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Abstract

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Development of rhamnose-rich hydrogels based on sulfated xylohamno-uronic acid toward wound healing application

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Abstract

An array of biological properties is demonstrated in the category of extracts broadly known as ulvans, including antibacterial, anti-inflammatory and anti-coagulant activities. However, the development of this category in biomedical applications is limited due to high structural variability across species and a lack of consistent and scalable sources. In addition, the modification and formulation of these molecules is still in its infancy with regard to progressing to product development. Here, a sulfated and rhamnose-rich, xylorhamno-uronic acid (XRU) extract from the cell wall of a controlled source of cultivated, Australian ulvacean macroalgae resembles mammalian connective glycosaminoglycans. It is therefore a strong candidate for applications in wound healing and tissue regeneration. This study targets the development of polysaccharide modification for fabrication of 3D scaffolds for skin cell (fibroblast) culture. The XRU extract is methacrylated and UV-crosslinked to produce hydrogels with tuneable mechanical properties. The hydrogels demonstrate high cell viability and support cell proliferation over 14 days, which are far more functional than comparable alginate gels. Importantly, an XRU-based bioink is developed for extrusion printing 3D constructs both with and without cell encapsulation. These results highlight the close to product potential of this rhamnose-rich XRU extract as a promising biomaterial toward wound healing. Future studies should be focused on in-depth *in vitro* characterizations to examine the role of the material in dermal extracellular matrix (ECM) secretion of 3D printed structures, and *in vivo* characterizations to assess its capacity in supporting wound healing.

Keywords: sulfated polysaccharide, rhamnose, ulvans, 3D printing, wound healing

Introduction

Ulvacean macroalgae are infamously associated with green tide phenomena and have been explored extensively as a potential renewable and economic biomass resource.¹ A key area of interest has been the utilization of the cell wall polysaccharides, which are broadly known as ulvans. Ulvans are naturally sulfated and demonstrate a number of biological activities.² In particular ulvans exhibit a similarity to the glycosaminoglycan polysaccharides in mammalian tissue; specifically dermatan sulfate and chondroitin sulfate.^{2,3} The fabrication of new biomaterials that might take advantage of this similarity should enable applications in tissue regeneration or wound healing. To date early research is promising yet underdeveloped.^{3,4} This is largely due to high variability of the chemical structure and composition across species and a lack of consistent and scalable sources.^{2,5} Further, fabrication into functional materials will require a number of processes, including for example structural modification to enable stable gel formation and transfer to scalable fabrication processes, and consistency of function through all of the stages.

Ulvans constitute primarily monosaccharide units of rhamnose, xylose and uronic acids, and sulfates. Structurally, ulvan is dominated by repeating disaccharide units, where uronic acid, either D-glucuronic acid or L-iduronic acid, or D-xylose is linked to L-rhamnose-3-sulfate through 1, 4-glycosidic bonds.^{2,5} Sulfate groups exist typically in the L-rhamnose units at the position C3 or in the D-xylose units.⁵ In comparison with other algal polysaccharide categories such as alginate⁶ and fucoidan⁷ from phaeophycean seaweeds, and carrageenan from rhodophycean red seaweed,⁸ ulvan is unique in its L-rhamnose-rich structure. Further there is a high degree of variability in the molecular ratio of rhamnose in ulvans from different species.⁹ Studies have shown that L-rhamnose-binding lectins are present on the cell membranes of human skin fibroblasts and keratinocytes whose cellular activities can be regulated by L-rhamnose-lectin interactions.^{10,11} Further, ulvan contains D-glucuronic acid and its epimer L-iduronic acid units, resembling the mammalian glycosaminoglycans (GAGs) that are found in human skin extracellular matrix (ECM), such as heparin, heparan sulfate,

dermatan sulfate and hyaluronic acid.² These mammalian GAGs play vital roles in regulating cellular events associated with wound healing.¹² It is for these properties that ulvans are considered a potential analogue to natural GAGs (Fig. 1), that include binding, structural support and protection of protein ligands such as collagen, growth factors and cytokines, with the benefits of increased stability¹³ in a proteolytic environment like a wound and avoidance of animal sources in medical devices. Lastly, ulvan exhibits diverse biological activities such as antibacterial, antiviral, anti-inflammatory and anticoagulant activities.² With this combination of features, ulvan makes a strong candidate for the application in wound healing.

