The molecular weight of ulvan affects the in vitro inflammatory response of a murine macrophage

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Abstract

Ulvan, a sulfated polysaccharide extracted fromthe green seaweed genus Ulva, has bioactive properties including an immunomodulating capacity. The immunomodulatory capacity of ulvan from Ulva ohnoi, however, has not been assessed in detail. We depolymerised purified ulvan from U. ohnoi to obtain a range of molecular weight fractions (Mw 7, 9, 13, 21, 209 kDa), which were characterised by constituent sugar analysis, SEC-MALLS, and NMR. Ulvan fractions contained 48.8–54.7 mol% rhamnose, 32.5–35.9 mol% glucuronic acid, 4.5–7.3 mol% iduronic acid, and 3.3–5.6mol% xylose. 1Hand13C NMRwas consistentwith hydrolysis occurring at the anomeric centre without further modification to the oligosaccharide structure. The in vitro immunomodulatory effect of ulvan fractions was quantified by measuring levels of inflammatory-mediating signalling molecules released from LPS-stimulated RAW264.7 murine macrophages. All ulvan fractions showed no toxicity on RAW264.7 cells at concentrations below100 µgmL−1 over 48 h. Secreted interleukin-10 and prostaglandin E2 demonstrated an anti-inflammatory effect by higher molecular weight ulvan fractions at 100 µgmL−1. To a lesser extent, these fractions also enhanced the LPS-induced inflammation through minor increases of IL-1β and IL-6. This study confirms that ulvan from U. ohnoi has a mild in vitro immunomodulatory effect.

Keywords: Ulvan, RAW264.7, immunomodulation, molecular weight, seaweed, sulfated polysaccharide

1.0 Introduction

Sulfated polysaccharides are complex, bioactive macromolecules featuring sugar residues with sulfate esters[1]. In mammals, sulfated polysaccharides modulate a range of biological functions including blood coagulation, cell adhesion, and cell growth; in addition to contributing structural support to the extracellular matrix and cartilage[2-4]. Sulfated polysaccharides containing the monosaccharide rhamnose induce a host of immunostimulating reactions in human skin[5]. Rhamnose is uncommon in nature; only being found in bacterial exopolysaccharides, plant pectic (non-sulfated) polysaccharides, and ulvan - a sulfated polysaccharide from green marine algae of the genus *Ulva*[6]. Ulvan is composed predominantly of sulfated rhamnose, uronic acids (glucuronic and iduronic), and xylose. Featuring a rare bioactive monosaccharide and exhibiting a wide range of bioactive properties ulvan has potential for human health applications[7-10].

To assess the impact of ulvan on human health, ulvan (or extracts containing ulvan) has been assessed for the capacity to alter the inflammatory response both *in vitro* using macrophages (e.g. RAW264.7 cells)[11-18] and *in vivo* using animal models[19-21]. *In vitro* assessment of the effect of ulvan on inflammatory responses is often evaluated by quantifying the levels of inflammatory cytokines secreted from macrophages such as interleukin (IL)-1β, 4, 5, 6, 10, 12, 18, and tissue necrosis factor alpha (TNF-α), and other inflammation markers such as prostaglandin E2 (PGE_2) and nitric oxide (NO)[13, 18]. Despite the increasing number of reports on the immunomodulating properties of ulvan, little is known about the structure-activity relationships of this class of sulfated polysaccharide. However, molecular weight (Mw) is known to be critical to the efficacy of bioactive polysaccharides[1, 4, 7, 8, 18, 22]. Ulvan extracts naturally exhibit a high level of polydispersity (which may be exacerbated by the extraction conditions employed), a feature that hinders precise assignment of immunomodulating activity.

The aims of this study were, therefore, to elucidate the effect of the molecular weight of purified ulvan, extracted from *Ulva ohnoi*, on the inflammatory response of RAW264.7 murine macrophages. To achieve these aims, we (i) characterised 'native' and depolymerised ulvan from *U. ohnoi*; (ii) assessed toxicity of ulvan on murine macrophages; (iii) assessed the effect of 'native' *U. ohnoi* ulvan on the inflammatory response of murine macrophages; and (iv) assessed the effect of molecular weight of ulvan has on the inflammatory response of murine macrophages.

2.0 Methods

2.1 Ulvan Preparation

2.1.1 Algal Cultivation & ulvan extraction

Ulva ohnoi (Gen-bank accession number KF195509, strain JCU 1[23]) was cultivated and supplied by Pacific Biotechnologies ABA facility, a land-based aquaculture facility near Ayr, Queensland, Australia (19°29'S, 147°28E). Crude ulvan was extracted using a proprietary extraction procedure (Marinova Pty. Ltd.) and purified as described below.

2.1.2 Purification & Fractionation

Crude ulvan (2 g L^{-1}) was dissolved in type one water prior to vacuum filteration (Filtech, 453) and diafiltration (ÄKTA flux 6 system fitted with a 10,000 NMWC filter, UFP-10-E-4X2MA) with five volumes of type one water. Protein was separated from the diafiltered retentate via anion exchange chromatography (AEC) using an ÄKTA pure 150 L coupled with a single wavelength (280nm) UV detector. 400 mL of retentate was loaded into an equilibrated (type one water, 5 column volumes (CV); 2 M NaCl, 5 CV; type one water, 5 CV) XK 50/30 column (GE Healthcare Life Sciences) packed with Q Sepharose XL media (bed height = 24.5 cm). The column was eluted using a stepwise gradient (0 M NaCl, 2 CV; 0 – 0.5 M NaCl, 2 CV; 0.5 – 1 M, 2 CV; 1 – 1.75 M NaCl, 3 CV; 1.75 – 2 M NaCl, 5 CV) at a flow rate of 20 mL min^{-1}. A chromatogram was produced by analysing collected fractions (13 mL) for uronic acid colourimetrically using the *m*-phenol phenol method with glucuronic acid as standard[24]. Fractions containing uronic acid that did not overlap the major peak in the UV trace (attributed to protein) were pooled and concentrated by diafiltration with type one water until permeate conductivity was < 5 µS cm^{-1}. The purified ulvan was recovered via lyophilisation – this is referred to as 'native' ulvan.

2.1.3 Ulvan hydrolysis

Native ulvan solution (1 % w/v in Type one) was heated to 50°C under constant stirring before H_2O_2 was added to a final solution concentration of 2.5 % v/v. The hydrolysis reaction was held at 50°C for 1, 2, 5, or 7 h before being quenched in an ice bath. The cold solution was diafiltered (Amicon Ultra-15, 10 kDa NMWL) with five volumes of type one water prior to recovery by lyophilisation. The permeate from all the hydrolysed ulvan samples was also collected and lyophilised. The 5 and 7 h treatments were combined due to low yield of hydrolysed ulvan and comparable molecular weight profiles. **Table 1** assigns sample labels used throughout the text.

Table 1. Sample labels used through the text for hydrolysed ulvan fractions

Sample ID	**Hydrolysis time (h)**
U209	0 (native ulvan)

U21	1
U13	2
U9	5 + 7
U7	Permeate

2.2 Chemical characterisation

2.2.1 Elemental characterisation

Elemental analysis (% C, H, N, S; n=1) and ash content (% w/w) of ulvan samples were measured commercially by OEA labs (www.oealabs.com, Callington, UK). Percent oxygen was calculated as % O = 100 – Σ(C, H, N, S, ash), where C, H, N, S, and ash are expressed as a percentage of the total mass. Sulfate content was quantified by ion chromatography (Metrohm 930 Compact IC Flex fitted with a Metrosep A Supp 5, 150 × 4.0 mm column and a Metrosep RP 2 Guard 3.5 mm guard column) by elution at 0.7 mL min^{-1} with 3.2 mmol L^{-1} Na_2CO_3 and 1.0 mmol L^{-1} $NaHCO_3$ using K_2SO_4 as a standard. Protein content was estimated using the nitrogen-to-protein conversion factor of 5 (% N x 5)[25].

2.2.2 Molecular Weight

Molecular weight (MW) profiles were determined using size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS). Samples (5 mg mL^{-1} in 0.1 M $NaNO_3$) were dissolved by heating at 80°C and then standing at room temperature overnight (~20 h). Soluble material was separated on three columns (TSK-Gel $G5000PW_{XL}$, $G4000PW_{XL}$ and $G3000PW_{XL}$, 300 x 7.8 mm, Tosoh Corp., Tokyo, Japan) connected in series, eluted with 0.1 M $NaNO_3$ (0.5 mL min^{-1}, 60°C). The eluted material was detected using a variable wavelength detector (280 nm), a SDL7000 MALLS detector (PSS Polymer Standards Service GmbH, Mainz, Germany) and a refractive index monitor (Agilent Technologies, Santa Clara, USA). The data for Mw determination was analysed using Win GPC Unichrom software (v8.2.1, PSS Polymer Standards Service) using a refractive index increment, dn/dc, of 0.146 mL g^{-1}.

