Addition of hyaluronic acid improves cellular infiltration and promotes early-stage chondrogenesis in a collagen-based scaffold for cartilage tissue engineering

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Abstract

The response of mesenchymal stem cells (MSCs) to a matrix largely depends on the composition as well as the extrinsic mechanical and morphological properties of the substrate to which they adhere to. Collagen-glycosaminoglycan (CG) scaffolds have been extensively used in a range of tissue engineering applications with great success. This is due in part to the presence of the glycosaminoglycans (GAGs) in complementing the biofunctionality of collagen. In this context, the overall goal of this study was to investigate the effect of two GAG types: chondroitin sulphate (CS) and hyaluronic acid (HyA) on the mechanical and morphological characteristics of collagen-based scaffolds and subsequently on the differentiation of rat MSCs *in vitro*. Morphological characterisation revealed that the incorporation of HyA resulted in a significant reduction in scaffold mean pore size (93.9 μm) relative to collagen-CS (CCS) scaffolds (136.2 μm). In addition, the collagen-HyA (CHyA) scaffolds exhibited greater levels of MSC infiltration in comparison to the CCS scaffolds. Moreover, these CHyA scaffolds showed significant acceleration of early stage gene expression of SOX-9 (approximately 60-fold higher, $p<0.01$) and collagen type II (approximately 35-fold higher, $p<0.01$) as well as cartilage matrix production (7-fold higher sGAG content) in comparison to CCS scaffolds by day 14. Combining their ability to stimulate MSC migration and chondrogenesis *in vitro*, these CHyA scaffolds show great potential as appropriate matrices for promoting cartilage tissue repair.

Keywords: Chondrogenesis, scaffold, collagen, glycosaminoglycan, chondroitin sulphate, hyaluronic acid.

1. Introduction

Degeneration of cartilage and bone leading to osteoarthritis (OA) within human joints remains one of the most common causes of disability in the adult population. Approximately 12% of adults aged over 25 years in the United States of America were diagnosed with symptoms of OA in 2005 (Lawrence et al., 2008). Articular cartilage is a highly specialised tissue which is organised into distinct layers. These include an overlying articular cartilaginous layer, an intermediate calcified cartilage layer and an underlying layer of subchondral bone. Superficial articular cartilage injury eventually causes permanent changes to the underlying bone tissue and subsequently results in OA, characterised by chronic regional pain and dysfunction (Dieppe and Lohmander, 2005). The response of cartilage to injury differs from that of other tissue types because of its inability to heal even the most minor defects. This has been mainly attributed to its avascular nature, limited mobility of chondrocytes and poor proliferative capacity of mature chondrocytes (Newman, 1998). In the hip and knee joints, severe OA generally leads to the requirement for arthroplasty or replacement of the joint with artificial prostheses (Culliford et al., 2010). Numerous problems exist with arthroplasty and patients are often faced with living with pain, reduced mobility and eventually revision arthroplasty. In recent years, the focus has moved to regenerating cartilage through surgical repair techniques such as osteochondral grafting (mosaicplasty), bone marrow stimulation techniques and autologous chondrocyte implantation (ACI). These approaches have shown promise by promoting synthesis of *de novo* tissue and restoring some joint function over a short term period. However, long term performance of *de novo* tissue is still a major challenge with none of these approaches encouraging total repair of tissue with physiologically similar hyaline-like cartilage and as a consequence, surface fibrillation and subsequent degeneration often occurs (Buckwalter, 1997, Gobbi et al., 2006, Gross et al., 2008). The potential of tissue engineering (TE) to promote defect healing has been recognised (Cascio and Sharma, 2008, Getgood et al., 2009), although the ideal scaffold-based tissue engineered solution has yet to be realised.

A major challenge in cartilage TE is the design and fabrication of biomaterials that are instructive for specific cellular functions, thereby regulating cellular adhesion, proliferation and synthesis of cartilage

matrix in a controllable manner (Miot et al., 2005). Various natural materials are derived from components of the extracellular matrix (ECM), with polypeptides and polysaccharides being the most widely used due to their biodegradability and non-toxic degradation products. Collagen has received a lot of attention in TE owing to its abundance and its mechanical properties which exceed those of other natural materials (Aigner and Stove, 2003, Harley et al., 2007). Collagen possesses an abundance of functional groups along its structure allowing interaction with other molecules such as polysaccharides and protein-based growth factors (Getgood et al., 2009). The biofunctionality and regenerative capacity of collagen can be significantly improved by the addition of glycosaminoglycans (GAGs) (Tierney et al., 2009). Scaffolds fabricated from copolymers of collagen and GAGs have been successfully applied *in vitro* and *in vivo* for skin, tendon, nerve, conjunctiva and more recently for bone and cartilage repair applications (Yannas and Burke, 1980, Louie et al., 1997, Chamberlain et al., 1998, Pieper et al., 2000, Hsu et al., 2000, Farrell et al., 2006, Lyons et al., 2010a).

