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Multifunctional biomaterials from the sea: Assessing the effects of chitosan incorporation into collagen scaffolds on mechanical and biological functionality

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ABSTRACT

Natural biomaterials such as collagen show promise in tissue engineering applications due to their inherent bioactivity. The main limitation of collagen is its low mechanical strength and somewhat unpredictable and rapid degradation rate; however, combining collagen with another material, such as chitosan, can reinforce the scaffold mechanically and may improve the rate of degradation. Additionally, the high cost and the risk of prion transmission associated with mammal-derived collagen has prompted research into alternative sources such as marine-origin collagen. In this context, the overall goal of this study was to determine if the incorporation of chitosan into collagen scaffolds could improve the mechanical and biological properties of the scaffold. In addition the study assessed if collagen, derived from salmon skin (marine), can provide an alternative to collagen derived from bovine tendon (mammal) for tissue engineering applications. Scaffold architecture and mechanical properties were assessed as well as their ability to support mesenchymal stem cell growth and differentiation. Overall, the addition of chitosan to bovine and salmon skin-derived collagen scaffolds improved the mechanical properties, increasing the compressive strength, swelling ratio and prolonged the degradation rate. Mesenchymal stem cell (MSC) attachment and proliferation was most improved on the bovine-derived collagen scaffold containing a 75:25 ratio of collagen:chitosan, and when MSC osteogenic and chondrogenic potential on the scaffold was assessed, a significant increase in calcium production ($p < 0.001$) and sulfated glycosaminoglycan (sGAG) production ($p < 0.001$) was observed respectively. Regardless of chitosan content, the bovine-derived collagen scaffolds out-performed the salmon skin-derived collagen scaffolds, displaying a larger pore size and higher percentage porosity, more regular architecture, higher compressive modulus, a greater capacity for water uptake and allowed for more MSC proliferation and differentiation. This versatile scaffold incorporating the marine biomaterial chitosan show great potential as appropriate platforms for promoting orthopaedic tissue repair while the use of salmon skin-derived collagen may be more suitable in the repair of soft tissues such as skin.

Statement of Significance

Collagen is commonly used in tissue engineering due to its biocompatibility; however, it has low mechanical strength and an unpredictable degradation rate. In addition, high cost and risk of prion transmission associated with mammalian-derived collagen has prompted research into alternative collagen sources, namely, marine-derived collagen. In this study, scaffolds made from salmon-skin collagen were compared to the more commonly used bovine-derived collagen with a focus on orthopaedic applications.

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To improve the mechanical properties of these scaffolds, another marine biomaterial, chitosan, was added to produce scaffolds with increased mechanical stability. The collagen-chitosan composites were also shown to support mesenchymal stem cell differentiation towards both bone and cartilage tissue. This multi-functional scaffold therefore has potential in both bone and cartilage regeneration applications.

1. Introduction

Biomaterial scaffolds for orthopaedic tissue engineering applications should be capable of integrating with native tissue, allowing for cell infiltration and proliferation, and to degrade at a rate proportional to new tissue formation, all without causing an immunological response. The material composition of the scaffold plays an important role in directing cell behaviour; natural biomaterials such as collagen show promise due to their inherent bioactivity [1–4]. The mechanical properties of biomaterials for orthopaedic applications must also be considered; in seminal work by Engler and colleagues, it was shown that mesenchymal stem cells (MSCs) differentiate toward different phenotypes depending on the stiffness of the substrate upon which they are seeded [5]. As well as influencing cell behaviour, scaffolds must be strong enough to withstand physical manipulation subjected during implantation and be straight-forward to fabricate, sterilize and shape to the size required [6]. The main limitation of natural materials, such as collagen, in orthopaedic tissue engineering applications, is their low mechanical strength and somewhat unpredictable degradation rate; however combining two or more materials can reinforce the scaffold mechanically and may slow the rate of degradation.

Chitosan is a biocompatible, biodegradable polysaccharide derived from chitin, which can be isolated from many marine species [7]. The most common source of chitin is crab and shrimp exoskeletons, of which at least 2.3 million metric tons are produced each year as food waste [8]. Chitosan is a very interesting material as it can be processed into hydrogels [9,10], nanofibers [11], beads [12], microparticles [13], nanoparticles [14–16] and porous scaffolds [17–19] and has been used in a wide range of tissue engineering applications such as in wound healing [20], and in drug and gene delivery [16,21–24]. Of particular interest in orthopaedic tissue engineering, chitosan has been shown to promote MSC osteogenesis [25] and, being a linear polysaccharide, has an analogous molecular structure to hyaluronic acid, a non-sulfated glycosaminoglycan which is a major component of articular cartilage, and has been shown to support MSC chondrogenesis.

Collagen is the most abundant protein found in the human body, serving as the major component of the extracellular matrix [4,26,27]. It is biocompatible and biodegradable and readily allows for cell adhesion, proliferation and differentiation [28]. For this reason collagen is in widespread use in tissue engineering research, however, it has low mechanical strength and rapid degradation rate, which are limiting its use commercially. Cross-linking methods can increase the mechanical properties of collagen however the incorporation of another material, forming a composite has shown the most promise in improving the scaffolds characteristics [29–31]. Due to the interesting properties of chitosan outlined above, the main aim of this study was to investigate the potential of collagen-chitosan composite scaffolds for orthopaedic tissue engineering applications.

A further caveat in choosing biomaterials for tissue engineering is the source; as collagen is such a ubiquitous material, it can be extracted from numerous sources, most commonly bovine and porcine connective tissue such as skin and tendon. However, while mammalian-derived tissues are subject to extensive processing to reduce risk of disease transmission and immunogenicity, there remain concerns about transmission of prions such as Creutzfeldt-Jakob Disease (CJD), although there have been no reports of this to date [32–35]. Religious restriction is also an important issue as three major religions; Judaism, Islam and Hinduism prohibit the use of products derived from either bovine or porcine origin [36]. For these reasons, there has been interest in alternative sources of collagen. While recombinant technology can be used to make collagen, it is extremely expensive and yields are low and inefficient for most tissue engineering applications [37]. Alternatively, marine-derived collagen is an easily accessible source of collagen and can be obtained from many different sources including fish skin, jellyfish and marine sponges [38–40]. As up to 75% of fish weight is discarded as a food waste, it is possible to obtain large amounts of collagen cheaply [41]. One drawback to marine-derived collagen is that it has lower hydroxyproline content than the more commonly used bovine-derived collagen. Hydroxyproline functions to stabilise the collagen triple helix conformation and a high hydroxyproline content indicates greater thermal stability [42]. The denaturation temperature of marine-derived collagen is thus reported to be approximately 40 °C [41] whereas bovine-derived collagen has a reported denaturation temperature of 95 °C [43].

