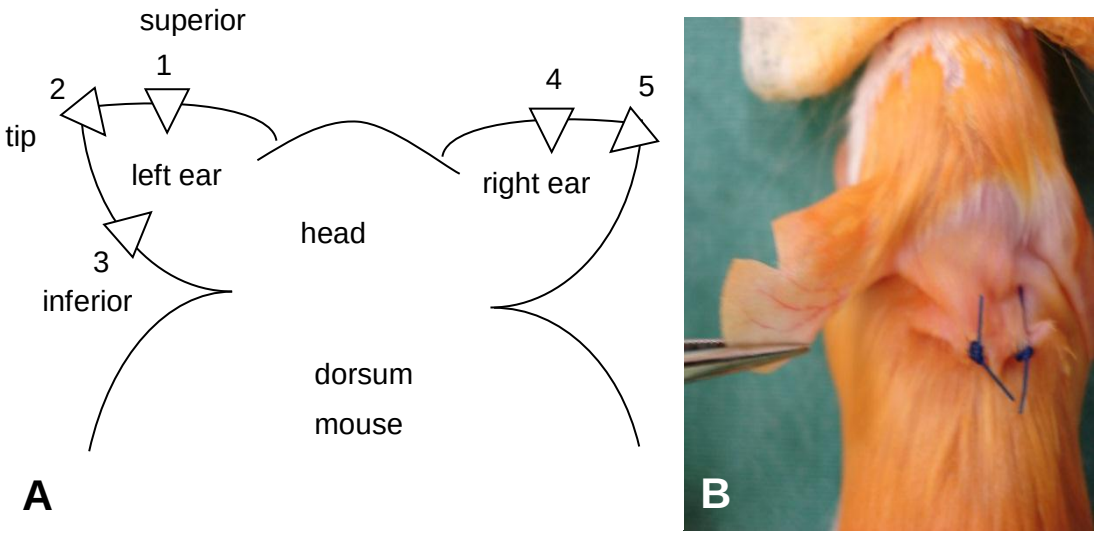


Plate 4.3: Individual identification of mice by ear notching: (A) location of identifying notices and (B) an example of an identifying notch in left superior position correlating with mouse numbers 1 or 6.

1. left superior
2. left tip
3. left inferior
4. right superior
5. right tip

In cages containing mice numbers 6-10, notch positions correlated as follows: 1~6, 2~7, 3~8, 4~9, 5~10.

4.2.2.9 Sacrifice

At time intervals of three, 14 and 28 days to follow the known phases of inflammation, implanted mice were euthanized with carbon dioxide gas. Implants were removed aseptically with a cuff of surrounding tissue and removed specimens prepared for histological examination to assess microscopic markers of biocompatibility. Animal carcasses were bagged and placed in the animal carcass bin in the cold room of the Necropsy Building at the School of Veterinary and Biomedical Sciences. Contractors removed the animal carcass bin and deep buried the contents when the bin was full.

4.2.2.10 Implant harvest (explantation)

Implantation scheduling was designed to allow all implantation and harvesting to be completed within one month of the first implant. Scheduling had to allow for availability of mice in batches over the course of the month. Nested implantation and harvesting meant

some groups (three day group) were implanted after and harvested before groups containing implants for longer durations (14 and 28 day groups).

Implant harvest was carried out in the Animal Surgery (room DB085-008), School of Veterinary and Biomedical Sciences at James Cook University. Mice containing subcutaneous implants of either PTFE alone or PTFE coated with plasma polymer were euthanized with carbon dioxide gas at planned time points (three, 14 and 28 days labeled as 3/7, 14/7 and 28/7 respectively) to correlate with normal phases of wound healing. Immediately post mortem, mice were placed onto a sterile sheet. Surgical wounds were examined macroscopically for adverse outcomes such as infection, sinus formation, dehiscence, implant extrusion or any other unusual or unexpected findings. Results of macroscopic examination were recorded manually in a logbook and photographic documentation obtained in cases of abnormal wound or mouse appearance. Inter-scapular soft tissue was palpated to localize subcutaneous implant in preparation for excision. Observing aseptic technique, *en bloc* excision of implant and surrounding soft tissue with a one half centimeter cutaneous margin was performed including all soft tissue layers to muscle (Plate 4.4). Harvested specimens were placed directly into individual, labeled, sterile 50 ml plastic vials containing 10% formaldehyde. Specimens were fixed for 24 hours prior to histological processing.

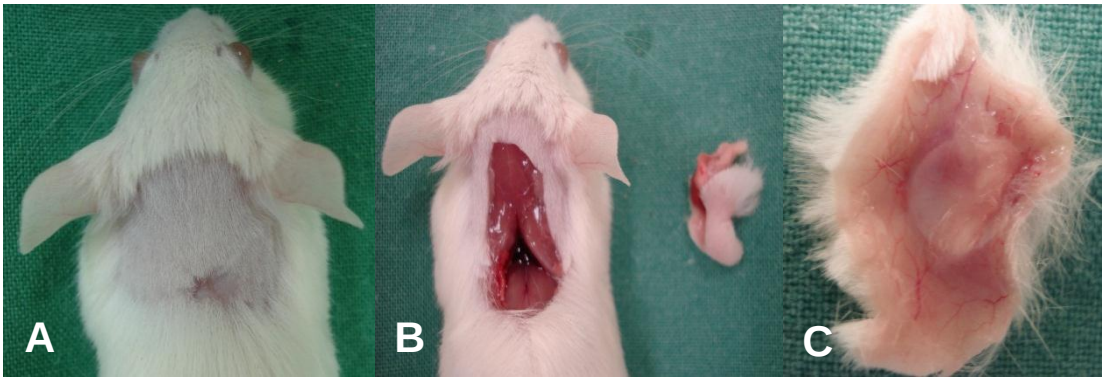


Plate 4.4: Implant harvest from euthanized BALB/c mouse:
(A) macroscopic inspection wound
(B) *en bloc* explantation with surrounding soft tissue to muscle
(C) implant within soft tissue specimen

4.2.2.11 Histological processing and preparation of explanted specimens

Post explantation of implant and surrounding tissue *en bloc* from murine host, samples were fixed in 10% formalin for 24 hours. After fixation, specimens were sharply bisected through the center of the implant disc and the halves placed together into labeled biopsy cassettes. The cassette contents were dehydrated with graded ethanol and xylene over 16 hours then embedded in paraffin wax as per standard protocols. Once hardened, paraffin blocks were trimmed and 5 µm thick ribbons were sectioned from the blocks using a Leica RM2135 Rotary Microtome. Sectioned specimen within paraffin ribbon were placed into a water bath at 45^{o}C and mounted onto standard glass laboratory slides. Finally sections were air dried for 45 minutes then baked at 45^{o}C in an oven overnight. Sections were routinely stained with hematoxylin and eosin (Appendix 4E). Once processed specimens were stored in the histology laboratory at the School of Veterinary and Biomedical Sciences at James Cook University until the conclusions of experimentation at which time they were loaded into biological waste bags and incinerated.

4.2.3 Histological assessment and scorecard for implant biocompatibility

Biocompatibility was determined by histological examination of sectioned subcutaneous implants and surrounding murine tissue at time intervals of three, 14 and 28 days after insertion (Section 4.2 Biocompatibility of Plasma Polymer Thin Films). Histology slide examination was performed in the microscope room, School of Veterinary and Biomedical Sciences at James Cook University using an Olympus® System BX41 BX2 Series optical laboratory microscope (1.25-100 x magnification). Specimen assessment was performed by two qualified, independent assessors (Professor P. Summers and Dr Emma Igras) and was not blinded. Scores that did not correlate between assessors were discussed and an agreed revised score was applied.

Appendix 4B contains the scorecard for histological categorization of subcutaneous implant location and tissue response. The scorecard details the semi-quantitative scoring system used to categorize histology results for the purpose of microscopic biocompatibility assessment of implants. The categorization was adapted from Parker's study of soft tissue response to silicone and poly-L-lactic acid polymer implants and was employed because the system was applicable, easy to implement and made use of available resources (Parker et al., 2002). The histological scorecard was developed in combination with Professor P. Summers from the School of Veterinary and Biomedical Sciences at James

Cook University who had extensive experience as a diagnostic veterinary pathologist. Computer based implant assessment software was not available at James Cook University, is expensive to procure and so was not implemented despite its advantages of quantification and lack of subjectivity.

The scorecard for histological categorization of subcutaneous implant location and tissue response broke observations down into three categories: implant location, implant-skin interactions and reaction zone response. Reaction zone responses consisted of assessment of capsular maturity, total capsule thickness measured as layers of fibrocytes around the implant, thickness of mature (or dense) capsular layer, interface qualities associated with a foreign body response and the nature of surrounding tissue cellular infiltrate.

4.3 Inspection of Postoperative Mice: Results and Discussion

4.3.1 Perioperative complications

The first three mice implanted (control 14/7 1-3) had wound closure performed with Tyco AutoSuture™ 35 disposable staples. Rationale behind stapled as opposed to sutured primary closure was to reduce operative time and therefore anesthetic time by using a quicker wound closure technique. Reduced anesthetic time aimed to reduce anesthetic complications. However, as stapled mice awoke from anesthetic, wounds primarily dehisced. Technical failure was due to correctly applied staples tearing through murine skin or dropping out due to relatively large staple weight and size compared to mouse host. Mice with dehisced wounds required a second anesthetic and wound closure with 3/0 interrupted polypropylene sutures. Subsequent murine wound closures were all performed with interrupted 3/0 polypropylene sutures.

Two perioperative deaths occurred. The 10 mice implanted with 25 W tea tree oil plasma polymers intended for harvest at 14 days became hyperactive and agitated soon after induction of anesthesia via intra peritoneal injection of ketamine and xylazine. Hyperactivity and agitation lasted less than one minute in all cases and effected mice along a spectrum with some showing brief hyper vigilance whilst others thrashed violently against the wall of their poly(styrene) holding container prior to becoming fully anesthetized. A single mouse (25 W 14/7 6) died during induction of anesthesia. Post mortem of this mouse revealed fractured ribs and a left traumatic pneumothorax. It is unclear whether the traumatic injury resulted from the reaction to anesthetic medications or due to the injection itself. All other mice injected with the same batch of anesthetic mixture went on to uneventful recovery. Abnormal induction behavior in mice may have resulted from contaminated or incorrectly proportioned anesthetic mixture. No obvious abnormality could be found on examination of anesthetic drugs nor were drugs expired but as anesthetic mixture was suspect it was discarded and replaced. No subsequent similar events transpired. The second perioperative death was due to exsanguination. 50 W 28/7 3 was successfully anesthetized and implanted but died during recovery from anesthesia. Post mortem showed a massive hemoperitoneum. A specific focus was not found but bleeding was presumably from vascular or solid organs injured by the intra peritoneal injecting needle. Both dead mice were immediately replaced within the experiment as deaths were due to perioperative complications unrelated to polymer implant.

4.3.2 Complications detected on daily inspection

Mice were individually inspected on a daily basis from the first post-operative day to sacrifice. Mice sacrificed at day three retained sutures. Mice implanted for longer periods shed sutures by the seventh postoperative day as wound healing progressed. Throughout the observation period mouse behavior, activity and general well-being was maintained within normal limits by all individuals. No unplanned deaths occurred outside of the perioperative period. Logbook data for wound inspection is found in (Appendix 4A).

4.3.2.1 Sinus formation and implant extrusion

Sinus between skin and implant cavity was the most common macroscopically detectable local wound complication (Plate 4.5, Table 4.4 and Table 4.5). Sinus was defined as a visible tract between the skin surface and the implant cavity. Some sinuses were transient and others persisted to animal sacrifice. No mice sacrificed at the three day time interval had evidence of sinus formation probably because it was too early in the wound healing process for sinus formation. There were no synchronous or metachronous sinuses within any individual mouse.

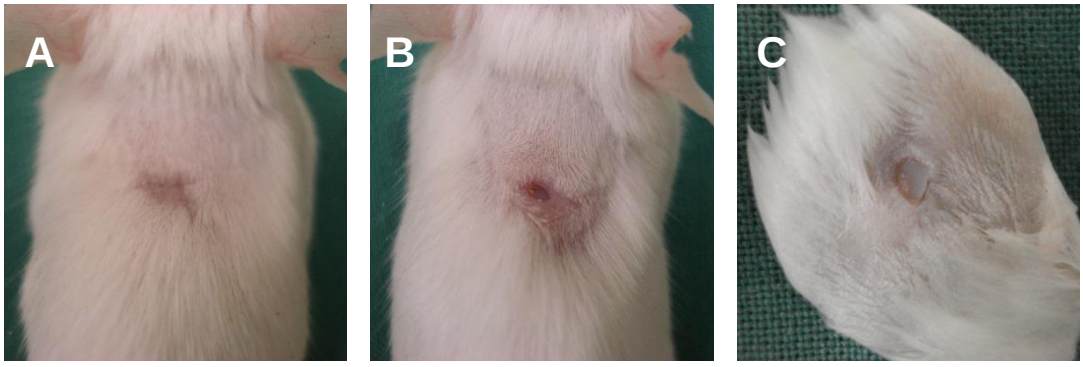


Plate 4.5: Sinus complications post implantation: (A) well healed wound, (B) wound sinus and (C) a partial implant extrusion. These changes occurred along a spectrum.

Of mice planned for sacrifice at 14 days, no control animals developed sinuses but all three polymer groups contained examples of sinus development. In the 25 W 14/7 group, sinuses formed in two mice on day nine, one healed over three days and the other persisted to sacrifice. In the 50 W 14/7 group, two sinuses developed on day nine and three sinuses on day 11 with all five sinuses persisting to sacrifice. Of this 50 W 14/7 group of mice with sinuses one had an obvious partial disc extrusion on day nine and the second (initially found to have a sinus on day 11) had completely extruded its

implant at sacrifice. Three sinuses developed in the 75 W 14/7 group. Two sinuses were found on day eight, one healed after four days and the other persisted to sacrifice. The third sinus developed on day 13 and persisted to sacrifice.

Mice planned for sacrifice at 28 days had sinuses develop in control, 25 W and 75 W groups with the 50 W group demonstrating no sinus development. A single control animal developed a sinus on day eight that healed after five days. In the 25 W 28/7 group three sinuses formed, two sinuses developed on day nine and were healed three days later. A final late sinus occurrence was found on day 25 and persisted to sacrifice. Two sinuses happened in the 75 W 28/7 group, both appeared on day eight. The first resolved over five days but the second was associated with delayed wound dehiscence followed by exuberant granulation tissue within the wound and finally complete implant extrusion.

Table 4.4: Sinus development during implantation period

Implanted material	Implant group (day at sacrifice)			Total
	3	14	28	
control PTFE	0	0	1	**1**
25 W plasma polymer	0	2	3	**5**
50 W plasma polymer	0	5	0	**5**
75 W plasma polymer	0	3	2	**5**
Total	**0**	**10**	**6**	**16**

Table 4.5: Sinus present at sacrifice

Implanted material	Implant group (day at sacrifice)			Total
	3	14	28	
control PTFE	0	0	0	**0**
25 W plasma polymer	0	1	1	**2**
50 W plasma polymer	0	5	0	**5**
75 W plasma polymer	0	2	1	**3**
Total	**0**	**8**	**2**	**10**

4.3.2.2 Isolated complications

Excluding sinus formation and implant extrusion, two further macroscopic complications were encountered amongst the 120 implanted mice. On day of sacrifice

(day three) a single mouse (75 W 3/7 6) wound became erythematous and indurated without evidence of collection or discharge. The mouse appeared systemically well. Differential diagnoses included infection, exuberant inflammation, foreign body reaction or allergy to either suture material or implant or mechanical interference by other mice. On explantation, surrounding soft tissue was injected and edematous. Changes were photo-documented and are displayed below next to a normal healing wound and explant for comparison (Plate 4.6). Abnormal specimen was evaluated histologically with hematoxylin and eosin staining and with Gram-Twort stain in an attempt to determine cause. Although the Gram-Twort stain was negative for bacteria, histology demonstrated a dense neutrophil infiltrate. The exact cause of this complication was not discovered.

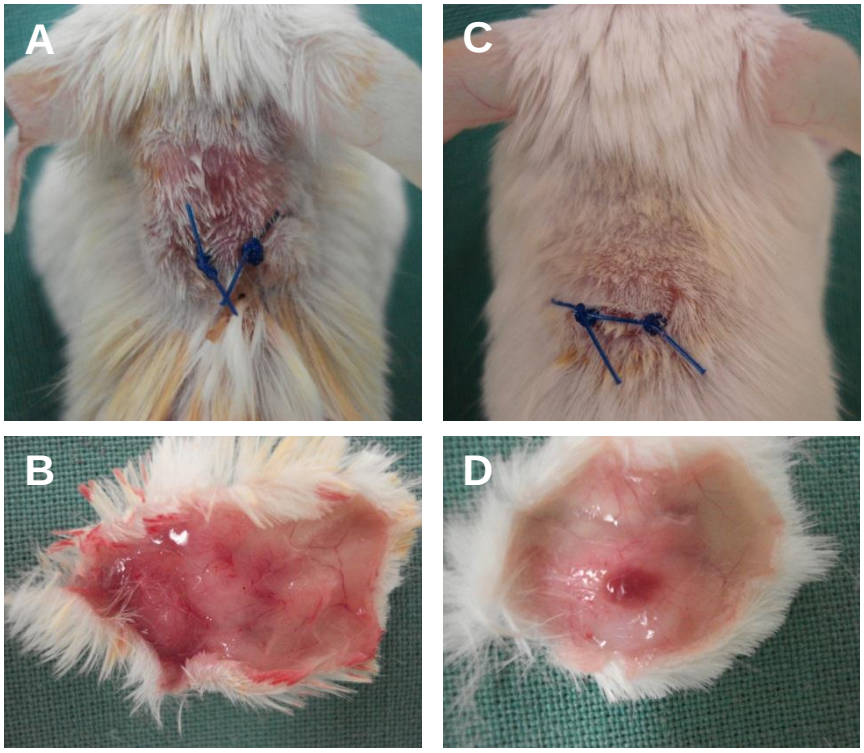


Plate 4.6: Isolated mouse complication: (A) erythematous wound seen in mouse 75 W 3/7 6 and (B) injection and edema in the subsequent explant. (C) and (D) show a normally healing wound and explant for comparison.

The second presumed isolated complication occurred distant to site of surgical implantation. A single mouse (50 W 28/7 1) developed hair loss and crusting of skin around the right eye 21 days post implantation. The eye itself was unaffected and the mouse was otherwise well (Plate 4.7). Changes persisted but did not progress. Possible causes for the isolated peri-orbital changes included atopic dermatitis,

eczema, fungal infection, mite infestation, autoimmune disease or trauma however polymer implant causation could not be ruled out.

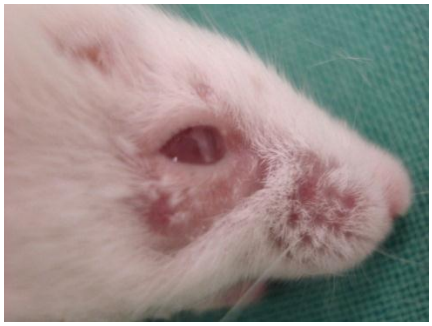


Plate 4.7: Peri-orbital hair loss and cutaneous crusting occurring on day 21 post implantation in mouse 50 W 28/7 1

4.3.3 Statistical evaluation of sinus results

As sinus formation was the most prevalent complication noted during implantation experimentation, an attempt was made to statistically analyze effect of substrate on sinus development. SPSS™ statistics software package calculated Pearson's chi-square test and Fisher's Exact test for independence and goodness of fit of implant effect on categorical variable sinus present or absent. When groups were separated according to time of sacrifice (and all polymer variants were combined) only two contingency tables demonstrated significant results. Mice sacrificed at 14 days (Pearson chi-square value 0.033) and 28 days (Pearson chi-square value 0.000) showed sinus formation was more frequent in plasma polymer groups at sacrifice than in control groups. These results needed to be interpreted with caution however as expected cell frequencies were less than five in many instances and total for all cells was less than 50. In an attempt to circumvent statistical problems associated with small numbers, all polymers were considered in one group and compared to control substrate across all time points (N=120). Fisher's Exact test result of 0.049 showed a significant association between plasma polymer implant and sinus formation in mice.

4.3.4 Results summary

Polymer groups all developed more sinuses than control. Six of 16 sinuses resolved (none took longer than five days to do so). Control group developed only one sinus (28/7 group) that resolved spontaneously over five days. Each polymer power category (25, 50 and 75 W) developed 5 sinuses, most occurring in the 14 day sacrifice group. Of the polymer associated sinuses, three out of five healed in the 25 W polymer group,

none of the 50 W polymer sinuses healed and two out of five sinuses associated with 75 W polymer implants healed. As a percentage, 20% of sinuses healed by day 14 post implantation and 66% by day 28.

Although the statistical data was weak due to small enrolment numbers, implant experiments demonstrated tea tree oil derived plasma polymers produced significantly more sinuses in mice than bare PTFE control implants at sacrifice times points of 14 and 28 days. Because 15 of 16 sinuses developed between days eight and thirteen it is valid to combine the 14 and 28 day sacrifice groups and analyze them together for statistical purposes. Taking this approach a definite significant association is seen between polymer implant and sinus formation.

The significance of isolated complications of possible would infection and eye dermatitis was difficult to determine in the context of such a small trial.

4.3.5 Discussion

Macroscopic biocompatibility results were inconclusive and need further investigation. Sinus development is not favorable however if sinuses heal spontaneously their impact on biocompatibility is lessened. Although plasma polymer implants seemed to create more sinuses than control, two thirds of sinuses healed by 28 days post implantation and no sinuses became infected or impacted the general well-being of mice hosts. In mice, the panniculus carnosus layer of skeletal muscle immediately beneath skin gives the cutaneous layer greater ability to heal by wound contraction and infection rates are in turn reduced (Machado et al., 2011). Because panniculus carnosus is essentially absent in humans, human ability to heal sinuses may however be reduced. Sinus formation in mice occurred after the acute inflammatory phase of wound healing was complete suggesting association with proliferation and remodeling or chronic inflammatory processes.

Sinus formation could not be statistically linked to the power parameter at which plasma polymers were generated. This may be a limitation of a small data set rather than an absolute result. Better data on effects of polymer power and other production parameters on sinus formation and better delineation of effect of structure and functional properties on biocompatibility are required to further investigate tea tree oil derived plasma polymer thin film biocompatibility profiles.

4.4 Histological Assessment of Biocompatibility: Results and Discussion

(Appendix 4C)

4.4.1 Implant location

Implant location on histology slides was sought partly as a quality control measure to ensure the correct tissue was being examined and partly to identify implant migration or extrusion. Of the 120 histology specimens examined, only 77 demonstrated an implant to be present and *in situ.* Of the remaining 43 specimens, 11 showed partially displaced implants or completely displaced implants overlying the slide and 30 specimens were devoid of implant material but had well defined “footprints” formed by lost implant (Plate 4.8). Only two specimens lacked both implant and implant “footprint”. These two specimens had contained a 50 W tea tree oil plasma polymer disc (sacrificed at 14 days) and a 75 W tea tree oil plasma polymer disc (sacrificed at 28 days).

Macroscopic examinations of mice confirmed only two hosts extruded their implants. Why then did so many slides harbor partially or completely displaced implants or clear footprint evidence of implant material not seen on the slide? The answer lay in a processing artifact. PTFE has a very low co-efficient of friction and therefore implant sections adhered poorly to glass laboratory slides. Sectioned PTFE discs frequently washed away during histological processing or were washed out of their “footprint” and deposited elsewhere on the tissue sample or slide. Taking into account a well-defined “footprint” of soft tissue changes as indirect evidence of *in situ* implant, all specimens had direct or indirect evidence of implant within the specimen. Histological examination also paralleled macroscopic confirmation of two polymer implant extrusions. Extrusion specimens histologically demonstrated partially obliterated capsular “footprints” with organization of implant cavity, implant cavity extending outside of the subcutaneous plane and in particular implant cavity extending to the skin surface (Plate 4.9).

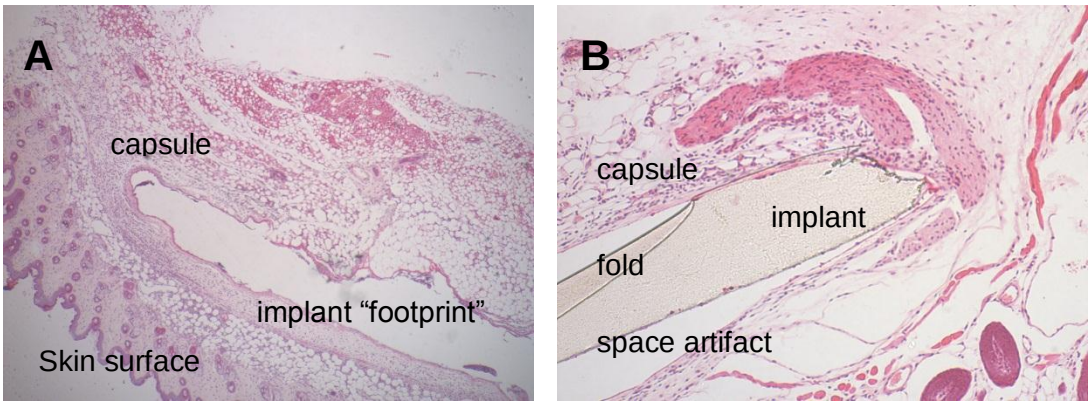


Plate 4.8: Histological processing artifacts: (A) well defined soft tissue cavity or "footprint" remained where implant was washed away and (B) implant is present but displaced and folded onto itself. (Light microscopy of standard hematoxylin and eosin stained specimens at (A) 4x magnification and (B) 10x magnification).

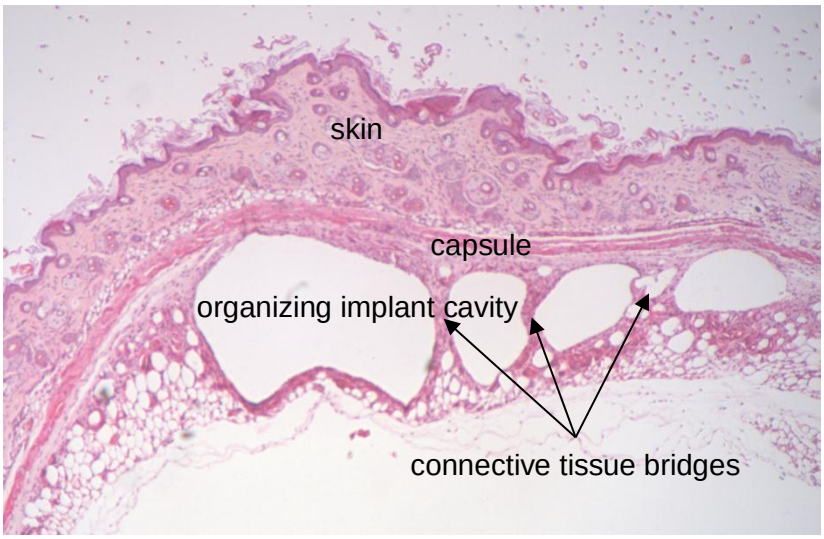


Plate 4.9: Histological soft tissue evidence of implant extrusion rather than processing artifact: note organization of the implant cavity associated with absent implant. Specimen was from a mouse known to have extruded implant (75 W polymer sacrificed at 28 days). (Light microscopy of standard hematoxylin and eosin stained specimen 4x magnification)

4.4.2 Implant-skin interactions

The primary complication of implantation noted on macroscopic examination of live mice was sinus formation. Histological analysis of implant-skin interactions was designed to pick up microscopic evidence of sinus formation to corroborate findings of macroscopic sinuses (Plate 4.10). Other implant-skin interactions sought to reflect poor host tolerance of implant were cutaneous ulceration adjacent to implant (without sinus formation) or direct adherence of dermis to implant via fibrous connective tissue. Adherence of implant to skin, ulceration without sinus and sinus formation occur along a spectrum and may indicate infection, pressure or mechanical effects of implant, florid inflammation, foreign body response, rejection or abnormal stimulation of the immune system. Fibrous connective tissue tethering implant to skin is unfavorable because it predisposes to ulceration and sinus formation and represents significant tissue response to implant. All of these responses are aligned with poor implant biocompatibility.

The optimal biocompatible result was considered to be no interaction between skin incision and implant and minimal or absent tissue response to implant. Animals sacrificed at the three day time point were expected to demonstrate an acute incisional wound without direct implant communication. Biocompatible implants sited for a longer duration were expected to show wound healing with minimal reaction around implant. Microscopic implant-skin interactions were grouped as pathological or non- pathological. Pathological skin interaction included fibrotic adherence of implant to skin, cutaneous ulceration or sinus formation. Non-pathological skin interactions encompassed implants in a remote subcutaneous pocket distant from the skin incision and those implants that were adjacent to incision without fibrotic adherence, ulceration or sinus formation. Originally implants were sited in subcutaneous pockets distant to cutaneous incisions in an attempt to separate host reactions incited by implants from those induced by incisional wounding of skin and subcutaneous tissue. Loose rodent skin however, allowed free movement of PTFE discs along implantation tracts. Location of wholly subcutaneous implants next to skin incisions was not therefore considered pathological implant migration but rather a reflection of murine anatomy.

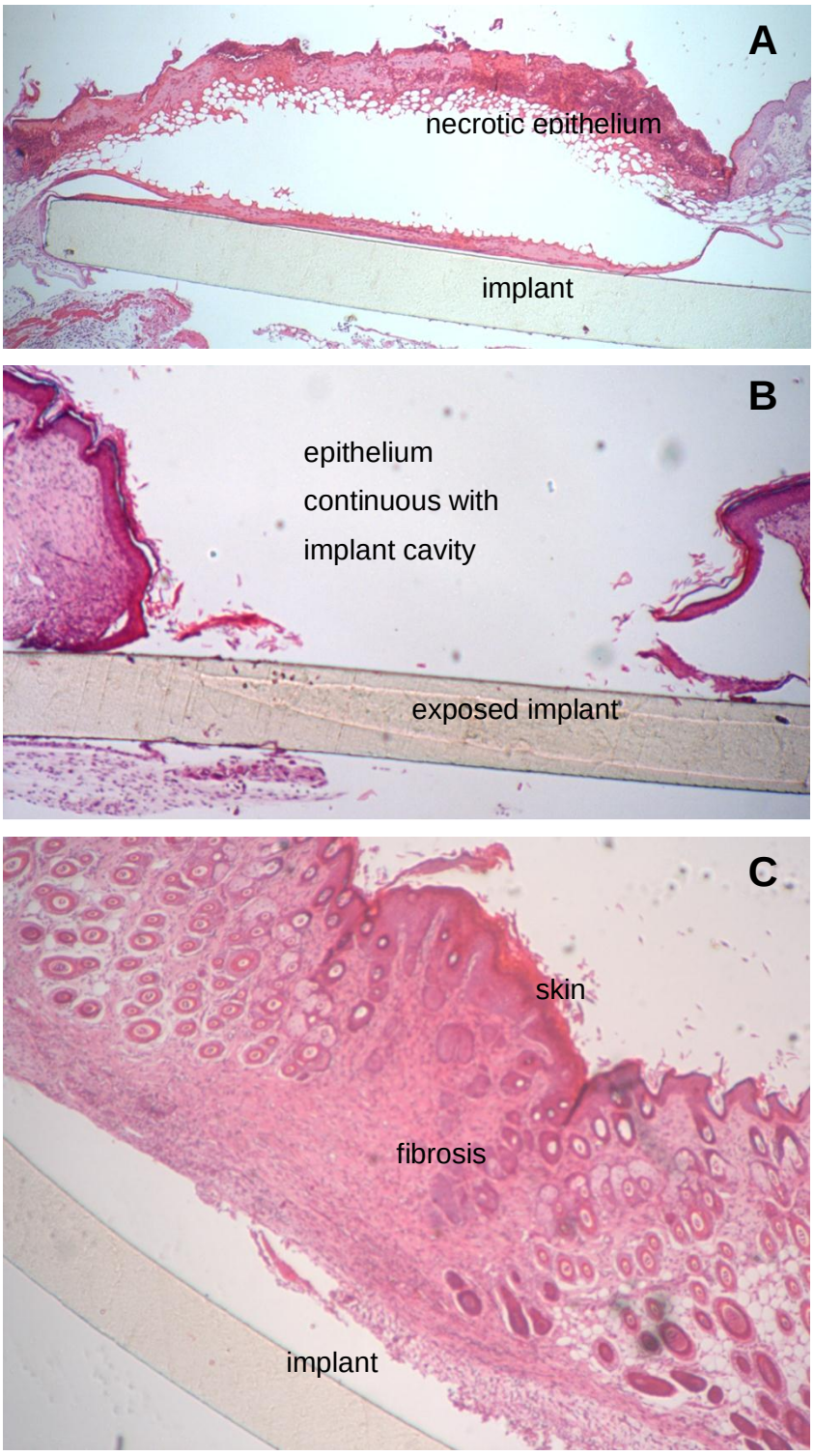


Plate 4.10: Pathological implant-skin interactions: (A) full thickness necrosis of skin overlying implant, (B) sinus formation between skin and implant cavity and (C) fibrotic adherence of implant to skin (Light microscopy 4x magnification).

Results for three day old implants (40 specimens) revealed no sinus formation and correlated with macroscopic inspection of mice. Cutaneous ulceration was seen overlying a single 50 W polymer implant and two implants (control and 75 W tea tree oil plasma polymer) incited fibrotic adherence to dermis. The remaining 37 three day old implants demonstrated non-pathological implant-skin interactions. Three day implants were deemed too premature to demonstrate more chronic sequelae of sinus formation. Overall three day implants were universally well tolerated by murine hosts without statistically significant differences between implant-skin interactions of any polymer implant group and control or between polymer groups ($p>0.9999$ Fisher's Exact test for categorical variables 2 tails).

Implants harvested at fourteen days (40 specimens) demonstrated an increase in pathological implant-skin interactions. Again cutaneous ulceration was seen overlying a single 50 W polymer implant. Fibrotic adherence of implant to skin was seen in eight samples: three controls, three 25 W polymer implants and two 75 W polymer implants. Sinus formation was found in six polymer implant specimens: two in 25 W samples, three in 50 W samples and one in a 75 W sample. Non-pathological implant-skin interactions occurred in the remaining 25 specimens. When polymer groups were compared to control using Fisher's Exact test for categorical values (2 tails) there were no significant differences in occurrence of pathological implant-skin interactions nor was there any significant differences between polymer groups ($p=0.6499->0.9999$).

Finally 28 day old implants (40 specimens) showed more pathological implant-skin interactions than three day specimens but fewer pathological interactions than 14 day specimens suggesting some implant complications may resolve with time. No 28 day specimens demonstrated cutaneous ulceration. Six polymer implants were fibrosed to dermis: four 25 W samples and one each of the 50 W and 75 W samples. Two sinuses were seen, one in a control and one in a 25 W specimen. The remaining 32 specimen slides were consistent with non-pathological implant-skin interactions. When polymer groups were compared to each other or to control using Fisher's Exact test for categorical values (2 tails) there were no significant differences in occurrence of pathological implant-skin interactions ($p=0.1409->0.9999$).

A statistical analysis was performed to compare pathological implant-skin interactions associated with polymer implants to control implants regardless of time of sacrifice (three, 14 or 28 days). Using this approach, Fisher's Exact test for categorical values (2 tail) showed no significant difference between groups (p=0.2326->0.9999). Even when all polymers were assessed collectively against control the difference was non-significant (p=0.4512). Overall there was no statistically definable difference between implant-skin interactions associated with any of the tea tree oil plasma polymer implant groups and control or between different polymer implant groups. Some form of pathological implant-skin interaction occurred in approximately one third of mice.

Exact correlation of sinus detection by macroscopic inspection and microscopic analysis did not occur but results were similar (Table 4.6). Differences in sinus reporting can be explained by missed small sinuses at macroscopic inspection and by sampling error in histological examination.

Table 4.6: Correlation of macroscopic (macro) and histologic (micro) sinus formation detected at animal sacrifice

Implanted material	Implant duration (days)					
	3		14		28	
	macro	micro	macro	micro	macro	micro
control PTFE	0	0	0	0	0	1
25 W polymer	0	0	1	2	1	1
50 W polymer	0	0	5	3	0	0
75 W polymer	0	0	2	1	1	0

4.4.3 Reaction zone responses

Reaction zone responses described several features of host tissue reaction in the immediate vicinity of implant including overall capsular maturity, capsule thickness measures, nature of the foreign body response at the interface between host and implant and assessment of surrounding tissue cellular infiltrates. All reaction zone responses were assessed at both superficial and deep implant surfaces.

4.4.3.1 Capsule maturity

Implant capsules were assessed to determine maturity along a scale documented in Appendix 4B. The scale described a spectrum from immature capsules of inflammatory cells with little or no connective tissue element to mature capsules resembling established fatty or connective tissue in non-injured regions (Plate 4.11).

Of the implants harvested at three days (40 specimens) all superficial implant surfaces (control, 25, 50 and 75 W) demonstrated immature capsules with fibroblasts, collagen and frequent acute inflammatory cells. One control implant and one 50 W tea tree oil derived polymer implant showed reduced inflammatory response at the deep implant surface with immature capsules of fibroblasts and collagen only. Fisher's Exact test for categorical values (2 tail) showed no significant difference between any polymer group and control or between polymer groups ($p>0.9999$). This finding was expected as at three days the normal acute inflammatory response was present and insufficient time had passed to allow for capsular maturation.

Implants harvested at 14 days (40 specimens) had more mature capsules than three day harvest specimens in keeping with the known phases of the inflammatory response and wound healing. Mature capsules were documented in four control samples, 11 25 W polymer samples, 16 50 W polymer samples and 15 75 W polymer samples. Mature capsules were significantly more frequent in all polymer variants compared to control PTFE implants (Fisher's Exact test for categorical values (2 tail) $p=0.0004$-0.0484) however no significant difference in effect was seen between polymer groups (Fisher's Exact test for categorical values (2 tail) $p=0.176$->0.9999).