2.2.3 Constituent sugar composition

Constituent sugar composition was determined by high-performance anion-exchange chromatography (HPAEC) after hydrolysis of the polysaccharides present to their component monosaccharides. Samples were hydrolysed with methanolic HCl (3 N, 80 °C, 18 h), followed by aqueous TFA (2.5 M, 120 °C, 1 h). The resulting hydrolysates were analysed on a CarboPac PA-1 (4 x 250 mm) column equilibrated in 20 mM NaOH and eluted with a simultaneous gradient of NaOH and NaOAc at a flow rate of 1 mL min^{-1}. The sugars were identified from their elution times relative to a standard sugar mix, quantified from response calibration curves of each sugar and expressed as µg of the anhydro-sugar (as this is the form of sugar

present in a polysaccharide) per mg of sample; the normalised mol % of each anhydro-sugar was also calculated.

2.2.4 NMR Spectroscopy

Ulvan samples were dissolved in D_2O and ^{1}H and ^{13}C (both ^{1}H coupled and decoupled) spectra were recorded on a Bruker Avance DPX-500 spectrometer. Assignments were made by comparison to literature values.

2.3 Immunomodulating Activity

2.3.1 Cell Culture

RAW264.7 murine macrophages were purchased from The European Collection of Authenticated Cell Cultures (ECACC) (Merck, USA) and maintained in a clean culture of Dulbecco's modified eagle medium (DMEM) with GLUTAMAX with the addition of 0.5 M HEPES buffer (HEPES) and 10 % foetal bovine serum (FBS) (Gibco, ThermoFisher scientific, Australia). Cells were cultured in T75 flasks at 37°C in an atmosphere of 5 % CO_2 with 100% humidity until reaching 80-90 % confluence before passage with cell scraper to dislodge cells. RAW264.7 cells were regularly screened for mycoplasma.

2.3.2 Cell viability assay

Toxicity of 2 to 2000 µg mL^{-1} of ulvan fractions was assessed over 48 h. Cells were seeded into a 96-well plate at a density of $3x10^5$ cells/well in DMEM supplemented with FBS, HEPES, 100 U penicillin/100 µg mL^{-1} streptomycin (pen/strep), and allowed to adhere for 16 h. Ulvan (dissolved in DMEM containing HEPES and pen/strep) was added through a media change in concentrations from 2 to 2000 µg mL^{-1}. The cells were then incubated for 6, 24 or 48 h. Following incubation, PrestoBlue™ reagent (Invitrogen, Thermofisher, Australia) was added to the wells to 10 % v/v. The cells were incubated for a further 60 min before fluorescence was measured (excitation, 544 nm; emission 620 nm) on a BMG POLARstar Omega microplate reader. The relative fluorescence units (RFU) were used to calculate macrophage viability by: viability % = RFU_t/RFU_c x 100, where RFU_t and RFU_c are the intensity of test and control groups, respectively. Ulvan concentrations were log_{10}-transformed, fitted using a variable slope four-parameter equation with the top value constrained to 100% using a least squares (ordinary) fit model in GraphPad Prism (v. 8.1.0).

2.3.3 Lipopolysaccharide (LPS) stimulation assay

The immunomodulating effect of ulvan fractions was assessed in lipopolysaccharide (LPS)-stimulated RAW264.7 cells by measuring the concentrations of secreted cytokines (IL-1β, IL-6, IL-10, IL-12, and TNFα) and PGE_2 in the cell culture supernatant by enzyme-linked immunosorbent assay (ELISA) (**Figure 1**). RAW264.7 cells were seeded into 24-well plates at a density of $1x10^5$ cells/well in 1000 µL of DMEM supplemented with FBS, HEPES, and pen/strep; cells were allowed to adhere for 16 h. Ulvan fractions (dissolved in DMEM

supplemented with FBS, HEPES, pen/strep, and absent phenol red) in concentrations of 1, 10 or 100 µg mL^{-1} were added through a media change. It was determined during optimisation that FBS is required for the successful release of cytokines by LPS-stimulated RAW264.7 cells (**Supp. 1**). 1 µg mL^{-1} dexamethasone (Merck, USA) served as a positive control; wells without the addition of ulvan served as negative controls.

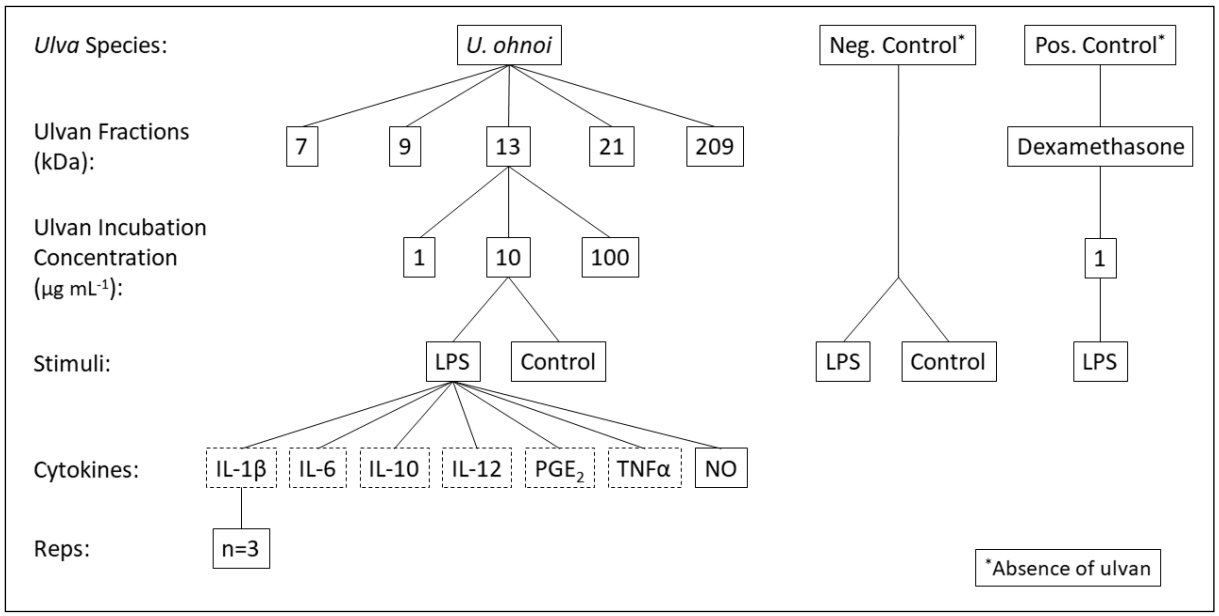


Figure 1. Experimental design of LPS stimulation assay. "Cytokines" collectively refers to cytokines and other immunological markers such as PGE_2 and NO. Cytokines with dashed borders were quantified by ELISA; NO was quantified through the Griess test.

Cells were pre-treated with ulvan or dexamethasone for 2 h before LPS from *Escherichia coli* (0111:B4; Merck, USA) was added to a final concentration of 1 µg mL^{-1}. There were an equal number of wells across all treatments that did not receive LPS-stimulation to act as internal controls. Cells were incubated for 46 h (48 h was necessary for secretion of IL-1β – see **Supp. 2B**), after which the supernatant was collected and stored at 4°C. The concentration of cytokines in the cell culture supernatant was analysed using respective ELISA kits within 6 hours of separation from cells by following the manufacturer's instructions (Abcam, UK: ab208348, 1:2-1:10 dilution; ab197742, 1:1-1:2 dilution: ab46103, 1:1-1:2 dilution; ab222503, 1:2-1:50 dilution; ab136949, 1:1 dilution; ab236717, 1:1-1:2 dilution). The optical density (OD) or relative fluorescence units (RFU) for each of the cytokine assays was read on a BMG POLARstar Omega microplate reader at the wavelength recommended by the assay manufacturer.

2.3.4 Nitric oxide assay

Nitric oxide (NO) secretion was estimated through quantification of its decomposition product, nitrite (NO_2^-) (**Figure 1**). The concentration of NO_2^- in cell supernatant was determined using the Griess reagent (modified) (Sigma, G4410). Cell supernatant collected as outlined in section 2.3.3 was mixed with equal volumes of Griess reagent (modified). The

absorbance was read at 540 nm after 15 min on a BMG POLARstar Omega microplate reader. Concentration of nitrite in cell supernatant was calculated from 0-100 µM sodium nitrite standards.