The objectives of this study was thus to investigate the effect of the incorporation of chitosan on the morphological and mechanical properties of collagen scaffolds and also to determine if marine-derived collagen, isolated from salmon skin, might serve as a viable alternative to mammal-derived collagen, isolated from bovine tendon, in tissue engineering applications. In addition, the potential of the resultant collagen-chitosan composite scaffold to enhance both MSC-mediated osteogenesis and chondrogenesis was assessed.

2. Materials and methods

All materials were provided by Sigma Aldrich, Ireland unless otherwise stated.

2.1. Isolation of collagen from salmon skins

Salmon skins were obtained from a local market and kept frozen until use. Scales and muscle were removed and skins were further washed with water and cut into pieces of about 2 × 2 cm. Fats were removed from salmon skins by immersion in 10% ethanol for 48 h, under stirring (with change of solution at least twice a day). Salmon skins were then treated with 0.1 M NaOH (1:10 w/v), during 3 × 2 h, to remove non-collagenous proteins as described previously [41,44–46]. After thorough washing with water, salmon skins were dissolved in 0.5 M acetic acid (HOAc) (1:10 w/v), during 72 h, under stirring. The resulting mixture was centrifuged and the supernatant, containing the acid soluble collagen, was further vacuum filtered to remove non-soluble impurities. Salmon-skin collagen was recovered by salting out and after centrifugation; collagen was resuspended in 0.5 M HOAc, dialysed against 0.1 M HOAc and freeze-dried until further use. All the extraction procedure was conducted at 4 °C and the resulting

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marine salmon skin collagen is consistent with type 1 collagen (Supplementary Fig. 1).

2.2. Fabrication of collagen and collagen-chitosan scaffolds

Bovine tendon collagen (Integra Life Sciences, USA) and marine salmon skin collagen were used to make a series of collagen-based scaffolds either containing collagen alone or as composites with chitosan derived from crustacean shells. Bovine and salmon-derived collagen slurries were made by adding 1.8 g of collagen to 360 mL of 0.5 M HOAc and blended at 15,000 rpm for 90 min using an overhead blender (Ultra Turrax T18 Overhead Blended, IKA Works Inc., USA) at a constant temperature of 4 °C [47]. Medium molecular weight chitosan (Mw 110–160 kDa; DD 75–85%), purchased from FMC Biopolymer, Norway, was added to collagen in ratios of 100:0, 90:10, 75:25 and 50:50 collagen:chitosan. Chitosan was first dissolved in 0.5 M HOAc by stirring for 3 h. The appropriate amount of collagen was then added to the dissolved chitosan and blended at 15,000 rpm for 90 min as described above. Each slurry was degassed under a vacuum prior to freeze-drying (Advantage EL, Vis-Tir Co., Gardiner NY) to a final temperature of -40 °C using a previously optimised freeze-drying process [47,48]. Collagen and chitosan interact via electrostatic interactions and hydrogen bonding as has been shown previously [49–51], and by adding chitosan to collagen slurries, the compressive strength increased 2-fold. However, as the scaffolds were made to be used in bone and cartilage applications, the scaffolds were further sterilised and cross-linked dehydrothermally (DHT) at 105 °C for 24 h at 0.05 bar in a vacuum oven (Vacucell 22; MMM, Germany) [52], followed by chemical cross-linking using a mixture of 6 mM N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and 5.5 mM N-Hydroxysuccinimide (NHS). Both DHT and EDC cross-linking methods act on lysine, arginine, serine, glutamic acid, aspartic acid and threonine amino acid residues on collagen [52,53]. While chitosan also contains amino groups, the lack of carboxyl groups indicates that EDC does not affect chitosan [54]. Cylindrical scaffolds (10 mm diameter), cut using a biopsy punch, were used in experiments.

2.3. Effect of chitosan incorporation on scaffold morphology and architecture

2.3.1. Effect of chitosan incorporation on scaffold pore size

Pore size analysis was carried out using a histological technique previously described [55]. 10 mm scaffold samples were obtained from three locations on each scaffold sheet. The samples were embedded in JB-4® glycolmethacrylate (Polysciences Europe, Eppelheim, Germany) and serially sectioned longitudinally and transversely on a microtome (Leica RM 2255, Leica, Germany) to give 10 μm thick sections, which were stained with toluidine blue. Digital images were captured at 10 x magnification using an optical microscope (Eclipse 90i, Nikon, Japan) and a digital camera (DS Ri1, Nikon, Japan). Pore size analysis was carried out on MATLAB (MathWorks Inc, MA, USA) using a pore topology analyser developed in conjunction with the Sigmedia Research Group in the Electrical Engineering Department at Trinity College Dublin, Ireland [48]. The programme transformed the images into binary form and calculated the average pore radii based on best-fit elliptical lengths.

2.3.2. Effect of chitosan incorporation on scaffold porosity

Total percentage porosity (P) of the scaffolds was measured according to the following equation

$$Porosity\ (\%) = \frac{\rho\ scaffold}{\rho\ material} \times 100 \tag{2.1}$$

where $\rho\ material$ is the density of the material of which the scaffold is fabricated and $\rho\ scaffold$ is the apparent density of the scaffold measured by dividing the weight by the volume of the scaffold.

2.3.3. Effect of chitosan incorporation on scaffold architecture

In order to examine the effect of chitosan incorporation on scaffold architecture, scanning electron microscopy was carried out. Scaffolds were manufactured as described in Section 2.5.1. The samples were dried before being mounted onto metallic studs using carbon cement and sputtered with gold/palladium alloy and imaged using a Zeiss Ultra Plus scanning electron microscope (SEM) (Zeiss, Germany).

2.4. Effect of chitosan incorporation on scaffold mechanical properties

2.4.1. Effect of chitosan incorporation on scaffold bulk compressive modulus

Uni-axial, unconfined compressive testing was carried out to determine the compressive elastic modulus of each scaffold with diameter of 10 mm and a height of 4 mm. Samples were pre-hydrated in phosphate buffered saline (PBS) for 1 h prior to testing. A mechanical testing machine (Z050, Zwick-Roell, Germany) was fitted with a 5 N load cell and used in the procedure. The pre-hydrated samples were immersed in PBS throughout the tests. The tests were conducted in triplicate for each scaffold type at a strain rate of 10% per minute. Stress was calculated from scaffold surface area and applied force, whilst strain was calculated from displacement of the scaffolds in relation to the original thickness. The compressive modulus was defined based on the slope of a linear fit to the stress-strain curve over 2–10% strain.