Articular cartilage ECM is composed of water (approximately 60-80 wt%), collagen (approximately 20 wt%, 90% of which is collagen type II) and GAGs (approximately 8 wt%). The GAG content is predominantly composed of chondroitin sulphate and keratan sulphate which are held together into aggregates by hyaluronic acid by means of specialised link proteins (Temenoff and Mikos, 2000). From a TE perspective, the interaction of GAGs with other ECM molecules and cells as well as their role in improving the response of collagen-based scaffolds for tissue regeneration, is of great interest. In particular, the addition of GAGs to collagen has the effect of altering the physical characteristics of collagen fibres, the morphology and subsequent cell response (Harley et al., 2007). Previous work in our laboratory has led to the development of novel collagen-GAG (CG) scaffolds optimised for orthopaedic applications which are fabricated using a freeze-drying technique that produces highly porous matrices with an interconnected pore structure (Haugh et al., 2009). These scaffolds have been shown to support mesenchymal stem cell (MSC) differentiation down an osteogenic lineage and to a lesser extent a chondrogenic lineage *in vitro* (Farrell et al., 2006). One of the main advantages of the GAGs within scaffolds is the potential for cells to interact with them through cell surface receptors, which may influence cell behaviour and direct MSC differentiation (Chung and Burdick, 2008). Studies previously carried out

on cartilage regeneration have shown that GAGs are fundamental for presenting motifs for cell attachment and migration. In addition, GAGs have also been attributed to activating pathways that govern proliferation and differentiation as well as assembly of the ECM (Pieper et al., 2000, Banu and Tsuchiya, 2007, Wu et al., 2010, Correia et al., 2011). Specifically, chondroitin sulphate (CS) which is the main type of GAG in cartilage ECM has been shown to stimulate chondrogenesis *in vitro* and promotes cellular ingrowth and cartilaginous tissue formation *in vivo* (van Susante et al., 2001, Buma et al., 2003). However, another GAG type, hyaluronic acid (HyA) has been shown to contribute to the migration and proliferation of MSCs and chondrocytes via association with specific cell surface receptors such as CD44 and RHAMM (Receptor for Hyaluronic Acid Mediated Migration) (Turley et al., 1993, Zhu et al., 2006, Toole, 2001). Moreover, the presence of HyA within scaffolds has been shown to create a chondro-inductive environment *in vitro* and *in vivo* promoting synthesis of cartilage tissue (Wu et al., 2010, Correia et al., 2011, Nehrer et al., 2006, Welsch et al., 2010).

In this study, we hypothesised that the presence of different GAGs within a CG scaffold would markedly alter MSC proliferation, differentiation and production of synthesised matrix. The aim was to elucidate the influence of two different GAG types (CS and HyA) incorporated within CG scaffolds on the mechanical and morphological properties and response of MSCs. Specifically, we investigated the effect of GAG type on the compressive modulus and mean pore size of scaffolds. We also examined how the presence of the two different GAG types affects the cross-linking mechanism after dehydrothermal (DHT) treatment. Subsequently, we investigated the ability of these GAG types to affect MSC proliferation and differentiation down a chondrogenic lineage (through chondrocyte-specific gene expression) and matrix production *in vitro* after 14 and 28 days.