2.4 Data & Statistical analysis

Cytokine concentrations were calculated from either 4-parameter logistic regressions or linear regressions derived from the assay standards. The fit with the highest r^2 value was used. Recorded sample OD values less than three standard deviations of the mean ELISA assay blank OD value were considered to have a concentration of zero. In the rare occurrence that a treatment OD was outside the standard range of the 4-parameter fit, linear regression was used to estimate a corresponding concentration. Prior to statistical analysis, cytokine, PGE_2, and NO_2^- concentrations were normalised to a percent of the respective ulvan-free control for each plate.

The effects of ulvan Mw and concentration on RAW264.7 cells were analysed with two-factor permutational analysis of variance (PERMANOVA)[26]. Molecular weight and ulvan concentration were treated as fixed factors. Cytotoxicity of ulvan on RAW264.7 cells was analysed by a three-factor PERMANOVA with ulvan Mw, ulvan concentration, and exposure time as fixed factors. All analyses were performed in Primer v6 (Primer-E Ltd., UK) using Euclidian distance similarity and 9,999 unrestricted permutations of the raw data. *a posteriori* pair–wise comparisons were used to determine the contribution of factors to significant results. Monte Carlo P-values were used in pair-wise comparisons with less than 100 unique permutations[26].

3.0 Results

3.1 Chemical Characterisation

3.1.1 Elemental Analysis

Ulvan elemental composition, ash, sulfate ester and protein contents are presented in **Table 2**. The ratio for C, H, O, N, and S are similar between samples, with significantly lower contents of N (and protein) in the chromatographed samples compared to the crude extract. Variations in the content of ash account for the absolute content of C, H, O, N, and S. Sulfate content was the same for samples hydrolysed for up to 2 h (U209, U21, U13), reducing after 5 to 7 hours of hydrolysis (U9), and higher in crude ulvan and the permeate sample (U7).

Table 2. Elemental analysis of native and hydrolysed ulvan. Average %w/w (± S.D, where applicable). Protein content calculated from % N.

Sample ID	%C	%H	%O	%N	%S	%Ash	%Sulfate	%Protein[1]
U209 (native)	25.92	4.77	42.07	0.10	5.47	21.67	16.4 ± 0.5	0.5
U21	26.66	4.84	41.30	0.09	5.65	21.45	16.3 ± 0.7	0.5
U13	26.16	4.88	42.68	0.10	5.44	20.75	16.3 ± 0.6	0.5
U9	29.69	4.99	43.94	0.34	5.90	15.14	13.7 ± 3.8	1.7
U7	26.48	4.22	35.40	0.09	6.32	27.47	22.4 ± 1.7	0.5

[1]Based on % N x 5

3.1.2 Molecular weight

Native ulvan (U209) had a average molecular weight (Mw) of 209 kDa (**Table 3**). The average Mw and polydispersity of the hydrolysed ulvans decreased with increasing time of hydrolysis (**Table 3**, **Figure 2**).

Table 3. The duration of hydrolysis applied to crude ulvan, resulting molecular weights and polydispersity index.

Sample ID	Hydrolysis time (h)	Mw (g mol^{-1})	Mn (g mol^{-1})	Polydispersity Index
U209 (native)	0 (native ulvan)	208,936	94,954	2.2
U21	1	21,271	12,316	1.7
U13	2	13,499	9,618	1.4
U9	5 + 7	8,933	6,539	1.4
U7	Permeate	6,788	5,399	1.3

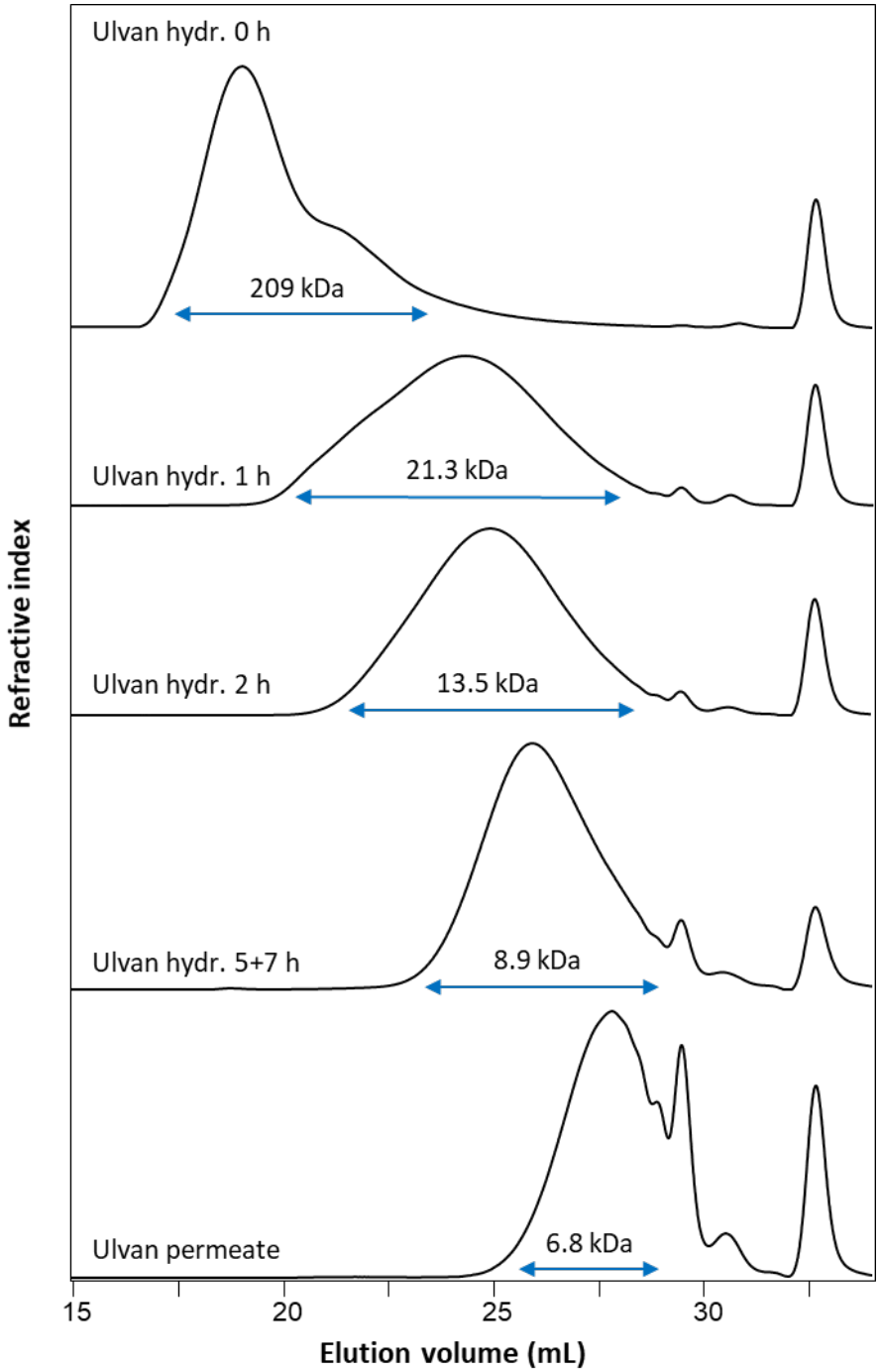


Figure 2. SEC-HPLC-RI chromatograms of the native ulvan, partially hydrolysed fractions and the permeate showing the weight average molecular weights determined by MALLS.

3.1.3 Constituent sugar composition

The total sugar content of the ulvan fractions ranged from 367 to 486 µg mg^{-1} of sample (**Table 4**). Native ulvan had the highest total sugar content (486 µg mg^{-1} sample) with the three partially hydrolysed ulvans having similar total sugar contents (453 – 478 µg mg^{-1} sample). U7 had the lowest total sugar content (367 µg mg^{-1} sample).

The ulvan fractions contained 48.8-54.7 mol % rhamnose, 32.5-35.9 mol % glucuronic acid, 4.5-7.3 mol % iduronic acid, and 3.3-5.6 mol % xylose (**Table 5**). U9 had the highest content of rhamnose (54.7 mol %), with the lowest content of xylose (3.3 mol %), glucuronic acid (32.5 mol %), iduronic acid (4.5 mol %), in addition to the highest content of trace sugars. Smaller amounts of other sugars including fucose, arabinose, galactose, glucose, and mannose were also present in all fractions.

Table 4. Constituent sugar composition and total sugar content (μg mg^{-1} sample) of purified, native ulvan, partially hydrolysed fractions and the permeate.