Conditions of chronic wounds necessitate clinical intervention to accelerate wound healing processes.¹⁴ To this end, skin grafts and bioengineered skin substitutes have been developed. While traditional skin grafts have their inherent limitations,¹⁴ skin tissue engineering offers wider options and have already delivered many skin substitutes for clinical use.¹⁵ However, these skin products also face miscellaneous problems arising from the use of animal-derived biomaterials, such as collagen and decellularized skin tissue *etc.* The potential safety concern raised by these biomaterials is considered as the first issue coming before their mechanical weakness and lack of long-term biostability.^{16,17} In this respect, algae-derived biomaterials, such as alginate, have already been developed into wound healing products due to low immunogenicity and versatile properties.⁶ However, alginate is primarily used in passive wound dressings to absorb excessive exudates,¹⁸ due to its poor cell-matrix interactions.¹⁹

The water-soluble extract used in this study is a rhamnose-rich xylorhamno-uronic acid (XRU) that is extracted from a genetically barcoded species of Australian ulvacean macroalgae. The objective of this work is to provide a foundation for our ongoing research program to develop marine-based bioprinting platforms for wound healing applications. Specifically, we address the limitations in the modification and formulation of XRU with regard to progressing to product development. XRU was

modified by methacrylation, and then photo-crosslinked to produce XRU hydrogels. The hydrogels were assessed in terms of physicochemical properties and cytocompatibility against a relevant cell model, human dermal fibroblasts (HDFs). To enable fabrication of 3D XRU-based structures in a spatially controlled manner, a XRU-based bioink was developed for 3D extrusion printing. Both 3D printed hydrogel scaffolds and cell-laden constructs were developed with excellent cytocompatibility with HDFs. These results highlight the potential of this sulfated, rhamnose-rich XRU extract as a promising biomaterial toward wound healing applications.

Materials and methods

Materials

The sulfated, rhamnose-rich xylorhamno-uronic acid (XRU) extract (PhycoDerm™) with a molecular weight of > 600 kDa was provided by Venus Shell Systems Pty. Ltd. (VSS, Australia). The proximate composition and key characteristics are provided in Table 1. N,N-dimethylformamide (DMF), ethanol, sodium hydroxide and calcium chloride dihydrate were purchased from Chem-Supply. Methacrylic anhydride, deuterium oxide (D₂O), alginic acid sodium salt (4-12 cP, 1 % in H₂O, 25 °C), gelatin from porcine skin (gel strength 300, Type A) and lithium phenyl-2, 4, 6-trimethylbenzoylphosphinate (LAP) were purchased from Sigma-Aldrich. All reagents were used as received.

Modification of the XRU extract with methacrylic anhydride

Methacrylation of the XRU extract was conducted using a method previously described^{1,20} with some minor modification. Briefly, the XRU extract was dissolved in a binary solvent system composed of Milli-Q water and DMF (50/50, v/v), followed by the addition of methacrylic anhydride (MA, 5 molar equivalents of disaccharide repeating units of XRU). The reaction mixture was stirred at room temperature, and the pH was maintained at 8.0-8.5 for the first 2 h by the addition of 5 M NaOH. The reaction mixture was then stirred for another 22 h at room temperature before being precipitated into

ethanol. The precipitate was collected by centrifugation, redissolved in deionized water, dialysed against deionized water for 48 h, and then lyophilized. The methacrylated XRU extract (XRU-MA) was stored at -20 °C for further analysis.

Structural characterization was performed by Fourier Transform Infrared Spectroscopy (FT-IR) and ^1H nuclear magnetic resonance (NMR). FT-IR spectra were recorded in the range of 4000–700 cm^{-1} at a resolution of 4 cm^{-1} on an IRPrestige-21 spectrometer (Shimadzu, Japan) attached with an attenuated total reflection (ATR) component. Prior to FT-IR analysis, the sample was dried in a vacuum oven at 40 °C overnight. ^1H NMR spectra were recorded in D_2O at 60 °C using a Bruker Avance III 400 MHz spectrometer (Bruker, Germany). The degree of substitution (DS), defined as the average number of introduced methacrylate group per XRU disaccharide unit, was estimated approximately by ^1H NMR by calculating the ratio of integration areas of proton peaks from the methacrylate groups to that of the methyl groups of the L-rhamnose in the polymer backbone.^{1,21}

Preparation of molded XRU hydrogels

XRU-MA was dissolved in phosphate buffered saline (PBS) at various concentrations (5%, 7.5% or 10%, w/v). LAP was added to each solution at a final concentration of 0.06% (w/v). Then, XRU hydrogels were prepared in an acrylic mold (8 mm diameter, 1.5 mm thickness) via photo-crosslinking using a Lumen Dynamics OmniCure LX400+ (400 nm light source) at various photo-exposure energies (792, 1524 or 2220 mJ). The hydrogel was washed with deionized water for 48 h to remove any un-crosslinked polymer residue.