2. Materials and methods

2.1 Fabrication of Collagen-Glycosaminoglycan scaffolds

CG scaffolds were fabricated using a freeze-drying method that has been previously described (O'Brien et al., 2004). CCS scaffolds were composed of collagen type I derived from bovine Achilles tendon (Collagen Matrix, USA) and CS derived from shark cartilage (Sigma-Aldrich, Arklow, Ireland). CHyA scaffolds were composed of collagen type I derived from bovine Achilles tendon (Collagen Matrix, USA) and HyA sodium salt derived from *streptococcus equi* (Sigma-Aldrich, Arklow, Ireland). Both CCS and CHyA suspensions were made by dissolving collagen and GAG in 0.5 M (pH 2.8) glacial acetic acid separately and blended at 15,000 rpm (IKAT18, IKA Works Inc, NC, USA). The blending process was carried out for 3.5 hours in a cooling system (WKL230, Lauda Brinkman, Westbury, CT, USA) at 4°C in order to prevent denaturation. The final concentrations of the suspensions were composed of 0.5% (w/v) collagen and 0.05% (w/v) CS or HyA. A GAG-free collagen suspension with a final concentration of 0.5% (w/v) collagen was also fabricated. Subsequently, the suspensions were freeze-dried (Virtis Genesis 25EL, Biopharma, Winchester, UK) at a constant cooling rate of 1°C/min to a final temperature of -40°C. After freeze-drying, the scaffolds were cross-linked using DHT treatment under vacuum of 50 mTorr and a temperature of 105ºC in a vacuum oven (VacuCell, MMM, Germany) for a duration of 24 hours.

2.2 Mechanical and morphological characterisation

2.2.1 Effect of GAG type on mean scaffold pore size

Pore size analysis was carried out using a histological technique previously described (O'Brien et al., 2005). Scaffold samples were obtained from three fixed locations on each fabricated sheet of the scaffolds. These samples were cut using a metal punch with a diameter of 9.5 mm and 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. Histological sections were stained using toluidine blue (Sigma-Aldrich, Arklow, Ireland). Digital images were captured at 10x magnification using a 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 (Haugh et al., 2009). The programme transformed the images into binary form and calculated the average pore radii based on best-fit elliptical lengths.

2.2.2 Effect of GAG type on molecular interaction and cross-linking mechanism after dehydrothermal treatment

Fourier Transform Infrared Spectroscopy (FTIR) microscopy was carried out using a BRUKER Tensor-27 FTIR spectroscopy machine (Bruker Tensor 27, Bruker Optik, Ettlingen, Germany) utilizing the attenuated total reflectance technique and OPUS data collection software (Bruker Optik). Cylindrical samples (8 mm diameter) were used for the analysis and milled with potassium bromide which is transparent to infrared. The spectra were collected over a range of 400–4000 cm^{-1} to monitor differences in the biochemical compositions of scaffolds before and after cross-linking as well as the cross-linking mechanism involved. The spectra were obtained at a resolution of 4 cm^{-1} and the samples were scanned 32 times to increase the signal-to-noise ratio. Formation of cross-links through DHT treatment was monitored by analyzing the characteristic amide I and II band absorbance peaks at 1630 cm^{-1} and 1550 cm^{-1} respectively. The band at 1550 cm^{-1} is proportional to the amount of amine NH_2, which is converted to amide NH during the formation of cross-links through condensation reactions. Additionally, absorbance between 970 cm^{-1} and 1300 cm^{-1} was assessed to observe the presence of ester bonds which correspond to GAG content within the scaffold structure.

2.2.3 Effect of GAG type on scaffold compressive modulus

Uni-axial, unconfined compressive testing was carried out to determine the compressive elastic modulus. Samples were pre-hydrated in phosphate buffered saline (PBS, Sigma-Aldrich, Arklow, Ireland) for 1 hour 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 kept immersed in PBS throughout the tests. The tests were conducted at a strain rate of 10% per minute and each sample was tested three times. 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 2-5% strain.

2.3 In vitro analysis

2.3.1 Cell isolation

MSCs were isolated from the bone marrow of three month old wistar rats (approximately 250-300 g) after ethical approval from an internal ethics committee. The femora and tibiae were dislocated and the bone marrow was flushed out and MSCs isolated on the basis of adherence to tissue culture plastic (Duffy et al., 2011). The cells were incubated with Dulbecco's modified eagles medium (DMEM, Sigma-Aldrich, Arklow, Ireland) supplemented with 10% fetal bovine serum (Biosera, Ringmer, UK), 100 U/mL penicillin/streptomycin (Sigma-Aldrich, Arklow, Ireland), 2 mM glutamax (Gibco-Biosciences, Dun Laoghaire, Ireland), 1 mM L-glutamine (Sigma-Aldrich, Arklow, Ireland) and 1% non-essential amino acids (Gibco-Biosciences, Dun Laoghaire, Ireland). Once the cells reached a confluence of between 80-90%, they were passaged and re-plated onto T-175 (175 cm^2 growth area) flasks. For the entirety of this study, cells were cultured onto scaffolds at passage number 3.