Sample ID	Sugars (μg mg^{-1} preparation)[a]									
	Rha	Xyl	GlcA	IdoA	Fuc	Ara	Gal	Glc	Man	Total
U209 (native)	218.6	22.5	194.0	38.7	0.5	0.2	10.3	1.2	-	486.1
U21	223.2	21.9	183.6	36.5	0.5	0.2	10.1	1.5	0.5	478.1
U13	213.1	18.4	176.7	31.7	0.6	0.2	9.9	1.6	0.4	452.6
U9	233.4	12.7	167.1	22.9	2.1	0.6	14.8	4.3	1.7	459.5
U7	178.3	15.1	137.0	29.8	0.1	0.2	4.7	0.7	1.1	367.0

[a] Values are the averages of duplicate analyses

Table 5. Constituent sugar composition (normalised mol %) of purified, native ulvan, partially hydrolysed fractions and the permeate.

Sample ID	Sugars (normalised mol %)[a]								
	Rha	Xyl	GlcA	IdoA	Fuc	Ara	Gal	Glc	Man
U209 (native)	48.8	5.6	35.9	7.2	0.1	0.1	2.1	0.2	-
U21	50.5	5.5	34.5	6.9	0.1	tr	2.1	0.3	0.1
U13	51.0	4.9	35.1	6.3	0.1	0.1	2.1	0.3	0.1
U9	54.7	3.3	32.5	4.5	0.5	0.1	3.1	0.9	0.4
U7	52.5	4.9	33.5	7.3	tr	0.1	1.2	0.2	0.3

[a] Values are the averages of duplicate analyses

3.1.4 NMR spectroscopy

Both ^{1}H and ^{13}C NMR spectra presented here are consistent with characteristic ulvan spectra[9, 27-34]. Spectra from native and partially hydrolysed ulvan samples were recorded in D_2O (**Figure 3** and **Figure 4**) without solvent suppression hence the large resonance recorded at δ 4.7 ppm for HDO.

The ^{13}C NMR shows the anomeric signals for glucuronic acid (104 ppm) and rhamnose (100 ppm) of the major ulvan disaccharide component, A_{3s} [-4-GlcA-1,4-Rha(3S)-1-] (**Figure 3**). Signals for rhamnose C5, C2, and C3/C4 from A_{3s} are also visible at 68, 69, and 78 ppm, respectively. The major peak at 74 ppm is consistent with C3/C2 of glucuronic acid in A_{3s}, while C5 and C4 of the same monosaccharide are responsible for the minor peaks at 76 and 79 ppm[9, 31]. The minor peak at ~71 ppm, particularly visible on the U13 spectra, is consistent with C2/C5 of iduronic acid from the other major ulvan disaccharide component, B_{3s} [-4-IdoA-1,4-Rha(3S)-1-][9, 31]. The intensity of rhamnose and glucuronic acid signals is consistent with the large proportion of these sugars reported in **Table 4**. Further, it is also consistent with a high proportion of the A_{3s} disaccharide.

The strong ^{1}H resonance at δ 1.3 ppm from the protons of the rhamnose methyl group (C6) is consistent with the large proportion of this sugar reported in **Table 4** (**Figure 4**). The ^{1}H NMR peaks at 3.3 ppm and 3.7 ppm are due to H2 and H3/H4 of glucuronic acid, respectively[9, 31]. Proton resonances at 3.8-3.9 ppm are likely a combination of a H4 peak from rhamnose and H5 peak of glucuronic acid[9, 31]; these peaks are more highly resolved and separated in the U13 and U7 spectra. The major peak at 4.2 ppm is due to H2 of rhamnose, and the minor peak to the right at 4.1 ppm due to H5[9, 31].

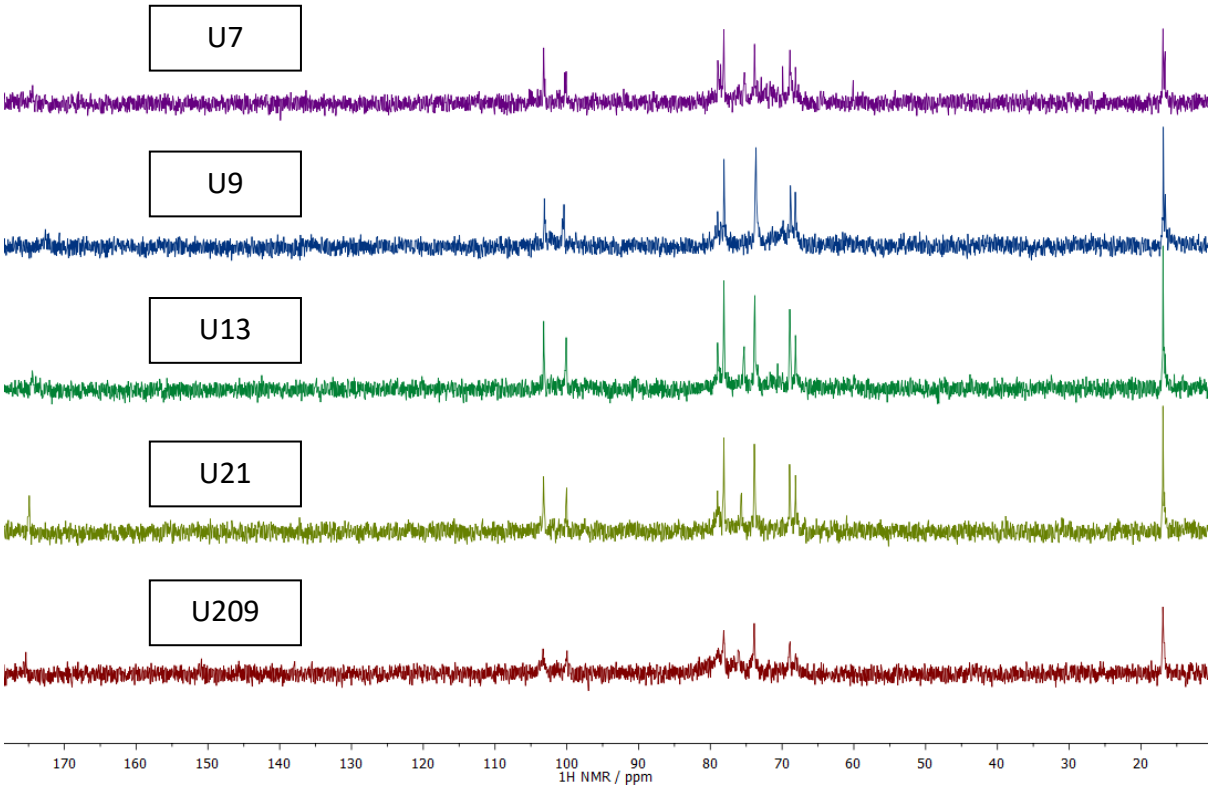


Figure 2. ^{13}C NMR data of native and hydrolysed ulvan preparations (25 mg mL^{-1}, D_2O, 25°C, 125 MHz).