2.4.2. Effect of chitosan incorporation on scaffold swelling ratio

The scaffold swelling ratio, or ability to retain water, is an important mechanical property as higher swelling ratios is associated with increased cell adhesion and infiltration. The function of glycosaminoglycans in orthopaedic tissue is also to retain water, which increases the compressive strength of the structure. To assess if chitosan can increase the swelling ratio of collagen scaffolds, dry scaffolds were first weighed (d) before being hydrated in PBS for 24 h at room temperature. After removal of excess surface water with filter paper, the wet scaffolds (w) were weighed again. The experiment was done in triplicate for each type of scaffold and the swelling ratio of the scaffolds was calculated using the following equation;

$$Swelling\ Ratio = \frac{w - d}{d} \tag{2.2}$$

where w = weight of wet scaffold and d = weight of dry scaffold

2.4.3. Effect of chitosan incorporation on scaffold degradation rate

It is hypothesised that the addition of chitosan to the collagen scaffold will improve the degradation characteristics of the scaffold. To test this hypothesis, the scaffolds were incubated at 37 °C for 28 days in serum-containing media in an attempt to replicate physiological conditions. After 28 days, samples were removed from the medium, rinsed with distilled water, lyophilized and weighed. The experiment was done triplicate for each scaffold type. The total percentage of scaffold remaining was calculated. Using the following equation;

$$Degradation\ Rate = d0 - \left(\frac{d28}{d0}\right) \times 100 \tag{2.3}$$

where d0 = weight of dry scaffold at day 0 and d28 = weight of dry scaffold at day 28.

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2.5. Effect of chitosan incorporation on MSC viability within scaffold

2.5.1. Cell culture

Primary rat MSCs were cultured in Dulbecco's modified eagles medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (Labtech, UK), 100 U/mL penicillin/streptomycin, 2 mM glutamax (Gibco- Biosciences, Ireland), 1 mM L-glutamine and 1% non-essential amino acids (Gibco-Biosciences, Ireland). Scaffolds of 10 mm in diameter and 4 mm in height were placed in 24 well-plates and seeded with 5×10^5 MSCs. Supplemented DMEM growth medium was added to each well and the scaffolds were incubated at 37 °C with 5% CO_2 for 14 and 28 days with media changes every 3 days.

2.5.2. Quant-iT dsDNA PicoGreen assay for cell viability

DNA quantification was carried out using a Quant-iT™ PicoGreen® dsDNA kit (Invitrogen, UK). After 14 and 28 days in culture, media was removed from the wells and the scaffolds were placed in tubes containing 1 mL of lysis buffer. The samples then underwent three freeze-thaw cycles at −80 °C before the assay was performed as per manufacturer's instructions. The DNA concentration was determined using a standard curve.

2.6. Assessment of potential of collagen chitosan scaffold to support MSC osteogenesis

2.6.1. Osteogenic cell culture

MSCs were cultured and seeded onto scaffolds (10 mm X 4 mm) as described in Section 2.5.1. After 7 days of culture in supplemented DMEM growth media, growth media was replaced with osteogenic media which contains DMEM supplemented with 10% FBS (Labtech, UK), 1% penicillin/streptomycin, 10 mM β-glycerophosphate, 50 μm ascorbic acid 2-phosphate and 100 nM dexamethasone. Media was replaced every 3 days for the duration of the culture period (14 and 28 days).

2.6.2. Alizarin red staining for calcium

After 14 and 28 days in osteogenic media, samples were fixed with 10% formalin for 30 min and processed overnight using an automatic tissue processor (ASP300, Leica, Germany). The constructs were embedded in paraffin wax before sectioning. 5 μm sections were cut using a rotary microtome (Microsystems GmbH, Germany) and sections were mounted on poly-L-lysine coated glass slides (Thermo Scientific, Ireland). Samples were deparaffinised with xylene and rehydrated in descending grades of alcohol (100% to 70%). Sections were stained in 2% alizarin red for 3 min. Digital images of all stained sections were obtained using an imaging system (AnalySIS, Nikon, Japan) in conjunction with a microscope (Olympus IX51, Olympus, Japan).

2.6.3. Calcium quantification

The scaffolds were assayed for calcium deposition as a measure of osteogenesis using a Stanbio calcium assay (Calcium CPC Liquicolour, Stanbio Inc., USA) following 14 and 28 days post cell seeding as per manufacturers' instructions. A Quant-iT™ PicoGreen® dsDNA assay (Invitrogen, UK) was also carried out at each time point as described in Section 2.5.2 to normalise levels of calcium to cell number.

2.7. Assessment of potential of collagen chitosan scaffold to support MSC chondrogenesis

2.7.1. Chondrogenic cell culture

Cells were cultured and seeded onto scaffolds (10 mm × 4 mm) as described in Section 2.5.1. After 7 days of culture in supplemented DMEM growth media, growth media was replaced with chondrogenic media which contains high glucose DMEM supplemented with 1% penicillin/streptomycin, 1% non-essential amino acids, 1% L-Glutamine, 50 μg/mL ascorbic acid, 40 μg/mL L-proline, 100 nM dexamethasone, 1x ITS (Insulin, Transferrin, Selenium) (BD Biosciences, UK), 0.11 mg/mL sodium pyruvate and 20 ng/mL of transforming growth factor-β3 (TGF-β3) (Prospec, Israel). Media was replaced every 3 days for the duration of the assay (14 and 28 days).

2.7.2. Safranin O staining for sGAG

Safranin O staining was used to investigate the level of sulfated GAG deposition within the scaffolds, which is representative of MSC chondrogenesis. Samples were fixed with 10% formalin for 30 min and processed overnight using an automatic tissue processor (ASP300, Leica, Germany). The constructs were embedded in paraffin wax before sectioning. 5 μm sections were cut using a rotary microtome (Microsystems GmbH, Germany) and sections were mounted on poly-L-lysine coated glass slides (Thermo Scientific, Ireland). Samples were deparaffinised with xylene and rehydrated in descending grades of alcohol (100% to 70%). Harris haematoxylin was used to stain cell nuclei, followed by a 0.2% Fast Green counter stain. The samples were then stained in Safranin O for 60 min. Digital images of all stained sections were obtained using an imaging system (AnalySIS, Nikon, Japan) in conjunction with a microscope (Olympus IX51, Olympus, Japan).

2.7.3. sGAG quantification

Cell-seeded and cell-free control scaffolds were washed in PBS before digesting in a solution prepared from papain enzyme solution containing 0.5 M EDTA, cysteine-HCl and 1 mg/ml papain enzyme (Carica papaya, Sigma-Aldrich, Ireland) at 60 °C for 12 h. sGAG content was assessed using a Blyscan Sulfated Glycosaminoglycan Assay kit (Biocolor Life Sciences, UK) following manufacturers' instructions.

2.8. Statistical analysis

Results are expressed as mean ± standard deviation. Statistical significance was assessed using two-way ANOVA analysis followed by Bonferoni post hoc analysis. The sample size was $n = 3$ and $p \leqslant 0.05$ values were considered statistically significant where $^{*}\ p < 0.05$, $^{**}\ p < 0.01$ and $^{***}\ p < 0.001$.