For *in vitro* cell culture, alginate hydrogels and collagen-coated XRU hydrogels were also prepared for comparison. Alginate hydrogels (8 mm diameter, 1.5 mm thickness) were prepared by crosslinking of an aqueous solution of alginic acid sodium salt (2% w/v) in calcium chloride (2% w/v) for 10 min. For preparation of collagen-coated XRU hydrogels, the molded XRU scaffolds were

freeze-dried and saturated with a collagen solution (Type I, 0.5 mg mL⁻¹ in PBS) at 37 °C overnight. Then the coated hydrogels were washed with PBS to remove un-bound collagen.

Physicochemical characterization of the molded XRU hydrogels

Mechanical properties. Mechanical test by micro-indentation was carried out using an EZ-S mechanical tester (Shimadzu, Japan) in the compression mode with a 10 N load cell at a cross-head speed of 0.1 mm min⁻¹. The Young's modulus (E_2) of the sample was calculated using Equation (1).²²

$$F = 8/3rE_2d \quad (1)$$

where F is the force applied to the hydrogel sample, r is the radius (0.495 mm) of the indenter tip, and d is the indentation depth. The data collected were linearly regressed by plotting the applied force against the indentation depth. The gradient of the linear regression was then used for the calculation.

Water uptake capacity. Samples of the molded XRU hydrogels were freeze-dried and their dry weights (W_i) were recorded before being re-immersed in deionized water. At predetermined intervals of 3, 8, 11 and 24 h, the swollen hydrogels were dabbed dry with Kimwipes to remove surface water and the hydrated weights (W_s) were recorded at each time point. The equilibrium water content and water uptake capacity were calculated using Equation (2) and (3), respectively.

$$\text{Water content (\%)} = (W_s - W_i)/W_s \times 100 \quad (2)$$

$$\text{Water uptake capacity (\%)} = (W_s - W_i)/W_i \times 100 \quad (3)$$

Microstructure. The microstructure of the XRU hydrogels was characterized using a scanning electron microscope (SEM, JEOL JSM-6490LV, Japan). The hydrogels were freeze-dried, coated with platinum (15 nm) using a Dynavac sputter coater and observed under an accelerating voltage of 15 kV.

***In vitro* cell culture of HDFs on the molded XRU hydrogels**

HDFs obtained from Cell Applications, Inc. (San Diego, CA, USA) were used at passages 10-15 in the study. Cells were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen™) and 1% (v/v) penicillin-streptomycin (10,000 U mL⁻¹, Gibco™) in a 5% CO₂ humidified incubator at 37 °C. For *in vitro* cell culture, all the hydrogel scaffolds used in this study were freeze-dried and UV sterilized.

Cytocompatibility of the XRU hydrogels was assessed by live/dead staining. Alginate hydrogels were employed for comparison. Briefly, HDFs were seeded at a density of 0.5×10^6 cells per scaffold and incubated with 1 mL cell culture medium. At day 1 and 7 post cell seeding, the cell-seeded hydrogels were incubated with calcein AM (5 µg mL⁻¹) in the culture medium for 25 min, and then propidium iodide (PI, 1 µg mL⁻¹) in the culture medium for 5 min. The hydrogels were washed with PBS and cell culture medium. Images of live (green) and dead (red) cells were acquired using a confocal laser scanning microscope (CLSM, Leica TSC SP5 II).

Proliferation of HDFs cultured on the XRU hydrogels was evaluated using Quant-iT™ PicoGreen™ dsDNA assay (Thermo Fisher Scientific). The alginate hydrogels and collagen-coated XRU hydrogels were used as negative and positive controls, respectively. For each type of scaffolds, non-seeded hydrogels were used as blank controls. At predetermined time points, cell-seeded hydrogels and cell-free hydrogels were collected, broken up using a spatula and analyzed following the manufacturer's protocol. The fluorescence reading was recorded using a microplate reader

(POLARstar Omega) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Cell number in each scaffold was determined using a standard calibration curve established with serial cell lysates of known cell numbers.

Cell morphology of the HDFs cultured on the XRU hydrogels was analyzed by F-actin staining. The cells were fixed in 3.7% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. Then the samples were stained with Alexa FluorTM 488 phalloidin (InvitrogenTM, Life Technologies) in 1% bovine serum albumin (BSA, 1:40, v/v) for 50 min and counterstained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, 1 $\mu\text{g mL}^{-1}$ in PBS) (InvitrogenTM, Life Technology) for 10 min, then washed with PBS and imaged using CLSM. HDF morphology was also examined by SEM. After fixation, the samples were dehydrated in a graded ethanol series (30%, 50%, 70%, 90% and 100%) for 30 min at each concentration, freeze-dried and coated with platinum for SEM observation.