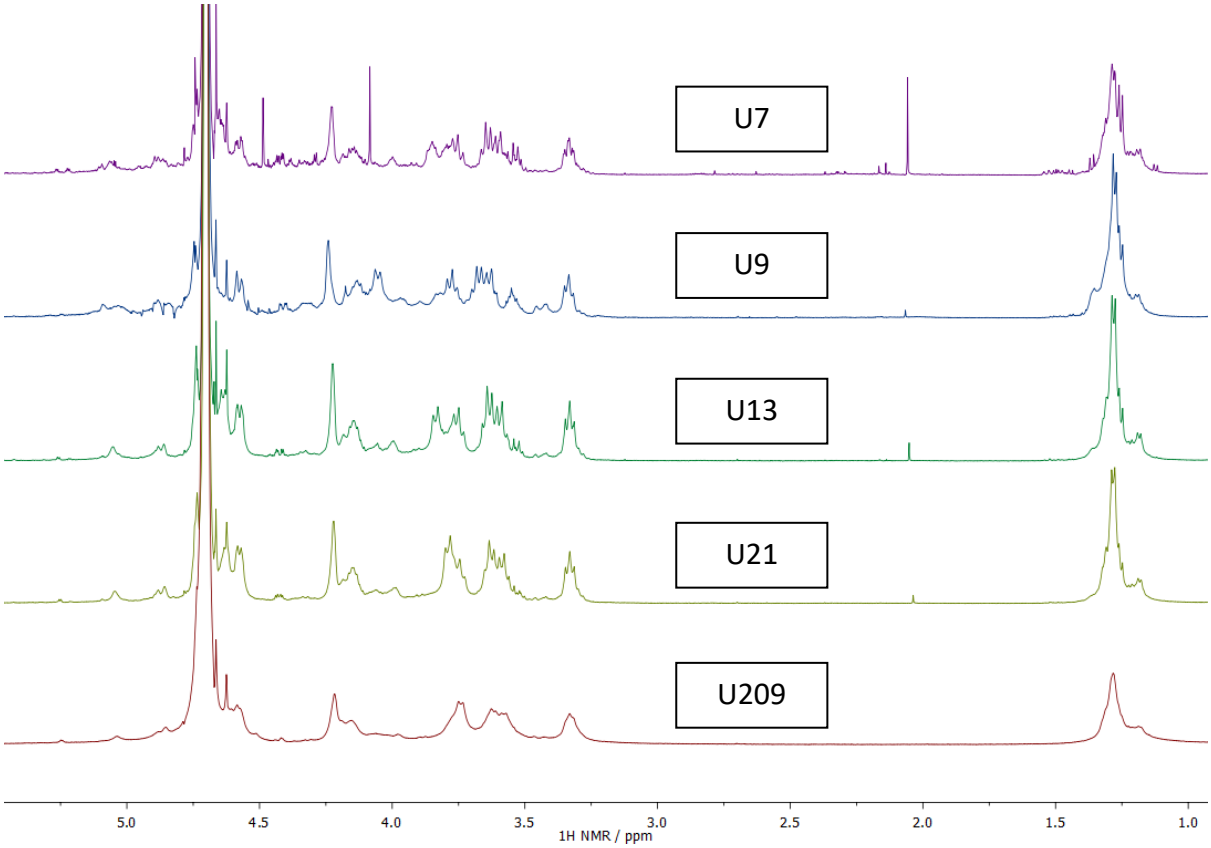


Figure 3. ^{1}H NMR data of native and hydrolysed ulvan preparations (25 mg mL^{-1}, D_2O, 25°C, 500 MHz, HDO peak not suppressed).

3.2 Immunomodulating activity

3.2.1 Ulvan cytotoxicity

All ulvan fractions showed no toxicity on RAW264.7 cells at concentrations below 100 µg mL^{-1} over 48 h (**Figure 5**). The cytotoxic effect of U9, U13, U21, and U209 increased with time for concentrations > 100 µg mL^{-1}, while the lowest Mw ulvan, U7, showed no cytotoxicity at any concentration up to 48 h (**Figure 5** A, B, C). This deviation in cytotoxicity with ulvan Mw, ulvan concentration and exposure time produced a significant three-way interaction (pseudo-$F_{88,360}$ = 13.15 $P < 0.01$).

After 6 h, > 100 µg mL^{-1} of ulvan induced cell proliferation, with up to 33 % increase in cell viability induced by 2000 µg mL^{-1} of U209 (**Figure 5** A). After 24 h, all ulvan fractions decreased cell viability at higher concentrations, except for U7. The lowest 24 h viability observed was 75% by 500 µg mL^{-1} of U21 and U209 ulvan (**Figure 5** B). With the exception of U7, the reduction in cell viability by ulvan became pronounced ≥ 100 µg mL^{-1} (**Figure 5** C). 500 µg mL^{-1} of ulvan was sufficient to reduce cell viability to 70 % for U9 and to 50 % for larger Mw ulvans (U12, U21, and U209); these reduced viabilities respectively remained at 70 % and 50 % up to the highest concentration of ulvan tested (2000 µg mL^{-1}). The dose-dependent

increase in cytotoxic effect of high concentration (100-500 µg mL^{-1}) ulvan through time was also confirmed during pilot assays (**Supp. 3**).

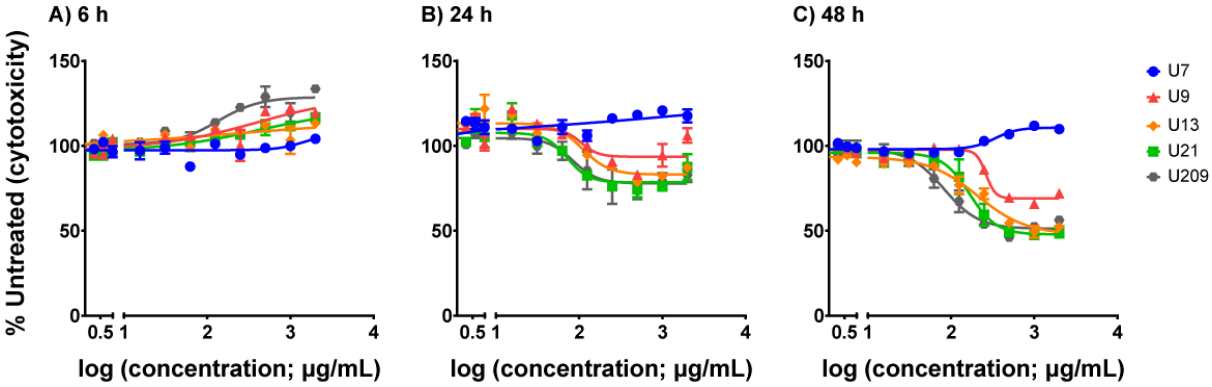


Figure 4. Effect of ulvan fractions on RAW264.7 cell viability. Cell proliferation was measured after incubation with ulvan of differing molecular weights (U7, U9, U13, U21, and U209) at concentrations of 2-2000 µg mL^{-1} for (A) 6 h, (B) 24 h and (C) 48 h.

3.2.2 Effect of ulvan in modulating inflammatory response

3.2.2.1 Inflammatory cytokines

High concentration of the two highest Mw fractions consistently resulted in the greatest levels of IL-1β, IL-6, IL-10 and IL-12 from RAW264.7 cells (**Figure 6** A-D). For IL-10, each concentration of U21 and U209 (1, 10, and 100 µg mL^{-1}) produced significantly higher levels of IL-10 compared to ulvan-absent controls (pseudo-$F_{13,42}$ = 4.28, $P < 0.001$), while the lower Mw fractions resulted in no change or a reduction in levels of IL-10 (**Figure 6** A).

High Mw ulvan fractions at high concentrations increased the secretion of IL-1β, IL-6, and IL-12 above that of ulvan-absent controls. The level of IL-1β was increased by 44 and 32 % in treatments with 100 µg mL^{-1} of U13 and U209, respectively (**Figure 6** B) (pseudo-$F_{13,46}$ = 15.52, $P < 0.001$). Additionally, 100 µg mL^{-1} of U209 also increased the level of IL-6 by 7% - the only treatment to significantly increase the level of IL-6 above the ulvan-absent control (**Figure 6** C) (pseudo-$F_{13,46}$ = 12.88, $P < 0.001$). As with IL-6, significantly more IL-12 was produced in treatments with 100 µg mL^{-1} of U13 and U21 (pseudo-$F_{13,46}$ = 7.95, $P < 0.001$). However, lower concentrations of high Mw ulvan also increased IL-12 levels (**Figure 6** D).

Low concentrations of ulvan, often combined with low molecular weights, resulted in the lowest levels of cytokine production. For example, IL-10 was reduced by 59 and 55 % from 1 µg mL^{-1} of U7 and U9, respectively (**Figure 6** A) (pseudo-$F_{13,42}$ = 4.28, $P < 0.001$). Levels of IL-6 were also reduced by low concentrations: by 10-15 % for U13, U21, and U209 (pseudo-$F_{13,46}$ = 12.88, $P < 0.001$) (**Figure 6** C). Contrary to IL-6 and IL-10, the lowest level of IL-1β (13 % lower than the ulvan-absent control) was at a high concentration (100 µg mL^{-1}) of low Mw ulvan (U7) (**Figure 6** B) (pseudo-$F_{13,46}$ = 15.52, $P < 0.001$).

Dexamethasone (1 µg mL^{-1}) significantly reduced the level of IL-1β, IL-6, and IL-12 to a greater extent than any ulvan treatment (**Figure 6** B-D). Conversely, dexamethasone significantly increased IL-10 level by 40% (**Figure 6** A). These effects of dexamethasone are consistent with the literature[35, 36]. Basal expression (cytokine levels in the absence of LPS) of IL-1β, IL-6, and IL-12 were at least an order of magnitude lower than LPS-treated cells. Further, basal IL-10 levels were 35% lower, on average, in the absence of LPS compared to LPS treated cells and showed no effect of Mw or concentration.

TNFα was released in concentrations far exceeding that recorded during optimisation trials (**Supp. 2**). The levels of TNFα for all LPS treatments in the LPS stimulation assay were above the OD detection limit for the microplate reader, resulting in unusuable data. No basal TNFα levels were recorded.