3. Results

3.1. Effect of chitosan incorporation on scaffold morphology and architecture

Scaffold pore size was quantified (Fig. 1A) and there was no significant difference in pore size between the bovine-derived collagen scaffolds containing chitosan at ratios of 100:0, 90:10 and 75:25 (161.1 μm ± 26.3 μm, 121.6 μm ± 4.5 μm and 130.6 μm ± 7.7 μm respectively), however when chitosan was incorporated at a ratio of 50:50, there was a significant reduction in pore size to 74.7 μm ± 2.3 μm ($p < 0.05$). In the salmon-derived collagen groups (Fig. 1A), pore size was smallest in the scaffold containing 100:0 ratio of collagen to chitosan (25.45 μm ± 2.4 μm) but pore size significantly increased with the incorporation of increasing amounts of chitosan, with a resultant pore size of 55.8 μm ± 2.3 μm, 61.8 μm ± 5.9 μm and 97.8 μm ± 12.3 μm in the scaffolds at 90:10, 75:25 and 50:50 respectively ($p < 0.01$). Overall, apart from the 50:50 group, the salmon-derived collagen scaffolds had significantly smaller pores than the bovine-derived collagen scaffolds ($p < 0.001$).

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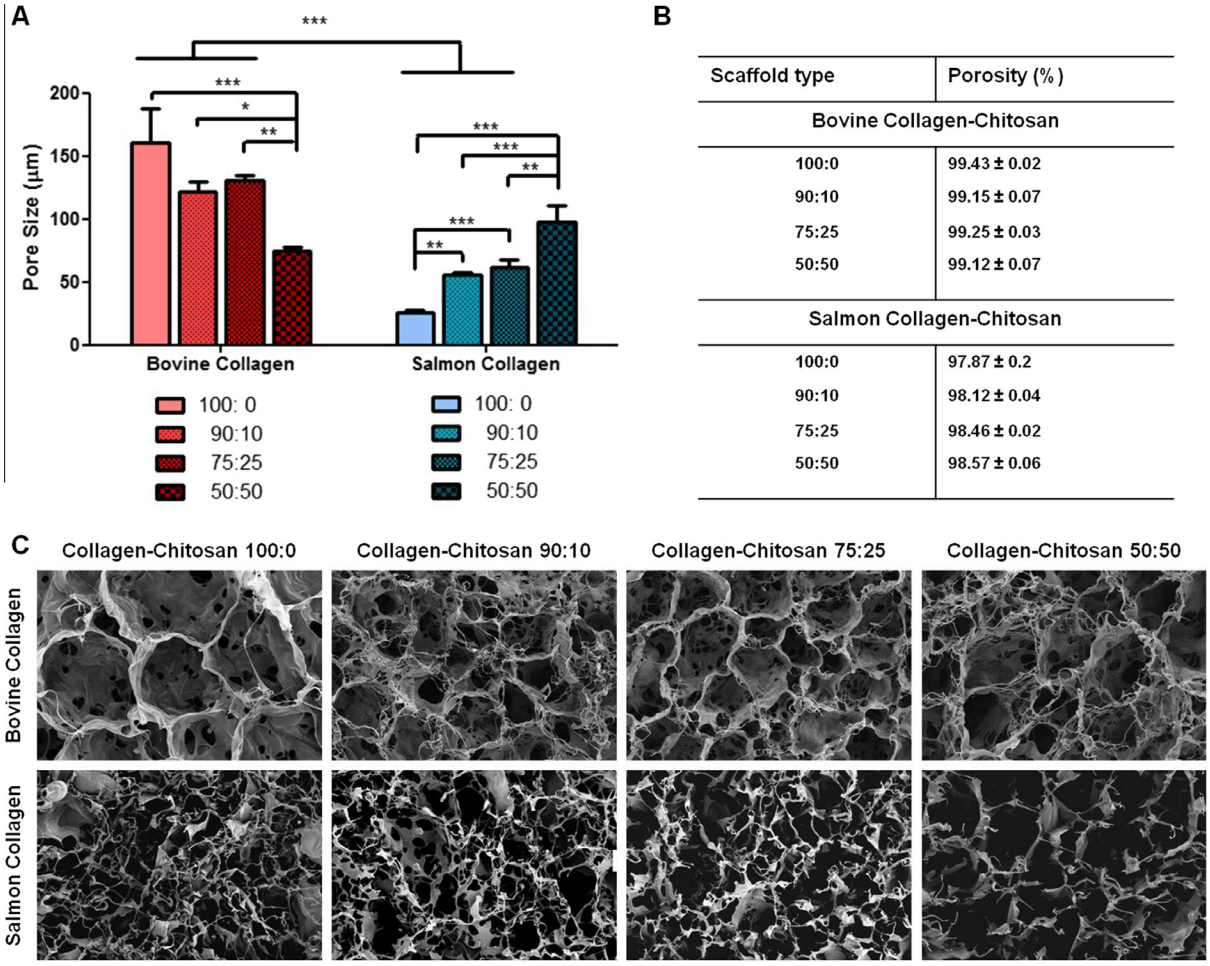

Scaffold type	Porosity (%)
Bovine Collagen-Chitosan	
100:0	99.43 ± 0.02
90:10	99.15 ± 0.07
75:25	99.25 ± 0.03
50:50	99.12 ± 0.07
Salmon Collagen-Chitosan	
100:0	97.87 ± 0.2
90:10	98.12 ± 0.04
75:25	98.46 ± 0.02
50:50	98.57 ± 0.06

Fig. 1. Effect of chitosan incorporation on scaffold morphology. Bovine and salmon-derived collagen scaffolds containing chitosan at ratios of 100:0, 90:10, 75:25 and 50:50 were assessed for pore size, porosity and porous architecture. The incorporation of chitosan into a bovine-derived collagen scaffold causes a decrease in pore size while the opposite occurs with salmon-derived collagen scaffold groups (A). Data plotted shows mean ± standard deviation (n = 3) and $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Percentage porosity was calculated as a relative ratio of scaffold density to the density of solid collagen and chitosan. All scaffolds had mean porosity of >97% (B). Statistical significance was measured using one-way ANOVA. Representative images of bovine and salmon-derived collagen scaffolds with increasing amounts of chitosan show porous structure and pore interconnectivity (C). The images were taken at 9 kV at a magnification of 300x. Scale bar represents 20 µm.

Each scaffold was highly porous with porosities above 97.5% (Fig. 1B). The bovine-derived collagen scaffold had the highest level of porosity at 99.4% and while the incorporation of chitosan caused a slight reduction in porosity, the porosity of the 90:10, 75:25 and 50:50 collagen-chitosan scaffolds remained above 99%. The salmon-derived collagen scaffolds had a lower overall porosity, which increased proportionally with the addition of chitosan from 97.8% (100:0) to 98.6% (50:50). All scaffolds retained a highly porous interconnected architecture as shown in representative SEM images in Fig. 1C.