Bioink formulation and rheology characterization

A composite bioink was formulated in PBS comprising 10% (w/v) XRU-MA, 5% (w/v) gelatin and 1% (v/v) penicillin-streptomycin. LAP was then added to a final concentration of 0.06% (w/v). The rheological properties of the bioink were characterized on an AR-G2 Rheometer (TA Instruments) using a 15 mm plate geometry with a 2° cone, fitted with a Peltier temperature controlled stage. The geometry gap between plates was set to 55 μm . Temperature sweep was conducted from 40 °C to 8 °C at a ramp rate of 1.5 °C min⁻¹, 1 Hz frequency and 1% strain. The sample was loaded at 37 °C and equilibrated for 2 min prior to the temperature sweep measurement. Viscometry was performed at 23 °C and 15 °C respectively with the shear rates ranging from 0.1 to 1000 s⁻¹. The sample was allowed to gel at room temperature (22-23 °C) before loading onto the Peltier plate at 23 °C. A conditioning step with 5 min pre-shear at a shear rate of 5 s⁻¹ and 5 min equilibrium both at 23 °C was applied before viscosity measurement.

3D printing (cell-containing) XRU-based hydrogels

Cell-free XRU-MA constructs were firstly fabricated by extrusion printing in a layer-by-layer fashion using a 3D Bioplotter (EnvisionTEC, Germany). The printer is housed within a biosafety cabinet to ensure sterility. All printing was conducted at 23 °C with the substrate set to 15 °C. The printing pressure and speed was 3.5 bar and 10 mm s⁻¹, respectively. An extrusion needle of 200 µm in diameter was used. A cuboid, 4-layered scaffold (10 × 10 mm) was designed, exported as a Standard Tessellation Language (STL) file and sliced using the Bioplotter RP[®] software. The infill of scaffolds was set to angles of 0° and 90°, with a print height of 0.130 mm and line space of 1.0 mm in the Virtual Machines software (EnvisionTEC, Germany). Once printed, the scaffolds were crosslinked with 400 nm light for 60 s (792 mJ). The printed scaffolds were incubated in the culture medium of HDFs at 37 °C for more than two days to remove the entrapped gelatin, freeze-dried and UV sterilized for 30 minutes on each side. The 3D printed scaffolds were then seeded with HDFs, and live/dead staining and cell proliferation assay were performed using the same procedure as detailed above.

For cell printing, HDFs were loaded to the bioink at a cell density of 1 × 10⁶ mL⁻¹, and the cell-laden XRU-based constructs were printed using the same procedure as described above. Upon printing, the 3D-printed cell-laden constructs were crosslinked with 400 nm light for 60 s (792 mJ), and incubated in the culture medium. Live/dead staining was performed at day 1 and day 7 post the printing.

Statistical Analysis

Data were presented as mean ± standard deviation (SD), where n ≥ 3. Statistically significant differences were obtained using Student's *t*-test (two-tailed). Values with p < 0.05 and p < 0.001 indicating significant and very significant differences separately were denoted as “*” and “**” respectively in the figures.

Results and discussion

Structural characterization of the modified XRU extract

The present study aims to provide a foundation for our ongoing research program to develop marine-derived bioprinting platforms for wound healing application. XRU is readily soluble in water, for which modification is required to render the structures stable in physiologically relevant conditions. A photo-crosslinkable XRU was developed by reacting XRU with methacrylic anhydride (Fig. 2A). Here a binary solvent of Milli-Q water and DMF (50/50, v/v) was employed, as it has been previously shown to improve the efficacy of methacrylation as a result of improved solubility of methacrylic anhydride in the reaction system.^{20,23}

The modified structure of XRU was verified by FT-IR (Fig. 2B) and ¹H NMR (Fig. 2C). In the FT-IR spectra, the peak at ~1713 cm⁻¹ for XRU-MA, not found in the spectrum of the unmodified XRU extract, corresponded to the stretching vibration of C=O from the conjugated ester linkage. In the ¹H NMR spectra, methacrylation of the XRU extract was demonstrated by the appearance of distinctive peaks in the double bond region (between 5.8 and 6.8 ppm) and a sharp peak that corresponded to the -CH₃ of the methacrylate groups (2.3 ppm). The estimated degree of methacrylation was ~0.8-0.9 per XRU disaccharide unit.

Physicochemical properties of the molded XRU hydrogels

XRU hydrogels were prepared by photo-crosslinking and subjected to mechanical and water-uptake studies. The mechanical property of the XRU hydrogels prepared with various XRU-MA concentrations (5%, 7.5%, or 10%, w/v) and photo-crosslinking energies (792-2220 mJ) showed Young's moduli ranging from ~18 kPa to ~309 kPa (Fig. 3A). The Young's modulus increased with increasing the XRU-MA concentration and photo-crosslinking energy. For example, the Young's modulus of the XRU hydrogels crosslinked with a photo energy of 792 mJ increased from ~18 kPa to ~182 kPa when the polymer concentration increased from 5% to 10% ($p < 0.001$, Fig. 3A).