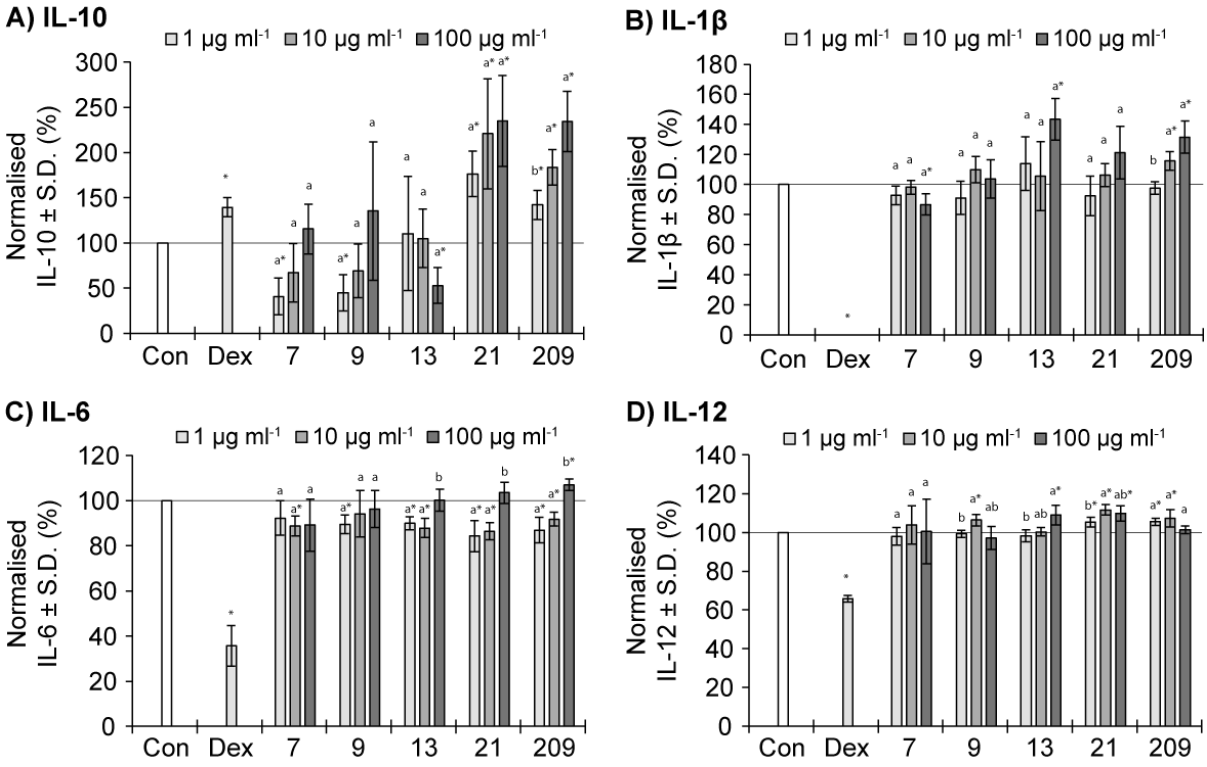


Figure 5: Percent normalised concentrations of interleukins (IL) (A) IL-10, (B) IL-1β, (C) IL-6, and (D) IL-12 (±S.D., n=3) measured in RAW264.7 cell supernatant following exposure to ulvan of differing molecular weights (7, 9, 13, 21, 209 kDa) at 1, 10 and 100 µg mL^{-1} in the presence of 1 µg mL^{-1} LPS. 1 µg mL^{-1} Dexamethasone (Dex) used as a positive control. Control (Con) contained 1 µg mL^{-1} LPS in the absence of ulvan or dexamethasone. Superscript letters (a, b) indicate significant difference between concentrations within a molecular weight fraction; Asterisk (*) indicates significant difference from control.

3.2.2.2 PGE_2 and NO_2^-

The levels of PGE_2 were consistently reduced when ulvan was applied to RAW264.7 cells stimulated by LPS; further reductions occurred at higher concentrations of ulvan (**Figure 7** A). A dose-dependent reduction in PGE_2 occurred when averaged across ulvan molecular weights (pseudo-$F_{3,45}$ = 8.21, P < 0.001), culminating with 100 µg mL^{-1} of U9, U21, and U209 inducing reductions of 24, 29, and 39 %, respectively. The proportional reduction in PGE_2 induced by 100 µg mL^{-1} of U209 (39 %) was very close to the 42 % reduction induced by 1 µg mL^{-1} of dexamethasone. In contrast, lower concentrations of ulvan resulted in reduced concentrations of nitrite (NO_2^-), on average (**Figure 7** B). 1 and 10 µg mL^{-1} of ulvan resulted in significantly lower nitrite levels compared to either ulvan-absent controls or 100 µg mL^{-1} of ulvan (pseudo-$F_{3,46}$ = 7.18, P < 0.001). There was no significant difference between 100 µg mL^{-1} of ulvan and the ulvan-absent controls.

Dexamethasone (1 µg mL^{-1}) significantly reduced secretion of PGE_2, but had no significant effect on nitrite secretion. On average, dexamethasone produced significantly less PGE_2 than 9, 13 and 21 kDa ulvan (pseudo-$F_{5,45}$ = 3.39, P < 0.05). The nitrite level in the absence of LPS increased by 2-8 times with 100 µg mL^{-1} of 9, 13, 21 and 209 kDa ulvan compared to any other respective concentration. No PGE_2 was detected in the absence of LPS.

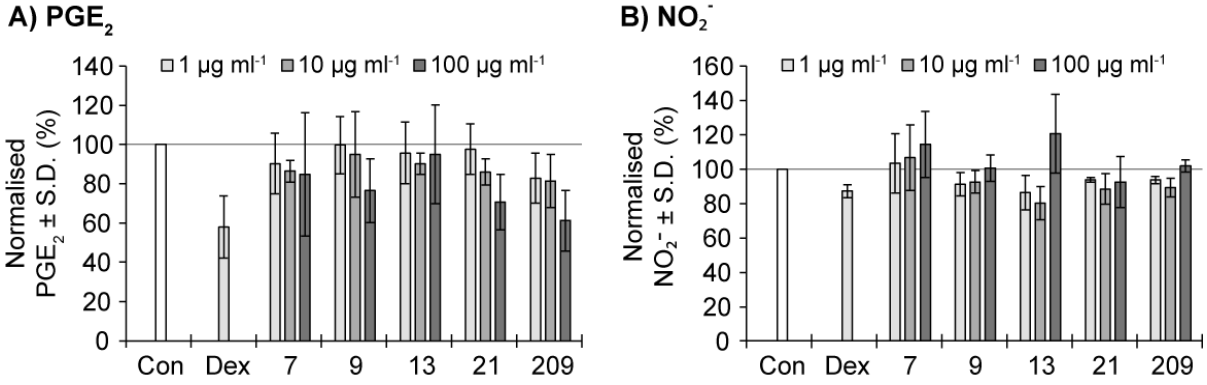


Figure 6. Percent normalised concentration of (A) prostaglandin E2 (PGE_2) and (B) nitrite (NO_2^-) (± S.D., n=3) measured in RAW264.7 cell supernatant following exposure to ulvan of differing molecular weights (7, 9, 13, 21, 209 kDa) at 1, 10 and 100 µg mL^{-1} in the presence of 1 µg mL^{-1} LPS. Nitrite measured with Griess reagent using $NaNO_2$ (0-100 uM) as standard. 1 µg mL^{-1} Dexamethasone (Dex) used as a positive control. Control (Con) contained 1 µg mL^{-1} LPS in the absence of ulvan or dexamethasone. Statistical analysis not possible due to lack of statistical interaction between ulvan concentration and Mw.

4.0 Discussion

Ulvan from *Ulva ohnoi* was depolymerised into fractions of 7, 9, 13, 21, and 209 kDa (named U7, U9, U13, U21, and U209, respectively). These fractions were chemically characterised and their cytotoxic and immunomodulating effect on RAW264.7 murine macrophages assessed. No ulvan fraction showed toxicity to RAW264.7 cells at concentrations up to 100 μg mL^{-1} over 48 h. Higher molecular weight fractions of ulvan elicited a greater immunomodulatory response at 100 μg mL^{-1} (by increasing IL-10, IL-1β and IL-6, and decreasing PGE2) compared to lower Mw ulvan fractions. Ulvan extracted from *Ulva ohnoi* is non-cytotoxic and has a mild anti-inflammatory capacity.