3.2. Effect of chitosan incorporation on scaffold mechanical properties

The bulk compressive modulus of each scaffold type was measured after chitosan incorporation and cross-linking with both physical (DHT) and chemical (EDC + NHS) treatments (Fig. 2A). The incorporation of chitosan into bovine-derived collagen scaffolds caused a significant increase in compressive modulus from 0.92 kPa for the collagen alone to 1.09 kPa and 1.23 kPa for the scaffolds containing collagen-chitosan at ratios of 75:25 and 50:50, respectively ($p < 0.05$ for 90:10 up to $p < 0.001$ for 50:50). An increase in bulk compressive modulus was also seen in the salmon-derived collagen group; scaffolds without chitosan (100:0) had a low compressive modulus of 0.13 kPa but after the incorporation of chitosan, a 3–4.5-fold increase was observed with increasing chitosan content at 0.38 kPa, 0.39 kPa and 0.56 kPa for 90:10, 75:25 and 50:50 chitosan-containing scaffolds respectively ($p < 0.001$). However, the salmon-derived collagen-chitosan scaffolds were significantly (50–80%) less stiff than the bovine-derived collagen-chitosan scaffolds overall ($p < 0.001$).

The scaffold swelling index, or the ability of the scaffold to absorb and retain water, was assessed by a standard swelling test. The incorporation of chitosan to both bovine and salmon-derived collagen scaffolds caused an increase in the hydrophillicity of the scaffolds ($p < 0.05$), with the scaffolds containing a 50:50 ratio of collagen to chitosan retaining the most water after 4 h (Fig. 2B). The incorporation of chitosan into collagen scaffolds also affected the scaffold degradation rate. The addition of even 90:10 chitosan was enough to significantly prolong the degradation rate of both bovine and salmon-derived collagen scaffolds ($p < 0.001$) (Fig. 2C). However, the salmon-derived collagen scaffolds containing 50:50 collagen-chitosan disintegrated after 14 days in serum-containing media.

Overall, the bovine-derived collagen scaffolds out-performed the salmon-derived collagen scaffolds in each test, displaying a larger pore size and higher percentage porosity (Fig. 1A and B),

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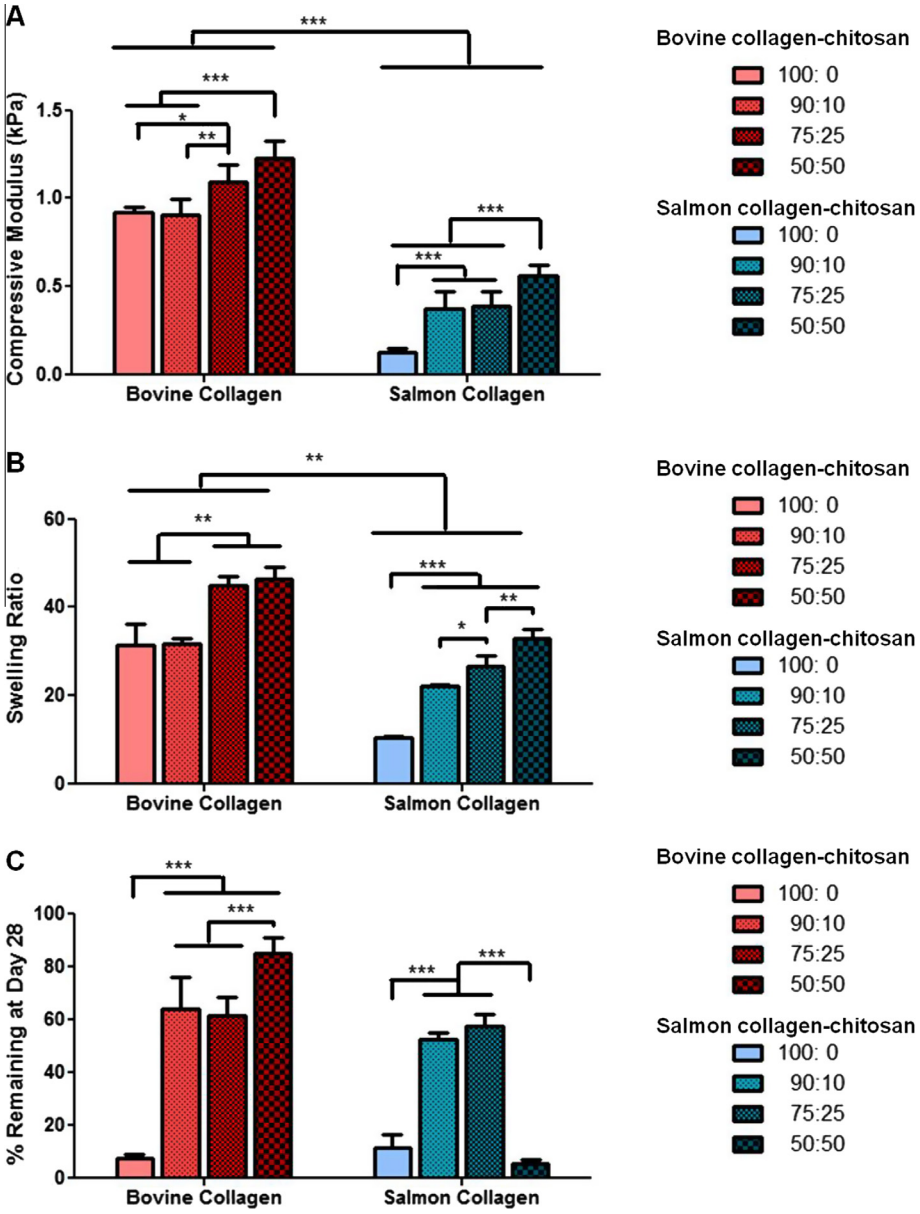

Fig. 2. Effect of chitosan on mechanical properties of collagen scaffolds. Scaffold compressive modulus (A), swelling ratio (B) and degradation rate (C) were assessed comparing both bovine and salmon-derived collagen-based scaffolds containing chitosan at ratios of 100:0, 90:10, 75:25 and 50:50. Unconfined uni-axial testing was carried on scaffolds and graph A shows an increase in compressive modulus in proportion with increasing chitosan content. A standard swelling test also revealed that increasing chitosan content causes an increase in the scaffolds hydrophilicity (B) and also prolongs the scaffolds degradation rate (C). Data plotted represents mean ± standard deviation (n = 3) and significance is indicated with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

more regular architecture (Fig. 1C), higher compressive modulus (Fig. 2A) and greater capacity for water uptake (Fig. 2B).