Increasing the photo-exposure energy from 792 mJ to 2220 mJ resulted in a 2.5, 1.7 and 1.7-fold increase in the Young's modulus of 5%, 7.5% and 10% XRU hydrogels, respectively. These correlations may result from an increase in crosslinking density as a result of higher concentrations of macromer and/or photo energy. Our results demonstrated that the Young's modulus of an XRU hydrogel can be easily tailored by manipulating polymer concentration and photo-crosslinking condition.

The water uptake capacity of a hydrogel is important for the targeted application in wound healing as the moisture level in a wound area is critical for the healing process.²⁴⁻²⁶ The XRU hydrogels demonstrated a high capacity to uptake and retain water, with ~30-80-fold increases in weight within 24 hours' hydration at room temperature (Fig. 3B). These samples reached their respective equilibrium water contents (> 96%) in less than 3 hours (Fig. 3C). Here, the samples prepared from 5% XRU-MA and crosslinked with a photo energy of 792 mJ were not included, as they were too fragile to maintain structural integrity during the course of study. Amongst the three groups, increasing the polymer concentration resulted in a small but significant reduction in the equilibrium water content. For instance, the equilibrium water content of the XRU hydrogels exposed to 1524 mJ decreased from 98.8% to 96.8% when the polymer concentration increased from 5% to 10% ($p < 0.001$, Fig. 3C). Within each group, the equilibrium water content decreased slightly with increasing the photo-crosslinking energy. It should be noted that the water contents for 7.5% and 10% XRU hydrogels were not significantly different when crosslinked with a photo energy of 1524 mJ and 2220 mJ, respectively.

These data evidenced that the XRU hydrogels had versatile physicochemical properties, which promised a wide spectrum of applications for the healing of different types of wound in the body.²⁷ Based on the results and reported Young's modulus of human skin,²⁸ the XRU hydrogels with Young's modulus of ~182 kPa (10% (w/v) XRU-MA, 792 mJ photo exposure, denoted as XRU₁₀ hereafter)

were selected for the following *in vitro* study. The morphology of this type of hydrogel, after being freeze-dried, was examined by SEM, which showed a highly porous microstructure in the cross section (Fig. 3D and E).

***In vitro* evaluation of cell-material interactions on the molded XRU₁₀ hydrogels**

The XRU₁₀ hydrogels were assessed for cytocompatibility against HDFs, one of the dominant resident cell types in skin tissue. HDFs cultured on the XRU₁₀ hydrogels demonstrated high cell viability (Fig. 4A and B). Also, the XRU₁₀ hydrogels supported cell proliferation of HDFs as demonstrated by a more densely populated cell colony at day 7 compared to day 1. This result proved the innate cytocompatibility of the XRU₁₀ hydrogels. By contrast, cells barely attached to the alginate hydrogel, and little cell growth was observed over 7 days (Fig. 4C and D).

Cell proliferation was further quantified by PicoGreen assay. Over 2 weeks' culture, HDFs grown on the XRU₁₀ hydrogels showed a 6.3-fold increase in cell number by day 14 (Fig. 4E). By contrast, alginate hydrogel showed a much smaller number of attached cells at day 1 compared to that of the XRU₁₀ hydrogel, and the cell number decreased over the culture time. The poor cell attachment and proliferation of HDFs observed on the alginate hydrogels is due to lack of cell adhesion motifs in natural alginate and subsequently poor cell-matrix interactions as reported in other studies.^{19,29,30} The higher cell proliferation rate observed for the XRU₁₀ hydrogels suggested that these hydrogels may interact actively with HDFs whereby cell attachment and subsequent cell proliferation took places. In this context, the reason for improved cell attachment remains to be elucidated, but might be due to the following two factors. Firstly, the sugar components in XRU, such as L-rhamnose, may facilitate cell attachment by interacting with HDFs through the cell membrane lectin sites.¹¹ For instance, Andres *et al.*³¹ reported that a rhamnose-rich polysaccharide RROP-1 was able to promote cell proliferation likely by interacting with the lectin sites present on human skin fibroblasts through the L-rhamnose moieties. Secondly, the protein component(s) present in the XRU extract may also

facilitate cell attachment by providing the cell binding ligands. From this point of view, it is of critical importance to study a protein-free XRU fraction for a better understanding of the interactions between HDFs and XRU.