4.1 Chemical Characterisation

Minor variation in elemental composition, ash, and sulfate ester content confirmed only small changes in the composition between ulvan fractions. The ash content for ulvan fractions ranged from 15-21 % ash for U9-U209, while U7 had an ash content of 27%. The latter was due to the ulvan being analysed and tested without desalting. The sulfate ester content ranged from 13.7-22.4 %, which is consistent with previously analysed ulvan from *U. ohnoi* (7.1 to 15.7 %)[27, 37] and the median from the ulvan literature (15.5 %)[7]. Depolymerisation had no effect on sulfate ester content for ulvan hydrolysed for 0-2 h (U209, U21, and U13), however a lower content was measured for U9 which was hydrolysed for 5-7 h. The apparent low sulfate content detected for U9 could be a result of the high variability in the sulfate measurement. In support of this, the S content for U9-U209 is consistent, reinforcing no change in sulfate ester content in ulvan fractions over the full 0-7 h hydrolysis period.

Chemical depolymerisation of ulvan with H_2O_2 was the chosen method as it has been used to selectively depolymerise related algal sulfated polysaccharides[37, 38]. In this study, SEC-MALLS measurements confirmed that H_2O_2 successfully depolymerised native ulvan affording five different molecular weight fractions ranging from 7 to 209 kDa. ^{1}H and ^{13}C NMR spectra of the fractions were both dominated by resonances consistent with ulvan samples that were rich in the disaccharide A_{3s} [-4-GlcA-1,4-Rha(3S)-1-]. The degree to which the spectra from the native and hydrolysed ulvan variants overlap in both ^{1}H and ^{13}C NMR indicate that the only major chemical modification is at the anomeric centre. However, further chemical characterisation of these fractions detected minor differences in composition; in particular, small variations in labile constituent sugars were detected.

Constituent sugar analysis confirmed that the sulfated polysaccharide extracted here from *U. ohnoi* and its hydrolysed products were consistant with the broader literature definition of ulvan[7, 27]. Furthermore, a high content of both rhamnose and glucuronic acid confirmed the NMR finding that ulvan from *U. ohnoi* is rich in the disaccharide A_{3s} [-4-GlcA-1,4-Rha(3S)-1-]. However, constituent analysis also detected incremental degradation of xylose and iduronic acid, with concomitant increase in the content of rhamnose, as the duration of hydrolysis was increased. This is consistent with previous reports that xylose and

iduronic acid are the most labile of the ulvan constituents[39]. The small changes in rhamnose and glucuronic acid (the two major constituent sugars) indicate only minor variations in constituent sugars between fractions, particularly relative to the differences in molecular weight.

Thus, in terms of cytotoxicity and immunomodulation, the composition of ulvan is considered to be equal between hydrolysed fractions. Differences in the bioactivity of these fractions are therefore interpreted on the basis of concentration and molecular weight.

4.2 Cytotoxicity and Immunomodulating Activity

All ulvan Mw fractions showed no cytotoxic effects on RAW264.7 cells after 48 h of exposure with < 100 μg mL^{-1}. Furthermore, for all fractions, cell viability was only reduced to 50 % with a 5- to 20-fold increase in ulvan concentration. These results are consistent with previous cytotoxic assessments of ulvan-containing extracts on murine macrophages and cancer cells[13-17]. In the present study, the lowest Mw ulvan (U7) displayed no cytotoxicity over 48 h (up to and including the highest concentration tested, 2000 μg mL^{-1}) and instead increased cell proliferation by 10 %, and 18 % for the same concentration over 24 h. A similar 20% increase in cell proliferation is also observed for the lowest molecular weight ulvan fraction (29 kDa) from *U. intestinalis*[16]. The low cytotoxicity of all ulvan Mw fractions allowed the immunomodulation capacity of ulvan to be assessed on RAW264.7 cells with an upper concentration limit of 100 μg mL^{-1}.

Immunomodulatory effects have been reported for ulvan samples from *U. armoricana*[40, 41], *U. clathrata*[42], *U. fasciata*[43, 44], *U. intestinalis*[12, 14, 16, 45], *U. lactuca*[19, 46, 47], *U. linza*[48], *U. ohnoi*[37], *U. australis*[15, 21], *U. prolifera*[17, 20], and *U. rigida*[18, 49]. In this study, ulvan from *U. ohnoi* had immunomodulatory effects on RAW264.7 cells that were both concentration dependent and influenced by Mw.

Increased levels of IL-10 and IL-1β, and to a lesser extent IL-6 and IL-12, were observed at high concentrations of the high molecular weight fractions (U21 and U209), a response that is consistent with the literature[13-15, 18, 45]. IL-10, a predominately anti-inflammatory cytokine that inhibits cytokine production and down-regulates inflammation, had the largest magnitude of change, increasing by 135 % from the treatment of U209 at 100 μg mL^{-1}, suggesting an anti-inflammatory effect of ulvan. PGE_2 is a principal protagonist in the inflammatory response due to its hyperalgesic, pyrogenic, and potent vasodilator capacities[50]. The dose-dependent reduction of PGE_2 levels below the control (up to 40% reduction with 100 μg mL^{-1} of U209) also suggests an anti-inflammatory response. The reduction in PGE_2 is particularly notable as in a similar study on RAW264.7 cells, ulvan from *U. rigida* stimulated a dose-dependent increase in PGE_2 levels from < 10 pg mL^{-1} up to ~270 pg mL^{-1} (a 2700 % increase) in treatments of 0 to 100 μg mL^{-1} of polysaccharide[18]. However, increased levels of the pro-inflammatory cytokines IL-1β, IL-6, and IL-12 detected in the current study would probably contradict the anti-inflammatory response of IL-10 and PGE_2. The precise

immunomodulatory nature of ulvan *in vivo* cannot be conclusively predicted from this study alone, however, these *in vitro* results support previous findings that ulvan possesses immunomodulating activity[13-16, 18].

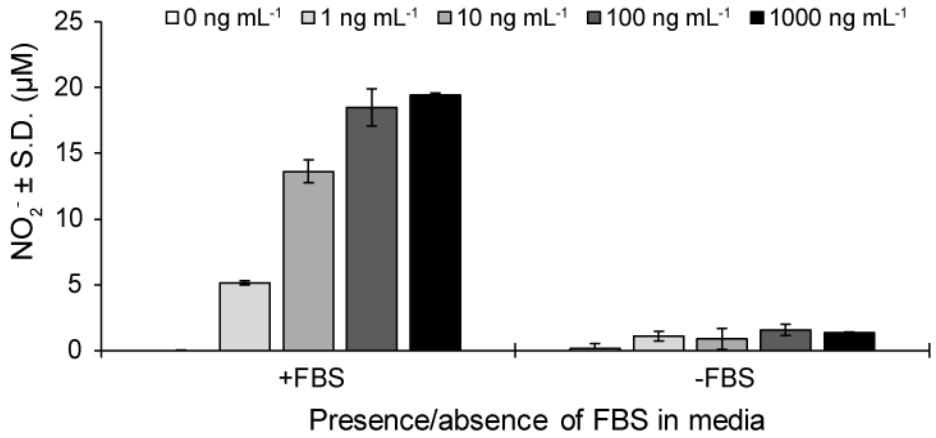
Although the mechanism of interaction between ulvan and LPS on RAW264.7 cells cannot be determined from the current data, molecular weight evidently plays a role in the cytotoxicity and immunomodulating capacity of ulvan. However, the apparent effect of ulvan Mw in the literature is contradictory, with some studies finding enhanced immunomodulating activity from higher Mw ulvan[13, 15, 37] while others found higher activity with lower Mw ulvan[12, 14, 16]. It is important to note that while these studies did use similar dose concentrations of ulvan, they did not study the effect of ulvan Mw in isolation. Furthermore, variation in the proportions of constituent sugars, proteins, and sulfate esters could affect immunomodulating activity. In the current study, we did assess the effect of ulvan Mw in isolation. The fact that the native (i.e. unhydrolysed) ulvan had the greatest immunomodulating effect is ideal for future studies and potential future commercial applications as minimal processing reduces production costs and time.

The mild immunomodulating activity and enhanced cell proliferation effects of ulvan, coupled with the capacity of mammalian skin fibroblast and keratinocyte cells to directly recognise and respond to rhamnose[5, 51, 52], suggest that ulvan may be applicable for topical treatments (e.g. wound dressing). However, while the *in vitro* results presented here are encouraging, *in vivo* assessment of ulvan will provide a more complete picture of ulvan immunomodulatory effects, and related anti-cancer[30, 53], anti-oxidant[22, 48], and antihyperlipidemic[54-56] effects.