3.3. Effect of chitosan incorporation on MSC viability within scaffold

To determine the effects of chitosan on cell viability and proliferation on the scaffolds, DNA content was quantified after 14 and 28 days in culture and an approximate cell number was determined (Fig. 3). In the bovine-derived collagen scaffolds there was no difference in cell growth after 14 days, however, following 28 days in culture, the bovine-derived collagen scaffolds containing 90:10 and 75:25 collagen-chitosan supported a 2.5-fold greater number of cells compared to scaffolds containing 100:0 and 50:50 collagen-chitosan (Fig. 3A). The incorporation of chitosan into the salmon-derived collagen scaffold initially caused a decrease in cell viability (Fig. 3B); however, after 28 days, there was a significantly higher number of cells on the salmon-derived collagen scaffolds containing 75:25 and 50:50 collagen-chitosan compared to collagen alone, although this number was 2-fold lower than that seen on the bovine-derived collagen groups.

Overall, the bovine-derived collagen scaffold composed of collagen-chitosan at a ratio of 75:25 supported more cell proliferation than any other group. Furthermore, cell numbers on the salmon-derived collagen-chitosan scaffolds were 50% lower than the bovine-derived collagen-chitosan scaffolds (90:10 and 75:25). For this reason, the bovine-derived collagen scaffold containing 75:25 ratio of collagen to chitosan was deemed the most favourable scaffold and was brought forward to assess its potential in bone and cartilage tissue engineering applications.

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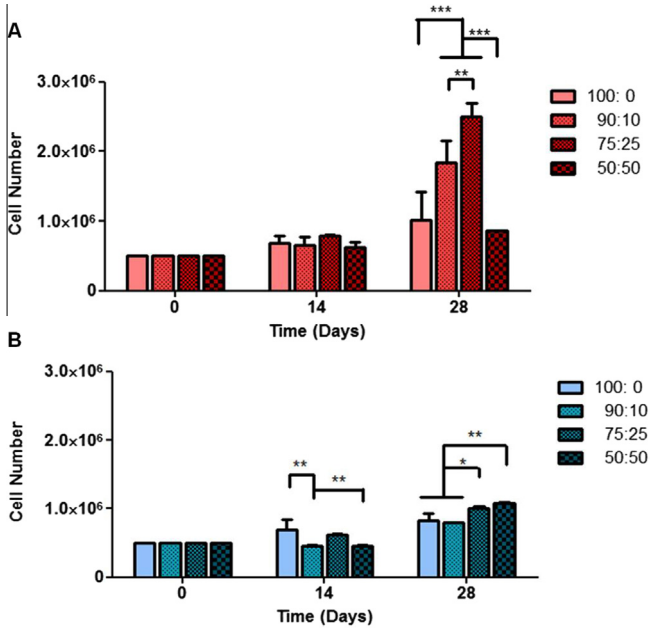

Fig. 3. Effect of chitosan incorporation into (A) bovine and (B) salmon-derived collagen scaffolds on MSC viability. $5X10^5$ cells were seeded onto 10 mm diameter discs of scaffold. After 14 and 28 days dsDNA was quantified. The dsDNA content in $5X10^5$ cells were also assessed and cell number on the scaffolds was calculated. The incorporation of chitosan into both bovine (A) and salmon (B) -derived collagen scaffolds cause an increase in cell proliferation with the bovine-derived collagen scaffold containing 75:25 collagen-chitosan supporting significantly higher cell proliferation than other groups. Cell number on the salmon-derived collagen-chitosan scaffolds was 50% lower than bovine collagen-chitosan scaffolds. Data plotted shows mean ± standard deviation (n = 3) and * indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3.4. Assessment of potential of collagen-chitosan composite scaffold to support MSC osteogenesis

MSCs were cultured on scaffolds composed of bovine-derived collagen-chitosan at a ratio of 75:25 and were compared to plain collagen scaffolds (100:0). Following 14 and 28 days in culture in osteogenic media, the scaffolds were stained with alizarin red as a marker of cell-mediated calcium deposition. The scaffold containing 100:0 collagen-chitosan had low levels of calcium deposition at both time-points (Fig. 4A and C) while the scaffold containing 75:25 collagen-chitosan (Fig. 4B and D) stained positively for mineralisation at both days 14 and 28. When calcium content within these scaffolds was quantified (Fig. 4E), the scaffold containing 75:25 collagen-chitosan caused the MSCs to deposit significantly more calcium than scaffolds composed of collagen alone (350 μg vs. 150 μg – $p < 0.01$).

3.5. Assessment of potential of collagen-chitosan composite scaffold to support MSC chondrogenesis

MSCs were cultured on scaffolds composed of bovine-derived collagen-chitosan at a ratio of 75:25 and were compared to plain collagen scaffolds (100:0). After 14 and 28 days in chondrogenic media, the scaffolds were stained using Safranin O which stains sulfated glycosaminoglycan (sGAG) pink. There were small amounts of sGAG evident around the periphery of the 100:0 collagen scaffold (Fig. 5A and B) while increased levels of sGAG can be seen in the scaffolds containing 75:25 collagen-chitosan (Fig. 5C and D). This observation was confirmed when sGAG was quantified showing significantly higher amounts of sGAG production on the scaffold containing 75:25 collagen-chitosan compared