Collagen coating has been widely employed in tissue engineering to improve the initial cell attachment to synthetic matrices.³² In this study, collagen was coated onto the XRU₁₀ hydrogel by simply soaking up collagen solution followed by fibrillogenesis at 37 °C. Compared to the pure XRU₁₀ hydrogels, collagen coating improved cell attachment as demonstrated by a higher cell number at day 1. Collagen coating further promoted cell proliferation with a 7.5-fold increase in cell number at day 14 compared to day 1 (Fig. 4E). As a native ECM component, collagen is known to carry cell adhesion peptides for cell recognition.^{33,34} The enhanced cell attachment and proliferation could be a result of improved cell-matrix interactions, thus pointing out a promising approach for further modification of XRU to improve its biological functions in wound healing applications. Importantly, the XRU scaffold offers an opportunity for the development on non-contracting collagen based scaffolds. Collagen hydrogels have been intensively explored in skin tissue engineering and skin repair.¹⁷ However, the force generated by the cultured cells causes severe contraction of the collagen gel, which would affect the normal healing process, due to insufficient wound covering after transplantation of the collagen-based structures.³⁵ In this study, the cell-seeded collagen-coated XRU₁₀ hydrogels did not show any notable dimension change within the period of study, and this lack of significant contraction was attributable to the ulvan structure.

The organization and distribution of cytoskeletal actin filaments and cellular morphology of HDFs cultured on the XRU₁₀ hydrogels were examined by F-actin staining and SEM, respectively (Fig. 5). Fluorescent images revealed that HDFs had densely packed network of actin cytoskeleton yet with one or two cytoskeletal extensions at day 1, whereas actin filaments were re-organized and distributed throughout the cellular body that possessed multiple cytoskeletal extensions at day 7 with well visible

bundles of actin filaments (Fig. 5A-F). This indicated that HDFs attached to and spread well on the XRU₁₀ hydrogel. F-actin staining exhibiting the cytoskeleton also revealed the cellular morphologies of HDFs. Overall, HDFs took on mainly rounded shapes at day 1 and then the typical elongated and spreading morphologies at day 7. Cell morphology of HDFs visualized by SEM showed rounded cell shapes at day 1 and well spread and flattened morphologies at day 7, supporting the F-actin staining results (Fig. 5G-J). These cell behaviors demonstrated that HDFs attached well on the XRU₁₀ hydrogel and achieved full spreading with extended culture time. This may provide a foundation for the observed high cell proliferation rate of HDFs cultured on the XRU₁₀ hydrogels, and substantiated the potential of the XRU-based hydrogel for promoting cell attachment and spreading of HDFs.

Fabrication of (cell-containing) XRU-based constructs by extrusion printing

For 3D printing XRU₁₀ hydrogels, a composite ink comprising 10% (w/v) XRU-MA and 5% (w/v) gelatin was prepared. The XRU-MA solution on its own behaved like a runny liquid, so gelatin was added to improve its printability. Gelatin has often been used in bioink formulation for 3D printing, and this is attributable to its thermo-responsive gelation behavior.³⁶ The thermo-gelling property of gelatin provides a means to engineer the viscoelastic properties and hence the printability of its containing inks. Further, gelatin has also been used as a sacrificial material in 3D printing as it can be readily removed post-printing.^{36,37} Fig. 6A shows the temperature sweep of the storage moduli (G') and loss moduli (G'') of the composite ink. Both G' and G'' increased with reducing temperature and crossed at ~ 23 °C as a result of temperature (T) induced sol-gel transition. When $T > 23$ °C, G' was less than G'' , indicating the presence of a viscous liquid. When $T \leq 23$ °C, G' was greater than G'' , indicating the presence of a viscoelastic solid. In this study, 23 °C was selected for extrusion printing as the ink behaved like a soft gel, which allowed it to be extruded as a consistent fiber. The ink was extruded onto a colder substrate at 15 °C to help retain the printed structure. G' at 15 °C was ~ 350 Pa, whereby the ink behaved more solid-like and was capable of retaining shape before photocrosslinking.³⁸

One key property that an ink should have for extrusion printing is shear-thinning. This was assessed here by performing the shear rate sweeps at 23 °C and a lower temperature of 15 °C, respectively (Fig. 6B). Fig. 6B shows that the ink exhibited a non-Newtonian, shear-thinning behavior as the polymer network aligned with a high shear stress present. The shear-thinning behavior can be described by the power-law model (the Ostwald-de Waele Model) as shown in Equation (4).³⁹ The flow behavior index, n , was determined to be 0.328 and 0.176 for the ink at 23 °C and 15 °C respectively, by fitting the viscosity-shear rate curve with the power-law model.

$$\eta = K\dot{\gamma}^{n-1} \quad (4)$$

where η is viscosity (Pa s), $\dot{\gamma}$ is shear rate (s^{-1}), and K is the flow consistency index (viscosity at a shear rate of $1 s^{-1}$).