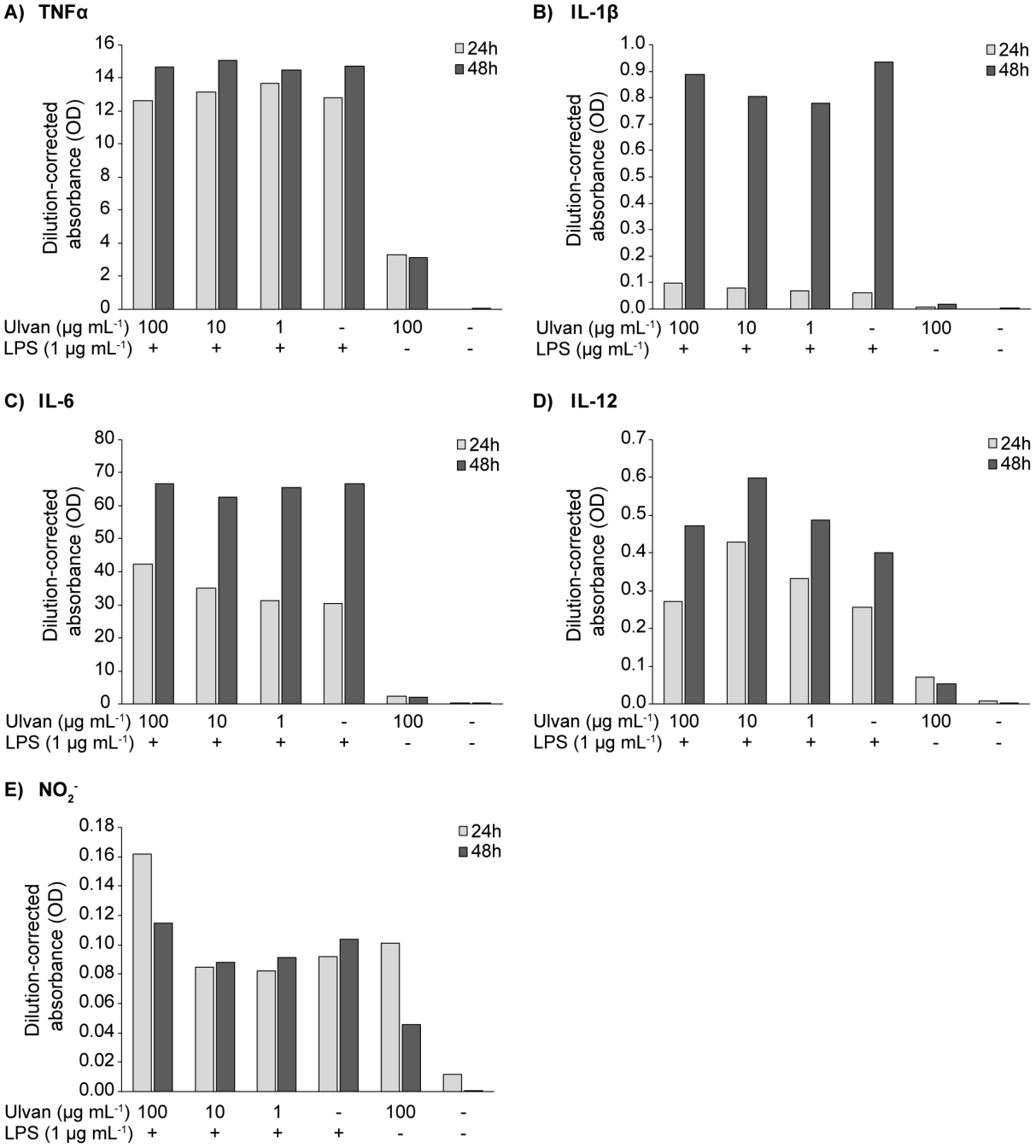
4.3 Summary and Conclusion

We assessed the influence of the molecular weight of ulvan, extracted from *Ulvan ohnoi*, on cytotoxicity and immunomodulatory activity. Ulvan Mw and concentration interacted to influence the cytotoxicity in RAW264.7 murine macrophages, and level of signalling molecules released. Depolymerisation appears to make the ulvan more benign; the unhydrolysed 'native' ulvan was the most bio-active, particularly at high concentrations. However, low molecular weight ulvan enhanced cell proliferation with little to no detectable immunomodulation activity. To summarise, due to the promotion of cell viability, low cytotoxicity at low concentrations, a moderate immunomodulating activity, and innate capacity to interact with human skin immunomodulatory cells, ulvan has multiple characteristics ideal for applications targeting the treatment of dermal diseases and wounds.

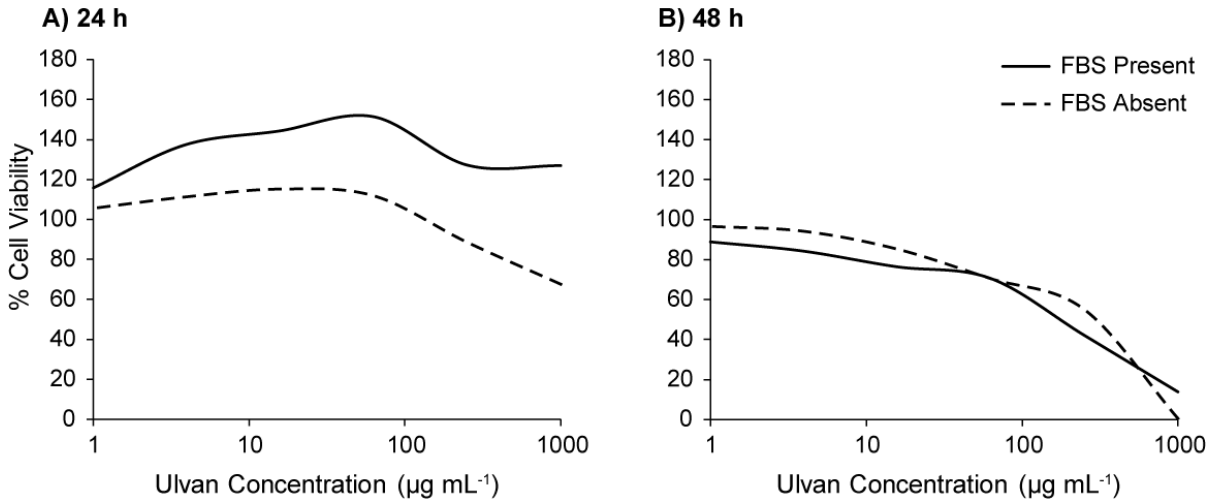
5.0 Supplementary Data



Supp. 1. The concentration of NO_2^- secreted into cell culture media in response to a range of lipopolysaccharide (LPS) concentrations (0, 1, 10, 100, 1000 ng mL^{-1}) in the presence or absence of foetal bovine serum (FBS) in the culture media during the assay. RAW264.7 cells seeded at $3x10^5$ cells mL^{-1} in 24-well plates; cells incubated in 100% humidity and 5% CO_2 for 16 h to allow for adherence and 24 h following addition of LPS; DMEM with HEPES and pen/strep with or without 10% FBS used as media. NO_2^- concentration measured by Griess reagent with absorbance at 540 nm. See main text for further definition of abbreviations.



Supp. 2. Cytokines secreted into RAW264.7 supernatant following exposure to ulvan of varying concentrations (-, 1, 10 or 100 μg mL^{-1}) in the presence (+) or absence (-) of 1 μg mL^{-1} of LPS for 24 and 48 hours. Absorbance for TNFα **(A)**, IL-1β **(B)**, IL-6 **(C)** and IL-12 **(D)** were determined by ELISA; absorbance for NO_2^- **(E)** determined by addition of Griess reagent. Absorbance presented as dilution-corrected units (i.e. absorbance multiplied by the dilution factor of the supernatant required to enable spectrophotometric analysis). RAW264.7 cells seeded at $3x10^5$ cells mL^{-1} in 96-well plates; cells incubated in 100% humidity and 5% CO_2 for 16 h to allow for adherence and 24 or 48 h following addition of ulvan and LPS; DMEM with HEPES, pen/strep and 10% FBS used as media. See main text for further definition of abbreviations.



Supp. 3 The effect of FBS during cytotoxic assessment of ulvan on RAW264.7 cells. Cell proliferation was measured after incubation with U209 ulvan at concentrations of 1-1000 µg mL^{-1} in the presence or absence of 10 % FBS for (A) 24 h and (B) 48 h. It was concluded that FBS was required for accurate ulvan cytotoxicity data, and that a dose-dependent increase in cytotoxic effect of high concentration ulvan through time was present.

6.0 References

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