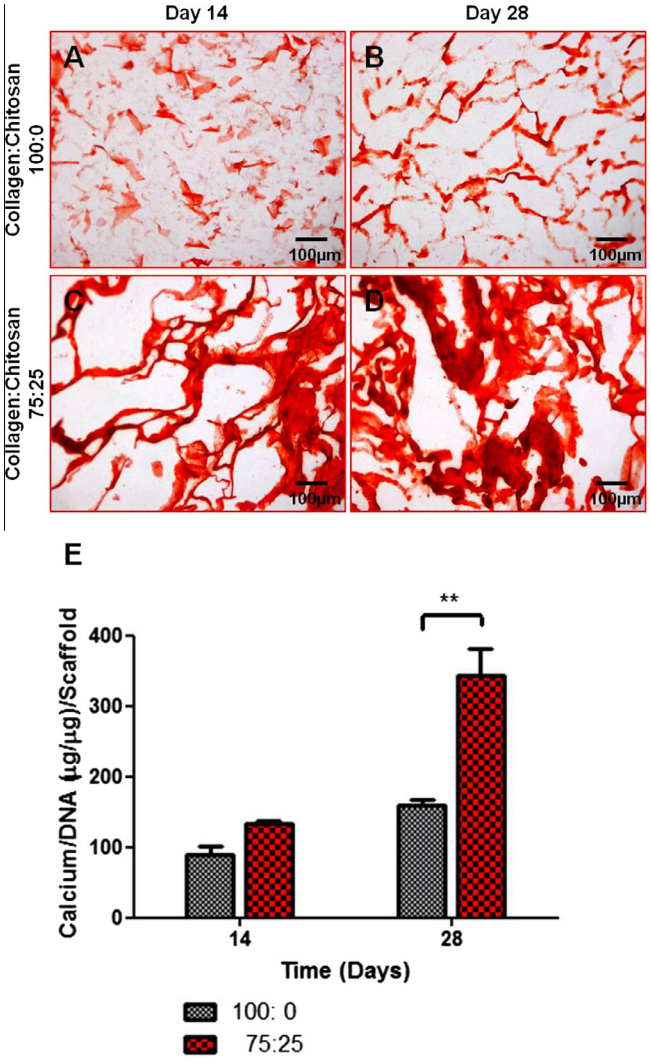

Fig. 4. Assessment of osteogenic potential of collagen-chitosan scaffold. Bovine-derived collagen scaffolds containing chitosan at ratios of 100:0 or 75:25 were seeded with MSCs and cultured in osteogenic media for 28 days. More mineral deposition (red) is seen with the scaffold containing 75:25 collagen-chitosan compared to the scaffold without chitosan (100:0). When quantified, MSCs cultured on the scaffold containing 75:25 collagen-chitosan deposited significantly more calcium compared to collagen alone (100:0). Data plotted shows mean ± standard deviation (n = 3) and * indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to the scaffold without chitosan (~40 μg per μg of DNA per scaffold compared to ~10 μg per μg of DNA per scaffold – $p < 0.01$) (Fig. 5E).

4. Discussion

The goal of this study was to investigate the effect of the addition of chitosan on the morphological and mechanical properties of collagen scaffolds and also to investigate if marine-derived collagen, isolated from salmon skin, might serve as a viable alternative to mammalian-derived collagen (from the bovine tendon) in orthopaedic tissue engineering applications. In addition, the potential of the resultant collagen-chitosan composite scaffold to enhance both osteogenesis and chondrogenesis was assessed. The results of this study demonstrated that the addition of chitosan improved the mechanical properties of both bovine and salmon-derived collagen scaffolds with bulk compressive modulus, swelling index and

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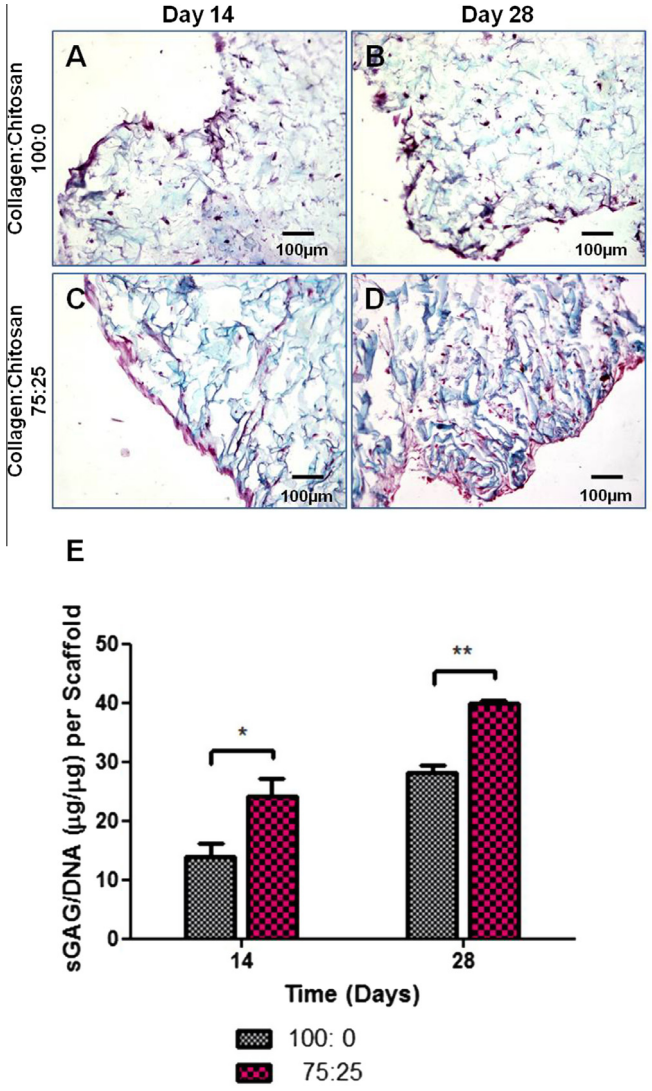

Fig. 5. Assessment of chondrogenic potential of collagen-chitosan scaffold. Bovine-derived collagen scaffolds containing chitosan at ratios of 100:0 or 75:25 were seeded with MSCs and cultured in chondrogenic media for 28 days. More sGAG deposition (pink) is seen with the scaffold containing 75:25 collagen-chitosan compared to the scaffold without chitosan. When quantified, MSCs cultured on the scaffold containing 75:25 collagen-chitosan deposited significantly more sGAG compared to collagen alone (100:0). Data plotted shows mean ± standard deviation ($n = 3$) and * indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

degradation rate increasing in proportion to chitosan content. However, overall, the bovine-derived collagen-based scaffolds were shown to be more suitable for orthopaedic tissue engineering than salmon-derived collagen-based scaffolds. Indeed, the bovine-derived collagen scaffold containing 75:25 collagen-chitosan supported both MSC osteogenesis and chondrogenesis with significantly more calcium and sGAG deposited respectively, when compared to collagen-alone (100:0) scaffolds. Taken together, the ability of chitosan to alter the mechanical and biological response of collagen scaffolds has led to the development of a biomimetic material highly suitable for orthopaedic tissue engineering.

The addition of chitosan to the bovine-derived collagen scaffold did not have a negative effect on scaffold architecture with all scaffolds retaining a highly porous interconnected microstructure. While the addition of chitosan caused a decrease in mean pore size in the bovine-derived collagen scaffold group, the pore size of the scaffold containing 75:25 collagen-chitosan was comparable to a previously developed collagen-GAG scaffold which has already shown success in bone tissue engineering applications [55–58]. Conversely, the incorporation of chitosan into salmon-derived collagen scaffolds caused a significant increase in mean pore size with increasing chitosan content from 90:10 to 50:50 collagen-chitosan. However overall, the salmon-derived collagen-derived scaffolds had a significantly lower mean pore size when compared to their bovine collagen derived counterparts. This is likely due to the lower density of the salmon-derived collagen which produces less viscous collagen slurry. In this study, the same freezing rate and heating phase was used for both bovine and salmon skin collagen. As the salmon skin collagen produced a less viscous slurry than the bovine collagen, the material responded differently to the freezing and heating steps, freezing faster than the more viscous bovine collagen and chitosan-containing slurries. Faster freezing leads to the formation of smaller ice crystals and therefore, smaller pores [47].