When a non-Newtonian power-law fluid flows in a cylinder tube such as an extrusion needle, according to the law of the wall, the fluidic portion near the wall of the tube moves the slowest and is subjected to the highest shear rate, when compared to the faster moving portion in the middle of the tube.³⁹ To estimate the maximum shear rate that the ink experienced during extrusion printing, i.e. the shear rate at the needle wall, the Rabinowitsch-Mooney equation was utilized as shown below.⁴⁰

$$\dot{\gamma} = (3n+1)Q/(\pi r^3) \quad (5)$$

where n is the flow behavior index, Q is the volumetric flow rate ($mm^3 s^{-1}$) and r is the radius of the extrusion needle (mm). For extrusion at 23 °C, with a 200 μm needle diameter and an extrusion rate of 10 $mm s^{-1}$ ($Q = \pi \cdot (0.1 mm)^2 \cdot 10 mm s^{-1} \approx 0.314 mm^3 s^{-1}$), the maximum shear rate during extrusion printing was estimated to be 604.9 s^{-1} , which correlated to a viscosity of $\sim 0.7 Pa s$.

Immediately after the extrusion, the bioink experienced negligible shear rates ($\leq 0.1 \text{ s}^{-1}$), and came into contact with the cold substrate at 15°C . Assuming a shear rate of 0.1 s^{-1} , the corresponding viscosity of the ink was $\sim 1070.7 \text{ Pa s}$ when touching the 15°C substrate, which was significantly higher than the one during the extrusion printing. The low viscosity during extrusion ($\sim 0.7 \text{ Pa s}$) enabled the smooth and continuous flow of the ink through the needle, while the high viscosity ($\sim 1070.7 \text{ Pa s}$) allowed it to maintain its shape after the extrusion.⁴¹ These data were in good agreement with those reported in the literature. Generally, the empirical ink viscosities for extrusion printing have been reported to range from 0.3 to 30 Pa s at the high shear rates (10^2 - 10^3 s^{-1}) during extrusion, and range from 10^2 to 10^3 Pa s at the low shear rates of $\leq 0.1 \text{ s}^{-1}$.^{38,40-43}

The XRU-based ink was tested firstly for 3D printing cell-free macroporous scaffolds. The 3D printed structures were stabilized by photo-crosslinking upon printing, and then incubated in the culture medium at 37°C to remove the entrapped gelatin. As shown in Fig. 7A, the 3D-printed XRU scaffolds have an average strut width of $180 \pm 24 \mu\text{m}$ and pore size of $1022.0 \pm 51.6 \mu\text{m}$. As with the molded XRU hydrogels, the 3D printed XRU scaffolds supported HDF attachment with high cell viability (Fig. 7B and C) and proliferation over a period of 2 weeks (Fig. 7D). This is quantitatively demonstrated by a PicoGreen assay that showed a 10.1-fold increase in the cell number by day 14 (Fig. 7D). The bioink was also assessed for 3D printing cell-laden structures. The 3D-printed HDFs-embedded constructs showed high cell viability over a period of 7 days (Fig. 7E and F). However, the encapsulated cells exhibited rounded morphologies even at day 7 with minimal cell growth. This is probably due to the formation of a densely packed polymer network which may impede to some extent cell-matrix interactions and cell metabolic activities. Our work demonstrates a highly cytocompatible nature of 3D printed XRU-based hydrogels as structural support. Optimization of the XRU ink formulation for direct cell printing is currently being undertaken in parallel with in-depth biological characterization, to understand the effects of scaffold structure on the functions of encapsulated HDFs at the molecular level.

The cell work presented here using HDF represents a first biologically relevant step to test the biocompatibility and safety of the biomaterial in wound healing. Future studies should focus on *in vitro* characterizations to understand the role of ink formulation in dermal ECM secretion, and subsequent optimization of 3D printing to produce skin like structures that imitate the cellular organization and functions of native skin. *In vivo* characterizations are equally important to assess tissue compatibility of the material firstly and then wound healing capacity of 3D printed cellular constructs.