By 28 days most implants (40 specimens) were associated with mature capsules at both superficial and deep surfaces. 17 control samples, 16 25 W samples, 16 50 W samples and 18 75 W samples were classed as having mature capsules with all other results falling into the immature capsule categories. There was no significant difference between any polymer group and control or between polymer groups (Fisher's Exact test for categorical values (2 tail) $p=0.3416$->0.9999).

The results indicated that capsular maturity proceeded according to known phases of inflammation in all groups. Three day sacrifice samples were predictably immature across the board. Polymer implants as a group produced mature capsules significantly more quickly than control but maturity was not dependent on the polymer power production parameter. Despite polymer samples maturing more quickly than control, by 28 days all polymer groups and control developed equally mature capsules.

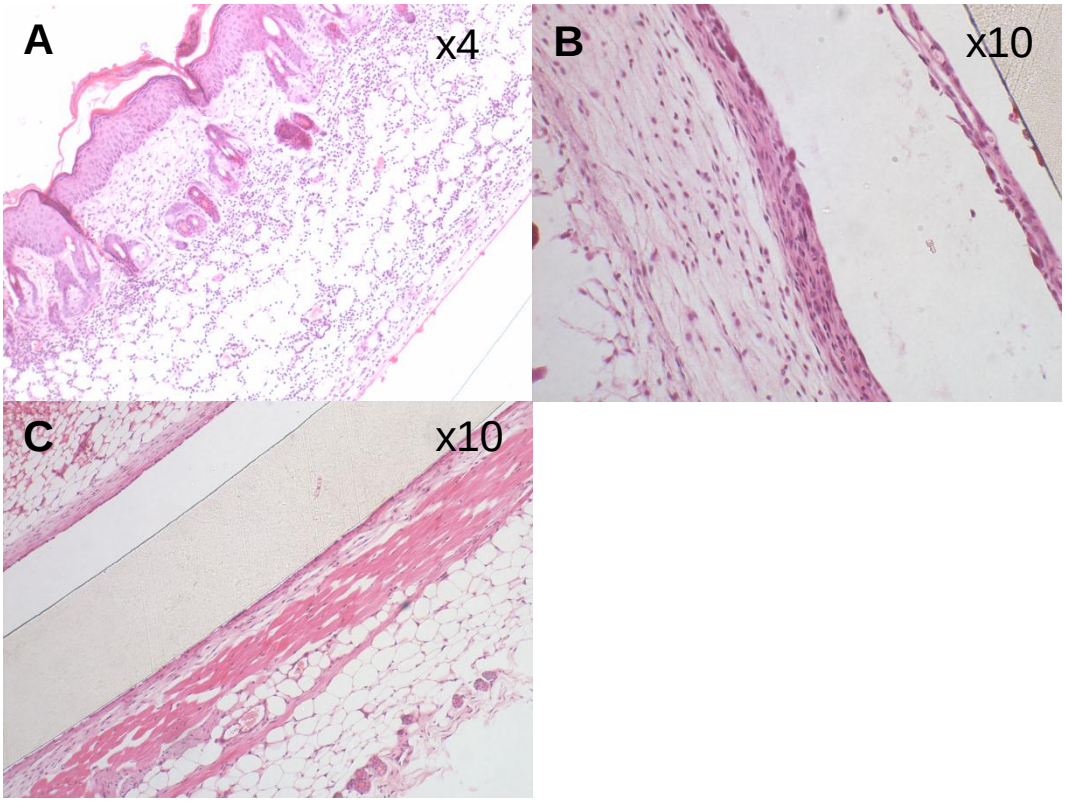


Plate 4.11: Capsule maturity: (A) immature inflammatory capsule (B) immature capsule of fibroblasts and collagen and (C) mature capsule resembling connective or fatty tissue in non-injured regions (hematoxylin and eosin stained samples at 4-10x magnification).

4.4.3.2 Capsule thickness

Capsule thickness was assessed in two ways: by measuring total connective tissue capsule thickness and by measuring thickness of the mature (dense) capsular component. Immature capsules were predominantly made of fibroblasts associated with formation of collagen and ground substance. Mature capsules consisted of fibrocytes and mature connective tissue or fatty elements. Fibrocytes are the resting form of fibroblasts and have less cytoplasm and more dense nuclei at histological examination. Measurements were made by estimating the average number of fibroblast/fibrocyte layers surrounding implant

at both the superficial and deep surfaces. Implants that produced thicker capsules of immature tissue were considered less biocompatible than those associated with thin, mature capsules.

Total capsule thickness at three days (40 specimens) had a median value of 10-30 fibroblasts/fibrocytes for control samples, 25 W polymer samples and 50 W polymer samples (ranges: control 1->30, 25 W 1->30, 50 W not applicable->30). 75 W polymer samples had a median thickness of 5-30 fibroblasts/fibrocytes (range: 1->30). When data was analyzed using 4x2 Fisher's Exact test for categorical values there was no significant difference in results produced by any polymer implant group when compared to control or between polymer implant groups (p=0.2486-0.8899).

Total capsule thickness at 14 days (40 specimens) had a median value of 10-30 fibroblast/fibrocytes for control samples (range: 5->30). 25 W, 50 W and 75 W polymer samples had median values of 5-9 fibroblast/fibrocytes (ranges: 25 W 1->30, 50 W not applicable->30, 75 W 1->30). 4x2 Fisher's Exact test showed no significant difference between control and 25 W polymer implant total capsule thickness. Both 50 W and 75 W polymer implants had significantly thinner capsules than control at 14 days (50 W p=0.0067 and 75 W p=0.0005). Analysis of effect between plasma polymers demonstrated no significant difference between total capsule thickness (p=0.0537-0.2495).

28 days explants (40 specimens) all had a median total capsule thickness of 5-9 fibroblasts/fibrocytes (ranges: control 1-30, 25 W 1->30, 50 W 1-30 and 75 W not applicable to 30). There was no statistically significant difference between control and any polymer implant group or between polymer groups (Fisher's Exact test p=0.1769->0.9999).

Results indicated that capsules became thinner with time across all specimens. This correlates with known phases of inflammation. 50 W and 75 W polymer implants produced thinner capsules at the half way point of 14 days when compared to control however by 28 days all implants produced the same thinned capsule. These outcomes echo the results of capsular maturity observations.

Dense or mature component of implant capsules was examined for three day explant specimens. Control samples universally showed no mature capsule component. Of the polymer implants, seven 25 W samples, four 50 W samples and four 75 W samples had between one and four mature fibrocyte layers around implant. The remaining samples were negative for mature capsular component. The 25 W polymer implants had significantly more mature component to their capsules than control (Fisher's Exact test $p=0.0083$). There was no statistically significant difference between 50 W or 75W plasma polymer implants and control or between polymer groups ($p=0.1060->0.9999$).

By 14 days mature capsular components were more frequent than at three day time intervals (40 specimens). 10 control samples had mature capsules of between five and nine fibrocytes and six had capsules of between one and four fibrocytes. Looking at the 25 W samples, six had mature components between five and nine fibrocytes and 10 had a mature layer around implant of only one to four cells. 17 of the 50 W polymer samples and 15 of the 75 W polymer samples had mature fibrocyte layers of one to four cells thick. The remaining samples did not demonstrate a mature (dense) capsular layer. Using Fisher's Exact test 4x2 there was no significant difference in mature capsule thickness between control and 25 W polymer sample however both 50 W ($p<0.0001$) and 75 W ($p=0.0002$) were significantly more mature than control. There was no significant effect demonstrated between 50 W and 75 W samples ($p>0.9999$) however both 50 W ($p=0.0072$) and 75 W ($p=0.0177$) samples showed significantly thinner dense capsular component than 25 W samples.

28 day sacrifice samples (40 specimens) demonstrated capsules with more mature components than 14 day sacrifice specimens in keeping with known phases of inflammation and wound healing. All groups demonstrated progressive capsular maturation. Every control specimen developed dense layers one to four cells thick on both superficial and deep surfaces. In the 25 W polymer group 17 samples were one to four cells thick, 2 were five to nine fibrocytes thick and one sample had a dense layer 10-30 cells thick. 18 50 W samples showed dense capsules one to four cells thick and one sample had a thickness of five to nine fibrocytes in the capsule. Finally in the 75 W samples, 15 demonstrated dense capsules one to four cells thick and three had fibrocyte layers five to nine cells thick. All groups performed equally as no significant statistical

difference was noted either between polymer and control or between polymers when groups were analyzed according to 4x2 Fisher's Exact test (2 tails) (p=0.2308->0.9999).

Results of mature (dense) capsule component assessment indicated that control and polymer samples matured in line with known phases of inflammation. 50 W and 75 W samples developed a mature capsular component sooner than control or 25 W specimens (by 14 days) however at 28 days all specimens developed equally mature and thin (one to four cells predominantly) dense capsule layers. These results were consistent with measures of morphological capsular maturity and total capsule thickness.

4.4.3.3 Interface qualities (foreign body effect)

Interface qualities were measures of foreign body cells found in contact with implant superficial and deep surfaces as scored in Appendix 4C. The foreign body reaction composed of macrophages and foreign body giant cells is the end-stage response of the inflammatory and wound healing cascade after medical device insertion. Bio-material surface properties play an important role in modulating foreign body reaction in the first two to four weeks following implantation of a medical device (Anderson et al., 2008). Foreign body response is not considered biocompatible and therefore more foreign body cells around implant were considered indicative of a less favorable response to bio-implant.

No specimens (control or polymer) examined at either superficial or deep surface demonstrated foreign body giant cells in animals sacrificed at three days, probably because it was too soon in the host response process. Even examining the 14 and 28 day specimens, only one 14 day control sample developed a single layer of foreign body giant cells at the implant surface. No other foreign body cells were found in any samples. Classical foreign body response was therefore a minimal component of host response to control and polymer implantation. There were no differentiating features between polymer and control or between polymer samples in foreign body response.

4.4.3.4 Surrounding tissue cellular infiltrate

Surrounding tissue cellular infiltrate was scored as described in Appendix 4C. Tissues immediately surrounding implants were examined for acute and chronic inflammatory

cells, foreign body cells or the absence of a cellular infiltrate. Active inflammation or foreign body cellular infiltrates were considered non-biocompatible and thus unfavorable.

Samples universally demonstrated either an acute inflammatory response with neutrophil predominance or no surrounding tissue cellular infiltrate. In keeping with results from interface quality analysis, no foreign body cells were identified in tissues surrounding any implants and only one specimen demonstrated foreign body cells at the implant surface (14 day control sample). With the exception of one control sample, three day harvest specimens all demonstrated a neutrophil predominant acute inflammatory response in line with known phases of the inflammatory response. By 14 days no control or 75 W samples exhibited an acute inflammatory response and only two 25 W and three 50 W samples demonstrated neutrophil predominant tissue infiltration around implant. Statistically there were no significant differences between any control or polymer group or between polymer groups at 14 days (Fisher's Exact test 2x2 2 tail p=0.2308->0.9999). By 28 days only one 25 W sample showed an acute inflammatory response around implant. Results indicated that as far as surrounding tissue cellular infiltrate assessment went, polymer and control groups behaved in a similar fashion and aligned with known phases of inflammation and healing.

4.4.4 Microscopic examination of isolated complication specimens

A single mouse housing a 75 W tea tree oil derived plasma polymer sample harvested at three days developed an erythematous and indurated wound without evidence of local collection or discharge (Section 4.3 Inspection of Postoperative Mice: Results and Discussion). The mouse appeared systemically well. Differential diagnoses included infection, exuberant inflammation, foreign body reaction or allergy to either suture material or implant or mechanical interference by other mice. On explantation, surrounding soft tissue was macroscopically injected and edematous. Microscopic examination of the specimen with standard hematoxylin and eosin staining showed a dense, neutrophil infiltration in the tissues surrounding implant (Plate 4.12). Although Gram-Twort stains for bacteria were negative, the most likely explanation was acute infection.

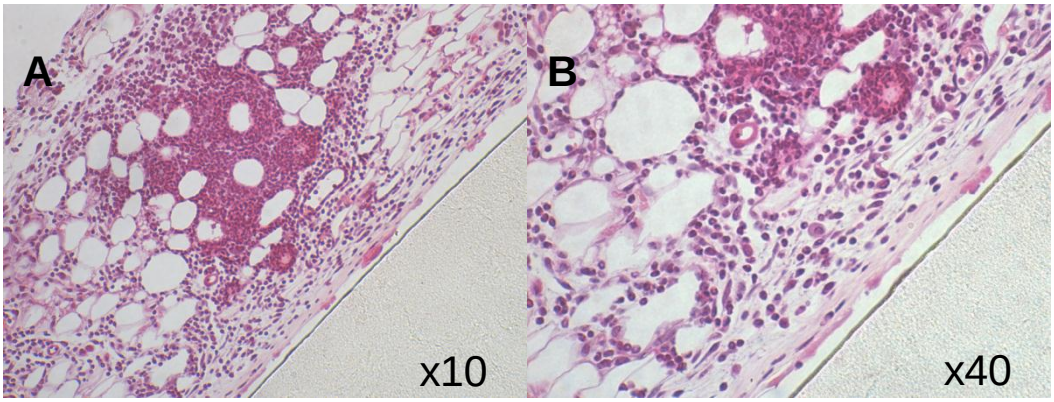


Plate 4.12: A dense neutrophil infiltrate is demonstrated around subcutaneous implant in a 75 W mouse explant harvested at three days. The mouse implantation site appeared macroscopically erythematous and indurated. Gram-Twort stains were negative.

4.4.5 Summary results and discussion of histological assessments

In summary, macroscopic and microscopic analysis of specimens showed direct or indirect evidence of implant within all specimens and acted as a quality control measure. The most common complication of implants was a spectrum of skin-implant interactions culminating in sinus formation. Sinus spectrum changes were more prevalent in mice sacrificed at 14 days with fewer changes occurring in 28 day specimens indicating a tendency towards resolution over time. All polymers and control groups developed sinuses and were assessed as equally biocompatible. Reaction zone analysis confirmed immature, thick capsules in most samples sacrificed at three days without differences between groups as was expected as part of the acute inflammatory response. Although polymer groups showed earlier capsule maturity (all polymer groups) and capsular thinning (50 W and 75 W groups) when compared to control, by 28 days the advantage of polymer over control was lost as all polymer and control groups displayed mature and thinned capsules. No significant difference between polymer groups was identified in any category. Foreign body reaction did not play a major role in host response to any of the implants and all implant groups generated an acute inflammatory response in keeping with known phases of inflammation after surgical wounding.

Polymer implants as a group were supported as biocompatible materials as they performed in an equivalent manner to biocompatible control implants made from PTFE. The hypothesis that plasma polymers generated under varied power parameters have different biocompatibility profiles was not supported. This may be because all tea tree oil

derived plasma polymers were equally biocompatible or it may reflect the limited number of power alterations used to manufacture tea tree oil derived plasma polymers. A direction for future research is further investigation of the effects of a broader range of power and other production parameters on the biocompatibility of tea tree oil derived plasma polymer thin films.

CHAPTER 5 ANTIBIOFILM EFFECT

5.1 Relevance of Antibiofilm Testing

Tea tree oil has documented antimicrobial activity (Halcon and Milkus, 2004). It is postulated that tea tree oil derived plasma polymer films may inherit antimicrobial properties from parent compound. Surface treatments that confer antimicrobial effects may protect sterile bio-implants from contamination and infection (Aslam et al., 2007, Bach et al., 1993, Bologna et al., 1999). As infection is a prominent cause of medical device related morbidity and implant loss, coatings that resist infection provide healthcare advantage (Antonois and Baddour, 2004). Tea tree oil derived plasma polymer coatings may therefore act to reduce medical implant infections and improve healthcare.

Microbe lifecycles include planktonic and biofilm stages that are phenotypically different. Where planktonic organisms are mobile individuals in solution, biofilm behaves as a heterogeneous, surface adherent colony encased in protective matrix (Donlan, 2001). Tea tree oil acts against both planktonic microbes and biofilm (Carson and Riley, 1998, Hammer et al., 2008). There are strong reasons however, to test antimicrobial properties of novel tea tree oil derived plasma polymers against biofilm as opposed to planktonic cells:

1. Problematic infection of implanted medical devices is caused by biofilm and results in substantial resource expenditure and human costs (Donlan and Costerton, 2002). As of yet there is no reliable means to eliminate biofilm on implants and the only definitive treatment option is device removal. Should tea tree oil derived plasma polymers exhibit antibiofilm properties, they will offer a novel means of preventing or eradicating biofilm on implants.

2. Tea tree oil is an essential oil in liquid form and cannot be used systemically due to its toxicity profile or as a surface coating due to its physical profile. Plasma polymers made from tea tree oil however, are solid essential oil derivatives constructed as films and are therefore localized, potentially avoiding the systemic toxicity hazards of liquid tea tree oil. These films require a foundation on which to be built such as medical implant surfaces. Surface coatings on medical implants

are localized to the site of biofilm initiation and maturation and are therefore well placed to act against biofilm.

3. A class property of plasma polymers is poor aqueous solubility and inability to diffuse in physiological solution. These baseline characteristics are due primarily to the highly cross-linked structure of plasma polymers. The practical result of poor aqueous solubility is that planktonic organisms in physiological solution are inaccessible unless at the polymer-solution interface. Standard, laboratory based antimicrobial testing of planktonic microbes is also water based relying on diffusion of antibiotic in agar or broth. These traditional antimicrobial sensitivity techniques will fail to demonstrate antimicrobial properties of plasma polymers due to lack of diffusion. Where poor water solubility hinders anti-planktonic activity, it may confer advantages in killing biofilm. Aqueous insolubility of plasma polymers localizes the compound to surfaces were biofilm is produced, reduces leaching into systemic compartments and limits systemic side effects. Biodegradation is directly related to aqueous solubility. Poor water solubility implies reduced biodegradation and improved longevity of surface film allowing longer lasting anti-biofilm effects. Tea tree oil derived plasma polymers thus may have physical and chemical characteristics more suited to leverage against biofilm than planktonic microbes.

In summary, biofilm is the cause of medical implant infection making it the relevant infective manifestation to target with antimicrobial treatments. Derived plasma polymer films may act as localized antimicrobial surface treatments with novel mechanisms of action against biofilm. Because of plasma polymer class attributes including lack of aqueous solubility, tea tree oil derived plasma polymers are more suited to test against biofilm and have specific advantages as anti-biofilm as opposed to anti-planktonic interventions.

5.2 Biofilm Culture

5.2.1 Introduction

Biofilm is the form bacteria take when infecting medical implants (Donlan and Costerton, 2002). Biofilm is a sessile community of microbes irreversibly attached to a substratum, interface or to each other with microbes embedded in self-produced matrix of extracellular polymeric substances (Costerton et al., 1978, Davey and O'Toole, 2000). Growing biofilm poses unique challenges in the laboratory environment. Artificial biofilm is difficult and laborious to culture and necessitates different techniques to culturing planktonic bacteria. A surface or substrate is required for adherence of biofilm. Tight parameter control is needed to force the bacterial equilibrium from planktonic toward biofilm formation and ideal culture parameters vary between bacterial species. In many cases these parameters are not fully elucidated. As well as nutrient, oxygenation and temperature control, quality biofilm requires consistent application of fluid forces such as shear and drag to the culture sample (Costerton, 2001).

Tea tree oil derived plasma polymer surface treatments are hypothesized to resist biofilm contamination due to inherited antimicrobial properties of parent compound tea tree oil. The goal of culturing biofilm was to determine if biofilm contamination on novel tea tree oil derived plasma polymer surfaces was less than biofilm contamination of bare PTFE control substrate in order to support or refute an antibiofilm effect for the novel polymer surface treatments. PTFE was employed as a control substrate because it is a polymer commonly used to produce a wide variety of medical implants, is known to have some anti-biofilm effect and is thus a good bench mark (Gore, 2011). PTFE is also inexpensive, easy to handle and shape and readily available.

Three tea tree oil derived plasma polymer variants were manufactured as thin films on PTFE squares under power conditions of 25, 50 or 75 W for the purpose of anti-biofilm plasma polymer substrate testing. Varied power settings were used to make plasma polymers in order to determine the effect power of production had on antibiofilm abilities of novel films. Higher power settings cause more extensive fracturing of parent monomers and plasma polymers produced at higher powers were therefore expected to inherit fewer intact anti-microbial functional moieties from parent compounds (Biederman and Slavinska, 2000). Plasma polymers produced at lower power settings were conversely anticipated to be more effective in preventing biofilm because they retain a greater number of intact functional moieties.

Staphylococci are the most common organisms to cause bacterial biofilm infection in medical implants and are ubiquitous in the hospital environment (Neely and Maley, 2000, Gristina, 1987). Because *Staphylococci* are the predominant bacterial cause of device infection, three subspecies were used in an attempt to produce biofilm on tested substrates. Sensitive *S.aureus* was enlisted because it is the most common cause of hospital acquired bacteremia, Methicillin Resistant *S.aureus* (*MRSA*) was selected because of increasing resistance to standard antibiotic chemotherapy and associated patient morbidity and mortality and finally *S.epidermidis* represented the coagulase negative *Staphylococci* and were the quintessential nosocomial pathogen characterized by biofilm development on indwelling medical devices (Vuong et al., 2004, Melzer et al., 2003, Engemann et al., 2003).

In order to produce biofilm, a closed culture circuit was designed to allow sterile substrate to be inoculated with one of the three pure, staphylococcal, planktonic bacterial broths under shear conditions. Shear was produced with a drip flow reactor component. After substrate inoculation with bacteria, specimens were continuously washed with nutrient broth to provide ongoing shear and sustenance and further propagate biofilm. At the end of each culture cycle, substrate and acquired biofilm were removed aseptically from the circuit. Each specimen was processed to enumerate biofilm organisms as a way of quantifying and comparing biofilm growth. Results were obtained for all bacterial species-substrate combinations, that is to say biofilms of sensitive *S.aureus, MRSA* and *S.epidermidis* were each grown on tea tree oil derived plasma polymer films produced under 25, 50 and 75 W power conditions as well as on bare control PTFE substrate.

Prior to formal biofilm culture experiments, the biofilm culture circuit was evaluated to confirm it was capable of producing uncontaminated biofilm of *Staphylococci*. Biofilm experiments of this nature had not been undertaken at James Cook University which meant there was little on site guidance on how to successfully grow bacterial biofilm. It was therefore mandatory to ensure the culture circuit made biofilm and was not simply producing a planktonic culture so trial specimens were subjected to imaging analysis in order to structurally confirm the presence of biofilm. Biofilm samples were also processed and plate cultured to exclude environmental circuit contamination.

5.2.2 Equipment and materials

Biofilm experimentation was performed in TV115 PC2 microbiology laboratory, School of Veterinary and Biomedical Sciences at James Cook University.

5.2.2.1 Glassware and accessories

- 5000 ml glass flask and modified airtight rubber stopper
- 1000 ml glass flask and modified airtight rubber stopper
- 250 ml glass bath

5.2.2.2 Tubing and tubing attachments

- 2 steel tubing clamps
- 8 glass tubing weights
- 2 Sarsedt syringe filters (0.2 µm)
- Tygon™ R3603 extension tubing 1.59 mm internal diameter
- Tygon™ FlowRate peristaltic pump tubing Gry/Gry 1 ml/min 1.30 mm internal diameter
- 4 PTFE T piece tubing connectors
- 4 PTFE in line tubing connectors
- poly(urethane) extension tubing 3 mm internal diameter
- poly(urethane) extension tubing 12 mm internal diameter
- 2 3000 ml effluent reservoirs with modified lids

5.2.2.3 Machinery

- Biosurface™ Technologies Corp. Model DFR 110 Drip Flow Reactor and attachments
- AGE magnetic stirrer (VELP Scientifica) and magnetic stirrer flea
- Gilson Minipuls 2 peristaltic pump
- Atherton Genesis series downward displacement sterilizer (autoclave)
- REVCO VS651 37 ºC incubator

5.2.2.4 Consumables

- glass microslides 25x75x1 mm VWR (+/- plasma polymer thin film coating)
- PTFE sheeting 25x25x1 mm (+/- plasma polymer thin film coating)
- Mueller-Hinton broth (MHB) (Oxoid)
- distilled water (sterile)
- 70% ethanol
- sterile gloves size 6.5

5.2.2.5 Planktonic bacteria

Planktonic bacterial isolates on plated blood agar were supplied by Dr R. Norton Director of Pathology at The Townsville Hospital:

1. *Staphylococcus epidermidis* ATCC 12228 (23/07/2007)
2. *Staphylococcus aureus* ATCC 29213 (23/07/2007)
3. methicillin resistant *Staphylococcus aureus* (MRSA) M29279 clinical isolate (23/07/2007)

Experimental bacteria were sub cultured fortnightly onto blood agar using dilution streak plating and stored at 4 ºC to maintain viability. Blood agar sub cultures were employed to produce planktonic bacterial broth for biofilm culture. Bacteria were also inoculated onto Microbank™ beads and stored at -80 ºC in case of failure or contamination of plated stocks.

5.2.2.6 Preproduction of bacterial broth

Bacterial broth cultures were produced in 1000 ml sterile glass flasks by inoculation of 600 ml Mueller-Hinton broth (MHB) with pure bacterial colonies grown on blood agar. Inoculated broth was incubated at 37 ºC for 24 hours under stationary conditions. Concentration of 24 hour bacterial broth ranged from 10^8 to 10^9 CFU/ml as assessed by viable plate count.

5.2.3 Biofilm culture circuit

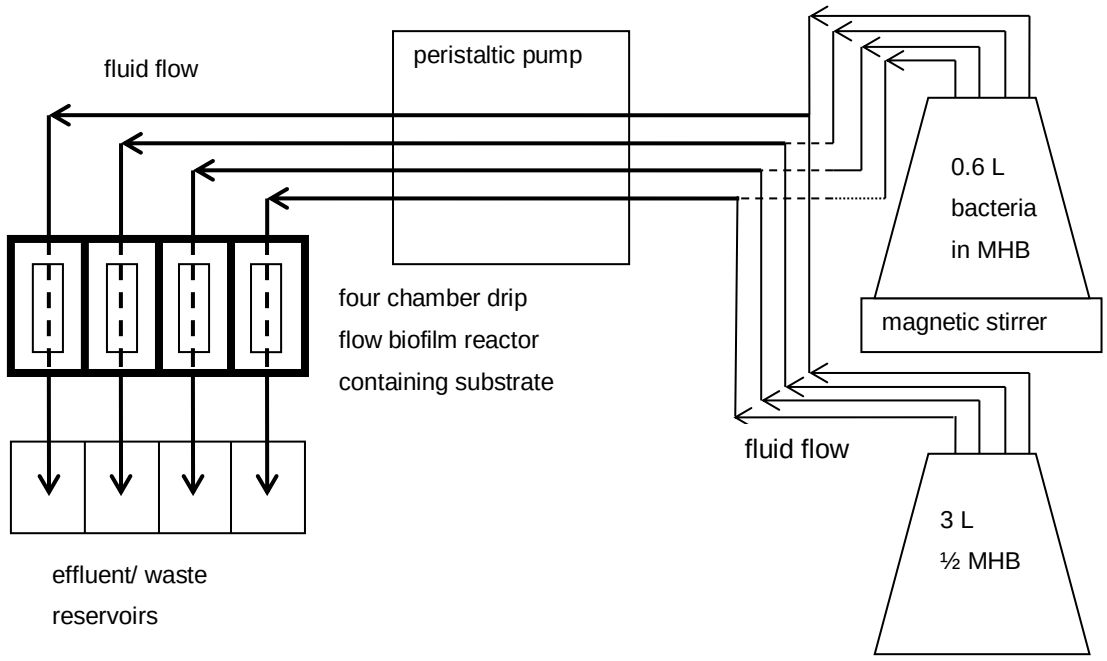


Figure 5.1: Schematic configuration of biofilm culture circuit

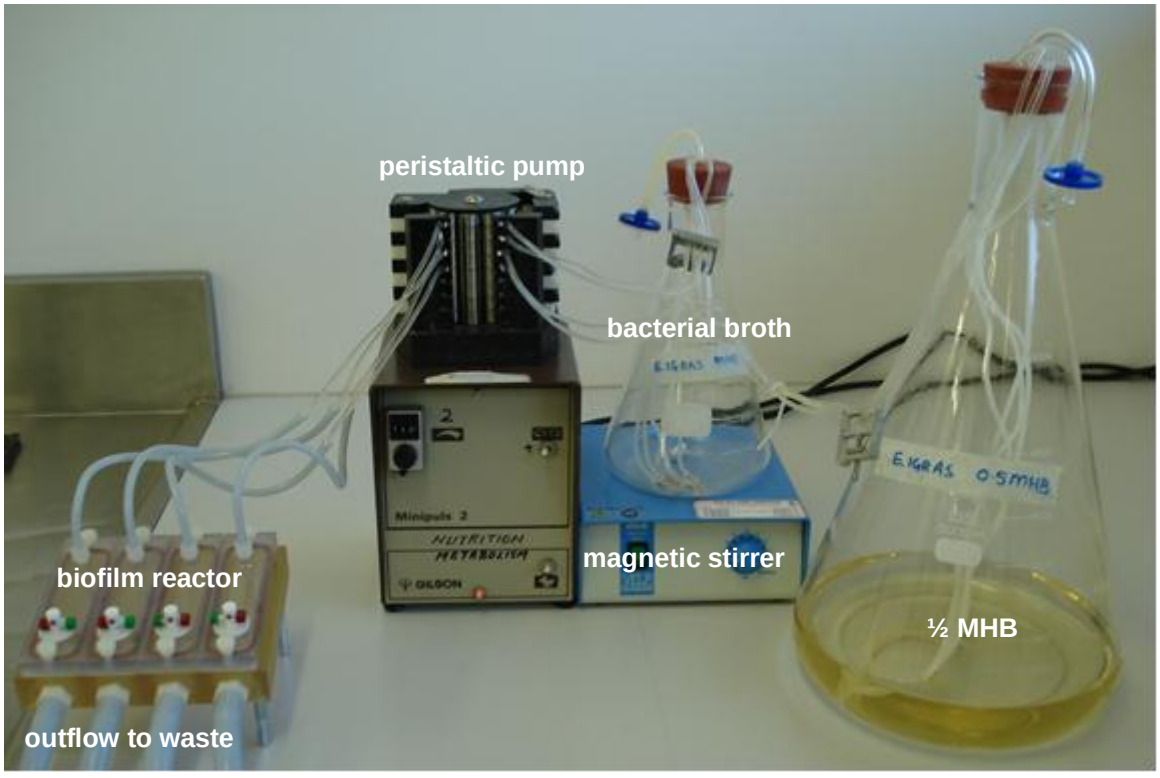


Plate 5.1: Photograph of biofilm culture circuit corresponding to Figure 5.1

Bacterial biofilms were grown at room temperature on substrate (bare PTFE sheeting or plasma polymer) using the *in vitro* model depicted (Figure 5.1 and Plate 5.1). Biofilm culture circuit was made up of four identical mini circuits arranged in parallel allowing simultaneous generation of biofilm specimens in quadruplicate.

Biofilm culture circuit originated at two points: 24 hour planktonic bacterial culture in 600 ml of MHB magnetically stirred within a one liter glass flask and secondly three liters of half strength MHB (biofilm nutrient source) contained within a five liter glass flask. Both flasks were fitted with tailored rubber stoppers drilled for passage of five tubes: four channels to extract fluid from the flasks (Tygon™ R3603) and one channel to air fitted with a syringe filter (0.2 µm Sarsedt syringe filter)(Plate 5.2). Syringe filter air channels allowed entry of uncontaminated air into glass flasks and thus permitted outward fluid flow. Each outflow tube from bacterial culture flask was linked at a "T" junction with a partner outflow tube from nutrient source flask to form four common channels. Fluid flow into common channels was controlled by two metal clamps (gates): one clamp encircling four extraction lines from bacterial broth and the other clamp encircling four extraction lines from nutrient source. Gating allowed control of fluid flow so either bacterial broth or nutrient broth entered common channels, but not both at once (Plate 5.4). Common channels (four channels) originating at "T" junctions consisted of specialized tubing (Tygon™ FlowRate) designed to be acted on by a peristaltic pump (Gilson Minipuls 2)(Plate 5.3). Specialized tubing channels passed through the peristaltic pump in parallel. Efferent peristaltic fluid was passed via three millimeter internal diameter poly(urethane) tubing to the inlet of one of four separate biofilm reactor chambers. Drip flow reactor chambers housed sterile biofilm culture substrate (either tea tree oil plasma polymer or bare PTFE sheeting). Peristaltic fluid was allowed to run down the 10 degree chamber gradient, wash over substrate and exit the reactor chamber via fluid/waste outlet under the influence of gravity. Waste fluid was continuously siphoned from each chamber via 12 mm internal diameter poly(urethane) tubing into separate sealed three liter waste reservoirs.

Plate 5.2: Tailored rubber flask stopper housing fluid extraction channels and a filtered air channel fitted with a syringe filter

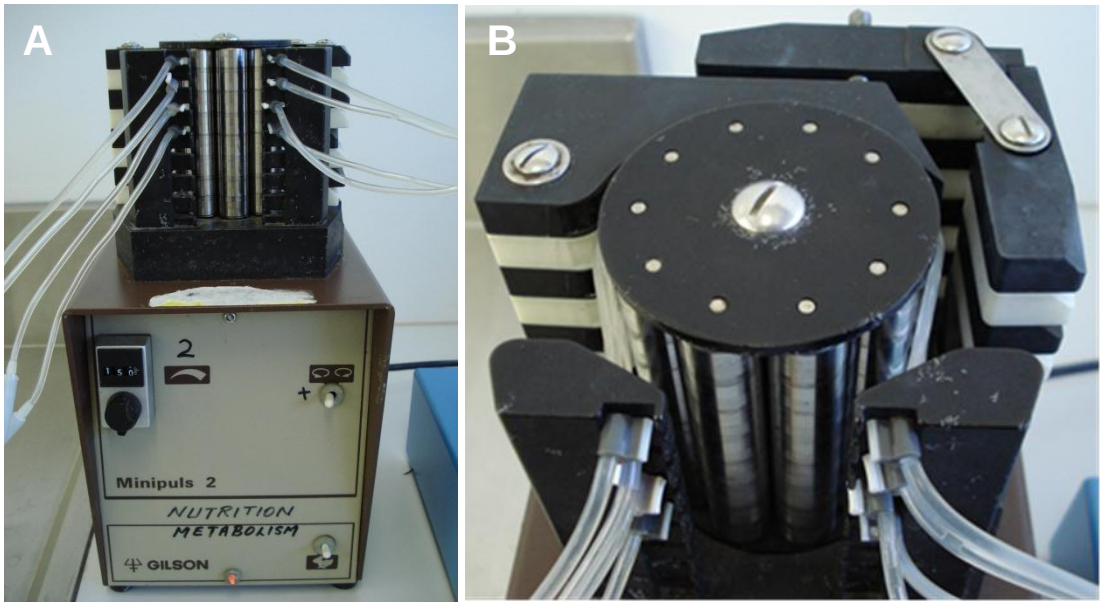


Plate 5.3: Gilson Minipuls 2 peristaltic pump containing Tygon™ FlowRate tubing:
(A) four common channels are processed in parallel through the peristaltic pump
(B) peristaltic flow is generated by pressure on tubing from a rotating variegated column

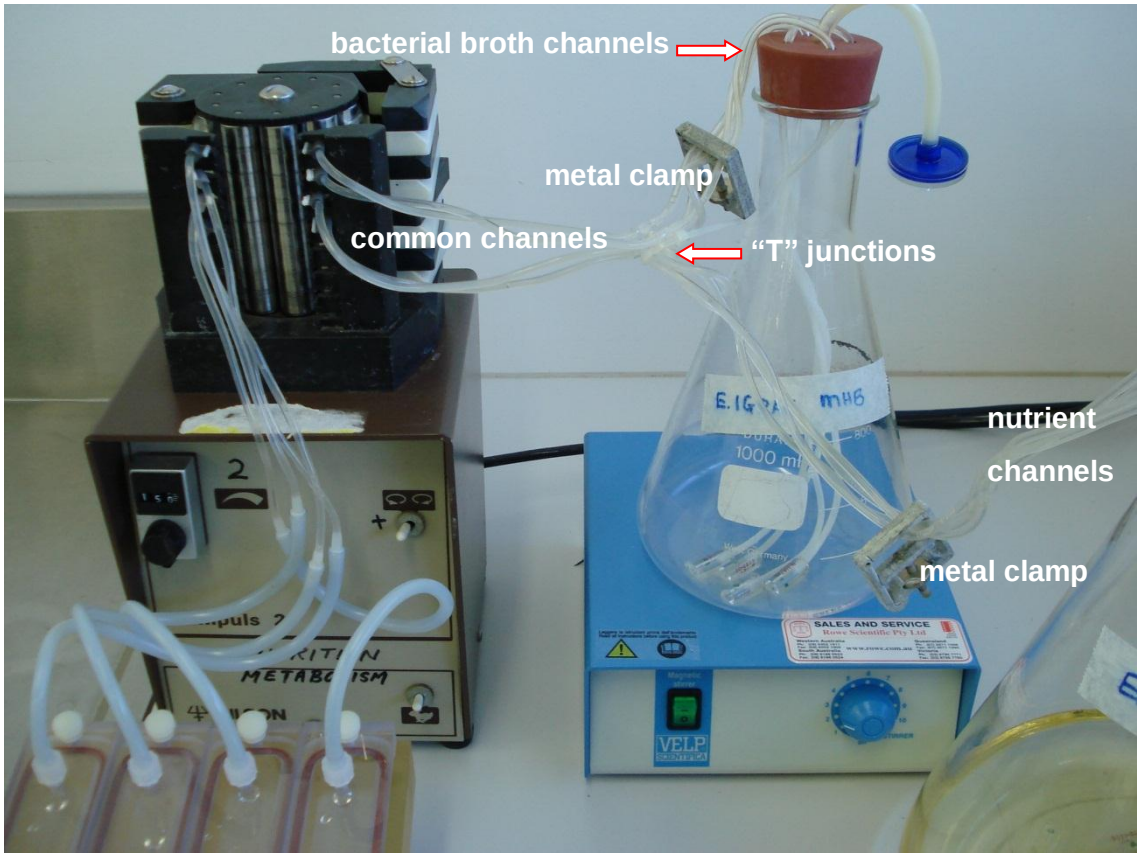


Plate 5.4: Biofilm circuit channel configuration and flow control

5.2.4 Drip flow biofilm reactor

The biofilm reactor component of biofilm culture circuit was a Drip Flow Biofilm Reactor DFR 110 from BioSurface™ Technologies Corp. (Appendix 5A and Appendix 5B). This reactor has been proven successful in biofilm culture of *Staphylococci* (Curtin and Donlan, 2006). Reactor was composed of a poly(sulfone body), poly carbonate lid, silicone gaskets, PTFE screws/connectors and four stainless steel legs and was easily disassembled for cleaning and sterilization (Plate 5.5 and Figure 5.2). Reactor was built with a 10 degree gradient to allow fluid flow with gravity and generation of shear forces.