As chitosan and collagen interact via electrostatic interactions and hydrogen bonding, it was unsurprising that the incorporation of chitosan into collagen scaffolds significantly increased the bulk compressive modulus of both the bovine and salmon-derived collagen-chitosan scaffolds [49–51,59]. However, overall the bulk compressive modulus of the salmon-derived collagen-based scaffolds was lower than the bovine-derived collagen group as evidenced by a maximum stiffness of 0.56 kPa in the scaffold containing 50:50 collagen-chitosan compared to 1.23 kPa in the equivalent bovine-derived collagen scaffold containing 50:50 collagen-chitosan. The stiffness of a material has an effect on cell behaviour with stiffer materials supporting MSC osteogenesis and chondrogenesis and less stiff materials supporting differentiation towards softer tissues such as skin or muscle [5]. While the bulk compressive modulus is still orders of magnitude lower than that of native bone or cartilage tissue, the purpose of highly porous scaffolds is to provide temporary support and allow for cell infiltration and vascularisation and endogenous healing rather than acting as a permanent structure [6,60]. As the salmon-derived collagen scaffolds were three times less stiff than the bovine-derived collagen scaffolds, they may prove more useful in soft tissue regeneration applications such as skin.

A major function of GAGs in cartilage is to retain water at the site which acts to increase the compressive resistance of cartilage. As chitosan is also a linear polysaccharide with a similar molecular structure to GAGs, we hypothesised that the incorporation of chitosan into collagen scaffolds might increase the hydrophillicity of the scaffold. In this study, as chitosan content was increased from 100:0 to 50:50, the ability of the scaffold to retain water increased in both bovine and salmon-derived collagen groups. The ability of a scaffold to retain water is an important feature when designing a scaffold for tissue engineering as it determines cell infiltration and proliferation. A high swelling ratio has also been shown to promote enhanced MSC osteogenesis and chondrogenesis when compared to scaffolds with lower swelling capacity [61,62].

One of the main limitations to using collagen-based scaffolds in tissue engineering applications is their rapid degradation rate in physiological conditions. While tissue engineered constructs do not necessarily need to exactly match the native tissues mechanical strength [6,60], the degradation rate must be relatively consistent with the rate of new tissue formation. Natural bone healing following fracture comprises a highly coordinated series of events which lead to new bone formation within 25–28 days [63]. While cross-linking the scaffolds using physical and chemical methods can improve the degradation rate [52,53], in this study, the interactions between collagen and chitosan materials through electrostatic activity lead to the formation of an even more stable construct, which degraded by 10–40% over 28 days, a big improvement compared to the collagen alone scaffolds, which degraded by

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90% over the same length of time [17]. There was no difference in the degradation rate of bovine and salmon-derived collagen containing 90:10 and 75:25 collagen-chitosan; however, the salmon-derived collagen scaffold containing 50:50 collagen-chitosan lost its structural integrity during the study. Excess electrostatic interactions between collagen and chitosan can lead to denaturation of the collagen so it may be that the improvement in mechanical properties caused by chitosan is concentration dependent and the addition of 50:50 chitosan to collagen is detrimental to the structural and mechanical properties of salmon-derived collagen chitosan composite scaffolds.

In this study, differences in cell response were evident depending on chitosan content and collagen source. After 28 days in growth media, MSC proliferation on the bovine-derived collagen scaffold containing 75:25 collagen-chitosan was significantly higher than any other scaffold including the scaffold without chitosan (100:0). Increasing chitosan content in the salmon-derived collagen scaffolds also caused an increase in cell proliferation but not to the same extent as the bovine-derived collagen scaffolds. This may be due to a combination of factors; the salmon-derived collagen-based scaffolds had very small pore sizes which may have limited cell infiltration to the edge of the scaffold [64]. The structural integrity of the salmon-derived collagen scaffolds was also lost over the course of the study, likely due to the low denaturation temperature of marine-derived collagen, preventing cells from adhering [35]. The number of viable MSCs on the bovine-derived collagen scaffold containing 75:25 collagen-chitosan was significantly higher than the collagen scaffold without chitosan while cell viability was reduced on the scaffold containing 50:50 collagen-chitosan. This may be due to excess complexation between collagen and chitosan, which can leave less sites available for cell attachment and can cause collagen to denature [59]. The bovine-derived collagen scaffold composed of 50:50 collagen-chitosan also had a reduced pore size ($p < 0.001$), which may have inhibited cellular infiltration [64].

As the bovine-derived collagen scaffold containing 75:25 collagen-chitosan was shown to have good morphological and mechanical properties, as well as supporting MSC attachment and proliferation, the potential of this scaffold for bone and cartilage tissue engineering applications was assessed by using differentiation media containing osteogenic or chondrogenic supplements. In the osteogenesis study, chitosan proved to have significant osteogenic potential as the levels of calcium deposited by MSCs on the chitosan-containing scaffold (75:25) was significantly higher than in the collagen alone scaffold (100:0). Chitosan has previously been shown to support osteoblast proliferation and mineralisation [65,66] and has been used to coat scaffolds to increase osteoconductivity [67]. However, this is the first time MSC osteogenesis has been reported on a collagen-chitosan scaffold. In the chondrogenesis study, MSC-mediated sGAG production on the collagen-chitosan scaffold (75:25) was significantly higher than the collagen alone scaffolds (100:0). Owing to the similar molecular structure of chitosan and glycosaminoglycans found native to cartilage tissue, particularly hyaluronic acid, it is thought that this is why cells behave in a similar manner on substrates composed of each material. Therefore, not only does the addition of chitosan improve the scaffold's mechanical properties, the environment presented by the scaffold containing 75:25 collagen-chitosan closely mimics the natural environment of mesenchymal stem cells and may lead to improved formation of bone or cartilage-like tissue, compared to a scaffold composed of collagen alone. Overall, the salmon-derived collagen scaffolds did not perform as well as the bovine-derived collagen scaffolds in this study. However, as the salmon collagen was sourced from skin, rather than orthopaedic tissue as is the case with the bovine collagen, the salmon collagen may be better suited for soft tissue regeneration such as skin or muscle tissue engineering.

5. Conclusion

In this study we have demonstrated that the incorporation of chitosan into collagen scaffolds can significantly improve the scaffolds mechanical and biological properties. With the addition of chitosan, the mechanical properties of the collagen scaffold are enhanced, cell proliferation was increased, and the presence of chitosan enhances both MSC osteogenesis and chondrogenesis. While the salmon-derived collagen scaffolds used in this study did not perform as well as the bovine-derived collagen scaffolds for orthopaedic tissue engineering, further studies to optimise the lyophilisation and cross-linking methods may lead to better results in the future. The biomimetic bovine-derived collagen-chitosan composite represents a versatile biomaterial platform which is capable of being applied to both bone and cartilage tissue engineering applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2016.07.009.

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