Conclusions

In this study we present the functionalization of a rhamnose-rich xylorhamno-uronic acid (XRU) extract in the context of wound healing. The XRU extract was methacrylated and XRU-based hydrogels were prepared via photo-crosslinking of the incorporated methacrylate groups. XRU-based hydrogels demonstrated high water uptake capacity and tuneable mechanical properties. The gels were found to be highly cytocompatible with high cell viability and supported cell attachment and cell proliferation when tested with HDFs. An XRU-based bioink has been formulated for enabling controlled fabrication of 3D XRU structures via extrusion printing both with and without cells. The 3D printed hydrogel scaffolds displayed high cytocompatibility with HDFs. These results highlight the potential of this sulfated, rhamnose-rich XRU extract as a promising biomaterial for dermal matrix, which provides a foundation for our ongoing research to develop marine-based bioprinting platforms for skin repair and regeneration.

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Tables

Table 1 The proximate composition and key characteristics of xylohamno-uronic acid (XRU) extract shown as wt. % of dry weight and elemental analysis.

Chemical components	XRU extract
Protein	13.2 ± 0.2%
L-rhamnose	47.5 ± 2.5%
Rhamnose:xylose	5-7
Total uronic acid	15.9 ± 0.4%
Sulfate	16.9 ± 0.4%
Elemental analysis	
C	31.1 ± 0.2%
N	3.6 ± 0.2%
S	5.6 ± 0.1%

Figure Legends

Fig. 1 Ulvans share a high structural similarity to mammalian glycosaminoglycans found in human skin, due to the presence of uronic acid units and sulfate groups.

Fig. 2 Structural characterization of XRU and XRU-MA: (A) Schematic representation of the XRU methacrylation; (B) FT-IR spectra confirmed the successful modification by the additional peak at $\sim 1713\text{ cm}^{-1}$ in XRU-MA; (C) ^1H NMR spectra obtained at 60°C confirmed the successful conjugation of the methacrylate groups. The proton(s) confined in boxes as shown in the formulae of XRU disaccharide repeating unit (denoted by a, b, c and d, respectively) were marked in individual spectrum.

Fig. 3 Physicochemical properties of the molded XRU hydrogels: (A) XRU hydrogels showed tuneable mechanical properties with Young's moduli in a range of $\sim 18\text{-}309\text{ kPa}$ by varying polymer concentration and photo exposure energy; (B) XRU hydrogels had high water uptake capacity ($\sim 3000\text{-}8000\%$) after 24 h hydration and reached the equilibrium water content within 3 hours; (C) XRU hydrogels showed a high water content of $> 96\%$ after 24 h hydration; (D,E) Representative images showing the microstructure of the cross section of the XRU₁₀ hydrogels (10% (w/v) XRU-MA, 792 mJ). E is the higher magnification of the confined area in D. * indicates $p < 0.05$ and ** indicates $p < 0.001$.

Fig. 4 HDFs cultured on XRU₁₀ hydrogels (10% XRU-MA, 792 mJ) after (A) 1 and (B) 7 days, and on alginate hydrogels after (C) 1 and (D) 7 days. Calcein AM/PI were used to stain live (green) and dead (red) cells, respectively. (E) Cell proliferation of HDFs on varying hydrogels including alginate, XRU₁₀, and collagen-coated XRU₁₀ (Col-XRU₁₀) hydrogels by PicoGreen assay. * indicates $p < 0.05$ and ** indicates $p < 0.001$.

Fig. 5 Cell morphology of HDFs cultured on the XRU₁₀ hydrogels (10% XRU-MA, 792 mJ) at day 1 (A-C, G-H) and day 7 (D-F, I-J) characterized by F-actin staining (A-F) and SEM (G-J), respectively. A & D: nuclei (blue); B & E: F-actin (green); C: merged image of A and B; F: merged image of D and E. H and J is the higher magnification of the confined area in G and I, respectively. Cells were highlighted by the yellow arrows in G-J.

Fig. 6 Rheological behavior of the XRU-MA/gelatin bioink demonstrated thermo-responsive phase change behavior (A) and shear-thinning property measured at 23°C and 15°C , respectively (B).

Fig. 7 Fabrication of (cell-containing) XRU-based constructs by extrusion printing: (A) A representative image of the 3D-printed XRU-based structures; (B, C) Representative live/dead staining images of human dermal fibroblasts (HDFs) cultured in the 3D-printed scaffolds at day 1 and day 7, respectively; (D) Cell proliferation of HDFs cultured in the prefabricated XRU hydrogels by PicoGreen assay. * indicates $p < 0.05$ and ** indicates $p < 0.001$; (E, F) Representative live/dead staining images of the 3D printed HDFs in the XRU hydrogels after day 1 (E) and day 7 (F) in culture. Inset image shows magnified live/dead staining from the same image. Living cells were stained green and dead cells red.

Figures

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Graphic abstract

3D printing of sulphated, rhamnose-rich hydrogels, using a seaweed extract from a controlled source of Australian ulvacean macroalgae, for wound healing.