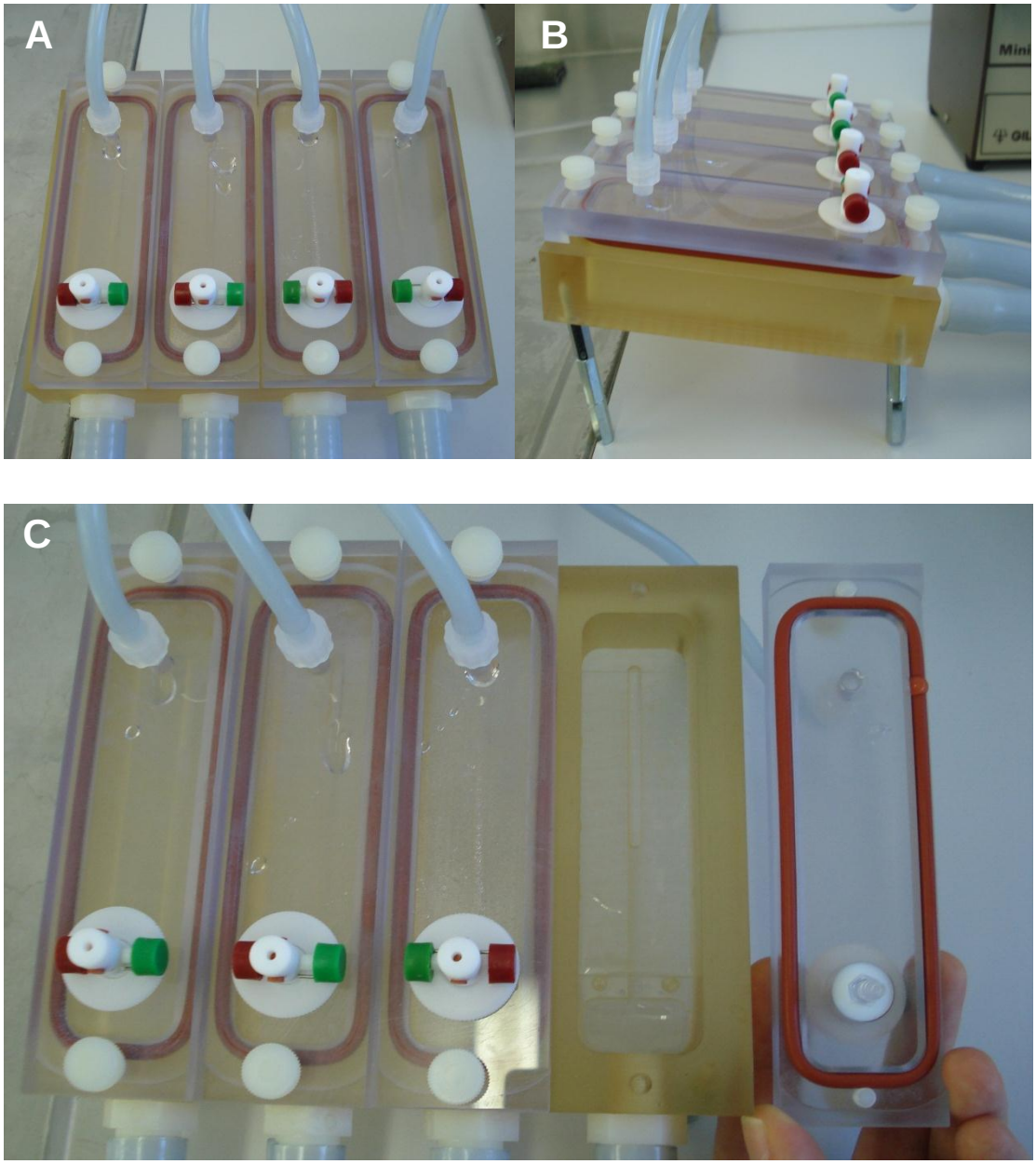


Plate 5.5: Biofilm reactor (see technical drawing below for explanation):

(A) viewed from above
(B) viewed laterally to demonstrate ten degree incline
(C) with the end chamber lid removed

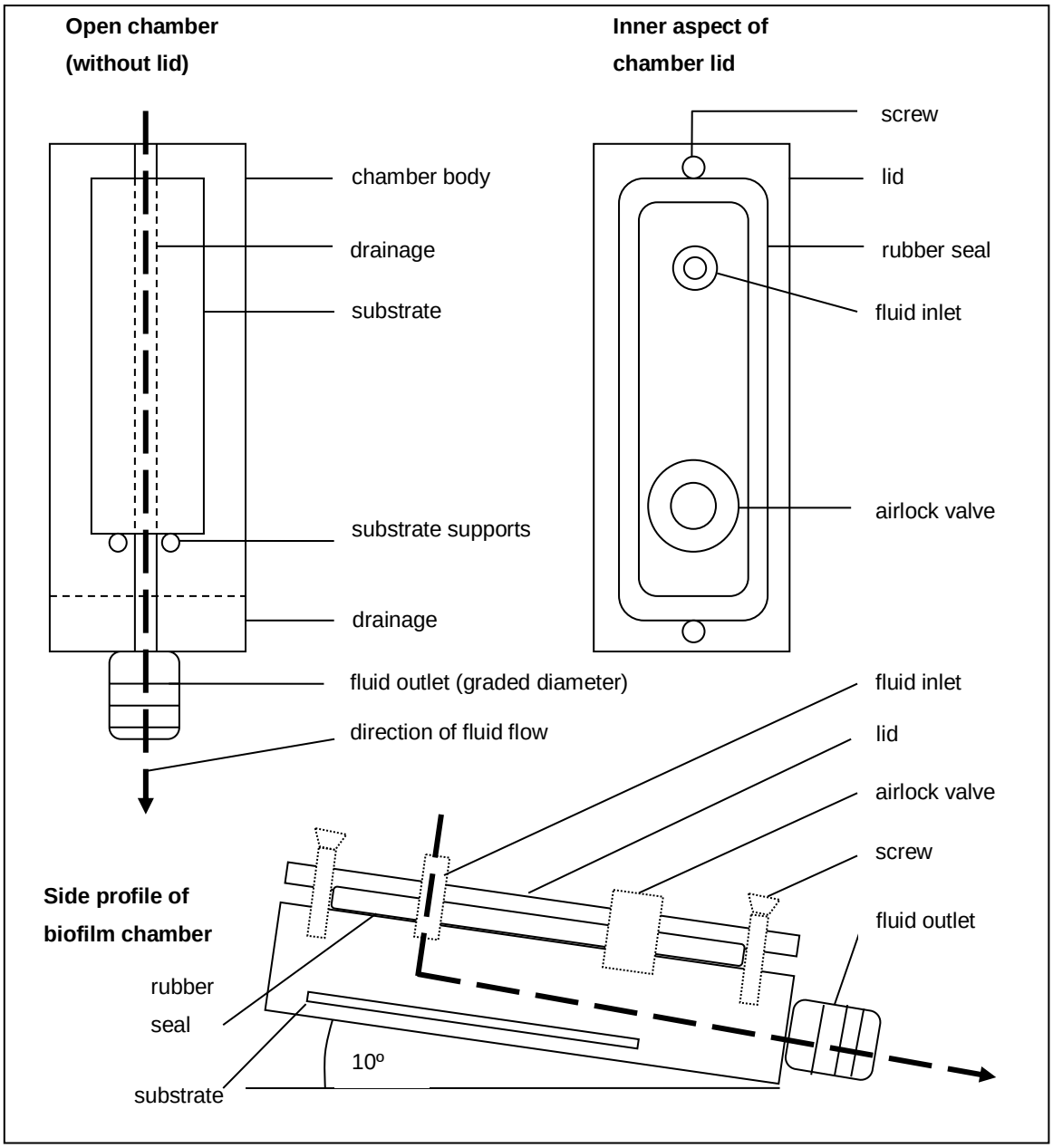


Figure 5.2: Technical drawing with features of Drip Flow Biofilm Reactor DFR 110

5.2.5 Protocol for culturing biofilm

Sterile substrate was placed aseptically into reactor chambers (four chambers) and chamber lids sealed. 600 ml of 24 hour old pure planktonic bacterial broth culture magnetically stirred in a one liter bacterial broth reservoir flask was linked into the biofilm culture circuit. Three liters of half strength nutrient broth within a five liter nutrient broth reservoir flask was added into the biofilm circuit. Tubing clamps were configured to allow bacterial broth to flow along the biofilm circuit whilst excluding flow from sterile nutrient flask. Bacterial culture was pumped through the chambers of the drip flow biofilm reactor for two hours at a rate of 1 ml/min/chamber in a peristaltic

fashion to allow irrigation of substrate with planktonic bacteria. At the two hour time point, tubing clamps were reconfigured preventing further bacterial broth entry to the circuit and allowing nutrient broth to flow. Sterile half strength MHB then irrigated substrate within biofilm reactor for 22 hours at a rate of 0.5 ml/min/chamber to establish a biofilm. At 24 hours biofilm specimens were aseptically harvested from the reactor for enumeration of organisms (Section 5.5 Biofilm Enumeration).

5.2.6 Sterilization of biofilm culture circuit

At the end of every culture run, biofilm culture circuit components were sterilized in preparation for the next culture cycle. Activities were carried out according to the following instruction list:

1. Mechanically clean all biofilm culture circuit components and flush thoroughly with tap water

2. Package biofilm culture circuit components in aluminum foil for autoclaving

    - Glass tubing weights, Tygon™ extension and peristaltic pump tubing, poly(urethane) inflow tubing, tubing connectors, rubber flask stoppers and steel clamps in continuity

    - Disassembled drip flow reactor

    - Poly(urethane) outflow tubing

    - Sealed three liter poly(urethane) waste reservoirs containing contaminated effluent

    - One liter glass flask containing magnetic flea (for bacterial broth culture)

    - Five liter glass flask (for nutrient broth)

3. Wet autoclave packaged components at 121 ºC and 240 kPa for 30 minutes (Atherton Genesis series downward displacement sterilizer). Allow to cool.

4. Remove pre prepared 24 hour bacterial culture from 37 ºC incubator (one liter glass flask containing 600 ml Mueller-Hinton broth inoculated with bacterium and flea for magnetic stirring)

5. Aseptically reassemble closed biofilm circuit incorporating pre-prepared bacterial broth culture and sterile nutrient broth

6. Decontaminate substrate +/- polymer by immersion in 70% ethanol prior to use allowing ethanol to completely evaporate

7. Commence new biofilm culture cycle (Section 5.2 Biofilm Culture)

5.3 Confirmation of Biofilm

Biofilm culture circuit testing was undertaken to confirm the ability of the constructed circuit to generate biofilm. Three fundamental stimuli drove confirmatory testing. Firstly, ability to culture biofilm was assumed in subsequent biofilm experiments. Secondly, though the reactor circuit was modeled on successful setups published by investigators in the U.S.A. and the DFR 110 Drip Flow Reactor (BioSurface™ Technologies Corp) was standard, other circuit components such as peristaltic pump and tubing were not standardized (Curtin and Donlan, 2006). Finally, no practical experience with biofilm generation existed at James Cook University at the time of experimentation which meant biofilm culture attempts needed validation.

Autoclaved standard glass laboratory slides were primed with *S.aureus A*TCC 29213 broth (test organism of interest) and processed as substrate within the biofilm culture circuit (Section 5.2 Biofilm Culture). Glass slides were utilized as substrate because they were compatible with analytical microscopy equipment. The processed slide and its bacterial load were collected aseptically from the biofilm reactor (PC2 Microbiology laboratory School of Veterinary and Biomedical Sciences), placed in sterile containers and immediately transported to the Advanced Analytics Centre (AAC), James Cook University. Specimens were then scrutinized for evidence of characteristic biofilm architecture using the following modalities: atomic force microscopy, scanning electron microscopy and confocal laser scanning microscopy.

5.3.1 Atomic force microscopy

Wet specimens were processed in air with the NTEGRA™ NT-MDT Prima Atomic Force Microscope (AFM)(Plate 5.6). NTEGRA™ NT-MDT Prima is a high-resolution, low-noise Scanning Probe Microscope (SPM) and was operated by experienced technical staff from AAC. For biofilm analysis a 10-15 µm probe (NSG10) with a tip radius of curvature of 10 nm was employed. AFM (semi-contact and contact), 3D

topographic images, magnetic force and phase images were acquired at magnifications ranging from 5-120 µm. Images that clearly demonstrated relevant specimen structure were retained.

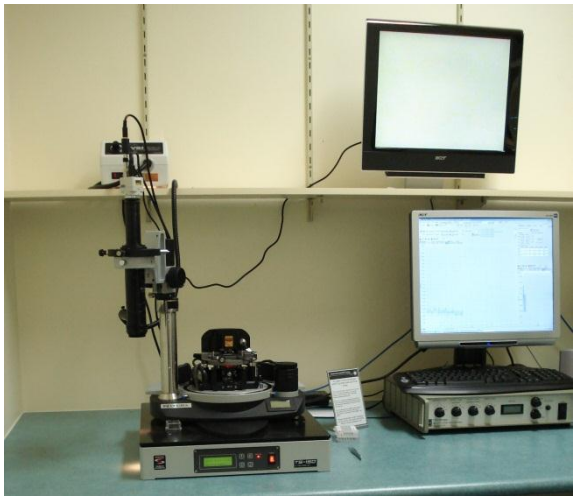


Plate 5.6: NTEGRA™ NT-MDT Prima Atomic Force Microscope at the Advanced Analytic Centre, James Cook University

5.3.2 Scanning electron microscopy with low vacuum

Surface topologies of uncoated specimens were examined in a low vacuum environment using JEOL JSM-5410LV Scanning Electron Microscope (SEM) (Plate 5.7). Coating was attempted but destroyed the specimens and therefore failed.

5.3.3 Confocal laser scanning microscopy

Wet biofilm specimens were stained with either 2% crystal violet (cells) or fluoresceine (matrix/extrapolysaccharide/voids) and examined for epifluorescence and with reflected light imaging using the BIO-RAD Radiance 2000 Laser Scanning Confocal System and Nikon Eclipse E600 microscope with a Vibroplane table (Plate 5.7). Excitation wavelengths of 488, 514, 543, 638 nm were implemented with an open emission filter. Specimens were imaged at 100 times magnification.

5.4 Confirmation of Biofilm Species

Purity and congruity of bacterial species in planktonic broth and resultant biofilm were confirmed for every biofilm specimen processed in order to avoid erroneous results due to circuit contamination. Biofilm circuit was constructed from delicate, mobile components with multiple interfaces requiring sterile and time consuming hand assembly. In addition equipment was exposed to recurrent potentially damaging autoclave cycles. Biofilm circuit was therefore particularly susceptible to environmental contamination due to disconnection of components or breaches associated with thermal or kinetic stress. Contamination carried over from bacteria in previous testing arms, source culture contamination or contamination during manipulation of biofilm and substrate were additional risks.

Planktonic broth and biofilm samples were examined to identify bacterial species. Planktonic broth organisms were first compared to source culture to exclude broth contamination. Species match between planktonic broth and manufactured biofilm was then sought as congruity was consistent with a closed, uncontaminated circuit. Biofilm species were checked by testing suspensions made at the time of viable plate count analysis (Section 5.5 Biofilm Enumeration). Planktonic and biofilm samples were lawn plated onto blood agar and Mueller Hinton agar then incubated at 37 ºC overnight (Plate 5.8). Plates were subsequently assessed as follows:

- Macroscopically uniform colonies indicated likelihood of pure culture and excluded gross contamination.
- *S.epidermidis* colonies appeared characteristically small, round and white. Cultures were non-hemolytic on blood agar.
- *S.aureus* colonies were larger than *S.epidermidis*, round and yellow. Cultures were ß hemolytic on blood agar.
- MSSA and MRSA were separated according to *in vitro* disc diffusion antimicrobial sensitivity testing protocols of Global Consensus Standardization for Health Technologies (previously NCCLS)(Figure 5.3)(Plate 5.9). Oxacillin discs were used as surrogates for methicillin sensitivity as methicillin discs were not available (MSSA source cultures were sensitive to oxacillin and MRSA source cultures were oxacillin resistant). Six millimeter diameter discs impregnated with one microgram of oxacillin (Oxoid) were placed onto a lawn inoculated Mueller Hinton agar plate and incubated overnight at 37 ºC. If disc

and clearance zone combined had a diameter >= 13 mm, *S.aureus* was defined as sensitive to oxacillin. Resistant *S.aureus* was defined when diameter <= 10 mm. Values in between were indeterminate.

Contamination was defined as lack of macroscopically pure culture, plated bacterial colonies inconsistent with *S.epidermidis* or *S.aureus* or any indication of incongruity between parent planktonic broth and biofilm plates. Contingency plans for contamination included formal identification of contaminating species via applicable standard techniques followed by a careful search for and nullification of contamination cause. Specimens from contaminated cycles were discarded and circuit sterilized prior to recommencement of culture activities.

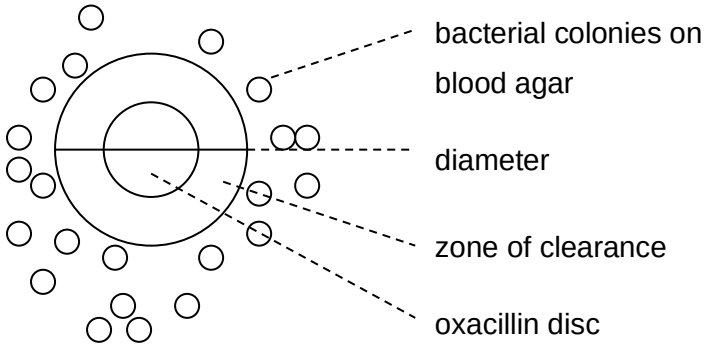


Figure 5.3: Determination of oxacillin resistance in S.*aureus* according to Global Consensus Standardization for Health Technologies. Diameter >=13mm represented oxacillin susceptibility and <=10mm represented oxacillin resistance.

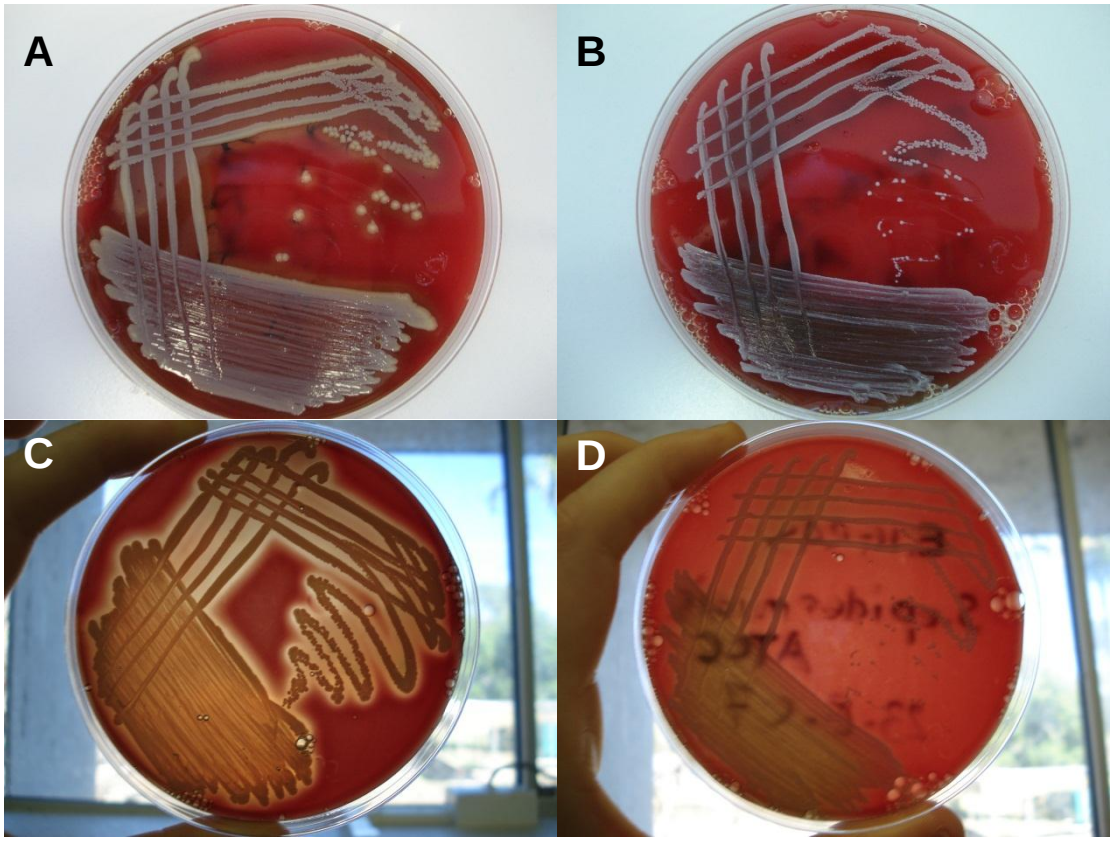


Plate 5.8: Dilution streak plates of bacterial isolates:
(A) dilution streak plate of *S.aureus* on blood agar. Individual colonies were rounded and yellow in color
(B) dilution streak plate of *S.epidermidis* on blood agar. Colonies were rounded, white and smaller than *S.aureus* colonies.
(C) *S.aureus* blood agar plate held up to light demonstrated β hemolysis
(D) *S.epidermidis* did not produce hemolysis on blood agar

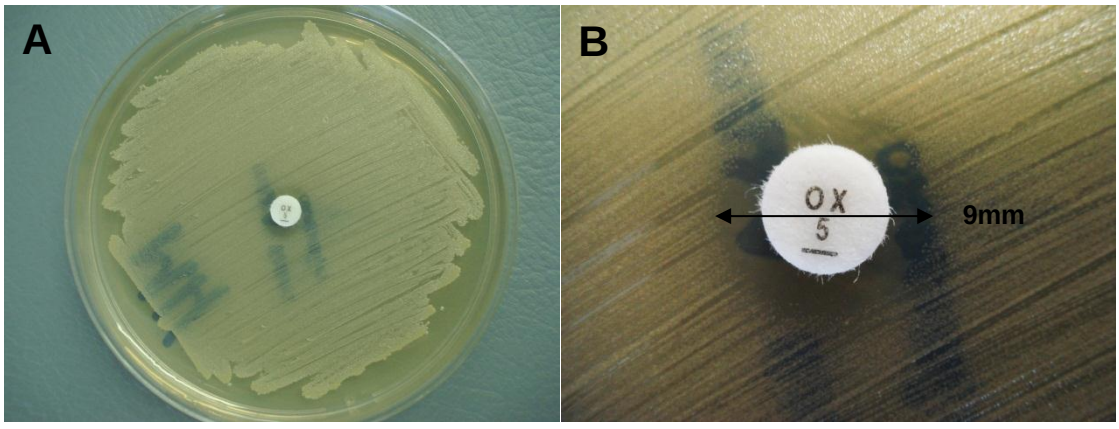


Plate 5.9: Oxacillin sensitivity of bacterial isolates:
(A) six millimeter diameter disc impregnated with 1 µgm oxacillin seated on an overnight lawn of *S.aureus* on Mueller Hinton agar
(B) clearance zone diameter is nine millimeter, consistent with oxacillin resistance

5.5 Biofilm Enumeration

5.5.1 Enumeration technique

A statistician was consulted to determine how many specimens needed to be analyzed to significantly enumerate organisms within a biofilm (Dr. Yvette Everingham School of Mathematics James Cook University). Greater than eight experimental repetitions supported implementation of 2-way ANOVA statistical test for each bacteria-substrate combination (Zar, 1999). ANOVA is a test of the hypothesis that the variation in an experiment is no greater than that due to normal variation of individuals' characteristics and error in their measurement. As biofilm culture circuit allowed four biofilm production runs to occur in parallel (biofilm reactor is built with four individual chambers), two biofilm culture cycles generated eight specimens. Eight iterations were therefore chosen to test each bacteria-substrate combination due to circuit design and statistical recommendation (Table 5.1). Stipulated regime required a total of 24 cycles using the biofilm culture circuit which were completed over a period of two months (Section 5.2 Biofilm Culture). 96 specimens were generated. SPSS™ 14.0 for Windows statistics software package was used to analyze data.

Table 5.1: Generation of sample numbers for biofilm enumeration experiments

Bacteria within biofilm	TTO plasma polymer on substrate				Total
	25 W	50 W	75 W	control PTFE	
S.epidermidis (CoNS) ATCC 12228	8	8	8	8	32
S.aureus (MSSA) ATCC 29213	8	8	8	8	32
S.aureus (MRSA) *Clinical isolate*	8	8	8	8	32
Total	24	24	24	24	**96**

One millimeter thick PTFE sheeting was hand cut into uniform 25 x 25 mm squares and cleaned (Section 3.3 Cleaning Construction Hardware). PTFE squares were used as substrate for plasma polymer thin films deposited at different power settings (25, 50 or 75 W) or the PTFE squares were left naked as controls. PTFE substrate squares of dimensions 25 x 2 5x 1 mm were chosen because they fit within biofilm reactor chambers and within vials used in vortexing and sonication. PTFE squares with or without polymer thin film coating were then processed through the biofilm culture circuit in an attempt to grow bacterial biofilm on the surfaces (Section 5.2 Biofilm Culture).

Once specimens were processed through the biofilm culture circuit they were removed aseptically from the biofilm reactor and independently processed according to the following steps:

1. Wash specimen gently by immersion in sterile phosphate buffered saline (PBS) pH 7.3 to remove planktonic bacteria, loosely adherent cells and debris associated with nutrient MHB
2. Place washed specimen into sterile 50 ml processing vial containing 20 ml sterile PBS
3. High speed vortex the specimen in 20 ml PBS for 15 sec (Plate 5.10)
4. Sonicate the specimen at 60 Hz for 15 min (Plate 5.10)
5. High speed vortex the specimen for 15 sec
6. Sonicate the specimen at 60 Hz for 5 min
7. Finally high speed vortex the specimen for 15 sec to obtain a bacterial suspension in PBS

Most cells were removed from solid biofilm phase without loss of viability and suspended in solution using this technique (Curtin and Donlan, 2006). Enumeration of biofilm cells was established by counting colony forming units per millilitre of suspension (CFU/ml).

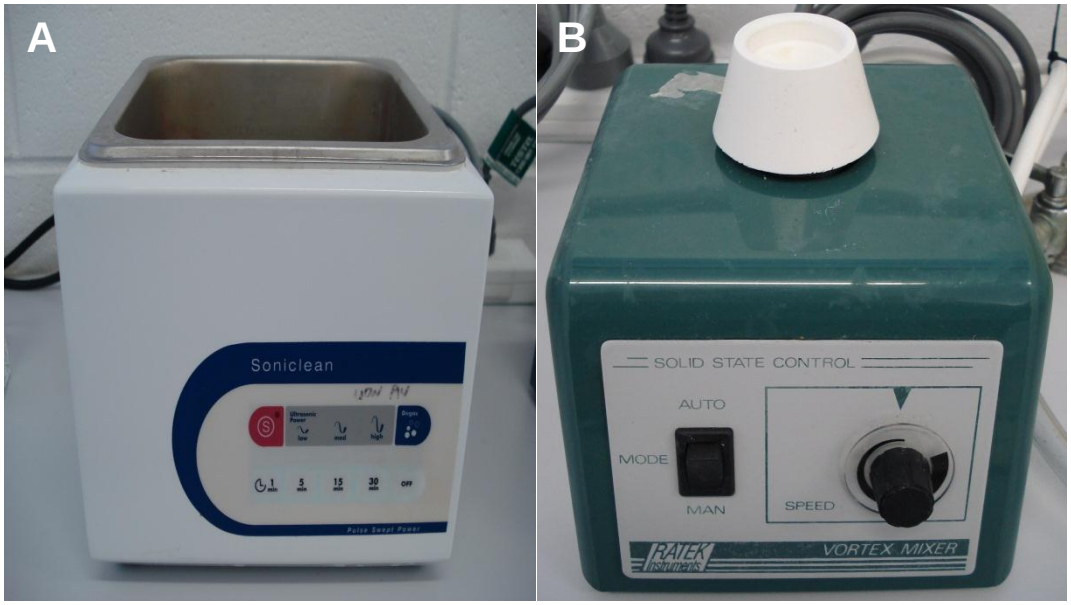


Plate 5.10: Sonication and vortexing hardware employed in enumeration of biofilm
(A) Soniclean 120T 60 Hz 60 W pulse swept sonicator
(B) Ratek VMI 50 Hz Vortex Mixer

5.5.2 Viable (standard) plate count

5.5.2.1 Equipment

- biofilm cellular suspension
- 10 ml sterile vials containing 9 ml of sterile normal saline
- pipette and sterile tips (1 ml and 100 µL)
- plates containing standard plate count agar

5.5.2.2 Technique

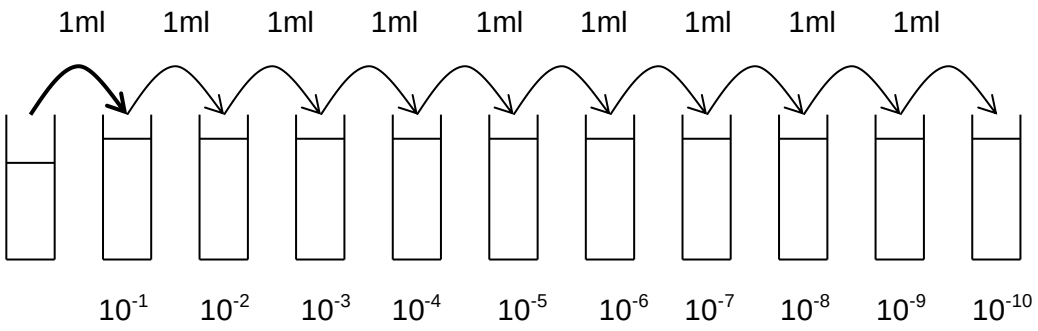


Figure 5.4: Diagram of dilution technique for determining viable plate count.
Each 10 ml vial contained nine milliliters of sterile normal saline prior to inoculation with diluted biofilm suspension.

One milliliter of neat biofilm suspension was aseptically transferred from 50 ml processing vial into a 10 ml vial containing nine milliliters of normal saline to make up a total volume of 10 ml (equating to a dilution factor of 10^{-1}). Bacteria were mixed through solution via gentle agitation of sealed 10 ml vial. One milliliter of newly diluted bacterial solution was then removed from first vial and added to a second vial containing nine milliliters of normal saline to make up a total volume of 10 ml within the second vial (equating to a dilution factor of 10^{-2}). Cycle was repeated until a dilution of 10^{-10} was achieved (10 vials of serially diluted biofilm suspension 10^{-1} to 10^{-10})(Figure 5.4). 100 µL of diluted biofilm suspension from each vial was plated onto standard plating agar and incubated overnight at 37 ºC. All 10 serial dilution plates were examined post incubation for evidence of colony formation. Each colony identified represented a single viable bacteria harvested from biofilm suspension. Colony populations per plate were counted with the aid of a Chiltern ZC301 colony counter. Concentrations of live organisms per ml (viable plate count) were ascertained by taking into account applied dilution factors. Number of colonies per plate were multiplied by 10 and then by 10^{x} where x was the number of serial dilutions performed to produce a plate with 30-100 CFU. Agar plates with a count of 30 to 100 colony forming units were selected to calculate CFU/ml as this range was least prone to counting error. Number of organisms derived from the original specimen was obtained by multiplying the CFU/ml by 20 as each specimen was originally processed in 20 ml of PBS (Plate 5.11).

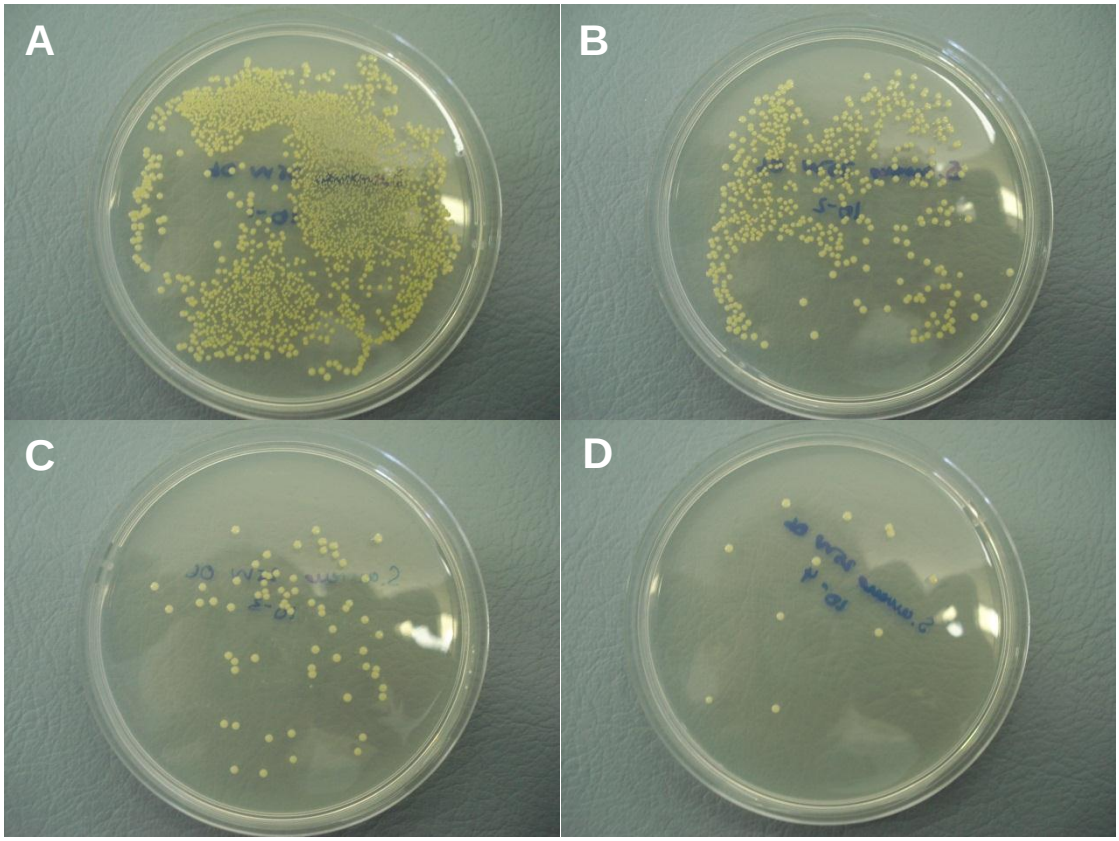


Plate 5.11: Plate counts derived from serial dilutions of *S.aureus* ATTC 12228 biofilm suspension:

(A) 1/10
(B) 1/100
(C) 1/1000
(D) 1/10000

Note that the 1/1000 dilution has a colony number within the CFU counting range (30-100 CFU per plate).

5.6 Confirmation of Biofilm: Results and Discussion

5.6.1 Atomic force microscopy

AFM analysis of *S.aureus* ATCC 29213 specimens grown using the biofilm culture circuit demonstrated biofilm. AFM employs a deflecting cantilever with a sharp tip (probe) at one end to scan a specimen surface. Forces measured by AFM include mechanical contact force, van der Waals forces, capillary forces, chemical bonding, electrostatic forces, magnetic forces and solvation forces (Butt et al., 2005). Semi-contact three dimensional (3D) topographic AFM represents specimen thickness along a “z” axis as well as by colour spectrum: dark colours indicating thinner and lighter colours thicker parts of the specimen. Using this technique, a 3D surface topography of biological specimens was generated without the damaging treatments or vacuum required by scanning electron microscopy. The 10 µm 3D topographic image in Plate 5.12 showed grape like clusters of cocci typical of the Genus *Staphylococci* (Ryan et al., 2004). Material being sampled was thus consistent with *S.aureus* ATCC 29213 and not indicative of contamination. Glass slide surface was seen as a smooth area coloured dark brown to black. With decreasing magnification (50 µm/100 µm/120 µm) a tertiary structure atop the glass surface emerged. Grape like clusters of cocci combined to form consistently peaked, umbilicated aggregations. This ordered and repetitive tertiary structure is not seen with free swimming planktonic bacteria and represented *Staphylococcal* biofilm.

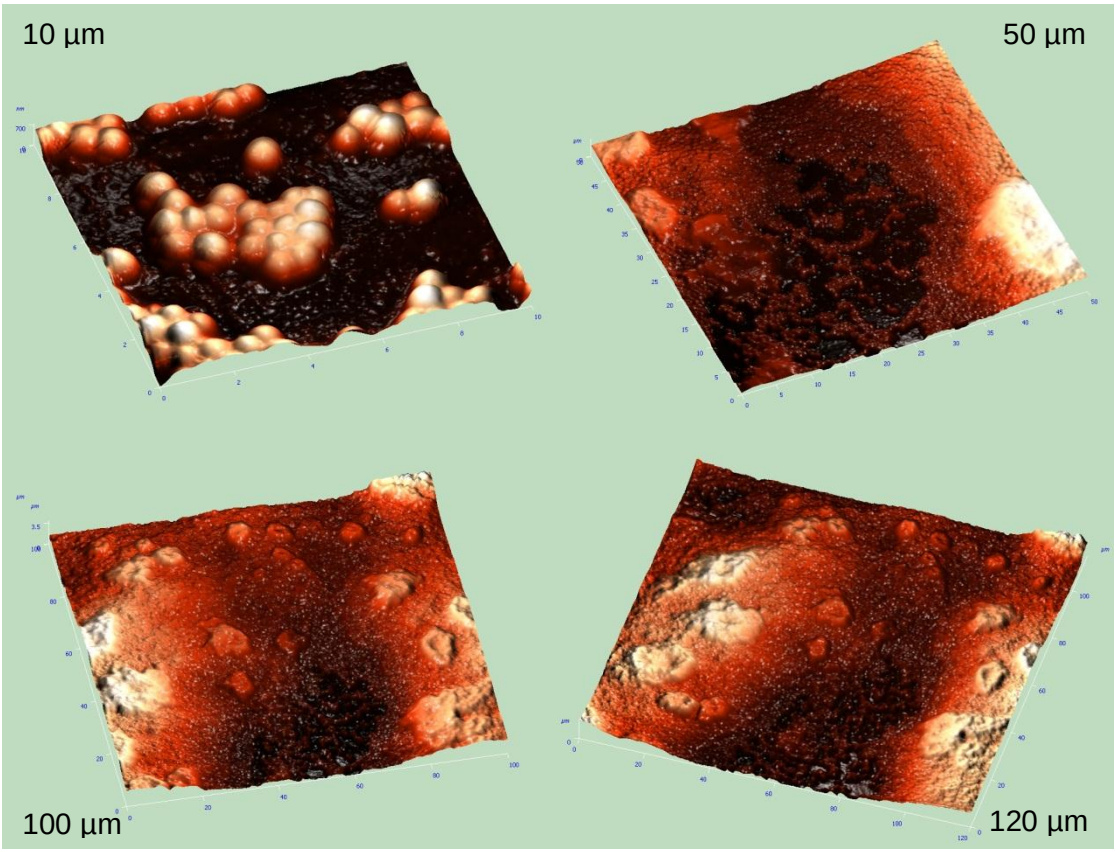


Plate 5.12: AFM semi-contact 3D topographic images of *S.aureus* ATCC 29213 (annotated dimensions represent the scale length of each imaging square)

Magnetic force microscopy (MFM) is a non-contact AFM variant that derives images from spatial changes in magnetic forces detected at the specimen surface. MFM images of *S.aureus* ATCC 29213 specimens generated by the biofilm culture circuit correlated with topographic AFM images (Plate 5.13). Again umbilicated biofilm towers were seen to project from the substrate surface on a background carpet of clustered cocci.

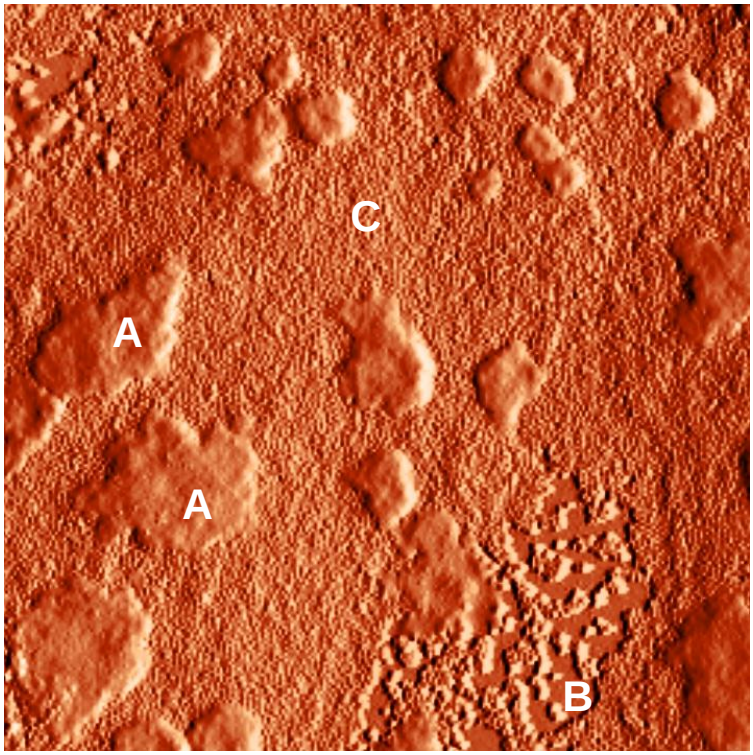


Plate 5.13: Magnetic force microscopy *S.aureus* ATCC 29213 biofilm at 100 µm resolution:

(A) biofilm towers
(B) exposed glass slide
(C) carpet of clustered cocci

Phase imaging was the final AFM modality employed to confirm the presence of biofilm. Phase imaging is a powerful extension of AFM that provides nanometre scale information about surface structure often not revealed by other AFM techniques (Magonov et al., 1997). It uses phase differences in cantilever oscillations to form highly contrasted images. Lower magnification (100 µm) phase contrast biofilm images such as that demonstrated in Plate 5.14 correlated with topographic AFM and MFM results to unveil tertiary biofilm towers. At higher magnification (10 µm) phase imaging also demonstrated biofilm matrix draping over and encasing individual bacterial cells (Plate 5.14).

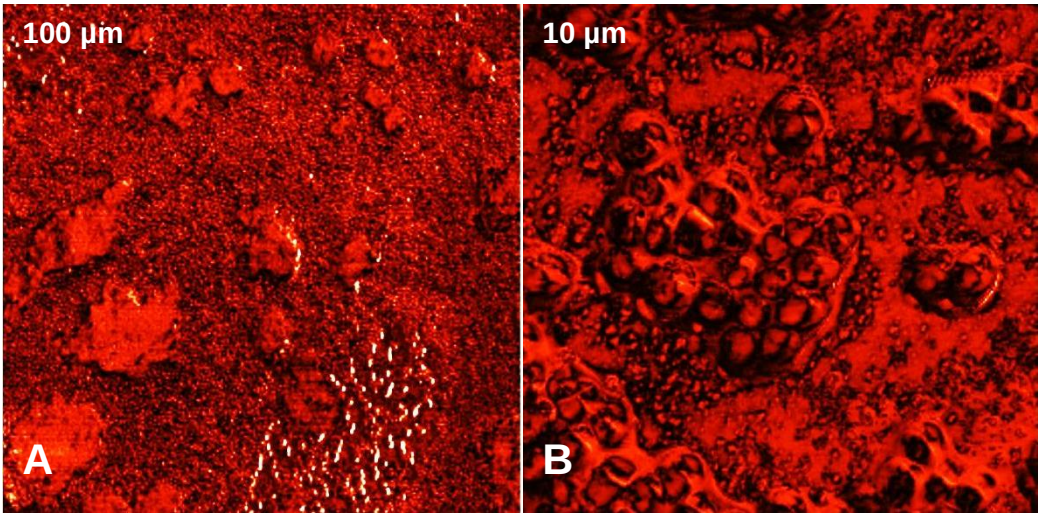


Plate 5.14: Phase contrast microscopy *S.aureus* ATCC 29213 biofilm: field diameter is as labelled. In (A) tertiary structure of biofilm is demonstrated whereas (B) shows clusters of cocci draped in biofilm matrix.

Contact AFM was attempted but failed as the process destroyed the delicate biofilms before adequate imaging could be achieved.

5.6.2 Scanning electron microscopy with low vacuum

Scanning electron microscopy (SEM) at low vacuum demonstrated bacterial cells associated with glass substrate but did not convincingly show the tertiary structure or matrix that defines biofilm and therefore was inconclusive (Plate 5.15). It may be that the metal coating treatment or vacuum environment required by SEM analysis destroyed biofilm components.

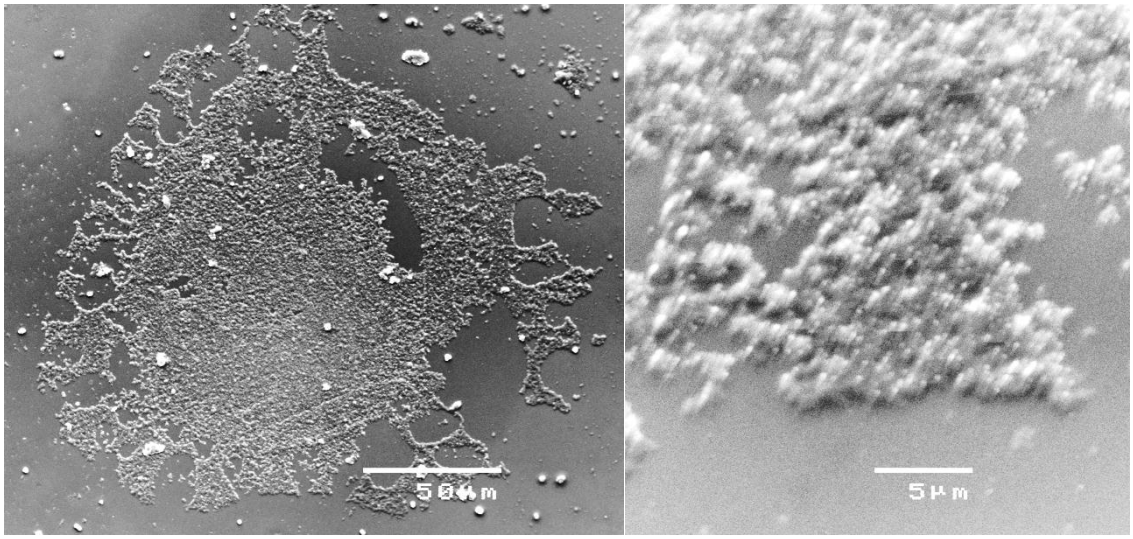


Plate 5.15: Low vacuum SEM of *S.aureus* ATCC 29213 cultured on glass. Single layers of clustered cocci are visualized but presence of biofilm matrix is not confirmed.

5.6.3 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) magnified live bacteria in real time and three dimensions without destroying the cells and was therefore a useful technique in biofilm analysis. CLSM (λ 534 nm) of two percent crystal violet stained wet specimens of *S.aureus* ATCC 29213 demonstrated focal epifluorescence (seen as bright white on imaging) within non-enhancing background material (Plate 5.16). Confocal microscopy therefore supported the presence of biofilm by highlighting its two major components: enhancing bacterial cells and non-enhancing matrix. Fluoresceine dye failed to epifluoresce matrix components at any employed wavelength (λ 488, 514, 543, 638 nm). Crystal violet was therefore the more effective epifluorescence agent.

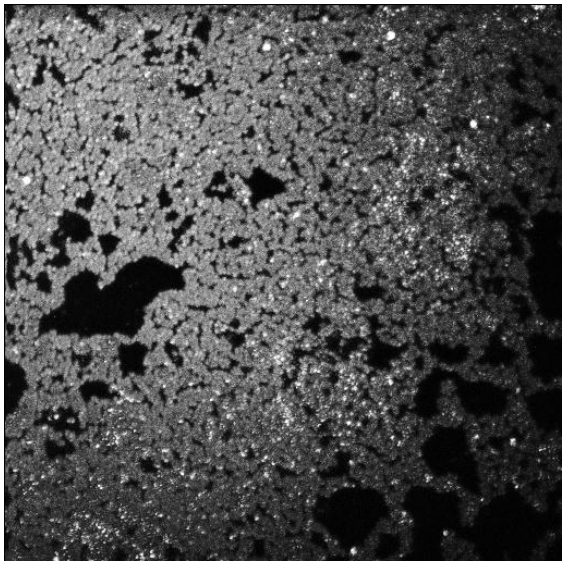


Plate 5.16: Confocal laser scanning microscopy of *S.aureus* ATCC 29213 biofilm stained with two percent crystal violet demonstrating epifluorescence (white scale) of bacterial cells and non-enhancement of matrix (grey scale). Exposed glass substrate is depicted as black.

5.6.4 Summary of imaging confirmation of biofilm

AFM and CLSM independently demonstrated *S.aureus* ATCC 29213 biofilm and therefore validated the biofilm culture circuit as a means to produce biofilm as opposed to no culture, contaminated culture or culture of planktonic bacteria alone. The imaging modalities were complimentary. AFM clearly showed primary, secondary and tertiary biofilm structure, demonstrated matrix and did not require stains or destroy specimens. CLSM allowed realtime and repeated 3D analysis of stained, wet biofilm preparations. SEM was an inferior modality to image biofilm probably due to effects of low vacuum and metallic coating required to undertake analysis.

5.7 Confirmation of Biofilm Species: Results and Discussion

During biofilm production, three culture cycles were contaminated. All three cycles were contaminated with *Bacillus*, a genus of bacteria commonly found in the environment and as a laboratory contaminant (Winn and Koneman, 2006). Contamination was initially detected when large colonies (Between two and seven millimeter diameter) with a frosted glass appearance formed on incubated SPA plates during the process of biofilm enumeration. Such colonies are typical of contamination with *Bacillus* species and co-existed with expected typical *Staphylococcal* colonies (HPA, 2007a, HPA, 2007b). Gram staining of foreign colonies was also consistent with *Bacillus* revealing large gram positive rods arranged in chains with rounded ends and an endospore (HPA, 2007a).

The first episode of contamination occurred early in experimentation, involved a cycle containing *S. epidermidis* and was probably due to handling error during cycle assembly causing breach in sterility and associated with the learning curve of biofilm culture technique. Second contaminated cycle occurred midway through culture experimentation and was subsequent to a small fatigue crack in peristaltic pump tubing which allowed entry of environmental bacteria into a closed *S.aureus* (MSSA) circuit. A final episode of contamination, again during *S.aureus* (MSSA) cycle happened when stretched tubing separated from its connector causing circuit breach. No further contamination occurred. After episodes of contamination, damaged peristaltic pump tubing and stretched circuit equipment were replaced as indicated, all contaminated specimens were discarded, circuit equipment was sterilized via autoclave and the laboratory area cleaned with bleach and alcohol. Successful culture then proceeded. When planktonic broth used to create biofilm was compared to output biofilm organisms all other experimental cycles were pure and congruent.

5.8 Enumeration of Biofilm Organisms: Results and Discussion

5.8.1 Results

Biofilm enumeration results are collated in Table 5.2 and represented graphically in Figure 5.5. The results showed bare PTFE control substrate grew the least biofilm when exposed to all three *Staphylococcal spp.* On each of the four substrates (polymers produced under powers of 25, 50 or 75 W and bare PTFE control), *S.epidermidis* formed less biofilm than either *S.aureus* strain. Further conclusions could not be drawn from data prior to statistical analysis (Appendix 5C).

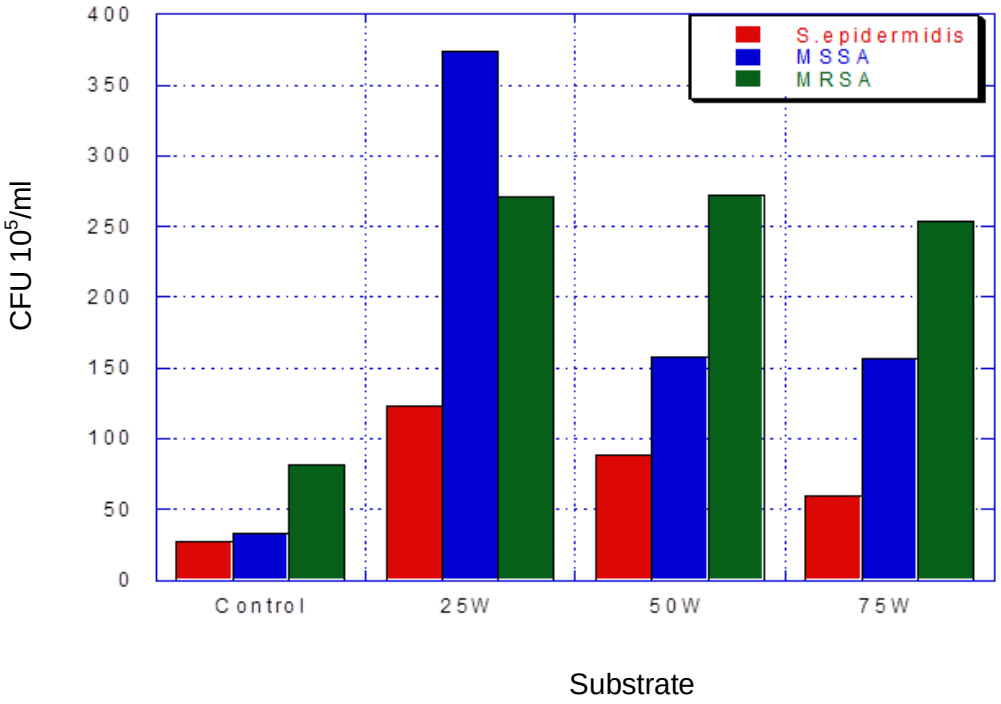


Figure 5.5: *S.epidermidis,* MSSA and MRSA biofilm CFU yield from bare PTFE control substrate and tea tree oil derived plasma polymer substrates generated at power settings of 25, 50 or 75 W

Biofilm enumeration data was analyzed with parametric two-way repeated measures ANOVA for independent variables with SPSS™ 14.0 for Windows using an alpha value 0.05. Independent variables were type of substrate and bacterial species and the dependent variable was biofilm formation as measured by number of CFU/ml derived from biofilm enumeration experiments (Section 5.5 Biofilm Enumeration). A general linear model was applied. No assumptions were violated.

Statistical tests were applied to answer the questions:

1. Did substrate type influence biofilm production?
2. Did bacteria type influence biofilm production?
3. Did influence of polymer on biofilm production depend on bacteria type?

Main effects of two-way ANOVA were:

1. The number of CFU (and therefore biofilm production) in each sample was influenced by the type of substrate ($F(3,21)=9.182$, $p<0.01$ Power 0.988)
2. The number of CFU (and therefore biofilm production) in each sample was also influenced by bacteria type ($F(2,14)=7.132$, $p<0.01$ Power 0.866)
3. The effect of polymer type on CFU did not depend on bacteria type ($F(6,42)=1.199$, p 0.325 Power 0.418).

Post hoc analysis by comparison of marginal means for significant main effects further elaborated on these results. Bare PTFE control polymer generated significantly less biofilm than any of the plasma polymer substrates ($p<0.05$). Furthermore there was no difference between effects of any of the plasma polymer substrates on biofilm production. *S.epidermidis* behaved differently to *S.aureus* variants in that *S.epidermidis* made less biofilm than either subspecies of *S.aureus* ($p<0.05$). No significant difference was noted in biofilm production between methicillin sensitive *S.aureus* and methicillin resistant *S.aureus.*

Table 5.2: Results of biofilm enumeration experiments: descriptive statistics

Substrate-bacteria Combination	Mean ($CFUx10^5$/ml)	Standard Deviation	Range ($CFUx10^5$/ml)	Number (N)
control PTFE				8
MSSA	33.1	43.2	6.6-132	8
MRSA	81.8	98.1	8-316	8
S.epidermidis	27.4	52.7	1.2-156	8
25 W polymer				8
MSSA	373.8	235	114-680	8
MRSA	271.5	113.9	112-462	8
S.epidermidis	123.7	158	2-370	8
50 W polymer				8
MSSA	158.1	92.8	56.6-302	8
MRSA	272.2	281.3	23.8-740	8
S.epidermidis	88.9	51.1	32.8-188	8
75 W polymer				8
MSSA	157.3	105.7	43.4-220	8
MRSA	254.3	273.9	54-730	8
S.epidermidis	59.2	55.2	14.2-184	8

5.8.2 Discussion

The results obtained from biofilm enumeration experiments did not support the hypothesis that tea tree oil plasma polymer thin films have bacterial anti-biofilm effect. More accurately, any anti-biofilm effect that may have been be provided by tea tree oil derived plasma polymer thin film surfaces was significantly less than that provided by bare PTFE polymer surface. Therefore the novel plasma polymers performed more poorly in preventing biofilms than PTFE (a currently used industry standard material deployed widely in medical implant production)(Gyo et al., 2008). In addition all tea tree oil plasma polymers performed equally poorly when compared to bare PTFE control. There was no difference in plasma polymer anti-biofilm performance according to plasma polymer power production parameters. Thus the hypothesis that altered power production parameters

changed plasma polymer surface moieties and in turn anti-biofilm capability was not supported in the power ranges utilized.

PTFE is used to make medical implants because it is biocompatible and resists biofilm formation. The anti-biofilm effects of PTFE are attributed to its low co-efficient of friction, reduced surface roughness, high hydrophobicity and surface charge which primarily dissuade bacterial adherence (Planchon et al., 2006, Gyo et al., 2008, Hallab et al., 2001). The physical properties of PTFE allow it to passively avoid biofilm colonization by reducing overall interactions with bacteria through being inert. Inert behavior of PTFE prevents both early physical-chemical and later molecular and cellular interactions that lead to bacterial adherence to surfaces (Katsikogianni and Missirlis, 2004). If bacteria cannot attach and interact with a surface they cannot create a biofilm on that surface.

Another theoretical approach to reducing biofilm formation is to actively engage bacteria and bacterial biofilm in a damaging or disabling way. The idea behind tea tree oil plasma polymers was to create novel surfaces laden with anti-biofilm moieties that harmfully interact with bacteria and actively lead to inhibition or destruction of biofilm cells or matrix. The plasma polymer active anti-biofilm components were in theory derived from parent compound tea tree oil (an essential oil known to have antibiofilm activity (Hammer et al., 2008)) and maintained through the process of plasma polymerization. Plasma polymers inherit intact portions of parent monomers according to production parameters, an important parameter being glow discharge power (Yasuda et al., 1978, Choukourov et al., 2010). Power settings were therefore altered during plasma polymer production in an attempt to discern a difference in antibiofilm performance based on power dependent retention of active moieties. As a class, plasma polymers are also thought to be antimicrobial due to their highly cross-linked hydrocarbon backbone creating a smooth, pinhole free, ultrathin film (Marx et al., 2011, Gomathi et al., 2008). The antibiofilm parent tea tree oil monomer building blocks, plasma polymerization technique and adjustable glow discharge power were therefore hypothesized to combine to create thin film surface treatments with antibiofilm actions.

Despite theoretical considerations, tea tree oil derived plasma polymers were not supported as antibiofilm in the enumerations experiments when compared to bare PTFE. In fact as a group, tea tree oil derived plasma polymers were equal and all significantly

worse than PTFE control in dissuading biofilm. Lack of demonstrable antibiofilm effect may have occurred for several reasons. The active antimicrobial components of tea tree oil are ill defined and there may be interplay between essential oil components in order to obtain biofilm killing (Carson et al., 2006). Plasma polymerization may have destroyed or inactivated vital antibiotic tea tree oil moieties or prevented interplay between components by physical separation of components within film. In order to overcome these limitations the mechanism of tea tree oil antimicrobial activity must first be better delineated then cross correlated with a structural and functional analysis of tea tree oil derived films to ensure common antimicrobial elements. If the exact mechanism of action of tea tree oil against bacteria were known then films could be generated at powers that maintain selected active moieties instead of using arbitrary power settings in a “hit and miss” fashion.

Plasma polymers generated under high powers are highly cross-linked, smooth and pinhole free with few branching side chains (Yasuda, 2005). Such polymers share physio-chemical characteristics with PTFE in that they are inert and therefore deter bacterial adherence. One explanation for enhanced biofilm formation on tea tree oil plasma polymers generated during this project was that the power settings of 25, 50 or 75 W were too low to invoke the characteristics typical of high power plasma polymer films. Lower power films retain fragments of monomers that may not be antimicrobial but still branch from film surface providing anchoring points for bacteria, increased roughness or greater surface area for adhesion. To resolve this possibility, tea tree oil plasma polymer films could be produced under higher power conditions and retested against biofilm. Such plasma polymers would be expected to reduce biofilm formation due to inert biochemical behavior and not due to preservation of active antibiofilm side chain moieties.

Even if the optimal power setting was known for plasma polymerization of tea tree oil with preservation of active antibiofilm components, the random recombination of monomer parts may present a substantial obstacle to designing surfaces with specific qualities. Due to the nature of plasma polymerization, monomer recombination to form surface polymer is random and unpredictable. Monomer is fragmented and pieces rejoined on a surface in a process of statistical recombination. If monomers are large (as are many organic molecules including those found in tea tree oil) and antimicrobial activity requires intact or long segments of intact monomer then plasma polymerization would be expected to

inactivate functional components and debunk antibiofilm capacity of tea tree oil and subsequent derived polymer films. This disabling effect would be even greater if interaction of multiple active intact tea tree oil components is needed to avert biofilm. Again a better understanding of the mechanism of tea tree oil antimicrobial activity and a structural analysis of plasma polymer film surfaces could contribute to answering these questions. Tighter production parameters and variation of productions parameters outside of simple power variation may also allow better control of plasma polymer structure. Clearly there is much work that needs to be done in both defining the source of tea tree oil antibacterial activity and translating the raw material into a designed capability thin film.

The bacterial dependent effects demonstrated by enumeration experiments were that *S.epidermidis* produced significantly less biofilm than either *S.aureus* subspecies regardless of substrate. This finding is explained by intrinsic differences in virulence factors and biofilm production ability known to exist between the *Staphylococcal* species (Gill et al., 2005). Similarly, both strains of *S.aureus* rely on machinery inherent to *S.aureus* in order to produce biofilm and so lack of difference in effect on biofilm production between these strains is not surprising. It is interesting to note that even subspecies of *S.aureus* are known to exhibit variability in biofilm production so the lack of difference in biofilm production seen between MSSA and MRSA in this enumeration experiment may be due to similarities in the specific isolates cultured and not a general condition of all *S.aureus* (O'Neill et al., 2007).

CHAPTER 6 FUNDAMENTAL PROPERTIES

6.1 Introduction

Identifying polymers for selection as biomaterials is challenging. A wide variety of polymers are available and no standardized set of ideal medical polymer properties exist to guide decisions (Angelova and Hunkler, 1999). In the context of medical implant medicine, a useful way to characterize polymers is to define properties that support polymers as biomedical materials by supporting biocompatibility, requisite application based criteria or beneficial commercial or production features.

Polymers with a wide variety of molecular structures are synthesized. Molecular characterization of these polymers is often extremely difficult owing to complicated morphology and intricate constituent interactions. Because polymers consist of a statistical distribution of chains of varying lengths, inter and intra-polymer forces and bonds make physical and chemical properties difficult to predict from polymer structure alone (Nojima et al., 2007). Useful characterization of polymers thus relies upon direct elucidation of chemical, physical and other baseline properties.

Baseline polymer properties fall into categories. Physical properties include degree of polymerization, molar mass distribution, crystallinity, stereo-regularity, water absorption and thermal phase transitions. Mechanical tests include tensile strength, viscoelasticity, ductile behavior and brittle failure, creep and relaxation (Phillips et al., 2008, Nielsen and Landel, 1994). Electrical factors encompass surface resistance, dielectric constant and dissipation. Other polymer properties include thermo-physical qualities, behavior in fields of force, transport properties and properties determining chemical stability and breakdown of polymers. The relevance of each property is contextual.

TTO derived plasma polymers are a completely new group of materials and as such are uncharacterized. Material characterization is important because it defines and identifies a material, suggests applications for the material and allows comparison to other materials and substances. Certain baseline properties are particularly informative in candidate bio-implant materials because they outline how a substance is expected to behave in a biological environment, how durable a material is and how efficiently it may be produced by industry. For example, mechanical properties of adhesion, hardness and internal stress, abrasion, friction and wettability indicate how a material

will perform as a device coating (Easton, 2005). Electromagnetic properties such as dielectric constant, magnetic permeability and conductivity will select polymer coatings for specialized applications such as implanted bio-sensors and heat conducting probes. Finally refractive index can be used as an identifying property and may support or refute materials as coatings in light transmitting applications such as lens systems (Easton, 2005). Parameters such as ease, speed and cost of production are important indicators of commercial viability.

A limited, focused and relevant subset of fundamental properties were analyzed in novel tea tree oil derived plasma polymer thin films as part of an initial characterization effort keeping in mind the context of bio-medical implants and using available resources. Initial property characterization of novel plasma polymers included:

- Thickness of polymer film as a function of deposition time
- Surface topography (roughness)
- Hardness
- Refractive index
- Dielectric constant
- Degradation and aqueous solubility

6.2 Plasma Polymer Film Thickness as a Function of Deposition Time

6.2.1 Introduction

Film thickness is an important specified property in surface coating industries. Film thickness influences film behavior, determines suitable applications for the film and contributes to production costs and timelines. Material properties of thin films and single elements differ substantially to those of related bulk materials (De Long 2003). As an example of this concept consider a material with structural disorder, impurity inclusion and random stoichiometric composition. A bulk material with these features would not imply reproducible optical and mechanical properties but sub-micrometer films with the same features routinely have controlled, reproducible film properties (De Long 2003). Film thickness is therefore not only a characterization tool but a functional determinant.

Film thickness parameters provide practical guides to industry and the research community. Thicker films require more monomer for production, more time for deposition and are therefore more expensive to manufacture. Maximum and minimum obtainable film thickness and parameters required to generate a range of film thicknesses are practical indices used in the commercial sector, in the production of polymer films for experimentation and are also part of polymer film characterization.

Information regarding *in situ* plasma polymer thin film formation, deposition and growth is required in order to control and implement plasma polymerization (Meichsner and Li 2001). Formation of plasma polymer is determined by the following parameters: geometry of the system, reactivity of the starting monomer, monomer flow rate, working gas pressure, power and frequency of excitation signal and temperature of substrate (Biederman and Slavinska 2000). For example, minimum wattage for plasma polymerization is monomer dependent because power needed to initiate glow discharge varies according to the monomer in the system (Gaur and Vergason 2000). As multiple production parameters contribute to the type of polymer generated these parameters must be thoroughly defined for a given polymeric material.

The ability to alter production parameters at this infant stage of novel tea tree oil derived plasma polymer thin film experimentation was limited by equipment and resources.

System geometry was held static as hardware components were custom made and could not be replaced. Tea tree oil monomer was a focus of experimentation and could not be altered within the context of this research. Monomer flow rate was controlled by a manual process that was difficult to quantitatively control and substrate temperature reflected room temperature. Power was the only production parameter that adjusted with ease and accuracy. Power adjustments were achieved simply by changing settings on a radiofrequency power generator. Because of these practicalities, the effect of changing power settings on polymer product was the only production variable pursued in any part of this thesis. All other production parameters were held constant to standardize production.

6.2.2 Assessment of film thickness as a function of deposition time

Tea tree oil derived polymer films were deposited on standard glass laboratory slides over time intervals of two, five, 10, 20, 30 and 60 minutes at radiofrequency powers of 25, 50 or 75 W (Section 3.2 Production of Tea Tree Oil Derived Plasma Polymer Thin Films). Thirty films were produced for each time period/ radiofrequency combination in order to have sufficient numbers to achieve statistically powerful results (Table 6.1). Resultant films were measured to determine thickness. Thickness measures were taken from the center of each laboratory slide using AvaSpec©-2048 spectrometer and accompanying AvaSoft© 6.1 thin film software. AvaSpec© thin film measurement system used mathematical functions to determine optical thickness from white light interference created by a film. For single layer systems the physical film thickness was calculated from optical thickness values using a function built into the same software package. AvaSpec© thin film system can measure film layers of 10 nm to 50 µm with a resolution of one nanometer (Avantes, 2011). Measurement hardware and software were already available at the School of Engineering at James Cook University. Measures were plotted graphically and statistically analyzed to determine the relationship between film thickness, deposition time and radiofrequency power stimulus.

Table 6.1: Number of samples generated for film thickness as a function of deposition time experiments

Deposition time	Radiofrequency power used to generate polymer			Total
	25 W	50 W	75 W	
2 min	30	30	30	90
5 min	30	30	30	90
10 min	30	30	30	90
20 min	30	30	30	90
30 min	30	30	30	90
Total	150	150	150	

Gradation of thin film deposition along the length of glass slides sometimes occurs during plasma polymerization. In order to adjust for this effect, thickness measures were taken from the center of each slide by aligning substrate with markers on AvaSpec© thin film stage.

AvaSoft© thin film measurement system was configured for glass substrate and essential oil thin film in the visible light spectrum (400-800λ). Lower and upper limits for thickness measures were adjusted until a good wave form fit in reflectance mode was attained. Film thickness was then recorded to one decimal place (Figure 6.1).

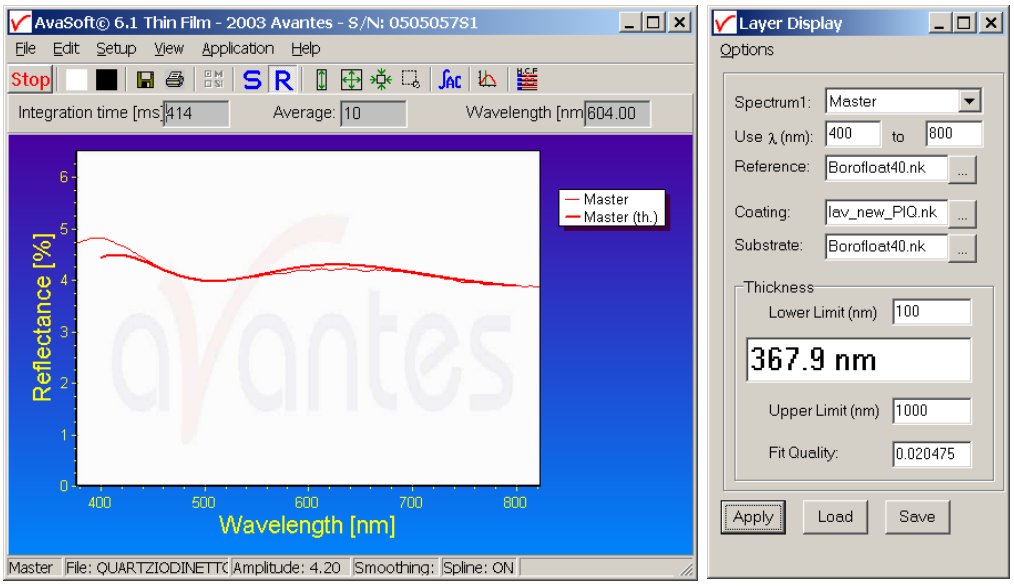


Figure 6.1: Example output from AvaSpec© thin film measurement system

6.2.3 Film thickness as a function of deposition time: results and discussion

6.2.3.1 Results

A predictable relationship between film thickness and deposition time was demonstrated in all three tea tree oil plasma polymer film variants (25, 50 and 75 W) (Figure 6.2 and Figure 6.3). 25 W thin films were deposited at an average rate of 100 nm per minute whilst both 50 W and 75 W films accrued at a slightly reduced rate approximating an average of 80 nm per minute (Appendix 6A).

Higher power cycles (50 and 75 W) produced incidental polymer deposition on the inner aspect of glass deposition chamber. During longer deposition cycles (30 minutes and longer), incidental polymer deposition on the inner surface of glass tube hardware became prominent. Incidental polymer flaking caused substrate contamination and bonding to hardware required extended mechanical and chemical cleaning to remove polymer from equipment surfaces.

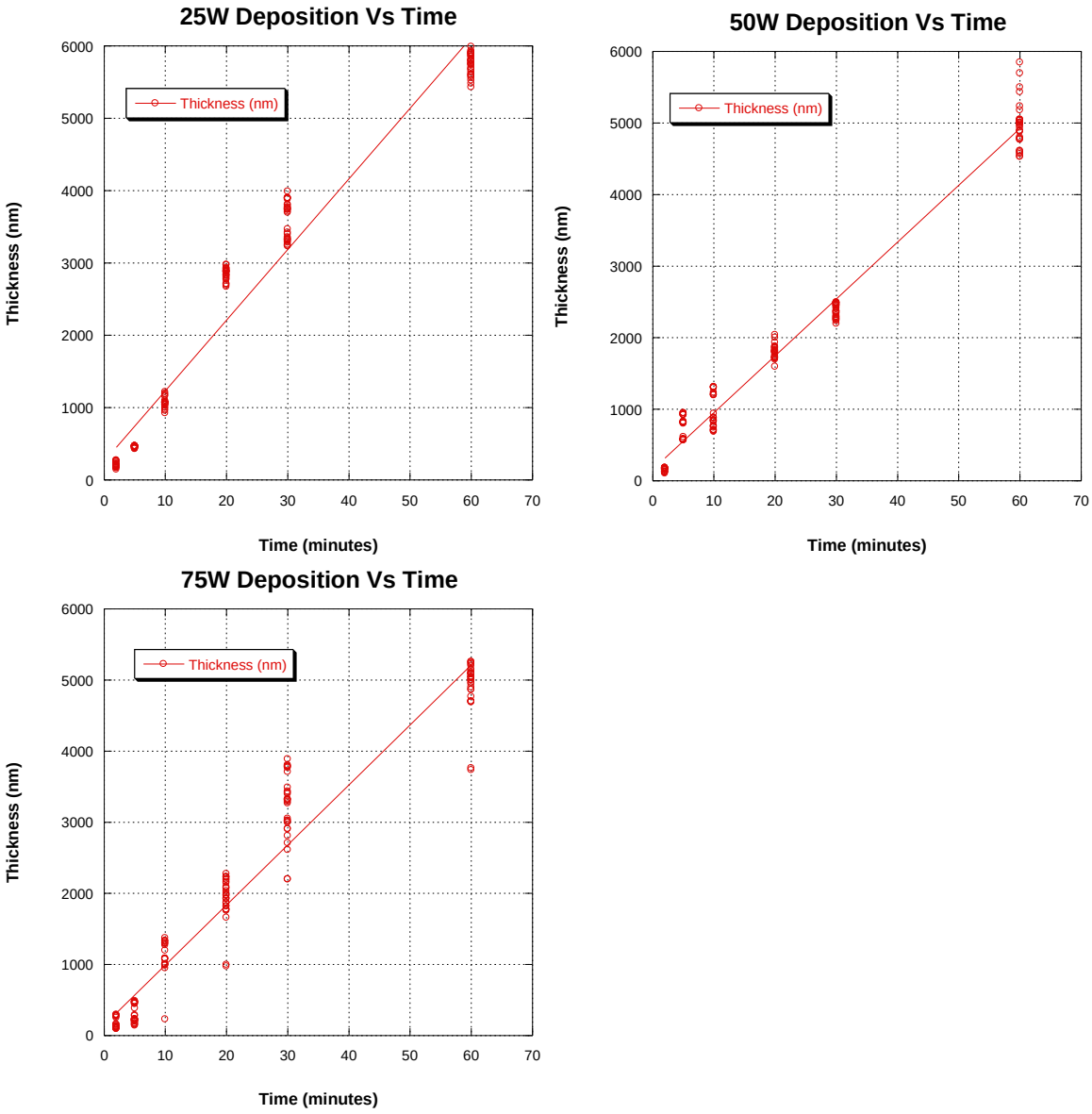


Figure 6.2: Graphical representation of the relationship between film thickness (nm) and deposition time (min) for plasma polymer thin film variants deposited under power parameters of 25, 50 and 75 W

At first glance, the graphically represented relationships between deposition time and plasma polymer film thickness appeared linear however statistical tests of between subject effects and lack of fit revealed quadratic relationships between film thickness and deposition time for all three plasma polymer variants (Figure 6.4)(Appendix 6B).

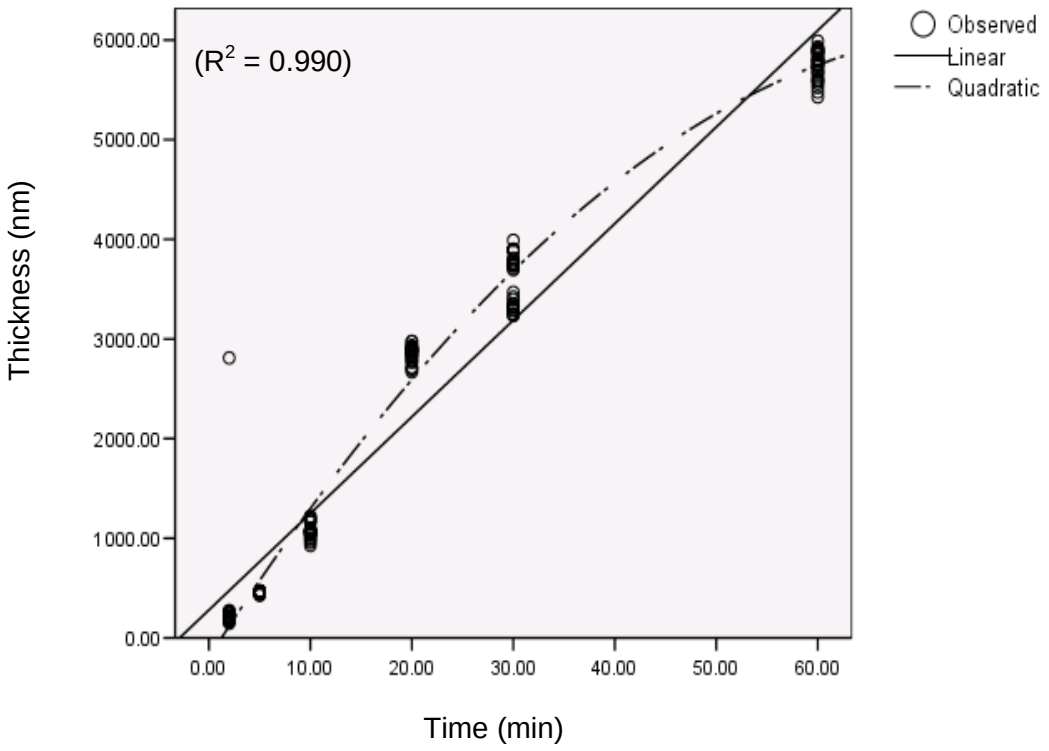


Figure 6.3: Plot of film thickness versus deposition time for plasma polymers produced under 25 W power conditions (R^2 = 0.990). Note that the best fit is quadratic. Results for 50 and 75 W polymer groups were similar (Appendix 6B)

6.2.3.2 Discussion

Deposition experiments running for longer than 60 minutes were not pursued as all films at one hour had achieved a thickness of several micrometers which is beyond the upper limit defined for thin films (thin films are typically stated to be less than one micron thick) and because flaking of films occurred at thicknesses over one micron. Thin films are advantageous as medical implant coatings over thick films because they allow for complexities in substrate geometry including miniaturization, avoid bridging across segments of implant, may allow for translucency in optical applications and aid in development of porous surfaces to facilitate timed release of drugs or intermittent sampling from a coated implant (Duka, 2008, Krebs, 2007).

Bonding and flaking seen in higher power tea tree oil polymers (50 W and 75 W) may have resulted from thermal annealing and free radical trapping within thin film. Plasma polymers can be stabilized by heat (annealed) as thermal treatment increases mobility of cross linked polymer chains, favours polymer recombination and reduces trapped free

radicals resulting in firmer bonding of polymer to substrate (Biederman, 2004). Annealing may also remove material strain, reduce hardness and increase ductility thereby creating a less brittle and more stable film that again improves bonding to substrate (Biederman, 2004). Conversely trapped free radicals lead to accelerated polymer degradation and flaking (Ali et al., 1994). Depending on the balance between thermal and free radical effects either bonding or flaking of plasma polymer prevailed. Excessive heat treatment for example, may cause polymer annealing but also leads to formation of new free radicals due to breaking of chemical bonds which may progress to film cracking and flaking (Biederman, 2004).

Differing rates of film thickness acquisition can be explained in several ways. The most likely explanation is a known property of plasma polymers. Plasma polymer films produced at higher powers are typically more dense than films produced at lower powers and therefore manifest as thinner films (Schreiber et al., 1980). Inconsistencies in production parameters may also cause thickness variability. Some controls such as monomer release into vacuum (monomer flow) were undertaken manually and were subject to human error. A +/-10% error in spectrometry thickness measures is accepted for the AvaSpec© system and can account for inconsistency in film thickness assessments. These points could be clarified by analysing the density of resultant films, excluding manual controls during polymer production and implementing more accurate measuring systems. Established results are sufficient however to conclude that tea tree oil plasma polymer thin films are deposited in a predictable, quadratic fashion over a short time period. Films in the micron thickness range were produced with in a 10 to 15 minute deposition period for all variants. Such information is important for industry feasibility studies and allows for estimation of polymer thin film production timelines.

6.3 Surface Topography (Roughness) and Hardness

6.3.1 Background on surface topography and hardness

Atomic force microscopy (AFM) is a high resolution technique that resolves images as small as the atomic lattice in real space (Hansma and Hoh, 1994). AFM works by bringing a cantilever tip extremely close to the surface to be imaged. As the tip traverses the surface, ionic attractive and repulsive forces on the scanned surface deflect the cantilever tip. The degree of tip deflection is measured by reflection of a laser spot onto a split photo detector and is proportional to the surface ionic forces. Summation of tip deflection data is collated and translated into a pictorial representation of the scanned surface using conversion software.

Surface roughness is a measure of the texture of a surface. It is quantified by vertical deviations of a real surface from its ideal form. Roughness is an important material feature because it effects fundamental properties such as friction, contact deformation, heat and electric current conduction, tightness of contact joints and positional accuracy (Gadelmawla et al., 2002). Real surface geometry is complex and a finite number of numeric parameters cannot provide a full description of surface roughness however summary measurements are useful in combination with image data (Gadelmawla et al., 2002). AFM imaging software automatically generates data such as average roughness, maximum and minimum peak heights and space between peaks to aid in quantifying and comparing surface topography.

Hardness is resistance to permanent and in particular plastic deformation. It is the measure of a locally loaded region of material to withstand external force by keeping its structure and form unchanged (NDT, 2011). Hardness is important in biomedical materials as it reflects the ability of a coating or implant to resist structural damage and is thus related to durability.

Numerous operative definitions of hardness exist and subsequently there are a large number of hardness-testing methods. The three main groups of hardness tests are scratch, indentation and rebound assessments. Within these categories lie multiple measurement scales. Mohs scale is the most commonly used scratch test (Williams, 1996). Indentation scales include those of Rockwell, Vickers, Shore and Brinell

(Sanponpute and Meesaplak, 2010, Hill et al., 1989, Altindag and Güney, 2006). Rebound testing applies the Leeb and Bennett hardness scales (Sanponpute and Meesaplak, 2010). In general there is no simple relationship between the results of these hardness tests making cross-comparison difficult and the techniques work poorly on thin films.

Indentation testing is the study of the plastic deformation of materials by indenters in order to measure hardness (Tabor, 1956). Using AFM and a specially prepared diamond tip probe, indentation testing can be miniaturized to measure hardness with an indentation depth as shallow as one nanometer (Bhushan and Koinkar, 2009). The AFM indentation technique allows hardness measurements of surface monolayers and ultrathin films in multilayered structures at very low loads. It can also measure rough surfaces to determine hardness (Bhushan and Koinkar, 2009). Because of these unique capabilities, AFM nano-indentation is the only accurate method of hardness determination for polymer thin films (Herrmann et al., 2000). Nano-indentation requires no special sample preparation and tests can be performed quickly and inexpensively. It is also a versatile technique for measuring mechanical properties other than hardness such as elastic modulus, creep/relaxation, interfacial adhesion, surface roughness, friction, wear and film fracture toughness (Duka, 2008, Bhushan, 2011).

AFM nano-indentation assessment of hardness is a well described technique (Bhushan and Koinkar, 2009). A typical nanoscopic indenter probe is composed of a pyramidal, single crystal diamond tip. The indenter stresses thin film surfaces at exactly maintained forces for one to two seconds. Indentation pits formed on polymer surface have unique geometry that is characterized with AFM surface topography imaging software. Nano-hardness is calculated by dividing loading force by the projected residual area of the indentation using a function embedded in AFM imaging software.

6.3.2 AFM surface topography and nano-indentation assessments

Atomic force microscopy was employed to determine surface topography (roughness) and hardness of novel tea tree oil derived plasma polymer thin films. Testing was performed at the Advanced Analytics Centre at James Cook University with assistance of onsite operational scientists. The scanning probe microscope was high resolution, low noise NTEGRA™ NT-MDT Prima AFM (Plate: 6.1).

Three tea tree oil derived plasma polymer thin film variants were tested. Each sample was generated under a different power condition (25, 50 or 75 W) to determine if power production parameters influenced film surface topography or hardness. Samples were deposited onto standard glass laboratory slides over 10 minute intervals (Section 3.2 Production of Tea Tree Oil Derived Plasma Polymer Thin Films). One centimeter diameter metal discs were adhered to the underside of each slide with cyanoacrylate glue to secure samples to the magnetic AFM stage. Magnetic discs were required because the AFM stage was not designed to house standard glass laboratory slides (Plate 6.1). All analyses were performed in air.

To assess surface morphology, plasma polymer samples were imaged with NT-MDT NTEGRA™ Prima Atomic Force Microscope in semi-contact mode using a 10-15 µm probe (NSG10) with a tip radius of curvature of 10 nanometers. Contact mode was attempted but failed due to specimen destruction. Images were optimized and analyzed with Image Analysis™ programs built into AFM control software. Image Analysis™ software derived information from surface images including maximum, minimum and average film height, peak-to-peak distance and average roughness (Appendix 6C: AFM data surface topography (roughness)) Each of the three plasma polymer thin film samples were analyzed twice at random positions along the films to test homogeneity.

Nano-indentation testing to determine film hardness required replacement of the AFM probe and head assembly with Hysitron Triboscope Nano-mechanical Test Instrument and Berkovich tip. Hardness was calculated as a function of load force and contact area where contact area was not directly measured but inferred from contact depth using a sixth order tip area function ingrained in AFM control software. Nano-mechanical testing of hardness was performed on a bare glass control slide using nineteen indents with loading forces ranging from 500-5000 µN. Tea tree oil derived plasma polymer thin films were each tested in triplicate using twenty-four indents per test cycle. Loading forces started at 100 µN with incremental increase in load of 50 µN until an indentation force of 450 µN was obtained (Appendix 6D: AFM data nano-indentation assessment of hardness). Loading forces were applied at 50 μNs^{-1}. Hardness measures were calculated in GPa.

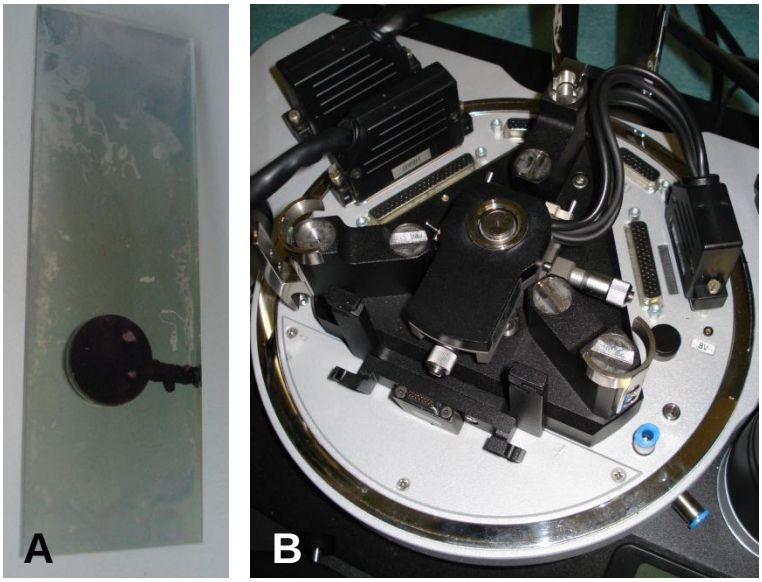


Plate 6.1: Atomic force microscopy configuration for surface topography and hardness testing:

(A) standard glass laboratory slide with deposited plasma polymer thin film mounted onto metallic disc

(B) mounting stage within NTEGRA™ NT-MDT Prima AFM

6.3.3 AFM nano-mechanical assessments: results and discussion

AFM structural investigation provided information on polymer homogeneity, roughness, height and hardness (via nano-indentation) which aided in classification and description of new thin film materials.

6.3.3.1 Surface topography (roughness) (Appendix: 6C)

The 25 W plasma polymer film had an average height of 3.04 nm (range 0-11.06 nm) and average roughness 0.46 nm (Figure 6.1). The 25 W film was only analysed in one location because the data file from the second location was corrupted.

The 50 W film was sampled successfully at two locations. The average height for the first location was 8.74 nm (range 0-20.67 nm) and for the second location was 2.31 nm (range 0-10.58 nm). Average roughness measures for the first and second sites were 0.31 nm and 0.30 nm respectively (Figure 6.2).

Topography results for the 75 W plasma polymer thin film were also obtained from two locations. The average height for the first location was 2.31 nm (range 0-6.57 nm) and for

the second location was 11.48 nm (range 0-127.31 nm). Average roughness measures for the first and second sites were 0.34 nm and 0.55 nm respectively (Figure 6.3).

Height measures from all films were comparable and consistent with ultrathin films (<100 nm film thickness). There was no obvious height pattern related to film power production parameters. All three films were discontinuous in places (height of 0 nm). Images revealed areas of discontinuity to be small enough however for films to be described as pinhole free. Roughness measures for film samples were similar and consistent with extremely smooth surfaces. Overall, film topography was therefore assessed as homogeneous, pinhole free and smooth. Power input made little difference to film surface topography.

Surface topography analysis confirmed the expected outcomes that tea tree oil derived plasma polymer thin films were homogeneous, pinhole free and smooth surface coatings. The results were expected because they are consistent with class properties of plasma polymer thin films (Biederman, 2004). Homogeneous, pinhole free surfaces are good candidates as coatings for two-phase medical implants because they are structurally consistent and act as effective barriers between implant bulk phase and host environment. In medical implant construction smooth surfaces are also advantageous because irregularities in polymeric surfaces promote bacterial adhesion and biofilm deposition whereas smooth surfaces dissuade bacterial adhesion and biofilm formation (Scheuerman et al., 1998). This may happen because rough surfaces have greater surface area and surface depressions provide more favourable sites for bacterial colonization and infection (Katsikogianni and Missirlis, 2004). Although power input seemed to make little difference to film surface topography, the small sample numbers hampered interpretation. Provisional plasma polymer film assessment suggested favorable surface topography for implementation of these films as medical implant coatings.

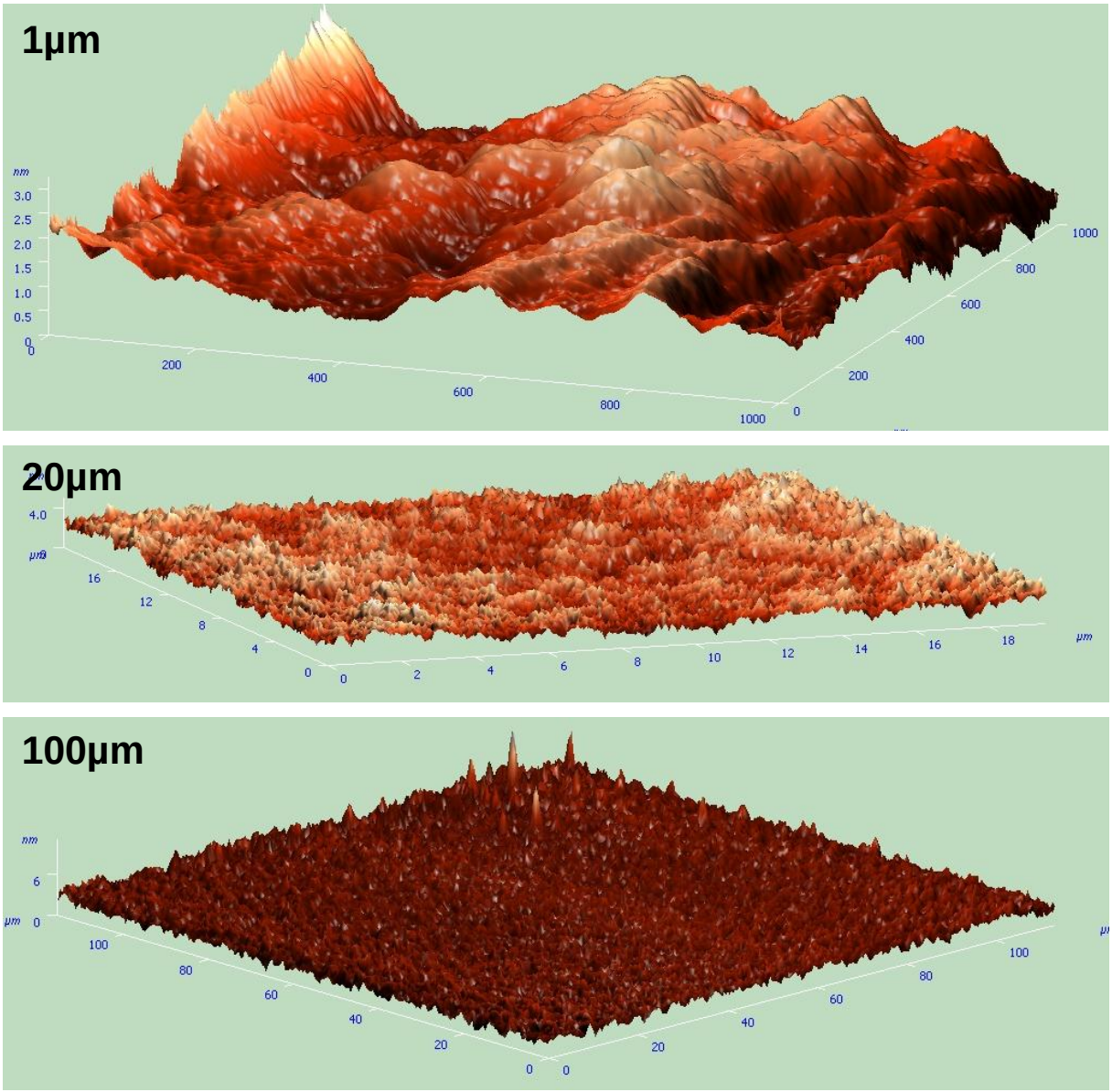


Figure 6.4: AFM images of 25 W tea tree oil derived plasma polymer thin films at magnifications of 1 µm, 20 µm and 100 µm (AFM image length and breadth dimensions)

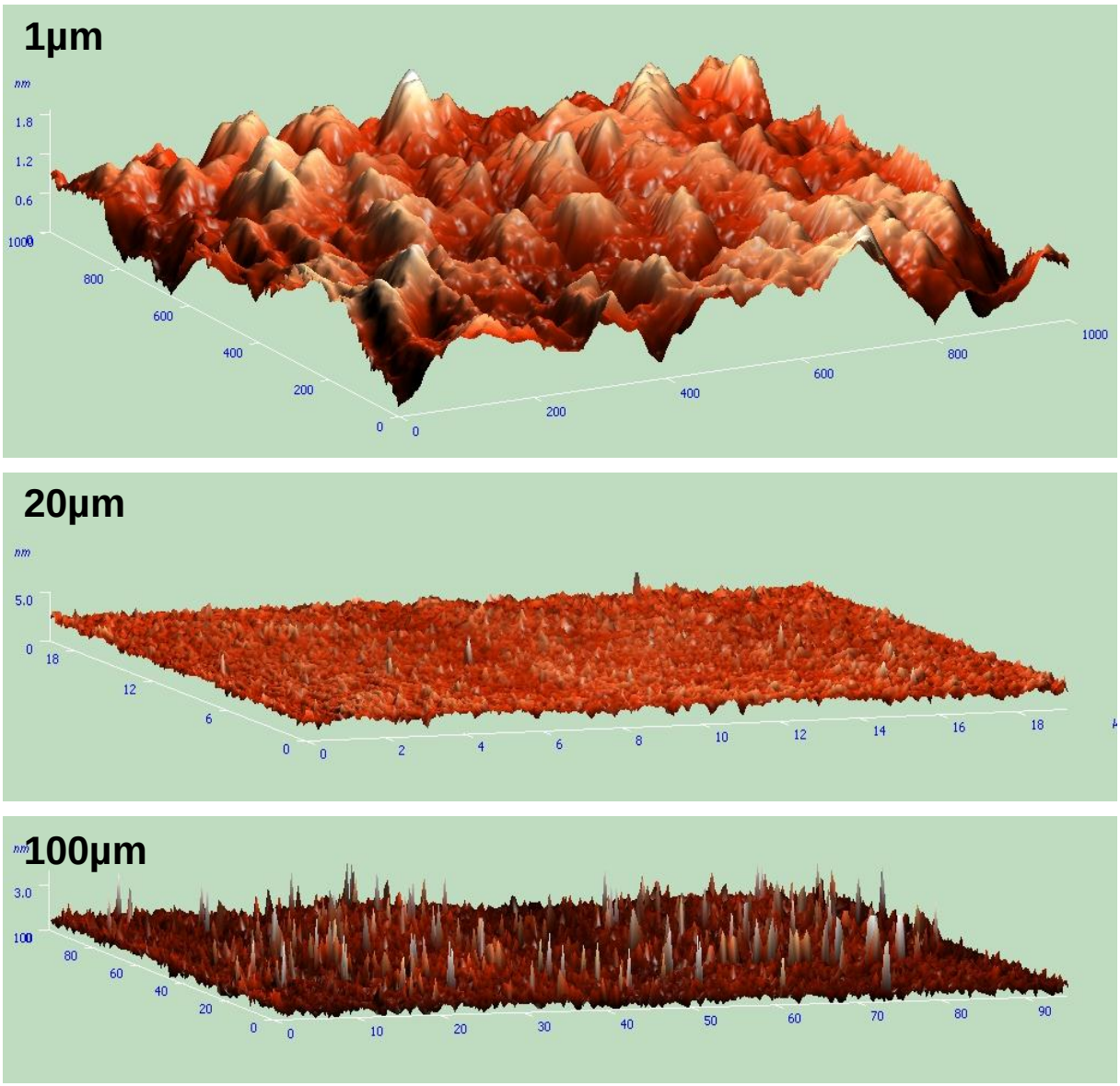


Figure 6.5: AFM images of 50 W tea tree oil derived plasma polymer thin films at magnifications of 1 µm, 20 µm and 100 µm (AFM image length and breadth dimensions)

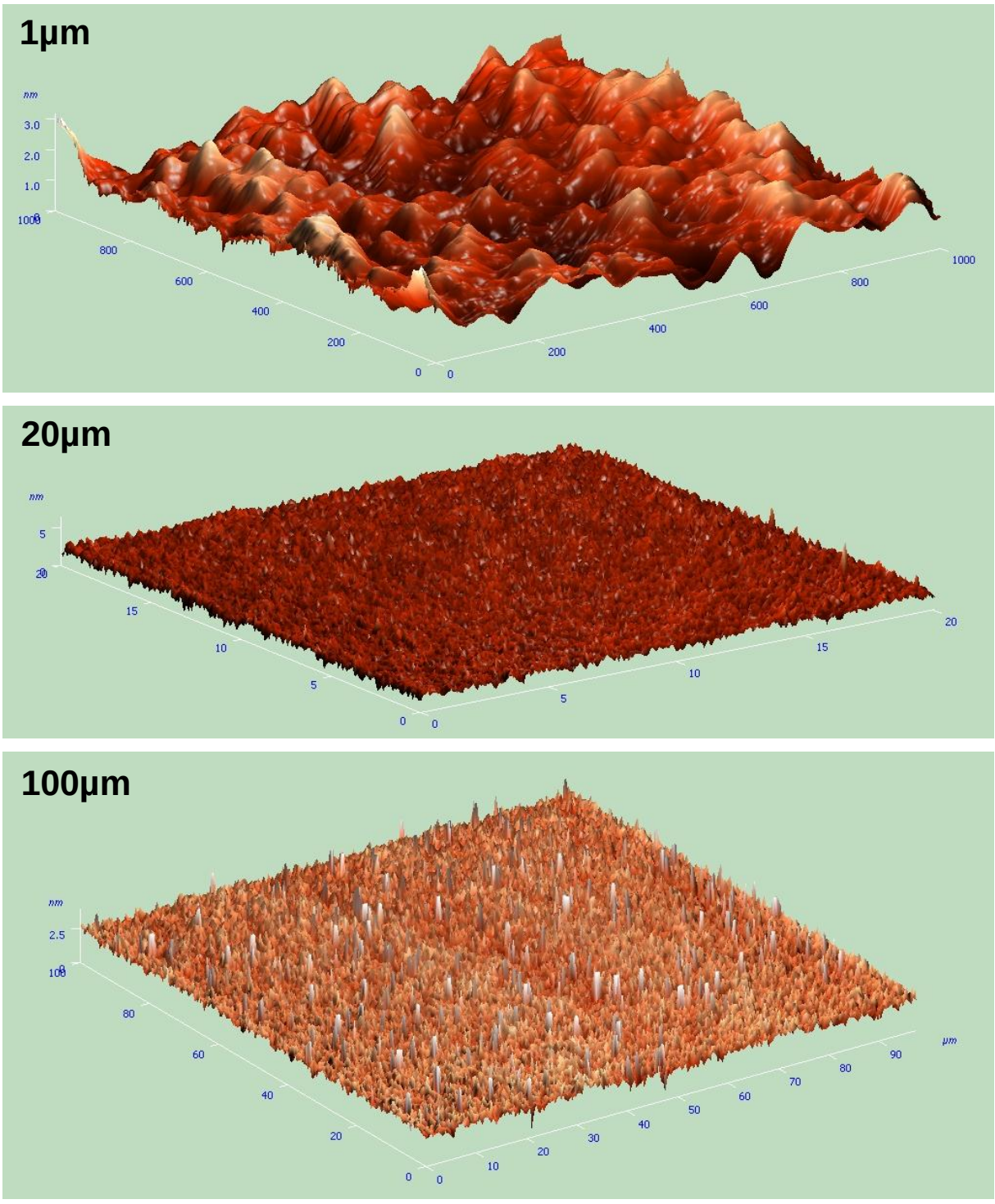


Figure 6.6: AFM images of 75 W tea tree oil derived plasma polymer thin films at magnifications of 1 µm, 20 µm and 100 µm (AFM image length and breadth dimensions)

6.3.3.2 Hardness (Appendix: 6D)

Images of nano-indented plasma polymer thin films showed characteristic distinct, pyramidal marks and three dimensional clefts left by the single-crystal diamond Berkovich tip. Progressive increases in indented surface area correlated with increasing load forces through the Berkovich tip and formed a graduated indentation grid on the surfaces of plasma polymer thin films (Figure 6.4A). Increasing indentation depth was also demonstrated in three dimensional views of the polymer thin film indentation grids (Figure 6.4B). Surface area and indentation volume were used by Image Analysis™ software attached to AFM to calculate hardness using load-displacement curves.

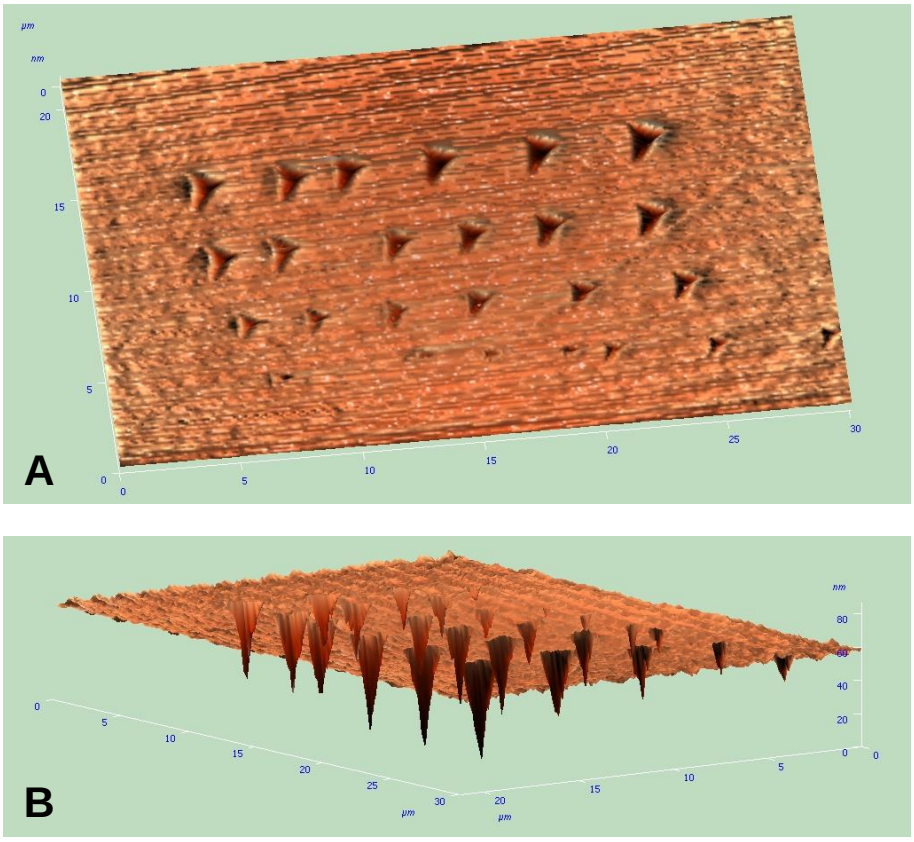


Figure 6.7: AFM images of 25 W tea tree oil plasma polymer thin films at magnification of 30 µm viewed (A) from above and (B) from below to demonstrate surface area and depth of probe indentation divot

A standard glass slide was used as a control surface for calculating hardness. Control slide had an average nano-indentation hardness of 5.07 GPa (Table 6.1). Plasma polymer thin film samples were much softer than glass control substrate. Average hardness values were 0.31 GPa, 0.42 GPa and 0.48 GPa for 25 W, 50 W and 75 W polymer films respectively (Table 6.1). Average nano-indentation hardness results for films therefore increased with increasing power production parameters.

Table 6.2: Summary of average nano-indentation hardness results

Material	Hardness (GPa)
glass slide substrate (control)	5.071962
25 W polymer	0.308723
50 W polymer	0.422373
75 W polymer	0.475502

Nano-indentation hardness for standard glass is usually quoted at 6.5 GPa (Tsui et al., 1997, Cao et al., 2006). The AFM was calibrated and functioning correctly so the less than expected hardness measurement of control glass slide (5.07 GPa) was either real or due to a substrate error. As the glass substrate was a brand new slide, substrate error was thought unlikely. Underestimation of glass hardness may have resulted from the sample being mounted with cyanoacrylate glue. By the same logic, underestimation of hardness results for tea tree oil derived plasma polymers may also have occurred.

Even allowing for 25% underestimation of hardness, novel plasma polymers were of comparable hardness to polymers commonly used in medical implant production (Table 6.2). Hardness of novel plasma polymer films was therefore considered adequate for use in medical implants.

Increase in hardness of polymer films with increase in power production parameters was in keeping with class properties of plasma polymers (Biederman, 2004). These results suggested that hardness of tea tree oil plasma polymer films can be titrated by controlling power input. Control of hardness is favourable as it means films can be tailored according to product hardness specifications.

Table 6.3: Nano-indentation hardness results of materials used in medical implants

Material	Hardness (GPa)	Reference
poly(propylene)	0.04	(Ramirez, 2010)
poly(vinyl chloride)	0.15	(Tommaso, 2011)
poly carbonate	0.22	(Briscoe et al., 1998)
poly(styrene)	0.3	(Briscoe et al., 1998)
poly(tetra fluoro ethylene)	0.3	(Heilmann, 2003)
poly(methyl methacrylate)	0.38	(Briscoe and Sebastian, 1996)
UHMW poly(ethylene)	0.6	(Briscoe et al., 1998)
stainless steel	3.0	(Cho and Chang, 2003)

6.4 Refractive Index

6.4.1 Introduction

Absolute refractive index is the ratio of speed of light in a vacuum to its velocity in a transmitting medium. Refractive index is constant for a defined pair of materials. By definition the refractive index of a vacuum is one. In practice, air makes little difference to light refraction with an absolute refractive index of 1.00029 thus air measures can be used as approximations of vacuum measures. As an exemplar, standard glass has a refractive index of 1.5, which means that light travels at 1 / 1.5 = 0.67 times the speed in glass as it does in air or vacuum. Yellow light emitted by a sodium lamp (average wavelength of 5.893 nm) is a standard light used to measure refractive index. Refractive index is a fundamental physical property and can therefore be utilized to identify materials or confirm their purity or concentration (Table 6.3).

Plasma polymers thin films are already being implemented as light transmitting thin films. For example, luminous chemical vapor deposition is used to fabricate transparent dielectric optical films for applications such as optical filters, antireflective coatings, optical waveguides and biosensors (Sakthi et al., 2003). Plasma polymer thin films are scratch and erosion resistant, surface adherent, stable and fluid impermeable which are beneficial qualities in light transmitting medical implants such as optical lenses (Martinu and Poitras, 2000). Refractive index is thus an important characterization tool because it is relevant to determining light transmitting applications for novel plasma polymer films.

Table 6.4: Absolute refractive indexes of common substances

Material	Absolute refractive index
vacuum	1.00000
air (STP)	1.00029
standard glass	1.5
diamond	2.417
ethyl alcohol	1.36
fused quartz	1.46
sodium chloride	1.544
water (STP)	1.333

Refractometry can be achieved in many different ways however spectroscopic ellipsometry has benefits when measuring thin films (McCrackin et al., 1963). Ellipsometry provides a robust, accurate and reproducible measure of refractive index, it is insensitive to scatter and fluctuations and requires no standard sample or reference beam (McGraw and Hill, 2003). In addition the technique allows analysis of complex, multilayered films with rough interfaces of unknown material composition. Ellipsometry is also nondestructive and contactless so specimens remain intact and can be analyzed multiple times.

Ellipsometry measures the change of polarization upon reflection or transmission of electromagnetic radiation on a plane surface. Subject to polarized light a thin film will absorb part of the light and reflect part of the light. Change in polarization is determined by thin film properties such as thickness, refractive index and dielectric function (McGraw and Hill, 2003). With ellipsometry, refractive index of a thin film is determined using the measured change in polarization in combination with predetermined formulas that compensate for variables such as film thickness and dielectric function.

6.4.2. Refractive index assessment

Refractive index was characterized using the technique of spectroscopic ellipsometry. Ellipsometry was expensive, costing AU$ 300 per hour and took three hours per sample to generate results (total cost AU$ 900 per sample)(Easton, 2005). It could not be performed at James Cook University. Because of involved costs and requirement for outsourcing, only three novel polymer samples were subjected to ellipsometry for determination of refractive index.

Tea tree oil derived plasma polymer thin films were deposited on individual, standard glass slides over ten minute time intervals (Section 3.2 Production of Tea Tree Oil Derived Plasma Polymer Thin Films). Each sample was generated under a different power parameter (25, 50 or 75 W) in order to assess the relative effect of power of production on film refractive index. Films were outsourced to to J.A.Woollam Co., Inc (645 M STREET, SUITE 102 LINCOLN, NE 68508-2243 USA), a North American based company that specializes in spectroscopic ellipsometry and thin film characterization. Refractive index was determined by onsite staff using the M-2000® rotating compensator spectroscopic ellipsometer.

6.4.3 Refractive index: results and discussion

Refractive index results for 50 W and 75 W tea tree oil derived plasma polymer thin films were obtained via ellipsometry (Figure 6.5). The 25 W film sample was smashed in transit and couldn't be assessed by the receiving facility.

At standard wavelength (5.893 nm), 50 W polymer absolute refractive index was 1.559 and 75 W polymer absolute refractive index was 1.548. Refractive index results were consistent with transparent materials and similar to refractive indices of typical glass (1.5) and fused quartz (1.46). Refractive index results for the novel polymers suggested suitability for light transmitting medical implant applications. The effect of production power on refractive index could not be implied from two samples.

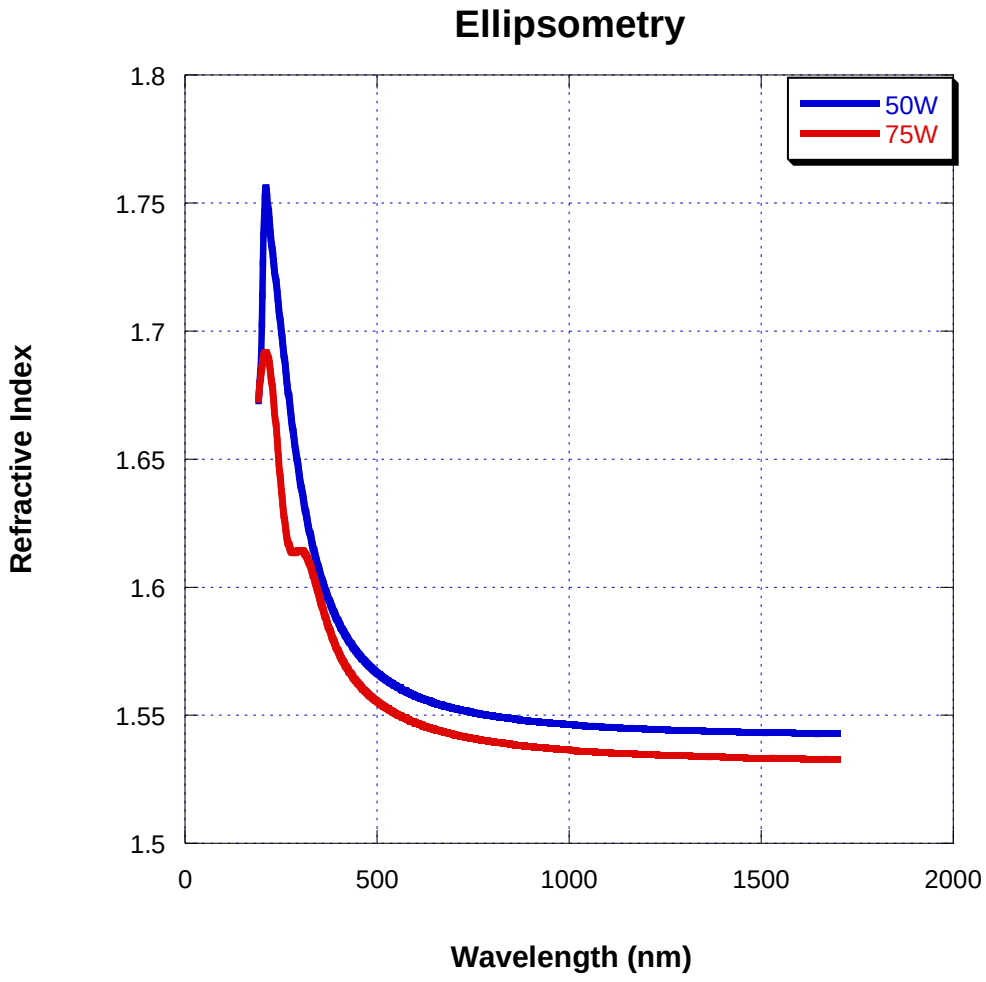


Figure 6.8: Refractive index results for 50 W and 75 W tea tree oil derived plasma polymer thin films determined via ellipsometry at different wavelengths of light

6.5 Dielectric Constant

6.5.1 Introduction

Dielectric constant is the ratio of electrical conductivity of a dielectric material to free space and represents a materials ability to resist the formation of an electric field within it. Dielectric constant is an essential piece of information when designing capacitors, electro-magnetic insulators, miniaturized electrical components and as part of accurate baseline electro-magnetic materials characterization. The lowest possible dielectric constant is 1.0 and the values for most medical polymers are between two and 10 which are relatively low values (Wang et al., 2002).

A “dielectric” is an electrical insulator that is polarized by an applied electric field. If a material with a high dielectric constant is placed in an electric field, the magnitude of that field will be measurably reduced within the volume of the dielectric. This is because electric charges do not flow through a dielectric but instead shift slightly from their equilibrium causing dielectric polarization. When dielectric polarization occurs, positive charges are displaced towards the electric field and negative charged shift in the opposite direction creating an internal electric field that reduces the overall electrical field within the dielectric (Huff and Gilmer, 2005). The term “dielectric” therefore describes materials with high polarizability and conversely low electrical conduction. Dielectrics are used in development of capacitors and electromagnetic insulation in medical devices.

Materials with low dielectric constants (low permittivity) are also advantageous in specific medical implants. With the development of microelectronic devices containing digital circuits, downscaling in size has led to contact between insulating dielectrics around conducting components. Because of miniaturization, insulating dielectrics have thinned to the point where charge build up adversely affects device performance (Sze and Ng, 2007). In microchips, insulators with low permittivity are used to reduce parasitic capacitance and improve circuit performance by increasing switching speeds and lowering heat dissipation (Maex et al., 2003).

Dielectric constant is an important property in bio-implant medicine because some implanted medical devices are electromagnetically sensitive and require electrical insulation. Examples of medical implants that benefit from electrical insulation are cardiac pacing wires and sleeve protectors for diathermy tips. Dielectric materials also

form capacitors in medical implants such as automated implantable defibrillators and may protect implanted medical devices exposed to magnetic fields used in diagnostic tests such as magnetic resonance imaging (Smith et al., 2000, Zhang et al., 2011). Materials with low dielectric constants are used to miniaturize diagnostic and therapeutic medical equipment such as micro-sensors and micro-biosensors for retinal implants, miniature piezo-resistive pressure sensors and medical nano-devices (Zhou and Greenberg, 2005, Van Hoof et al., 2008, Khine and Tsai, 2011). Given the influence of dielectric constant, the parameter was considered a significant characterization property for novel tea tree oil derived plasma polymers.

6.5.2 Dielectric constant assessment

A new and accurate technique to measure dielectric constants of low permittivity plasma polymer thin films was developed by researchers at the School of Engineering at James Cook University (Easton et al., 2007). This modified system employed split-post dielectric resonators at operating frequencies of 10 GHz to determine the relative permittivity of materials without destroying the samples. The split-post dielectric resonator was used as a tool for microwave characterization of novel plasma polymer thin films (Easton et al., 2007).

The scanning microwave microscope is a near field characterization tool employed to determine dielectric constant of a specimen film at microwave frequencies. Microwave microscope consists of a microwave resonator with a probe attachment coupled to electronic measurement devices such as voltage controlled oscillator and frequency counter. A film sample brought into close proximity of the probe attachment causes a reduction in the energy stored in the electromagnetic field depending on the sample permittivity (Talanov et al., 2006). The change in energy stored in the electromagnetic field results in a change in the resonant frequency and quality factor of the resonator (Wang et al., 2002). Resonant frequency and quality factor alterations can then be used to calculate dielectric constant of a material using predetermined equations (Courtney, 1998). Microwave resonant cavity techniques are considered the most sensitive measures of dielectric constants in thin films and have an accuracy of +/- 2% (Afsar et al., 1986, Easton et al., 2007).

Because results of microwave characterization were highly user dependent and necessitated meticulous technique by experienced operators and application of complex mathematical calculations, measurement of plasma polymer thin film permittivity was outsourced to scientists at the Electronic Material Research Laboratory

at James Cook University. Dielectric constant was obtained for tea tree oil derived plasma polymer thin films deposited onto GE-type 124 quartz slides (dielectric constant 3.75) at room temperature. Three samples were produced for the purposes of permittivity assessment: each sample under a different power parameter (25, 50 and 75 W) in order to determine the effect of power input on polymer thin film permittivity. Long deposition times (60 minutes) were needed to develop thick enough polymer films to acquire good frequency shifts and high quality measurements. Because of the duration of film production, time consuming measurement process and substrate expense more than three samples could not be processed. Film thickness was measured with optical spectrometry (Section 6.2.2 Assessment of film thickness as a function of deposition time).

6.5.3 Dielectric constant: results and discussion

6.5.3.1 Results

Dielectric constants for tea tree oil derived plasma polymers were derived using split-post dielectric resonator and microwave characterization (Section 6.5.2 Dielectric constant assessment). Dielectric constants were 2.46 for 25 W variant, 2.56 for 50 W variant and 2.63 for 75 W variant (Table 6.5). Permittivity increased with increasing power production parameters. Good frequency shifts were obtained for all three plasma polymer variants when deposited on quartz substrate (Table 6.5).

Table 6.5: Permittivity results for tea tree oil derived plasma polymer thin films

Power (W)	Film thickness (nm)	Frequency shift (MHz)		Unloaded Q (quality factor)		Dielectric constant
25	5800	19697.387	19694.576	7260	6908	**2.46**
50	4800	19695.164	19692.693	7114	6875	**2.56**
75	5100	19696.552	19693.802	7246	7009	**2.63**

6.5.3.2 Discussion

Commercial poly(propylene) capacitor film has a low permittivity of 2.2 and is the most commonly used dielectric in the film capacitor industry (Reed and Cichanowski, 1994). Tea tree oil derived plasma polymer permittivity is comparable to permittivity of commonly used medical dielectrics such as poly(propylene) (1.5), PTFE (2.05) and poly(ethylene) (2.20). Permittivity results suggested novel tea tree oil derived plasma

polymer thin films may be suitable for production of medical capacitors, electromagnetic insulators, low-permittivity thin films or for insulation of miniaturized medical device elements.

6.6 Effects of Ethanol and Water Exposure

6.6.1 Comment on polymer degradation and aqueous solubility

Polymer degradation entails change in polymer properties such as tensile strength, hardness, shape or molecular weight under the influence of environmental factors such as heat, light or chemicals (Halim Hamid, 2000). The type of environmental exposure directly influences degradation for example oxygen rich surroundings support oxidation and aqueous environments hydrolysis. Effects of polymer degradation may be undesirable or desirable depending on the resultant loss or gain of polymer properties. Polymer degradation may affect processes such as cell growth, tissue regeneration and host immune response in a local or systemic fashion. Degradation may also produce unfavorable toxic byproducts that harm host (Lykissa et al., 1997). In the bio-medical arena, degradation is thus significant because it contributes to polymer durability, determines functional polymer longevity in physiological environment and defines harmful polymer side effects.

Materials for implant applications are considered permanent if degradation is minimal or happens over a protracted time, eliminable if products are water soluble and cleared by host or truly degradable where polymer properties are changed by an environmental exposure. The three pathways of true polymer degradation are polymer crosslink degradation, side chain degradation and backbone degradation (Pielichowski and Njuguna, 2005). Polymer degradation requires energy derived from thermal processes, oxidation, photo degradation, hydrolysis, enzyme linked reactions or mechanical stressors (Vroman and Tighzert, 2009). In the setting of the implanted medical device, hydrolysis and enzymatic degradation dominate with mechanical stressors becoming prominent in devices with moving parts (Mano et al., 2004). Hydrolysis refers to random non-enzymatic chain scission that leads to reduction in molecular weight and is affected by the rate of diffusion of water through amorphous polymer (Kale, 2007). Enzymatic degradation relies on enzymatic catalysis of degradation reactions.

The steps of bio-implant polymer degradation occur in sequence and are frequently water dependent. When a polymer degrades in aqueous environment, water sorption is followed by a reduction in mechanical properties, reduction in molar mass and weight loss (Fambri et al., 2002). Factors that control polymer degradation rates include bond stability, hydrophobicity, steric effects, production of autocatalytic breakdown products and microstructure where microstructure encompasses porosity, crystallinity and phase separation (Gopferich, 2006). As a generalization, polymers are more resistant to

degradation if they are hydrophobic with greater phase separation and crystalline in structure without bulky pendent groups or autocatalysis (Gopferich, 2006). Polymer structure, surface area and porosity influence overall rate of water ingress and subsequent erosion, dissolution and diffusion (Braunecker et al., 2004). Polymer conformational flexibility increases water and enzyme access sites increasing polymer degradation (Kale et al., 2007). Polymer degradation is therefore strongly linked to aqueous solubility.

Aqueous solubility of polymers is important for many additional reasons: water solubility is a baseline property that describes the polymer; most substances are transported to and into living cells through water making aqueous solubility important in biomedical applications and rate of degradation via hydrolysis, photolysis, oxidation, reduction and biodegradation depend on a substance being soluble in water (Halim Hamid, 2000). Finally many chemical and health based tests rely on water solubility. For example, standard antimicrobial assessment methods rely on diffusion of product in water based substances such as agar or broth.

Plasma polymers have their own unique properties that influence degradation. Unlike traditional polymers, plasma polymers are not constructed of regular repeating units. Plasma polymers have a heterogeneous structure, are highly branched, amorphous and contain many double bonds (Yasuda, 2005). These irregular structural features originate as a result of radical termination reactions, chain transfer reactions and from impurities in monomer precursors (Biederman, 2004). Weak bonds in the irregular structures may be starting points for degradation of plasma polymer. Trapping of free radicals within polymer during film deposition is another source of possible film degradation. When polymer film and contained free radicals are exposed to water, transient formation of peroxy-radicals and hydro-peroxides can occur resulting in altered polymer properties (Biederman, 2004). Incorporation of oxygen into film surfaces also creates a hydrophilic interface that is more susceptible to degradation (Biederman, 2004). Degradation of plasma polymers may therefore be augmented in the aqueous environment.

Conversely plasma polymers may be degradation resistant because of their high molecular weight, surface configuration and hydrophobicity. High molecular weight polymers and synthetic polymers are as a rule water insoluble and therefore degrade less (Erik et al., 2004, Kwak, 1998). Luminous chemical vapor deposition classically produces imperturbable surfaces that are water insoluble (Yasuda, 2005). Finally, plasma polymers made from a lipophilic essential oil with poor aqueous solubility (such

as tea tree oil) may demonstrate poor aqueous solubility due to common functional groups. Degradation of plasma polymers therefore depends on the balance between factors that support and inhibit degradation.

6.6.2 Testing polymer degradation

Polymer degradation can be assessed by various methods. Determinants of polymer film degradation or loss include: visible morphological changes such as swelling and deformation, molecular structural changes, weight loss, loss of film thickness, thermal behavior changes and change in chemistry (Halim Hamid, 2000). Degradation can be induced *in vivo* (in the living organism) or *in vitro* (in the laboratory). *In vivo* degradation exposes an implant to several degradation mechanisms in concert and anticipates real life conditions. *In vivo* degradation occurs regardless of whether the method of degradation is known and is context specific. Degradation for example, will vary depending on the location of the polymer within the host and will change subject to individual host factors. *In vivo* studies are therefore the gold standard in bio-implant degradation testing. *In vitro* degradation studies attempt to delineate a single mechanism of degradation and give valuable baseline information on polymer degradation that is uniform, easy to measure and lends itself to comparison and replication.

Researchers at Swinburne University of Technology in Melbourne related polymer degradation to attachment of water (Kingshott et al., 2011). They assessed degradation by measuring uptake of water achieved by a polymer from dry state in an environment of constant humidity. Plasma materials were weighed at intervals to assess hydration and specimens were then dried to see if they retained their initial mass.

Another measure of polymer degradation is contact angle (wettability) with water. In 1986 Van Oss, Chaudhury and Good (VCG) applied the thermodynamic definition of solubility and developed a formula to measure free energy change using interfacial surface tension between solute and solvent (Good, 1992). The VCG technique stipulates that for a given solid-liquid system the interfacial tension can be determined directly by contact angle measurement (Good, 1992). Contact angle measurement can be derived from the sessile drop technique. Using this method the solubility of water soluble materials including biopolymers have been calculated (Shuhui and Shanks, 2004). If easily wetted a material will more likely degrade (Good, 1992).

Data on polymer degradation is also provided by examining molecular and chemical structures of polymers. Analytical techniques, such as Fourier transform infrared spectroscopy, UV-visible spectroscopy, gas chromatography mass spectroscopy, X-ray photoelectron spectroscopy, scanning near-field optical microscopy and ellipsometry have been employed (Halim Hamid, 2000). Suniket and Fulzele determined the average molecular weights of initial polymer films and degraded samples using gel permeation chromatography (Suniket and Fulzele, 2007). Specimens were subsequently analyzed with scanning electron microscopy and surface topographical changes were observed to monitor degradation. Degradation correlated with dry weight loss.

Many novel polymer degradation assessments have been put forward. Rehman studied degradation properties of polymers constructed from poly(lactic acid) via a soil burial test (Rehman, 1996). Specimens were exhumed at time points and degradation defined as loss of mass, molecular weight reduction, pH value changes and swelling index. Polymer discs placed in phosphate-buffered saline baths for up to seven months have been utilized to meter degradation (Lips, 2006). At experiment conclusion, discs were rinsed with demineralized water and dried under vacuum at room temperature. Subsequently, the dry weight, molar composition, molecular weight, and thermal properties of the specimen were determined (Lips, 2006). *In vitro* hydrolytic degradation has been assessed by immersing polymer films in alkaline solution and detecting changes is tensile strength, infrared spectroscopy and mass loss (Rehman, 1996). Ratner treated polymer films with enzymes characteristic of foreign body inflammatory cells in an attempt to replicate the environment of a foreign body reaction and associated enzyme induced degradation (Ratner, 1988). Molecular weights and poly-dispersities of polymers were then measured by gel permeation chromatography before and after treatment to detect degradation effects.

In vivo induced degradation requires an animal model or human host. Because host reaction forms a protein immune mediated coating around a medical implant, standard measures of film thickness and molecular weight are confounded unless the host derived coating is removed. If the host derived tissue can be detached without affecting the polymer then assessment can proceed as with *in vitro* studies. To monitor *in vivo* degradation of poly(bis(ethyl 4-aminobutyro))phosphazene, Satturwar and Fulzele implanted polymer films subcutaneously on the backs of male Wistar rats (Satturwar et al., 2003). Films were explanted at predetermined time points and assessed for weight change, molecular weight decline and surface morphological analysis (Satturwar et al.,

2003). Etienne, Picar et al placed poly(lactic acid) polymers in the mouths of humans and compared cheek and tongue sides of the polymers for the effects of degradation (Etienne et al., 2006). Degradation was then assessed with confocal laser scanning microscopy measurements of film thickness and by static contact angle measurements of surface wettability. Surface topography was also observed by scanning electron microscopy to allow direct visual observations of surface roughness and homogeneity (Etienne et al., 2006). Novel methods of determining *in vivo* degradation include Cabon14 acetylation labeling and histological assessment of absorption and structure with morpho-metrical evaluation using computer-based image analysis techniques (Leica® Qwin Pro-Image Analysis System) (Ruhe, 2005, Nieves Olmo, 1996).

Lack of universal approach to polymer degradation testing is reflected in the multitude of available testing strategies**.** International standards for degradation studies of polymers in contact with human tissue were published by the International Organization for Standardization paper ISO 10993: Biological Evaluation of Medical Devices (ISO, 2010). These guidelines cover general principles for design and performance of biodegradation studies and the systematic evaluation of the potential and observed biodegradation products related to medical devices (ISO 10993-13). They also describe tests on how much product is absorbed by the body and product effects on host over time (ISO 10993-9). ISO 10993 recommends degradation studies be considered if a device is absorbed, is intended to be implanted for more than 30 days, or if informed consideration suggests toxic substances may be released from the material during body contact (ISO, 2010).

The physiological environment of the human body can be aggressive to polymers. Most implanted polymers suffer some degree of degradation and the process can be affected by biologically active species such as enzymes, lipids, peroxides, free radicals, phagocytic cells and the aqueous physiological environment. Host tolerance of polymer degradation depends critically upon the type and effect of degradation. Degradation is thus an important fundamental property of any novel polymer thin film earmarked for use in implanted medical devices.

6.6.3 Assessment of ethanol and water effects on plasma polymers

Aqueous solubility is an important fundamental property in plasma polymers destined for medical implants because the physiological environment is water based and because aqueous solubility is directly related to polymer degradation and can therefore act as a surrogate polymer degradation assessment (Section 6.6.1 Comment on polymer degradation and aqueous solubility). The effect of ethanol on plasma polymers is important because alcohol is commonly used as a disinfecting and sterilizing solution in clinical practice.

The definitive assessment of polymer solubility in water or ethanol is probably contact angle analysis however this test is complex, expensive and the closest available laboratory providing the service was the Royal Melbourne Institute of Technology making the approach impractical. Instead effects of water or ethanol on tea tree oil derived plasma polymers were indirectly assessed by change in polymer film thickness after liquid immersion. The resources and expertise required for measuring polymer thickness were already available at the School of Engineering at James Cook University. Evidence of visible polymer change such as flaking or altered color was also sought. Polymers were produced under different power parameters (25, 50 or 75 W) to determine if this parameter changed polymer film interactions with ethanol or water.

6.6.3.1 Equipment

- standard glass laboratory slides coated with tea tree oil derived plasma polymer films (25, 50, 75 W) (10 minute deposition times)
- glass control slides
- 100% ethanol
- distilled water
- clean plastic Petrie dishes
- Avantes Avaspec-246 Fiber Optic Spectrometer and Avasoft © 6.1 software
- curvette for spectrometer to allow measurement of samples is transmission mode

6.6.3.2. Methods

Standard glass laboratory slides were cleaned, individually labeled and coated for 10 minutes with a tea tree oil derived plasma polymer (25, 50 or 75W) as per predefined

protocols (3.2 Production of Tea Tree Oil Derived Plasma Polymer Thin Films). Macroscopic appearance and plasma polymer film thickness were recorded for each sample using Avantes Avaspec-246 fiber optic spectrometer and Avasoft © 6.1 software. Measurements were collected from the center of each slide. Samples were then placed into Petrie dishes and immersed in either 100% ethanol or distilled water for 24 hours. Post immersion, specimens were air dried for 24 hours. Observations and measurements were repeated on post exposure polymer films. Macroscopic evidence of gross separation of polymer from substrate (flaking, sloughing) was sought. Pre and post exposure parameters were compared to assess alteration or loss of tea tree oil plasma polymer films due to the effects of either alcohol or water. Naked glass laboratory slides were used as controls. The experiment was repeated in triplicate for each tea tree oil plasma polymer variant.

6.6.4 Ethanol and water effects: results and discussion

6.6.4.1 Macroscopic Appearance

Pre-exposure polymer coated glass substrate had a characteristic homogeneous, transparent and yellow tinted appearance. Higher power deposition films (75 W) imbued a deeper yellow tint. Macroscopically films appeared uniform, in one piece and adherent to substrate (Plate 6.2).

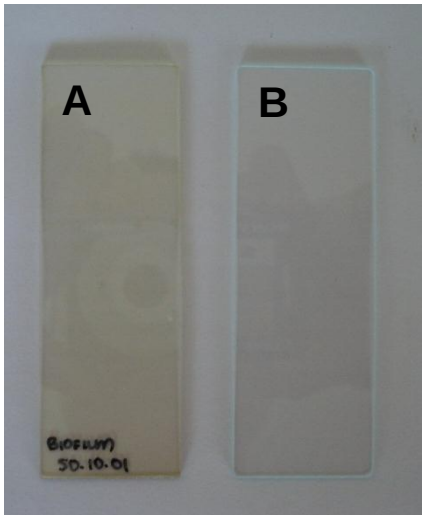


Plate 6.2: 50 W tea tree oil derived plasma polymer thin film on glass substrate (A). Note the characteristic yellow tint and homogeneous finish. Control slide without polymer coat is provided for comparison (B).

Both ethanol and distilled water changed plasma polymer film appearance on glass substrate (Appendix 6E)(Plate 6.3). Post ethanol exposure, films produced at 25 W

appeared clear and comparable to control slides. 50 W and 75 W films developed cracking, peeling and flaking and some areas of polymer film were completely sloughed. Homogeneity of these higher power films was also lost and color change was apparent. Although areas of film retained yellow tint, other parts of film became opaque and white or demonstrated a rainbow color spectrum. Distilled water altered the appearance of plasma polymer to a lesser extent than did exposure to ethanol. Films retained a homogeneous, transparent, yellow tinted appearance however water tracked between glass substrate and film and caused lifting, folding and wrinkling. Floating films sustained minor tears which led to small areas of denuded substrate.

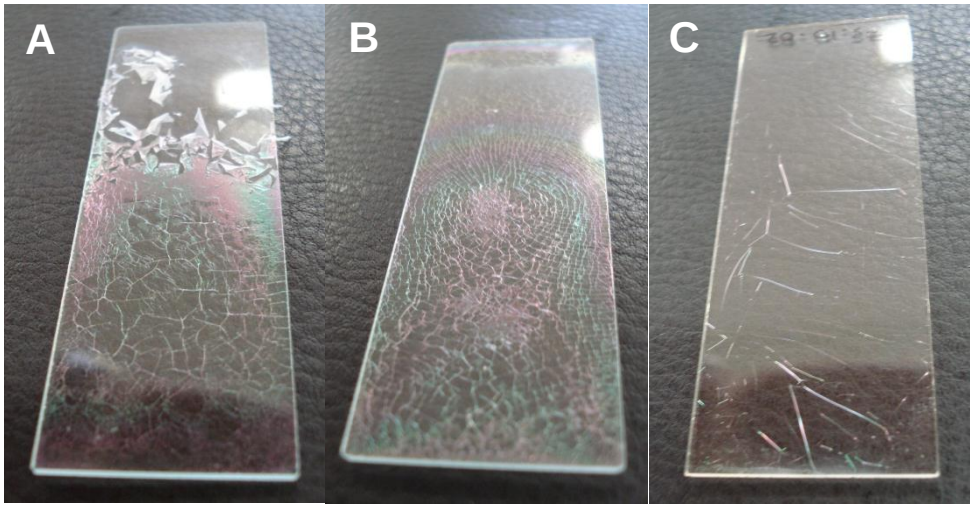


Plate 6.3: Film appearance post ethanol and water exposure: polymer films showed flaking (A) and cracking (A and B) with rainbow spectrum color change after 24 hours of ethanol exposure. Distilled water exposure separated polymer from substrate and resulted in wrinkling of film on substrate (C).

6.6.4.2 Thickness Measures

Film thickness was altered on exposure to ethanol (Appendix 6E). In the 25 W polymer group >96% of polymer thickness was lost. Across all polymers exposed to ethanol, percentage reduction in film thickness ranged from 19.2 to 97.8% with an average loss of 64.2%. Although some thickness measures may have represented cracked or missing sections of polymer it was clear that alcohol exposure has a deleterious effect on polymer preservation.

Distilled water caused separation, folding and displacement of plasma polymer film from substrate but film thickness was conserved (Appendix 6E). Percentage loss of film thickness ranged from 0.1-7.9% with an average loss of 2.4%. A single 75 W polymer film registered a gain in thickness of 0.04% which was within the range of

measurement error. Overall plasma polymers were preserved when immersed in distilled water but minimally displaced from glass slide deposition surface.

Glass control slides were unchanged in appearance and no significant film thickness was detected after ethanol or water exposure.

6.6.4.3 Discussion

Results suggested both ethanol and distilled water disturbed adhesion of tea tree oil derived plasma polymer thin films to glass substrate as demonstrated by flaking, cracking, folding and lifting of films. Altered thickness of polymers exposed to ethanol and water may have resulted from dissolved polymer loss, bulk shedding of polymer due to flaking and cracking or a mixture of these processes. Even allowing for a measurement error of 10%, reduction in film appearance and thickness on exposure to alcohol suggested significant degradation. Distilled water exposure caused much less alteration in polymer appearance and thickness suggesting a lesser degradation effect. Inert glass slide controls were (as anticipated) unchanged by exposure to either distilled water or alcohol consistent with uncontaminated solvent exposure.

During both liquid exposures, fluid was trapped between thin film and substrate causing macroscopically apparent sloughing of polymer. Several implications arose from this observation:

- The assumed plasma polymer class activity of excellent adherence to most surfaces may not apply to tea tree oil derived plasma polymers in general or perhaps more specifically to tea tree oil plasma polymers on glass substrate
- Ethanol is commonly used to decontaminate equipment prior to use. Protracted ethanol immersion may therefore be unsuitable as a decontamination method for tea tree oil derived plasma polymers and ethanol trapped under plasma polymer film may act as a confounding antibacterial agent in anti-biofilm testing of plasma polymers
- Poorly adherent tea tree oil polymer films may not endure biofilm tests that mandate exposure to dynamic fluid in order to produce shear and successfully culture biofilm
- A polymer that sloughs off substrate on exposure to water may behave similarly in physiological solution within host leading to lack of durability, migration, loss of function or toxic byproducts

Further and more detailed investigation of plasma polymer structure, adherence to substrate and solubility is needed to understand the mechanisms behind why and how much polymer was lost. The basic results obtained here indicate alternatives to protracted alcohol decontamination should be considered for tea tree oil plasma polymer products. *In vitro* testing on glass slides may in addition be augmented by using super clean, hydrophilic glass slides treated with plasma beam to generate a charged surface and encourage polymer film adherence (e.g. StarFrost®, poly(L-lysine), silane). Although the polymers did not bind well to plain glass, the substrate was employed for measurement efficiencies and is not utilized in medical implant applications. Other substrates may adhere more strongly to tea tree oil derived plasma polymers and materials used more widely in medical implants such as PTFE, titanium, nylon and poly(propylene) should be investigated to see if polymers adhere better to these more applicable deposition substrates than they do to glass.

CHAPTER 7 CONCLUSIONS AND RECOMMENDATIONS

7.1 Introduction

Novel tea tree oil derived plasma polymer thin films are potentially biocompatible and biofilm resistant coatings for two-phase implanted medical devices. As such the surface treatments provide a new avenue for surface optimization of medical implants to improve function, reduce morbidity and truncate healthcare costs. Tea tree oil derived plasma polymers have many possible applications within a large scale medical implant market with great commercial value. These essential oil based plasma polymer thin films therefore have potential to benefit human healthcare through improved biocompatibility and reduced infection of two-phase implanted medical devices.

7.2 Fabrication of Plasma Polymer Thin Films

Tea tree oil derived plasma polymer thin films were constructed within a basic laboratory using standard equipment and limited operator experience (Chapter 3). After working through a learning curve, plasma polymers were easily manufactured from cheap, renewable, Australian tea tree oil vapor without toxic constituents or byproducts using a simple, affordable process undertaken at room temperature and in a dry environment. These production factors are appealing to industry and consumers alike. Plasma techniques can also generate sterile polymer product, a useful side effect in medical implant creation (Biederman and Slavinska, 2000).

Power generation parameters were simple to change and allowed development of plasma polymer variants. Other production parameters were held constant due primarily to limitations in equipment. Visible micron range plasma polymer thin films were quickly and consistently produced. In general plasma polymer thin films were straightforwardly deposited onto both glass and PTFE substrate. Films produced at higher powers and for longer duration were seen to flake and bond to manufacturing equipment. Stringent cleaning protocols were designed to provide clean deposition surfaces for quality polymer creation and to decontaminate incidental polymer deposits on hardware.

A limitation of plasma polymer production was lack of rigorous control systems. Many production parameters other than power input alter plasma polymer product including system geometry, pressure, temperature and monomer flow rate (Yasuda, 2005). To define the spectrum of tea tree oil plasma polymers, controlled and expanded

production parameters are recommended so polymers can be specifically engineered. Knowledge about constraints on polymer product is equally valuable.

7.3 Biocompatibility Profile

In vivo biocompatibility was determined by surgical implantation of novel tea tree oil plasma polymer into subcutaneous pockets on the backs of BALB/c mice (Chapter 4). Novel polymer implants were compared to PTFE control implants both *in situ* and after histological examination of excised polymer specimens. Specimens were harvested from mice at time points of three, 14 or 28 days in keeping with phases of wound healing.

Overall tea tree oil derived plasma polymers were biocompatible as defined by implantation in the murine model. Plasma polymer variants compared favorably with PTFE control (a well-used biocompatible medical implant polymer) although PTFE performance was probably marginally superior (Gyo et al., 2008). Sinus formation was the predominant complication of implantation. Macroscopic inspection showed sinuses were slightly more prevalent in mice with plasma polymer implants than in controls however the statistical significance of these results was called into question by small experimental numbers. A third of sinuses were transient and resolved spontaneously. Semi-quantitative analysis of histology specimens showed no statistically significant difference between biocompatibility measures of plasma polymer and control. All plasma polymer variants behaved in a similar manner. Foreign body reaction did not play a major role in host response to any of the implants and all implant groups generated an acute inflammatory response in keeping with known phases of inflammation after surgical wounding.

Even if plasma polymer implants created marginally more sinuses than PTFE control, six out of 16 sinuses healed over time and no sinuses became infected or impacted the general well-being of mice hosts. Given more time perhaps an even greater proportion of polymer associated sinuses may have healed spontaneously. In mice, the panniculus carnosus layer of skeletal muscle immediately beneath skin gives the cutaneous layer greater ability to heal by wound contraction and infection rates are in turn reduced (Machado et al., 2011). Because panniculus carnosus is essentially absent in humans, human ability to heal sinuses may however be reduced. Sinus formation in mice occurred after the acute inflammatory phase of wound healing was complete suggesting association with proliferation and remodeling or chronic inflammatory processes. Plasma polymer related sinus formation is therefore a

potential biocompatibility problem for skin related implants but the magnitude of the problem was estimated to be small and overall the novel polymers were considered biocompatible.

7.4 Antibiofilm Effect

Because medical implant infection with biofilm is a major cause of device related morbidity, novel tea tree oil derived plasma polymers were tested for anti-biofilm properties (Costerton et al., 1995). Much work was undertaken to build and validate a biofilm culture circuit that consistently produced bacterial biofilms of staphylococcal species known to infect medical implants in clinical practice (Chapter 5). Experimentation was designed to determine if novel plasma polymers were antimicrobial as it was theorized tea tree oil derived plasma polymer films inherit antibacterial moieties from parent compound (Hammer et al., 2005).

Biofilm culture circuit was validated by imaging tertiary biofilm structure on circuit cultures using atomic force microscopy and confocal scanning laser microscopy. Multiple culture cycles were undertaken to grow *S.aureus* and *S.epidermidis* biofilm on plasma polymer thin films and PTFE control substrates. Biofilm was enumerated by following a sequence of mechanical biofilm breakdown steps followed by viable plate counts. Results from biofilm enumeration experiments did not support the hypothesis that tea tree oil plasma polymer thin films were anti-biofilm. Novel plasma polymer variants universally grew more biofilm than bare PTFE control. There was no difference in anti-biofilm performance between plasma polymer variants. Thus the hypothesis that altered power production parameters changed plasma polymer surface moieties and in turn anti-biofilm capability was not supported either.

PTFE is used to make medical implants because it is biocompatible and resists biofilm formation. The anti-biofilm effects of PTFE are attributed to its low friction co-efficient, reduced surface roughness, high hydrophobicity and surface charge which primarily dissuade bacterial adherence (Planchon et al., 2006, Gyo et al., 2008, Hallab et al., 2001). Physical properties of PTFE allow it to passively avoid biofilm colonization by reducing overall interactions with bacteria through being inert. Another approach to reducing biofilm formation is to actively engage bacteria cells and biofilm in a damaging or disabling way. The idea behind tea tree oil plasma polymers was to create novel surfaces laden with anti-biofilm moieties that harmfully interact with bacteria and actively lead to inhibition or destruction of biofilm. Plasma polymers inherit intact portions of parent monomers according to production parameters, an important

parameter being glow discharge power (Yasuda et al., 1978, Choukourov et al., 2010). Power settings were therefore altered during plasma polymer production in an attempt to discern a difference in antibiofilm performance based on power dependent retention of active moieties.

Despite theoretical considerations, tea tree oil derived plasma polymers were not supported as antibiofilm in the enumerations experiments when compared to bare PTFE. Lack of demonstrable antibiofilm effect may have occurred for several reasons. The active antimicrobial components of tea tree oil are ill defined and there may be interplay between essential oil components in order to obtain biofilm killing (Carson et al., 2006). Plasma polymerization may have destroyed or inactivated vital antibiotic tea tree oil moieties or prevented interplay between components by physical separation of components within film. In order to overcome these limitations the mechanism of tea tree oil antimicrobial activity must first be better delineated then cross correlated with a structural and functional analysis of tea tree oil derived films to ensure common antimicrobial elements. If the exact mechanism of action of tea tree oil against bacteria were known then films could be generated using parameters that maintain selected active moieties instead of using arbitrary settings in a “hit and miss” fashion.

Plasma polymers generated under high powers are highly cross-linked, smooth and pinhole free with few branching side chains and have inert surface structure and chemistry (Yasuda, 2005). Such polymers share physio-chemical characteristics with PTFE in that they are inert and therefore deter bacterial adherence. One explanation for enhanced biofilm formation on tea tree oil plasma polymers generated during this project is that the power settings of 25, 50 or 75 W were too low to invoke the characteristics typical of high power plasma polymer films. Lower power films retain fragments of monomers that may not be antimicrobial but still branch from film surface providing anchoring points for bacteria, increased roughness or greater surface area for adhesion. To resolve this concern, tea tree oil plasma polymer films could be produced under higher power conditions and retested against biofilm. Such plasma polymers would be expected to reduce biofilm formation due to inert biochemical behavior and not due to preservation of active side chain moieties.

Even if the optimal power setting was known for plasma polymerization of tea tree oil with preservation of active antibiofilm components, the random recombination of monomer parts may present a substantial obstacle to designing surfaces with specific structure. Due to the nature of plasma polymerization, monomer recombination to form

surface polymer is random and unpredictable. Monomer is fragmented and pieces rejoined on a surface in a process of statistical recombination. If monomers are large (as are many organic molecules including those found in tea tree oil) and antimicrobial activity requires intact or long segments of intact monomer then plasma polymerization would be expected to inactivate functional components and debunk antibiofilm capacity of tea tree oil and subsequent derived polymer films. This disabling effect would be even greater if interaction of multiple active intact tea tree oil components is needed to avert biofilm. Again a better understanding of the mechanism of tea tree oil antimicrobial activity and a structural analysis of plasma polymer film surfaces could contribute to answering these questions. Tighter production parameters and variation of productions parameters outside of simple power variation may also allow better control of plasma polymer structure. Clearly there is much work that needs to be done in both defining the source of tea tree oil antibacterial activity and translating the raw material into a designed capability thin film.

7.5 Fundamental Properties

Plasma polymers are a new class of polymer thin film with unique properties. Whereas conventional polymers comprise an ordered series of intact, repeating monomer units, plasma polymers are highly cross linked, disordered but stable, pinhole-free surface films that provide excellent barrier function. This new class of polymer tenaciously binds and conforms to most surfaces without altering material bulk properties and can act as an occlusive or selective barrier. Many of the unique characteristics of plasma polymers make them obvious candidates for design of novel surface treatments for IMDs. A basic subset of fundamental properties for novel plasma polymers was determined to evaluate congruency with general class properties of plasma polymers and to determine suitability for medical implantation (Chapter 6).

7.5.1 Film thickness as a function of deposition time

Films of several micrometers thick were easily and consistently obtained with the system described in Chapter 3. Results concluded tea tree oil plasma polymer thin films were deposited in a predictable, quadratic fashion over a short time period. Films in the micron thickness range were produced with in a 10 to 15 minute deposition period for all plasma polymer variants. Such information is important for industry feasibility studies and allows for estimation of polymer thin film production timelines. Thin films (less than one micron thick) are advantageous as medical implant coatings over thick films because they allow for complexities in substrate geometry including

miniaturization, avoid bridging across segments of implant, may allow for translucency in optical applications and aid in development of porous surfaces to facilitate timed release of drugs or intermittent sampling from a coated implant (Duka, 2008, Krebs, 2007). Bonding and flaking seen in higher power tea tree oil polymers (50 W and 75 W) may have resulted from thermal annealing and free radical trapping within thin film.

7.5.2 Surface topography (roughness)

Surface topography was examined with atomic force microscopy. Results confirmed tea tree oil derived plasma polymer thin films were homogeneous, pinhole free and smooth surface coatings. The results were consistent with class properties of plasma polymer thin films (Biederman, 2004). Homogeneous, pinhole free surfaces are good candidates as coatings for two-phase medical implants because they are structurally consistent and act as effective barriers between implant bulk phase and host environment. Smooth surfaces are also advantageous because irregularities in polymeric surfaces promote bacterial adhesion and biofilm deposition whereas smooth surfaces dissuade bacterial adhesion and biofilm formation (Scheuerman et al., 1998, Katsikogianni and Missirlis, 2004). Although power input seemed to make little difference to film surface topography the small sample numbers hampered interpretation. Provisional assessment suggests tea tree oil derived plasma polymer thin films have favorable surface topography for implementation as medical implant coatings.

7.5.3 Hardness

Hardness was assessed via nano-indentation using a variant of atomic force microscopy. Novel plasma polymer thin films were of comparable hardness to polymers commonly used in medical implant production (Table 6.2). Hardness of novel plasma polymer films was therefore considered adequate for use in medical implants. Plasma polymer films produced under higher power conditions were harder in keeping with known class properties (Biederman, 2004). The results suggested film hardness can be titrated by controlling power input. Control of hardness is favourable as it means a material can be built according to product specifications.

7.5.4 Refractive index

Refractive index of tea tree oil derived plasma polymer thin films was determined with ellipsometry. Results were consistent with transparent materials and similar to

refractive indices of typical glass and fused quartz. Refractive index results for the novel polymers suggested suitability for light transmitting medical implant applications.

7.5.5 Ethanol and water exposure as a measure of degradation

The effects of polymer exposure to water and ethanol were determined because water solubility is directly related to polymer degradation in physiological solution and ethanol exposure is commonly used as a disinfectant for medical devices (Halim Hamid, 2000). Experiments consisted of simple immersion experiments over 24 hours looking for changes in macroscopic appearance or measured thickness of plasma polymer films. Results confirmed both ethanol and distilled water disturbed adhesion of tea tree oil derived plasma polymer thin films to glass substrate as demonstrated by flaking, cracking, folding and lifting of films. Reduction in film thickness on exposure to alcohol suggested significant degradation. Distilled water exposure caused much less alteration in polymer thickness than ethanol suggesting a lesser degradation effect.

During both liquid exposures, fluid was trapped between thin film and substrate causing macroscopically apparent sloughing of polymer. Several implications arose from this observation:

- The assumed plasma polymer class activity of excellent adherence to most surfaces may not apply to tea tree oil derived plasma polymers in general or perhaps more specifically to tea tree oil plasma polymers on glass substrate
- Ethanol is commonly used to decontaminate equipment prior to use. Protracted ethanol immersion may therefore be unsuitable as a decontamination method for tea tree oil derived plasma polymers and ethanol trapped under plasma polymer film may act as a confounding antibacterial agent in anti-biofilm testing of plasma polymers
- Poorly adherent tea tree oil polymer films may not endure biofilm tests that mandate exposure to dynamic fluid in order to produce shear and successfully culture biofilm
- A polymer that sloughs off substrate on exposure to water may behave similarly in physiological solution within host leading to lack of durability, migration, loss of function or toxic byproducts

Further and more detailed investigation of plasma polymer structure, adherence to substrate and solubility is needed to understand the mechanisms behind why and how

much polymer was lost. The basic results obtained here indicate alternatives to protracted alcohol decontamination should be considered for tea tree oil plasma polymer products. Although the polymers did not bind well to plain glass, the substrate was employed for measurement efficiencies and is not utilized in medical implant applications. Other substrates may adhere more strongly to tea tree oil derived plasma polymers and materials used more widely in medical implants such as PTFE, titanium, nylon and poly(propylene) should be investigated to see if polymers adhere better to these more applicable deposition substrates than they do to glass.

7.6 Overall Conclusions and Recommendations

The aim of this research was to investigate the hypothesis that tea tree oil derived plasma polymer thin films are biocompatible surface treatments that resist biofilm formation and have potential as novel coatings for implanted medical devices. Some light can now be shed on the matter although investigations are preliminary.

Tea tree oil derived plasma polymer thin films were easy, cheap, quick and convenient to fabricate in an environmentally friendly way from local resources. These features auger well as far as mechanics of production and commercial viability go.

The new polymers were biocompatible in the murine implantation model but probably not as well tolerated as PTFE which is a standard medical implant construction polymer. In particular cutaneous sinus formation was concerning but the statistical significance of this complication was uncertain and the effect if present was minimal. Cutaneous sinus formation may be a problem in subcutaneous devices but may also be indicative of chronic inflammation which can be detrimental at any site within host. Further biocompatibility studies in porcine or ovine models that better approximate human host may clarify the relevance of sinus development.

It was disappointing to discover biofilm derived from *Staphylococci* (common human implant pathogens) were augmented by tea tree oil derived plasma polymer thin films. The result was not however definitive as such a limited subset of plasma polymers was investigated and alteration in production parameters may results in better anti-biofilm activity. Unfortunately the other option is that plasma polymers do not inherit the antimicrobial function of parent tea tree oil compound indicating any hope of antimicrobial effect will rely on class properties of high power plasma polymers to deter bacterial adhesion. The easiest way forward from this quandary would be to fabricate high-power tea tree oil derived plasma polymer films, confirm high power structural features using advanced topographical analysis and retest against biofilm. Of course

delineation of active antimicrobial constituents of tea tree oil would assist in making a more representative hybrid thin film.

Fundamental property analysis described smooth polymer thin films with similar hardness to other medical polymers and a refractive index consistent with transparency. These results implied the new polymers may be suited for optical and biosensor medical implant applications. Water and ethanol exposure studies indicated plasma polymer thin films did not adhere to all surfaces and were subject to degradation and loss. Degradation and loss in aqueous physiological environment means the plasma polymers may not be suited for permanent implant applications but may be useful when degradation is favorable such as in drug delivery matrices, dissolvable sutures or short term implants. Better assessment of degradation mechanisms are essential because bulk loss and shedding of polymer have different implications and effects on host than polymer dissolution and excretion. Aqueous solubility of polymers is best determined by contact angle analysis using the sessile drop technique and this assessment is recommended (Good, 1992).

Tea tree oil derived plasma polymer thin films may have a future as medical implant surface treatments to improve device biocompatibility and control infection however at this point it can only be stipulated as potential. First much work needs to go into understanding the behavior, strengths and limitations of these materials and how to control their function. The work completed in this thesis is expected to contribute towards the knowledge base and development of more effective biocompatible and biofilm resistant surface coatings for medical implants and may therefore lead to more efficient and improved healthcare.

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Appendix 2A: Methods for measurement of biofilm on implanted medical devices

Method	Protocol	Advantage(s)	Limitation(s)	Reference
roll plate	roll surface over blood agar plate	simple to implement, available, inexpensive	may not access organisms within matrix	(Maki et al., 1977)
vortex then viable plate count	surface in PBS vortexed prior to viable count on fluid	attempts to free bacteria from matrix	efficacy unknown	(Tenney et al., 1986)
sonicate, vortex then viable plate count	surface in PBS sonicated, vortexed prior to viable count on fluid	attempts to free bacteria from matrix	efficacy unknown	(Sherertz et al., 1990)
sonicate, vortex, homogenize then viable plate count	surface in PBS sonicate/vortex repeatedly then homogenise prior to viable count on fluid	frees bacteria from matrix and recovery efficacy determined	required extensive processing of sample	(Donlan et al., 2001)
acridine orange direct staining	direct staining of *in situ* microbes	direct examination of specimen	qualitative analysis that cannot enumerate organisms	(Zuffrey et al., 1988)
specimen brushing or scraping	surface brushed then brush sonicated in PBS prior to viable count on fluid	implanted medical device can be assessed *in situ*	efficacy and effect on implanted device unknown	(Kite et al., 1997)
alginate swab	surface swabbed then viable count on swab	implanted medical device can be assessed *in situ*	efficacy and effect on implanted device unknown	(Cercenado et al., 1990)
wash and count	shake surface in sterile water prior to viable plate count on fluid	simple to implement, available, inexpensive	may not access organisms within matrix	(Ballie and Douglas, 1998)

Continued:

Method	Protocol	Advantage(s)	Limitation(s)	Reference
air dried biofilm absorbance	surface scraped and biofilm allowed to air dry. Light absorbance measured at 492nm	limited processing required	requires specialized equipment and efficacy unknown	(Standar et al., 2010)
dry weight and epifluorescence	surface scraped and biofilm allowed to air dry. Specimen stained with fluorescent dye and examined under the microscope	dye can be inconsistent	requires specialized equipment and efficacy unknown	(Turakhia et al., 1983)
scraping and microscopic imaging	surface scraped and scrapings subjected to microscopy	limited processing required	qualitative analysis that cannot enumerate organisms	(Schwartz et al., 2010)
SEM/TEM	scanning or transmission electron microscopy	high resolution determination of biofilm structure	expensive, limited availability and may destroy specimen	(Stickler et al., 1999)
CSLM	confocal Scanning Laser Microscopy	biofilm examination *in situ* in a live culture allowing for functional analysis	reduced resolution compared to SEM and requires fluorescent markers	(Donlan and Costerton, 2002)

Appendix 3A: Sourcing of tea tree oil

100% fresh tea tree oil (TTO) was voluntarily donated by two vendors:

- Thursday Plantation TP Health Ltd. Pacific Highway Ballina NSW 2478

  Website: www.thursdayplantation.com

  Phone: +61 (02) 6620 5100

  Free call: 1800 029 000 (Australia only)

  Fax: +61 (02) 6620 5101

  Contact Person: Robert Riedl (Technical Manager)

  ABN 17 002 833 141

- G.R. Davis Pty. Ltd. 29-31 Princes St. Riverston NSW 2765

  Email: GRDAVIS@bigpond.com

  Phone: +61 (02) 9627 4537

  Fax: +61 (02) 9627 4537

  Conact Person: Sybil Davis

TTO was delivered to James Cook University in one liter, sealed, air tight and light proof containers. Thursday Plantation 100% tea tree oil conforms to the Australian Standard AS-2782, Oil of Melaleuca, terpinen-4-ol type (tea tree oil) International Standard ISO 4730-2004 and the tea tree oil monograph 04/2002:1837 in the European Pharmacopoeia. G.R. Davis Pty. Ltd tea tree oil is steam distilled from the leaves and terminal branches of *Melaleuca* species and tested to comply with the standard Oil of Melaleuca, terpinen-4-ol type (tea tree oil) International Standard ISO 4730-2004.

G.R. Davis Pty Ltd
ABN 93 001 859 543

P O Box 123 Riverstone NSW 2765 Australia

Telephone 61 2 9627 4537
Facsimile 61 2 9627 5468
Email GRDAVIS@bigpond.com
Web www.grdavis.com.au

Date of Issue: 28 February 2007

CERTIFICATE OF ANALYSIS

BATCH NO. **228130207**

Product Code 228 **Tea Tree Oil ISO 4730:2004**

Optical Rotation (° at 20° C)	+ 9.6
Refractive Index (20° C)	1.4765
Relative Density (20° C)	0.902
Miscibility in vols of 85% Ethanol (v/v at 20°C)	0.9 volumes
Appearance	Clear, colourless to pale yellow mobile liquid without traces of water
Odour	Characteristic
Flash Point (Closed Cup method °C)	59.0

Chromatographic Profile

α-Pinene % (m/m)	2.80
Sabinene % (m/m)	0.20
α-Terpinene % (m/m)	8.60
Limonene % (m/m)	1.30
ρ-Cymene % (m/m)	4.70
1,8-cineole % (m/m)	4.40
γ-Terpinene % (m/m)	22.50
Terpinolene % (m/m)	3.20
Terpinen-4-ol % (m/m)	43.40
α-Terpineol % (m/m)	2.40
Aromadendrene % (m/m)	0.20
Ledene % (m/m)	0.00
δ-Cadinene % (m/m)	0.30
Globulol % (m/m)	0.30
Viridiflorol % (m/m)	0.50
Date of Manufacture	February 2007
Date of Expiry (Under recommended conditions of MSDS)	February 2012

We certify that this batch conforms to specification for this product

Authorised by

Managing Director
G.R. Davis Pty. Ltd.

Appendix 4A: Surgical site outcomes observed in implanted mice

Key

- no abnormality detected, wound healing as expected

Dehis wound dehiscence

Extr partial disc extrusion

<Fur fur loss

Gran florid granulation tissue

Imp? implant not identified at sacrifice and presumed complete extrusion

Red wound erythematous and indurated

Sinus sinus connecting implant cavity to skin

PTFE control: 3 days to sacrifice

Day	Murine identification number									
	1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-

PTFE control: 14 days to sacrifice

Day	Murine identification number									
	1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-

PTFE control: 28 days to sacrifice

Day	Murine identification number									
	1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	Sinus	-	-	-	-	-	-	-	-	-
9	Sinus	-	-	-	-	-	-	-	-	-
10	Sinus	-	-	-	-	-	-	-	-	-
11	Sinus	-	-	-	-	-	-	-	-	-
12	Sinus	-	-	-	-	-	-	-	-	-
13	Sinus	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-

25 W TTO plasma polymer on PTFE substrate: 3 days to sacrifice

Day	Murine identification number									
	1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-

25 W TTO plasma polymer on PTFE substrate: 14 days to sacrifice

Day	Murine identification number									
	1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	Sinus	-	-	Sinus	-	-	-	-	-
10	-	Sinus	-	-	Sinus	-	-	-	-	-
11	-	Sinus	-	-	Sinus	-	-	-	-	-
12	-	-	-	-	Sinus	-	-	-	-	-
13	-	-	-	-	Sinus	-	-	-	-	-
14	-	-	-	-	Sinus	-	-	-	-	-

25 W TTO plasma polymer on PTFE substrate: 28 days to sacrifice

Day	Murine identification number									
	1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	Sinus	Sinus	-	-	-	-	-	-	-	-
10	Sinus	Sinus	-	-	-	-	-	-	-	-
11	Sinus	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	Sinus	-	-
26	-	-	-	-	-	-	-	Sinus	-	-
27	-	-	-	-	-	-	-	Sinus	-	-
28	-	-	-	-	-	-	-	Sinus	-	-

50 W TTO plasma polymer on PTFE substrate: 3 days to sacrifice

Day	Murine identification number									
	1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-

50 W TTO plasma polymer on PTFE Substrate: 14 days to sacrifice

Day	Murine identification number									
	1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	Sinus	-	Sinus	-	-	-	-	-	-	-
10	Sinus	-	Sinus	-	-	-	-	-	-	-
11	Sinus	-	Sinus	-	-	Sinus	-	-	Sinus	Sinus
12	Sinus	-	Sinus	-	-	Sinus	-	-	Sinus	Sinus
13	Sinus	-	Sinus	-	-	Sinus	-	-	Sinus	Sinus
14	Sinus/ Extr	-	Sinus	-	-	Sinus	-	-	Sinus	Sinus/ Imp?

50 W TTO plasma polymer on PTFE substrate: 28 days to sacrifice

Day	Murine identification number									
	1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-
21	<Fur	-	-	-	-	-	-	-	-	-
22	<Fur	-	-	-	-	-	-	-	-	-
23	<Fur	-	-	-	-	-	-	-	-	-
24	<Fur	-	-	-	-	-	-	-	-	-
25	<Fur	-	-	-	-	-	-	-	-	-
26	<Fur	-	-	-	-	-	-	-	-	-
27	<Fur	-	-	-	-	-	-	-	-	-
28	<Fur	-	-	-	-	-	-	-	-	-

75 W TTO plasma polymer on PTFE substrate: 3 days to sacrifice

Day	Murine identification number									
	1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	Red	-	-	-	-

75 W TTO plasma polymer on PTFE substrate: 14 days to sacrifice

Day	Murine identification number									
	1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	Sinus	Sinus	-	-	-	-	-
9	-	-	-	Sinus	Sinus	-	-	-	-	-
10	-	-	-	Sinus	Sinus	-	-	-	-	-
11	-	-	-	Sinus	Sinus	-	-	-	-	-
12	-	-	-	Sinus	-	-	-	-	-	-
13	-	-	-	Sinus	-	-	-	-	-	Sinus
14	-	-	-	Sinus	-	-	-	-	-	Sinus

75 W TTO plasma polymer on PTFE substrate: 28 days to sacrifice

Day	Murine identification number									
	1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	-	-	Dehis	Sinus	-	-	-	-	-	-
9	-	-	Gran	Sinus	-	-	-	-	-	-
10	-	-	Gran	Sinus	-	-	-	-	-	-
11	-	-	Sinus	Sinus	-	-	-	-	-	-
12	-	-	Sinus	Sinus	-	-	-	-	-	-
13	-	-	Sinus	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-
28	-	Imp?	-	-	-	-	-	-	-	-

Results summary tables

Surgical site outcomes observed in implanted mice (event/mouse/days)

Polymer	Days to sacrifice	Surgical site outcomes observed in implanted mice (event/mouse/days)							
		-	(%)	Sinus	Extr	Imp?	Dehis	Gran	Red	<Fur
PTFE control	3	30	(100)	-	-	-	-	-	-	-
	14	140	(100)	-	-	-	-	-	-	-
	28	274	(97.86)	6	-	-	-	-	-	-
25 W	3	30	(100)	-	-	-	-	-	-	-
	14	131	(93.57)	9	-	-	-	-	-	-
	28	271	(96.79)	9	-	-	-	-	-	-
50 W	3	30	(100)	-	-	-	-	-	-	-
	14	114	(81.43)	24	1	1	-	-	-	-
	28	272	(97.14)	-	-	-	-	-	-	8
75 W	3	29	(96.67)	-	-	-	-	-	1	-
	14	127	(90.71)	13	-	-	-	-	-	-
	28	268	(95.71)	8	-	1	1	2	-	-
Total		1716		69	1	2	1	2	1	8

Surgical site outcomes observed in implanted mice (individuals affected)

Polymer	Days to sacrifice	Surgical site outcomes observed in implanted mice (total number of individuals affected)							
		-	Sinus	Extr	Imp?	Dehis	Gran	Red	<Fur
PTFE control	3	10	-	-	-	-	-	-	-
	14	10	-	-	-	-	-	-	-
	28	9	1	-	-	-	-	-	-
25 W	3	10	-	-	-	-	-	-	-
	14	8	2	-	-	-	-	-	-
	28	7	3	-	-	-	-	-	-
50 W	3	10	-	-	-	-	-	-	-
	14	5	5	1	1	-	-	-	-
	28	9	-	-	-	-	-	-	1
75 W	3	9	-	-	-	-	-	1	-
	14	7	3	-	-	-	-	-	-
	28	7	2	-	1	1	1	-	-
Total		102	16	1	2	1	1	1	1

Appendix 4B: Scorecard for histological categorization of subcutaneous implant location and tissue response

Adapted from (Parker et al., 2002)

Key

Implant location	category
present and in situ	4
partially displaced	3
completely displaced but present on slide	2
absent from slide: footprint apparent	1
absent from slide: footprint not apparent	0

Implant – skin interactions	category
acute cutaneous incision wound adjacent to implant	4
cutaneous ulceration overlying implant	3
implant connected to epidermis/dermis by scar tissue	2
sinus formation	1
no direct implant – skin incision interaction demonstrated	0

Reaction zone response (at superficial and deep implant surfaces)	category
1. Capsule Maturity	
capsule is mature resembling connective or fatty tissue in non-injured regions	4
capsule in immature showing fibroblasts and collagen	3
capsule in immature showing fibroblasts, collagen and frequent inflammatory cells	2
capsule consists predominantly of inflammatory cells with little or no connective tissue elements	1

Continued:

Reaction zone response (at superficial and deep implant surfaces)	category
2. Total Capsule Thickness	
1-4 fibroblasts/ fibrocytes	4
5-9 fibroblasts/ fibrocytes	3
10-30 fibroblasts/ fibrocytes	2
>30 fibroblasts/ fibrocytes	1
not applicable	0
3. Thickness of Mature Capsular Layer	
1-4 fibrocytes	4
5-9 fibrocytes	3
10-30 fibrocytes	2
>30 fibrocytes	1
not applicable	0
4. Interface Qualities	
fibroblasts +/- hyaline membrane contact implant surface without the presence of foreign body giant cells	4
scattered foci of foreign body cells are present	3
one layer of foreign body cells are present	2
multiple layers of foreign body cells are present	1
cannot be evaluated due to infection or other factors	0
5. Surrounding Tissue Cellular Infiltrate	
acute inflammatory cellular infiltrate: predominantly neutrophils	4
acute inflammatory cellular infiltrate: predominantly macrophages	3
chronic inflammatory cellular infiltrate	2
foreign body cellular infiltrate / change	1
no cellular infiltrate	0

Appendix 4C: Histological categorization of subcutaneous implant location and tissue response results

Implant location

Polymer implant	Time to sacrifice (days)	Score				
		0	1	2	3	4
control PTFE	3	0	7	2	0	1
	14	0	4	0	0	6
	28	0	1	0	0	9
25 W	3	0	1	1	0	8
	14	0	1	0	1	8
	28	0	1	0	2	7
50 W	3	0	5	0	0	5
	14	0	1	0	1	8
	28	0	1	0	2	7
75 W	3	0	4	1	1	4
	14	0	1	1	0	8
	28	1	0	0	0	9

Implant-skin interaction

Polymer implant	Time to sacrifice (days)	Score				
		0	1	2	3	4
control PTFE	3	2	0	1	0	7
	14	7	0	3	0	0
	28	9	1	0	0	0
25 W	3	6	0	0	0	4
	14	5	2	3	0	0
	28	5	1	4	0	0
50 W	3	5	0	0	1	4
	14	6	3	0	1	0
	28	9	0	1	0	0
75 W	3	4	0	1	0	5
	14	7	1	2	0	0
	28	9	0	1	0	0

Capsule maturity

Polymer implant	Time to sacrifice (days)	Score				
		0	1	2	3	4
control PTFE	3	0	0	19	1	0
	14	0	0	0	16	4
	28	0	0	0	3	17
25 W	3	0	0	20	0	4
	14	0	0	1	8	11
	28	0	0	1	3	16
50 W	3	0	0	19	1	0
	14	0	0	0	4	16
	28	0	0	0	4	16
75 W	3	0	0	20	0	0
	14	0	0	0	5	15
	28	0	0	0	2	18

Total capsule thickness

Polymer implant	Time to sacrifice (days)	Score				
		0	1	2	3	4
control PTFE	3	0	6	6	5	3
	14	0	4	11	5	0
	28	0	0	3	10	7
25 W	3	0	4	7	5	4
	14	0	3	6	7	4
	28	0	1	2	14	3
50 W	3	2	5	4	4	5
	14	2	0	5	13	0
	28	0	0	3	9	8
75 W	3	0	2	8	3	7
	14	0	1	2	12	5
	28	2	0	2	10	6

Thickness of mature (dense) capsular layer

Polymer implant	Time to sacrifice (days)	Score				
		0	1	2	3	4
control PTFE	3	20	0	0	0	0
	14	4	0	0	10	6
	28	0	0	0	0	20
25 W	3	13	0	0	0	7
	14	4	0	0	6	10
	28	0	0	1	2	17
50 W	3	16	0	0	0	4
	14	3	0	0	0	17
	28	1	0	0	1	18
75 W	3	16	0	0	0	4
	14	5	0	0	0	15
	28	2	0	0	3	15

Interface qualities (foreign body effect)

Polymer implant	Time to sacrifice (days)	Score				
		0	1	2	3	4
control PTFE	3	0	0	0	0	20
	14	0	0	2	0	18
	28	0	0	0	0	20
25 W	3	0	0	0	0	20
	14	0	0	0	0	20
	28	0	0	0	0	20
50 W	3	0	0	0	0	20
	14	0	0	0	0	20
	28	0	0	0	0	20
75 W	3	0	0	0	0	20
	14	0	0	0	0	20
	28	0	0	0	0	20

Surrounding tissue cellular infiltrate

Polymer implant	Time to sacrifice (days)	Score				
		0	1	2	3	4
control PTFE	3	1	0	0	0	19
	14	20	0	0	0	0
	28	20	0	0	0	0
25 W	3	0	0	0	0	20
	14	18	0	0	0	2
	28	19	0	0	0	1
50 W	3	0	0	0	0	20
	14	17	0	0	0	3
	28	20	0	0	0	0
75 W	3	0	0	0	0	20
	14	20	0	0	0	0
	28	20	0	0	0	0

Appendix 4D: Gram-Twort stain

Twort's Stain

0.2% a/c neutral red	100 ml
0.2% a/c fast green	11.3 ml

keep as a stock solution and dilute 1:3 with tap water just prior to use

Lugol's Iodine

iodine crystals	1 gm
potassium iodide	2 gm
distilled water	100 ml

2% Crystal Violet

crystal violet	2 gm
95% alcohol	20 ml
ammonium oxalate	0.8 gm
distilled water	80 ml

dissolve crystal violet in alcohol. Dissolve oxalate in distilled water and then mix the two solutions together.

Method: Sequence of chemical exposure for specimen staining

1. Take sections to water
2. Stain with 2% crystal violet for two minutes
3. Wash off with Lugol's Iodine
4. Treat with Lugol's Iodine for three minutes
5. Rinse in water
6. Decolorize with acetone
7. Rinse immediately in water
8. Counter stain in Twort's (dilute stock solution 1:3) for 10 minutes
9. Rinse in water
10. Dehydrate quickly, cover and mount

Results

gram positive bacteria	dark blue
gram negative bacteria	pink
cytoplasm	light green
erythrocytes	green

Appendix 4E: Hematoxylin and eosin stain

Mayer's hematoxylin

Distilled water	2 L
Chloral hydrate	100 gm
Citric acid	2 gm
Aluminium ammonium sulphate	100 gm
Sodium iodate	0.4 gm
Haematoxylin	2 gm

Young's eosin

Distilled water	2 L
Calcium Chloride	5 gm
Erythrosin	5 gm
Eosin	15 gm

Scott's tap water substitute

Distilled water	2.5 L
Sodium bicarbonate	8.75 gm
Magnesium sulphate	50 gm

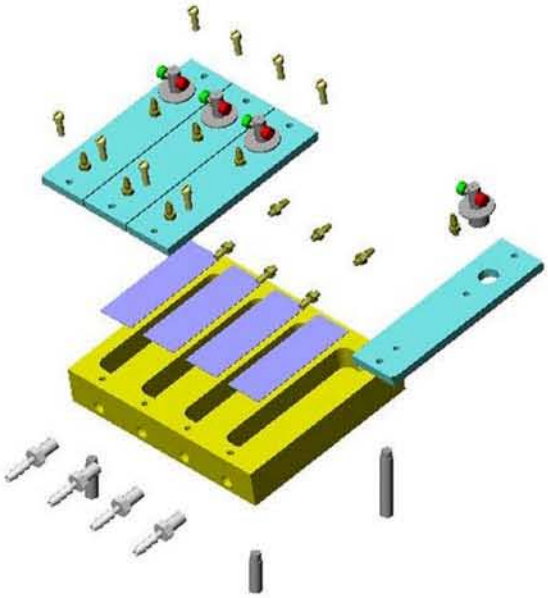
Sequence of chemical exposure for specimen staining

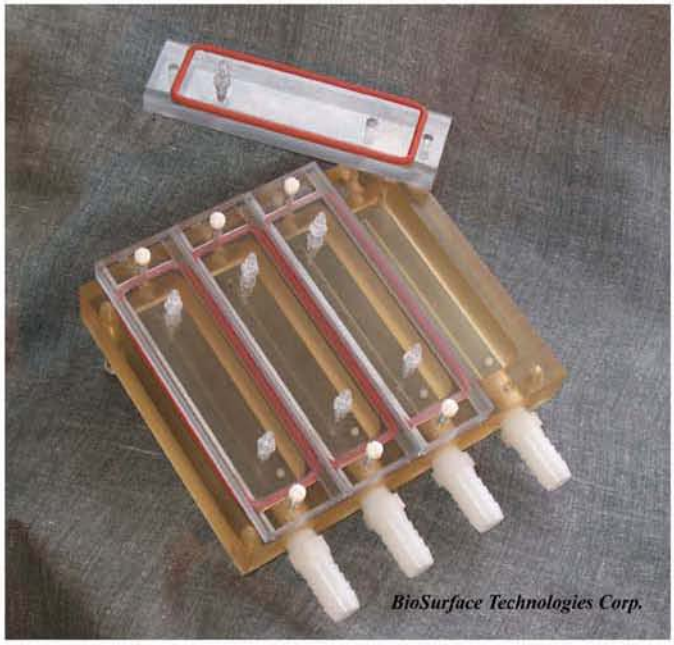
1.	xylene	2 min
2.	xylene	2 min
3.	absolute ethanol	2 min
4.	absolute ethanol	1 min
5.	absolute ethanol	1 min
6.	water wash	1 min
7.	Mayer's hematoxylin	8 min
8.	water wash	30 sec
9.	Scott's tap water substitute	30 sec
10.	water wash	2 min

11. Young’s eosin	4 min
12. water wash until sections are red-purple	~1 min
13. absolute ethanol	10 dips
14. absolute ethanol	10 dips
15. absolute ethanol	1 min
16. xylene	2 mins
17. xylene	1 min
18. xylene	1 min
19. mount cover slips with DPX	

BioSurface Technologies Corp.

Drip Flow Biofilm Reactor





The Model DFR 110 Drip Flow Biofilm Reactor consists of four (4) parallel test channels, each capable of holding one (1) standard glass microscope-slide sized coupon, or a length of catheter or stint. Media is provided by dripping over the coupon or catheter surface. Biofilm growth conditions provide low shear, short residence time (2 minutes). Catheter and stint studies can also utilize the alternate end media port to provide flow within the catheter or stint. Media flows via gravity to provide a very low shear condition.

The Drip Flow Reactor is ideal for microsensor monitoring, general biofilm studies, biofilm cryosectioning samples, high biomass production, medical material evaluations, and indwelling medical device evaluations.

Model DFR 110 Drip Flow Reactor **$949.00 US**

includes: Polysulfone reactor body with polycarbonate lid/silicone gaskets, glass slide coupons (1 bx), slide removal pick, bacterial vents (4)glass flow break (4).

Warranty is 6 months on all components. Normal delivery for reactors is 2-3 weeks. Special orders may require 30 days for shipment. All orders are FOB Bozeman, Montana. International sales require letter of credit, credit card, or prepayment prior to shipment.

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AMERICAN SOCIETY OF TESTING AND MATERIALS
100 Barr Harbor Dr., West Conshohocken, PA 19428

Designation:

DRAFT (January 2006)

Standard Test Method for the Quantification of a *Pseudomonas aeruginosa* Biofilm Grown with Very Low Shear and Continuous Flow using a Drip Flow Biofilm Reactor[1]

1. Scope

1.1 This test method is used for growing a repeatable[2] *Pseudomonas aeruginosa* biofilm close to the air/liquid interface in a reactor with a continuous flow of nutrients under low fluid shear conditions. In addition, the test method describes how to sample and analyze the biofilm for viable cells.
1.2 In this test method, biofilm population density is recorded as log colony forming units per surface area.
1.3 Basic microbiology training is required to perform this test method. This standard does not claim to address all of the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety practices and determine the applicability of regulatory limitations prior to use.

2. Referenced Documents

2.1 (Buffered dilution water preparation – Method 9050 C.1*a*)[3]

3. Terminology

3.1 Biofilm – an accumulation of bacterial cells immobilized on a substratum and embedded in an organic polymer matrix of microbial origin.

Note 1: Biofilm is a dynamic, self-organized accumulation of microorganisms and microbial and environmental by-products that is determined by the environment in which it lives. Definition 3.1 does not capture every biofilm type that is known to exist.

3.2 Coupon – biofilm sample surface.
3.3 Channel – rectangular well in reactor chamber (base) where sampling coupon is placed.

4. Summary of Test Method

4.1 This test method is used for growing a repeatable *Pseudomonas aeruginosa* biofilm in a Drip Flow Biofilm Reactor. The biofilm is established by operating the reactor in batch mode (no flow) for 6 hours. A mature biofilm forms while the reactor operates for an additional 48 hours with a continuous flow of the nutrients. The residence time of the nutrients in the reactor is approximately 2 minutes. During the entire 54 hours, the biofilm experiences very low shear caused by the gravity flow of media dripping onto a surface set at a 10° angle. At the end of the 54 hours, biofilm accumulation is quantified by removing coupons from reactor channels, rinsing to remove the

[1] This test method is under the jurisdiction of ASTM Committee E-35 on Pesticides and is the direct responsibility of Subcommittee E-35.15 on Antimicrobial Agents
[2] Ellison, S.L.R., M. Rosslein, A. Williams. (Eds.) 2000. Quantifying Uncertainty in Anyalytical Measurement, 2nd Edition. Eurachem.
[3] Eaton, A.D., L.S. Clesceri, A.E. Greenberg. (Eds.) 1995. Standard Methods for the Examination of Water and Waste Water, 19th Edition. American Public Health Association, American Water Works Association, Water Environment Federation. Washington D.C.

planktonic cells, scraping the biofilm from the coupon surface, disaggregating the clumps, then diluting and plating for viable cell enumeration.

5. Significance and Use

5.1 Bacteria that exist in a biofilm are phenotypically different from suspended cells of the same genotype. In addition, biofilm grown under different fluid shear conditions will exhibit different architectures that may make them more or less susceptible to disinfection. The study of biofilm in the laboratory requires protocols that account for these differences. Laboratory biofilms are engineered in growth reactors designed to produce a specific biofilm type. Altering system parameters will correspondingly result in a change in the biofilm. The purpose of this method is to direct a user in the laboratory study of biofilms by clearly defining each system parameter. This method will enable a person to grow, sample and analyze a laboratory biofilm grown under very low shear.

6. Apparatus

6.1 Teflon, metal or rubber spatulas, sterile, for scraping biofilm from coupon surface.
6.2 Inoculating loop.
6.3 Petri Dish, 100 by 15 mm, plastic, sterile and empty for transporting coupon holders from reactor to work station.
6.4 Culture tubes and culture tube closures – any with a volume capability of 10 mL and diameter no less than 6 cm. Recommended size is 16 by 125 mm borosilicate glass with threaded opening.
6.5 Beakers – 100mL containing 45mL sterile dilution water.
6.6 Conical-bottom disposable plastic centrifuge tubes – 50 mL containing 45 mL sterile dilution water.
6.7 Vortex – any vortex that will ensure proper agitation and mixing of culture tubes.
6.8 Homogenizer – any capable of mixing at 20,500 $\pm$ 5000 rpm in a 50 mL volume.
6.9 Homogenizer probe – any capable of mixing at 20,500 $\pm$ 5000 rpm in a 50 mL volume and can withstand autoclaving or other means of sterilization.
6.10 Sonicator – any noncavitating sonicating bath that operates at 50-60 hertz.
6.11 Bunsen or alcohol burner – used to flame inoculating loop and other instruments.
6.12 Stainless steel hemostat clamp or Forceps – for aseptic handling of coupons.
6.13 Pipettes – continuously adjustable pipette with volume capability of 1 mL (for dilutions and rinsing). Automatic or manual pipette with dispensing volume of 0.01 mL for drop plating.
6.14 Analytical balance sensitive to 0.01 g.
6.15 Sterilizers – any steam sterilizer capable of producing the conditions of sterilization.
6.16 Colony Counter – any one of several types may be used. A hand tally for the recording of the bacterial count is recommended if manual counting is done.
6.17 Peristaltic Pump with four pump heads – capable of holding tubing with ID 3.1 mm and OD 3.2 mm and operating at a flow rate of 200 mL per hour. Each channel will experience a flow rate of 50 mL per hour during continuous flow.
6.18 Environmental shaker capable of maintaining temperature of 35˚C $\pm$ 2˚C.
6.19 Silicon Tubing – two sizes of tubing: one with ID 3.1 mm and OD 3.2 mm and the other with ID 7.9 mm and OD 9.5 mm. Both sizes must withstand sterilization.
6.20 Glass Flow Break – any that will connect with tubing of ID 3.1 mm and withstands sterilization.
6.20.1 Clamp – used to hold flow break, extension clamp with 0.5-cm minimum grip size.
6.20.2 Clamp stand, height no less than 76.2 cm, used with clamp to suspend glass flow break vertically and stabilize tubing.
6.21 Reactor Components.[4]
6.21.1 Chamber (base) – polysulfone chamber with 4 channels and 4 effluent ports (one at the end of each channel). The underside holds 4 adjustable inserts (legs) providing a 10° angle of operation.

[4] CBE Drip Flow Biofilm Reactor is available commercially from BioSurface Technologies, Corp. www.imt.net/~mitbst, or the user may build the reactor.

6.21.2 Top – four polycarbonate tops each with 2 threaded holes for nylon screws to secure to reactor chamber (base). Two ports, one for influent media line attachment and another for bacterial air vent attachment. O-rings fitted underneath to seal top to chamber during operation.
6.21.3 Mini-nert valves – fit into each top as influent ports to allow inoculation and media line attachment.
6.21.4 Needle – 1 inch, 21 gauge; fit into mini-nert port.
6.21.5 Glass coupons – Four new rectangular glass microscope slides (or other similar shaped material) with a surface area of 18.75 cm^2 (25 x 75 x 1mm).
6.21.6 Teflon thread seal tape – to prevent leakage from effluent port connector.
6.21.7 Figure 3 illustrates an expanded view of the reactor and associated parts.
6.22 Carboys – two 15-20L autoclavable carboys, to be used for waste and nutrients.
6.22.1 Carboy Lids – one carboy lid with at least 2 barbed fittings to accommodate tubing ID 3.1 mm (one for nutrient line and one for bacterial air vent). One carboy lid with at least 2-1 cm holes bored in the same fashion (one for effluent waste and one for bacterial air vent).[5]
6.23 Bacterial Air Vent – autoclavable 0.2 micrometer pore size, to be spliced into tubing on waste carboy, nutrient carboy and each reactor channel top (use small air vent for reactor channel tops). Recommended diameter is 37 mm.
6.24 Figure 4 is a picture of the assembled system.

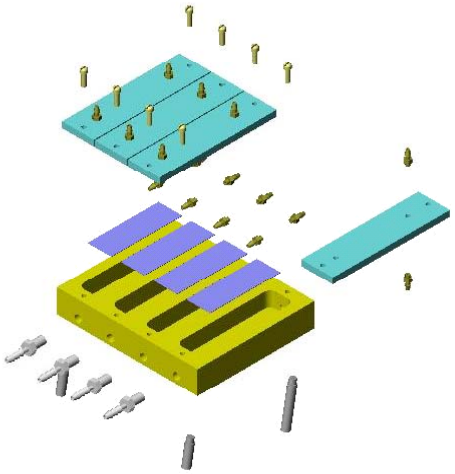


Figure 3. Expanded view of the reactor.

[5] Carboy tops can be purchased with fittings.

Figure 4. A picture of the DFR laboratory set-up.

7. Reagents and Materials

7.1 Purity of Water – all reference to water as diluent or reagent shall mean distilled water or water of equal purity.
7.2 Culture Media.
7.2.1 Tryptic Soy Broth (TSB) at a concentration equal to 3000 mg/L is used for the inoculum and batch reactor operation. TSB at a concentration equal to 270 mg/L is used during the continuous flow phase of reactor operation. Dissolve appropriate amount of TSB into one liter of distilled water. Sterilize for 20 minutes on liquid cycle.
7.2.2 Bacterial Plating Medium. R2A agar is recommended.
7.3 Buffered Water – 0.0425 g/L KH_2PO_4 distilled water, filter sterilized and 0.405 g/L $MgCl \cdot 6H_2O$ distilled water, filter sterilized.[6]

8. Culture/Inoculum Preparation

8.1 *Pseudomonas aeruginosa* (ATCC 700888) is the organism used in this test. An isolated colony is aseptically removed from an R2A plate and placed into 100 mL of sterile bacterial liquid growth broth (3000 mg TSB/L) and incubated in an environmental shaker at 35°C $\pm$ 2°C for 18-20 hours. Viable bacterial density should equal 10^8 CFU/mL, and may be checked by serial dilution and plating.

9. Reactor Preparation

9.1 Use new coupons (glass microscope slides) for every experiment.
9.2 Reactor Set-up.
9.2.1 Insert a pretreated coupon into each reactor channel, positioning the top of the slide directly under the influent media port and allowing the bottom of the slide to rest on the pegs at the bottom of the channel.
9.2.2 Place channel lids loosely onto base with nylon screws.
9.2.3 Attach the bacterial air vent.
9.2.4 Splice the glass flow break into the area of the media tubing line that will be near the carboy top when attached.
9.2.5 Configure the media tubing so that four individual lines result. The four lines will each be fed through a pump head then attached to a chamber as shown in Figure 4.
9.2.6 Wrap each effluent barbed fitting with Teflon tape and insert into end port. Clamp tightly each effluent line using stainless steal screw clamps. Re-tighten each barbed fitting after autoclaving to prevent any leaking during continuous flow.
9.3 Sterilizing the reactor system.

[6] Prepared according to the document referenced in 2.1.

9.3.1 Remove the adjustable legs from the reactor chamber. Wrap all exposed tubing ends and openings with aluminum foil and place assembled reactor into an autoclave tray. Cover entire tray with aluminum foil.
9.3.2. Sterilize the reactor system for 20 minutes on liquid cycle. Remove immediately after cycle is finished to prevent cracking of the reactor base.

10. Procedure

10.1 The batch phase.
10.1.1 Place the cooled reactor in a level position on the bench top.
10.1.2 Clamp flow break in upright position; leave other tubing clamped and foiled.
10.1.3 Aseptically add 15 mL of 3000 mg TSB/L (sec. 7.2.1,) and 1mL of inoculum (sec. 8.1) to each channel then tighten the lid securely with attached screws. Alternatively, pump 15 mL of the 3000 mg TSB/L media into each channel and add 1mL of inoculum through the influent port.
10.1.4 The reactor system is allowed to incubate in batch mode at room temperature (21°C $\pm$ 2°C) for 6 hours, remaining level.
10.1.5 Remove foil from the effluent tubing and place end into a waste carboy. Do not unclamp until continuous flow phase.
10.2 Continuous Flow (CF).
10.2.1 Prepare continuous flow nutrient broth by adding sterilized bacterial liquid growth medium to 20 L sterile reagent grade water so that final concentration is equal to 270 mg TSB/L (sec. 7.2.1). Dissolve and sterilize the medium in a smaller volume of water to prevent caramelization. Aseptically pour the concentrated medium into the carboy of sterile water to make a total of 20 L.
10.2.2 Aseptically connect the influent nutrient tubing line to the carboy containing the continuous flow nutrient broth. The nutrient tubing is spliced so that four lines result, feed each line through a pump head and connect a sterile needle on the end.
10.2.3 After 6 hours of batch conditions, aseptically attach the influent tubing by inserting the sterile needle through the mini-nert ports in the channel lids.
10.2.4 Adjust the legs in the reactor chamber so that it slopes downward 10°. Unclamp the effluent lines and turn on the pump, allowing media to slowly drip onto the bacterial cells attached to the coupon. A continuous flow of nutrient is pumped into the reactor through a pump set at a flow rate equal to 200 mL/hr (50mL/hr per channel).
10.2.5 The media should flow downward from the influent port to the effluent port. Periodically check the reactor for proper drainage and also check the effluent tubing for leaks.
10.2.6 The reactor is operated in CF mode for 48 hours.
10.3 Sampling the biofilm.
10.3.1 Prepare sampling materials: vortex, homogenizer, sterile beakers, sterile centrifuge tubes, culture tubes, pipettes, empty sterile petri dish, sterile spatulas, and flame sterilized stainless steel hemostat or forceps.
10.3.2 Carefully loosen channel lid screws and lift channel lid up. Aseptically remove one of the coupons by gently lifting up the coupon with sterile forceps. Hold coupon over a sterile petri dish while moving it to the sampling area.
10.3.3 Hold the coupon with sterile forceps or hemostat being careful not to disturb the attached biofilm. Be sure to wear gloves. Sterilize the hemostat between each coupon sampling.
10.3.4 Rinse the slide to remove suspended cells. Hold slide and centrifuge tube at a 45° angle.
Uncap tube and gently immerse slide with a fluid motion until slide is completely covered. Immediately reverse motion to remove the slide, being careful not to agitate liquid and biofilm.
10.3.5 Scrape the top of the coupon surface for approximately 15 seconds using the flat end of a sterile spatula or scraper held perpendicular to the coupon surface. The spatula or scraper is rinsed by stirring it in the in the beaker containing 45 mL of sterile dilution buffer. Repeat the scraping and rinsing process 3-4 times, ensuring full coverage of the coupon surface.
10.3.6 The coupon is rinsed by holding it at a 60° angle over the sterile beaker and pipetting 1 mL of sterile dilution water over the surface of the coupon. Repeat for a total of 5 rinses. The final volume in the beaker is 50 mL.
10.4 Analyze the biofilm sample.
10.4.1 The scraped biofilm sample is homogenized in the beaker at 20,500 $\pm$ 5000 rpm for 30 seconds. If more than one biofilm sample is taken rinse the homogenizing probe by homogenizing a dilution blank for 30 seconds at the same rpm, homogenize a tube containing 70% ethanol for 15 seconds, then remove the probe and let the probe set in the ethanol tube for 1 minute. Shake any remaining ethanol off the probe, reattach probe and homogenize a dilution

blank for 30 seconds. Homogenize a second dilution blank and then homogenize the next sample tube. Always repeat this cleaning process between samples. Discard 70% ethanol at end of sampling.

Note 2: Homogenizing the sample disaggregates the biofilm clumps to form a homogeneous cell suspension. Improper disaggregation will result in an underestimation of the viable cells present in the sample.

10.4.2 Serially dilute the sample 1:10 using sterile culture tubes.
10.4.3 Plate each dilution in duplicate for colony growth using an accepted plating technique such as spread or spiral plating.
10.4.4 Incubate the plates for 17-20 hours at 35°C $\pm$ 2°C.
10.5 Cell Enumeration.
10.5.1 Count the appropriate number of colonies according to the plating method used.
10.5.2 Calculate the arithmetic mean of the colonies counted on the duplicate plates.
10.6 The log density for one coupon is calculated as follows:

$$LOG_{10}\ (CFU/cm^2) = LOG_{10}\ [(\text{mean CFU/volume plated per sample})(\text{dilution}) \bullet (\text{volume scraped into/surface area scraped}^{7})] \quad \textbf{[1]}$$

10.7 Calculate the overall biofilm accumulation by geometric mean of the log densities calculated in section 10.6.

11. Precision and Bias

11.1 Randomization should be used whenever necessary to eliminate potential bias caused by subjective decisions.
11.2 The repeatability standard deviation was 0.28 for this protocol when the biofilm density was based on a single coupon. The sources of variability were:
65% attributable to with-in experiment sources,
35% attributable to between experiments sources.

[7] The surface area of the glass coupon is 18.75 cm^2 (SOP Section 6.21.5).

Appendix 5C: Enumeration of biofilm organisms

Substrate		Number of biofilm organisms x10^5 per ml		
		S.aureus ATCC 29213	MRSA clinical isolate	*S.epidermidis* ATCC 12228
control PTFE	01	8	60	1.22
	02	53	8	9.6
	03	10.8	49.6	2.12
	04	9.8	38.6	19
	05	34	58.2	4.08
	06	6.6	96	25.4
	07	10.6	27.8	156
	08	132	316	2.08

Substrate		Number of biofilm organisms x10^5 per ml		
		S.aureus ATCC 29213	MRSA clinical isolate	*S.epidermidis* ATCC 12228
25 W polymer	01	164	306	11.2
	02	228	462	7.8
	03	520	318	5.56
	04	542	112	2.0
	05	114	184	370
	06	136	340	232
	07	680	158	37.2
	08	606	292	324

Substrate		Number of biofilm organisms x10^5 per ml		
		S.aureus ATCC 29213	MRSA clinical isolate	*S.epidermidis* ATCC 12228
50 W polymer	01	170	23.8	100
	02	124	740	57
	03	100	660	126
	04	56.6	96	92
	05	302	82	188
	06	292	344	32.8
	07	78	146	37.2
	08	142	86	78

Substrate		Number of biofilm organisms x10^5 per ml		
		S.aureus ATCC 29213	MRSA clinical isolate	*S.epidermidis* ATCC 12228
75 W polymer	01	144	54	24.8
	02	88	58	56.4
	03	220	140	14.2
	04	166	730	32.8
	05	188	194	184
	06	45	660	84
	07	43.4	114	25
	08	364	84	52.6

Appendix 6A: Plasma polymer film thickness as a function of deposition time

25 W TTO plasma polymer film thickness (nm)

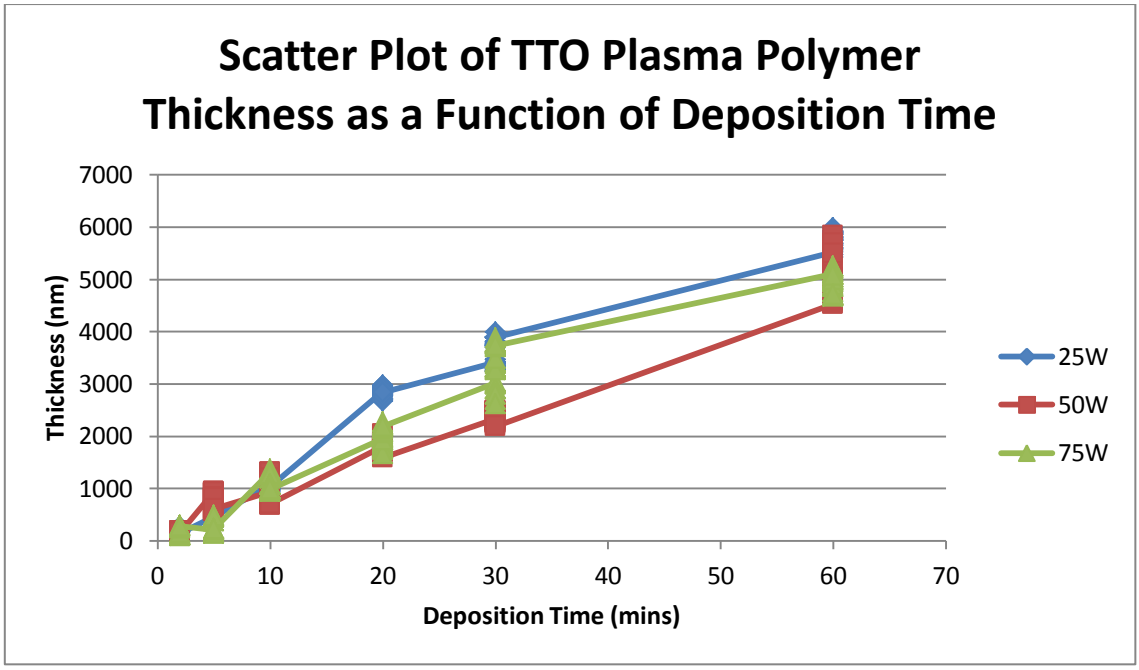
Sample number	Deposition time (mins)					
	2	5	10	20	30	60
1	185.5	469.2	1064.1	2870.0	3420.0	5525.8
2	172.4	468.1	1073.3	2885.1	3339.9	5674.9
3	182.4	472.9	1055.6	2885.0	3395.0	5563.0
4	188.7	456.2	1096.1	2900.1	3285.2	5747.0
5	191.2	463.1	1169.5	2915.2	3355.1	5757.0
6	198.2	466.1	1160.1	2889.9	3295.1	5814.3
7	197.7	465.1	1193.4	2970.0	3325.0	5912.4
8	202.1	435.8	1185.3	2820.0	3255.0	5899.4
9	206.1	456.1	1215.2	2935.1	3314.9	5879.3
10	212.3	459.2	1219.4	2885.0	3235.0	5858.0
11	188.0	466.2	1030.3	2900.0	3740.0	5988.0
12	210.6	466.6	975.8	2884.9	3695.0	5478.7
13	212.4	465.8	1048.4	2975.1	3770.1	5700.0
14	225.9	454.9	1070.9	2880.1	3740.0	5781.5
15	240.0	445.7	1067.4	2670.0	3809.9	5603.8
16	254.6	451.5	1075.6	2855.0	3749.9	5677.0
17	262.6	440.2	1062.9	2920.1	3880.1	5800.6
18	268.9	454.6	1071.4	2880.2	3765.1	5586.0
19	273.6	428.6	1066.6	2775.1	3895.0	5932.0
20	254.7	456.1	1075.6	2870.5	3795.1	5754.6
21	181.3	464.9	927.0	2915.2	3895.2	5793.9
22	196.4	465.6	958.3	2835.0	3468.0	5800.4
23	196.6	464.8	994.6	2715.1	3990.0	5428.9
24	182.0	467.0	1005.0	2795.1	3711.1	5599.0
25	181.5	467.8	1066.5	2710.1	3242.2	5895.9
26	186.1	468.4	1032.2	2830.1	3736.9	5879.0
27	161.2	465.0	1075.5	2693.5	3737.3	5898.7
28	189.6	464.8	1039.9	2810.0	3322.0	5639.8
29	187.5	464.7	1073.1	2770.0	3892.5	5740.0
30	147.5	466.4	1039.5	2840.0	3900.0	5849.0

50 W TTO plasma polymer film thickness (nm)

Sample number	Deposition time (mins)					
	2	5	10	20	30	60
1	147.5	945.3	939.1	1813.8	2337.9	4532.1
2	125.8	922.5	872.4	1791.2	2364.2	4531.0
3	128.5	948.0	885.3	1796.9	2344.0	4589.0
4	103.2	920.3	741.1	1694.1	2288.0	4936.5
5	119.9	922.7	846.6	1724.5	2276.0	4785.3
6	122.7	947.9	828.0	1759.8	2231.9	5049.7
7	120.7	922.8	842.2	1720.0	2267.8	5045.1
8	126.9	920.1	830.8	1877.7	2255.7	4569.2
9	118.1	930.4	839.7	1869.5	2251.5	5041.4
10	124.2	927.6	878.6	1712.0	2251.9	5036.1
11	177.0	829.9	1314.7	2039.9	2482.2	4878.0
12	185.0	830.0	1195.0	2037.7	2486.8	4999.0
13	162.0	827.5	1309.9	2002.3	2490.5	5181.8
14	172.4	816.1	1215.2	1934.6	2481.1	5844.4
15	155.4	822.5	1294.9	1866.9	2459.2	5698.3
16	166.0	816.1	1232.9	1824.8	2456.0	5233.3
17	153.9	814.2	1309.9	1834.6	2474.9	5434.3
18	154.0	805.6	1200.0	1802.9	2430.7	5005.5
19	158.3	802.4	1307.3	1812.1	2487.6	4605.9
20	156.8	814.8	1202.6	1713.1	2496.2	4612.5
21	165.2	569.6	768.6	1852.2	2447.9	4775.9
22	173.6	587.0	760.5	1856.1	2445.9	4767.0
23	178.9	571.1	763.5	1811.9	2448.0	4800.7
24	176.8	579.4	706.1	1803.0	2407.7	5000.0
25	182.4	568.9	705.9	1743.7	2376.2	5030.9
26	176.8	572.3	689.7	1717.7	2366.9	5500.1
27	180.4	577.1	691.9	1719.6	2461.9	4973.8
28	178.1	575.5	710.5	1729.8	2293.2	4985.5
29	162.5	569.9	708.2	1793.9	2313.4	4893.2
30	162.8	614.9	712.5	1596.0	2197.9	4888.5

75 W TTO plasma polymer film thickness (nm)

Sample number	Deposition time (mins)					
	2	5	10	20	30	60
1	123.1	205.8	1327.5	1962.0	3024.9	5109.9
2	95.8	208.9	1373.0	1926.0	3020.0	5034.8
3	108.2	198.9	1301.2	1923.9	2709.9	5222.3
4	97.2	196.0	1333.6	1816.0	2994.9	4690.8
5	116.2	171.7	1274.0	1874.0	3005.0	5094.1
6	94.3	159.8	1330.0	1860.1	3050.0	5257.1
7	116.4	176.8	1298.4	1756.0	2910.0	5259.6
8	108.1	151.5	1319.4	1819.9	2904.9	5239.3
9	128.8	149.6	1197.1	1770.0	2810.0	5047.1
10	120.3	143.1	1291.4	1775.5	2614.9	5000.2
11	113.4	382.6	1003.8	2275.0	3317.6	4700.7
12	110.5	448.3	994.8	2220.1	3320.2	4767.0
13	122.8	450.7	985.8	2035.9	3325.2	4699.8
14	154.6	474.9	994.8	2230.1	3304.9	5000.2
15	110.2	459.1	991.6	1974.5	3417.1	5138.6
16	113.7	474.1	994.1	2000.1	3394.4	5090.5
17	114.1	469.1	1001.3	1774.1	3287.5	4967.6
18	153.9	487.6	999.9	1660.0	3434.8	5200.4
19	138.7	461.9	1001.3	2235.0	3267.4	5150.7
20	157.7	473.6	1076.1	2195.1	3490.3	5043.0
21	281.1	228.0	1074.1	1979.9	3805.0	4692.8
22	294.1	223.5	1072.5	1929.0	3887.6	4866.0
23	279.9	232.1	1079.6	2076.0	3710.0	4864.5
24	289.2	218.9	1081.9	2074.1	3780.8	4945.7
25	248.8	219.8	1001.8	2092.1	3792.5	4900.0
26	267.1	212.4	946.8	2146.2	3792.5	5000.2
27	271.3	224.6	1083.5	2163.3	3767.5	5090.9
28	288.1	222.5	1085.8	2189.2	3777.5	5080.9
29	290.4	227.6	976.5	2199.0	3765.0	4678.0
30	276.1	230.1	995.7	2196.7	3735.5	4709.9


Scatter Plot of TTO Plasma Polymer Thickness as a Function of Deposition Time
Thickness (nm)
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
Deposition Time (mins)
25W
50W
75W

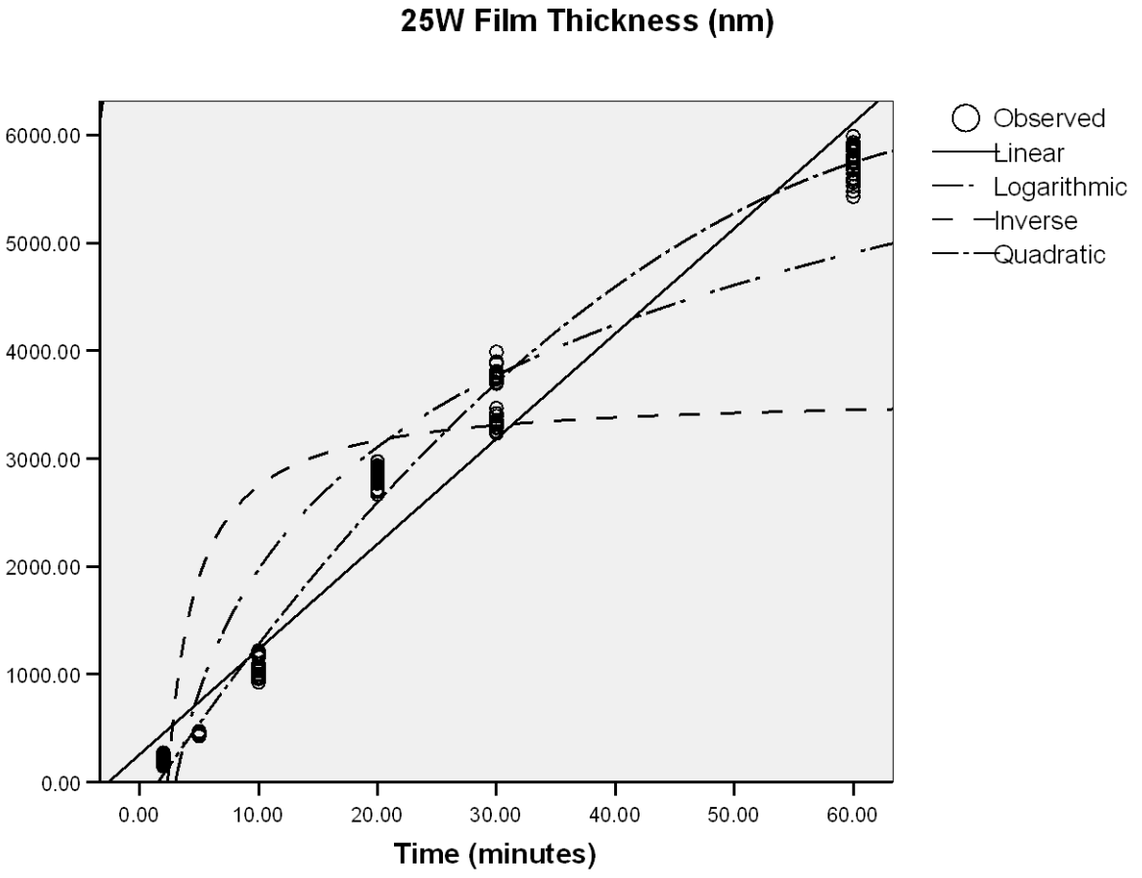
Appendix 6B: Statistical relationship between film thickness and deposition time

Model Summary and Parameter Estimates

Dependent Variable: 25W Film Thickness (nm)

	Model Summary					Parameter Estimates		
Equation	R Square	F	df1	df2	Sig.	Constant	b1	b2
Linear	.958	4061.487	1	178	.000	257.409	97.555	
Logarithmic	.888	1411.437	1	178	.000	-1799.371	1638.149	
Inverse	.521	193.528	1	178	.000	3592.666	-8468.891	
Quadratic	.990	8652.127	2	177	.000	-250.871	163.572	-1.061

The independent variable is Time (minutes).

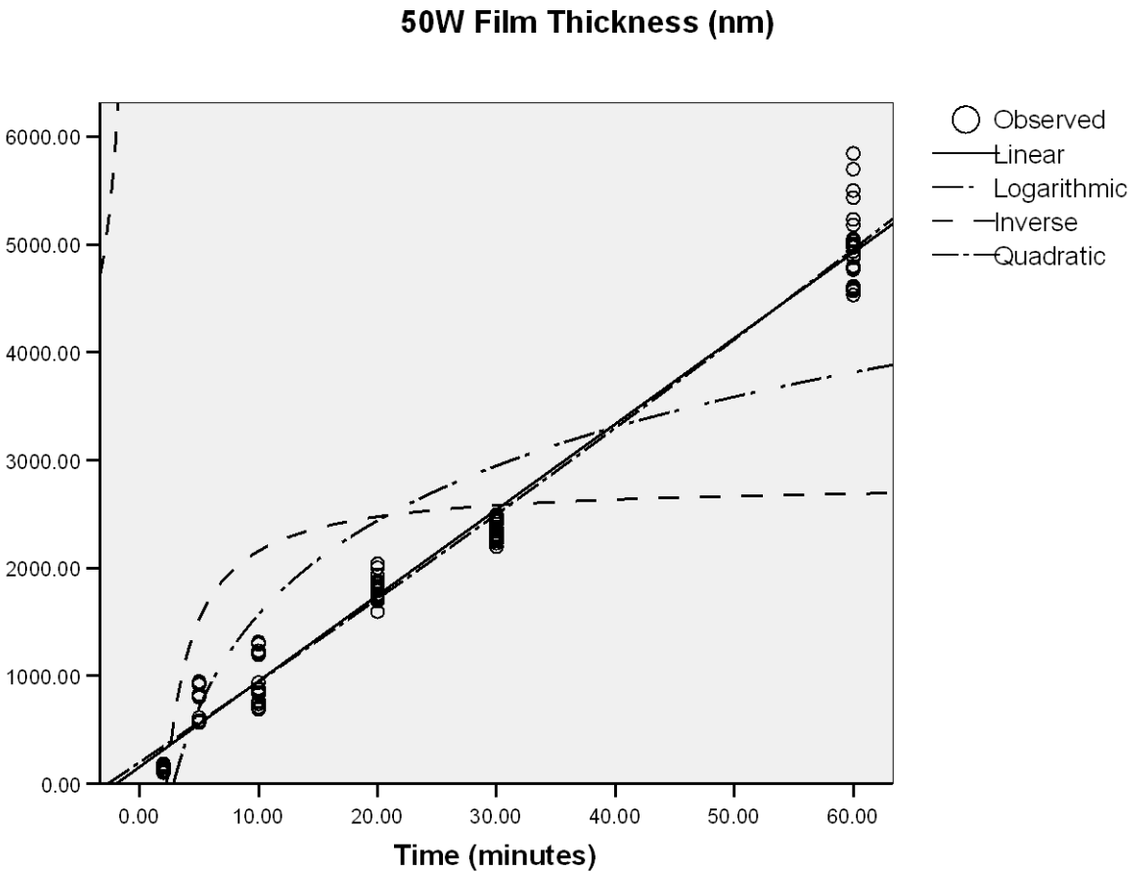


Model Summary and Parameter Estimates

Dependent Variable: 50W Film Thickness (nm)

	Model Summary					Parameter Estimates		
Equation	R Square	F	df1	df2	Sig.	Constant	b1	b2
Linear	.980	8639.443	1	178	.000	156.855	79.477	
Logarithmic	.800	712.072	1	178	.000	-1312.410	1252.558	
Inverse	.454	148.247	1	178	.000	2794.913	-6371.971	
Quadratic	.980	4370.750	2	177	.000	199.207	73.976	.088

The independent variable is Time (minutes).

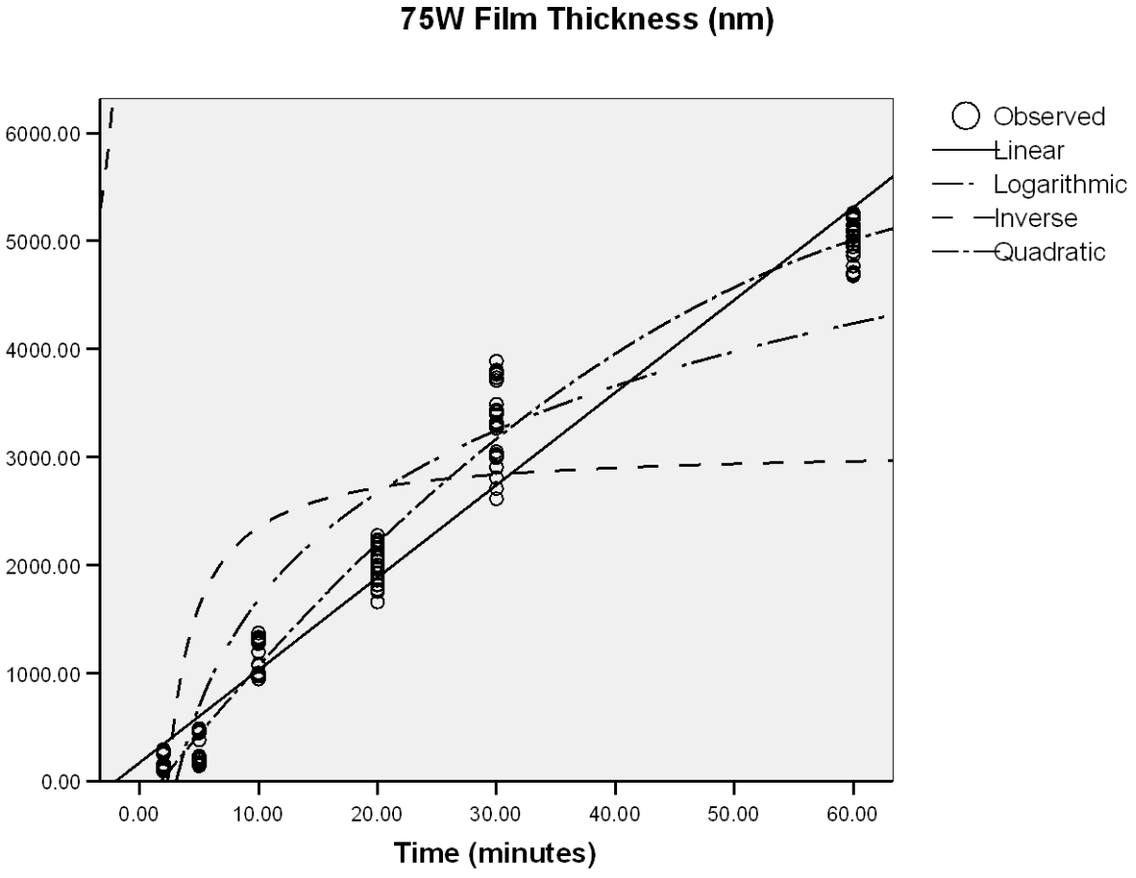


Model Summary and Parameter Estimates

Dependent Variable: 75W Film Thickness (nm)

	Model Summary					Parameter Estimates		
Equation	R Square	F	df1	df2	Sig.	Constant	b1	b2
Linear	.952	3528.002	1	178	.000	173.143	85.611	
Logarithmic	.869	1182.024	1	178	.000	-1604.474	1426.715	
Inverse	.502	179.718	1	178	.000	3083.543	-7321.985	
Quadratic	.980	4348.114	2	177	.000	-246.949	140.174	-.877

The independent variable is Time (minutes).



Appendix 6C: AFM data surface topography (roughness)

Measure	TTO derived plasma polymer (25 W)	
	Sample 1	Sample 2
amount of sampling	64256	sample failed
max height (nm)	11.0647	
min height (nm)	0	
peak-to-peak, sy (nm)	11.0647	
ten point height, sz (nm)	5.30132	
average (nm)	3.03802	
average roughness, sa (nm)	0.456971	
root mean square, sq (nm)	0.592159	
surface skewness, ssk	0.470669	
coefficient of kurtosis, ska	3.89809	
entropy	4.30047	
redundance	-0.290956	

Measure	TTO derived plasma polymer (50 W)	
	Sample 1	Sample 2
amount of sampling	62464	63232
max height (nm)	20.6703	10.5783
min height (nm)	0	0
peak-to-peak, sy (nm)	20.6703	10.5783
ten point height, sz (nm)	10.1649	4.96087
average (nm)	8.73914	2.31493
average roughness, sa (nm)	0.305444	0.297108
root mean square, sq (nm)	0.438139	0.398375
surface skewness, ssk	1.81744	0.987617
coefficient of kurtosis, ska	40.1976	16.2303
entropy	3.72847	3.69208
redundance	0.1325	-0.132618

Measure	TTO derived plasma polymer (75 W)	
	Sample 1	Sample 2
amount of sampling	65536	65536
max height (nm)	6.56586	127.305
min height (nm)	0	0
peak-to-peak, sy (nm)	6.56586	127.305
ten point height, sz (nm)	2.82089	56.9528
average (nm)	2.31022	11.4827
average roughness, sa (nm)	0.341509	0.548625
root mean square, sq (nm)	0.433569	2.56926
surface skewness, ssk	0.0453315	21.4529
coefficient of kurtosis, ska	0.400884	584.397
entropy	3.87815	4.1293
redundance	-0.565913	0.408474

Appendix 6D: AFM data nano-indentation assessment of hardness

Summary of nano-indentation results

Material	Average H(GPa)
Glass slide substrate (control)	5.071962
25 W TTO polymer	0.308723
50 W TTO polymer	0.422373
75 W TTO polymer	0.475502

Glass slide substrate (control) nano-indentation hardness results

Force (uN) applied at 50 uN/s	H(GPa)
500	5.348392
750	5.204212
1000	4.827698
1250	5.04145
1500	5.186837
1750	5.217099
2000	5.030537
2250	5.284514
2500	5.177931
2750	4.875928
3000	5.277833
3250	5.216362
3500	4.891995
3750	5.145324
4000	5.065151
4250	5.035899
4500	5.020815
4750	4.654867
5000	4.864427

Tea tree oil plasma polymer nano-indentation hardness results

Force (uN) applied at 50 uN/s	H(GPa) TTO derived plasma polymer		
	25 W	50 W	75 W
100	0.332576	0.449786	0.579031
	0.342281	0.508787	0.538583
	0.337429	0.470055	0.586825
150	0.333931	0.446334	0.541437
	0.338889	0.413473	0.503494
	0.33641	0.429904	0.481863
200	0.306359	0.406965	0.492489
	0.314453	0.426194	0.534691
	0.308031	0.416283	0.478376

Continued:

Force (uN) applied at 50 uN/s	H(GPa) TTO derived plasma polymer		
	25 W	50 W	75 W
250	0.299874	0.414303	0.443194
	0.301277	0.421791	0.460122
	0.296434	0.418047	0.442906
300	0.30145	0.386413	0.463245
	0.302871	0.415759	0.490519
	0.298885	0.404731	0.439263
350	0.299058	0.394208	0.422643
	0.293555	0.401029	0.418884
	0.287748	0.40512	0.427923
400	0.301336	0.424364	0.506652
	0.304786	0.421465	0.488569
	0.303061	0.422915	0.470389
450	0.291156	0.420547	0.404407
	0.287956	0.411013	0.404669
	0.289556	0.407465	0.391879

Appendix E: Effect of ethanol and water on tea tree oil plasma polymers

Ethanol exposure

Substrate		Thickness pre-exposure (nm)	Thickness post-exposure (nm)	Δ Thickness (nm)	Δ Thickness (%)	Appearance post-exposure
control PTFE	1	27.1	0.5	-26.6	-98.1	clear
	2	29.9	0.5	-29.4	-98.3	clear
	3	33.2	0.5	-32.7	-98.5	clear
25 W polymer	1	2615.0	97.6	-2517.4	-96.3	clear
	2	1580.2	52.3	-1527.9	-96.7	clear
	3	1809.0	40.5	-1768.5	-97.8	clear
50 W polymer	1	680.2	549.8	-130.4	-19.2	flaked/cracked
	2	1469.9	822.6	-647.3	-44.0	flaked/cracked
	3	1201.7	750.0	-451.7	-37.6	flaked/cracked
75 W Polymer	1	1612.1	37.3	-1574.8	-97.7	flaked/cracked
	2	412.9	139.1	-273.8	-66.3	cracked
	3	882.1	689.9	-192.2	-21.7	cracked

Water exposure

Substrate		Thickness pre-exposure (nm)	Thickness post-exposure (nm)	Δ Thickness (nm)	Δ Thickness (%)	Appearance post-exposure
control PTFE	1	10.3	0.5	-9.8	-95.0	clear
	2	10.3	0.5	-9.8	-95.0	clear
	3	10.0	0.3	-9.7	-97.0	clear
25 W Polymer	1	1644.2	1600.4	-43.8	-2.7	folded
	2	1083.1	1064.9	-18.2	-1.7	folded
	3	1439.5	1430.0	-9.5	-0.7	folded
50 W Polymer	1	1233.8	1205.0	-28.8	-2.3	folded, sloughed
	2	1585.4	1460.5	-124.9	-7.9	folded
	3	841.0	840.0	-1.0	-0.1	folded, sloughed
75 W Polymer	1	487.2	462.4	-24.8	-5.1	folded
	2	1637.9	1618.3	-19.6	-1.2	folded
	3	1663.5	1664.1	+0.6	+0.04	folded