2.3.2 Cell culture

Cells were detached from culture flasks using trypsin ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, Arklow, Ireland). Subsequently, cells were counted and re-suspended at a cell density of $5X10^6$ cells per mL and a total volume of 100 µL per scaffold. Scaffolds of 9.5 mm in diameter and 4 mm in height were pre-hydrated in PBS for 15 minutes, and placed in 6 well-plates. The cell suspension was then added to the scaffolds, 50 µL on one side of each scaffold and incubated for 15 minutes in a 5% CO_2, 37°C incubator to allow initial attachment. The seeded scaffolds were subsequently turned over and the procedure repeated. After the second incubation period, 5 mL of supplemented DMEM growth medium was added to each well and pre-cultured for 7 days (medium change on day 3). After 7 days, the growth medium was replaced with medium supplemented with chondrogenic factors. The chondrogenic factors included 20 ng/mL human TGF-β3 (Prospec, Rehovot, Israel), 50 µg/mL ascorbic acid (Sigma-Aldrich, Arklow, Ireland), 40 µg/mL proline (Sigma-Aldrich, Arklow, Ireland), 100 nM dexamethasone

(Sigma-Aldrich, Arklow, Ireland), 1X ITS (Insulin, Transferrin, Selinium) (BD Biosciences, Oxford, UK), and 0.11 mg/mL sodium pyruvate (Sigma-Aldrich, Arklow, Ireland). The wells were subsequently incubated for a further 14 and 28 days with medium being changed at day 3 intervals.

2.3.3 Effect of GAG type on cell density

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, Arklow, Ireland). Cell number was quantified using a Hoechst dye 33258 assay which fluorescently labels double stranded DNA as previously described (Murphy et al., 2010). Measurements were taken from a fluorometric plate reader (Wallac 1420 Victor^2 D, Perkin Elmer, MA, USA) at an emission of 460 nm and excitation of 355 nm, 1.0 s. The measurements were read against a standard curve to obtain the relative cell numbers per scaffold in terms of the DNA content.

2.3.4 Histological analysis of scaffolds

Samples were fixed in 10% formalin for 1 hour and using a histokinette tissue processor (Leica TP1050, Leica Microsystems, Wetzler, Germany), were dehydrated and paraffin wax embedded. Blocks of wax embedded samples were serially sectioned using a microtoming machine (Leica RM2255, Leica Microsystems, Wetzler, Germany) which sliced the samples at 10 µm thick sections and every 20th section was used to give a gap of 200 µm between sections. These sections were successively mounted on polysine-coated glass slides (Fisher-Scientific, Dublin, Ireland). The glass slides were then deparaffinised and hydrated before staining. Stains used in the histological analysis were haematoxylin which stains the DNA and RNA rich cell nuclei, eosin which stains the extracellular matrix, safranin-O which stains sulfated GAGs and fast green, a light counter stain for the sections.

2.3.5 RNA extraction and gene expression analysis

The total RNA was isolated using an RNeasy kit (Qiagen, Crawley, UK) as previously described (Duffy et al., 2011). 200 ng of total RNA was reverse transcribed to cDNA using a QuantiTect reverse transcription

kit (Qiagen) on an authorised thermal cycler (Mastercycler Personal, Eppendorf, UK). Real time polymerase chain reactions were run on 7500 real-time PCR System (Applied Biosystems, UK) using a QuantiTect SYBR Green PCR Kit (Qiagen, UK). The relative expression of mRNA was calculated by delta-delta Ct (ΔΔCt) method, where delta Ct (ΔCt) is the value obtained by subtracting the Ct value of a house-keeping gene GAPDH, from the Ct value of the target mRNA (collagen type I, collagen type II, collagen type X and SOX-9). These target genes were chosen since they are expressed in cells differentiating down a characteristic chondrogenic lineage.

The amount of target mRNA relative to GAPDH was normalised to a calibrator sample to generate ΔΔCt. This was then converted to a fold increase in expression using the formula: Fold increase = $2^{-(\Delta\Delta Ct)}$ (Dussault and Pouliot, 2006).

2.3.6 Effect of GAG type on sulfated GAG production

Scaffolds were digested in papain enzyme solution as described in the cell density assay. A solution of di-methyl-methylene blue (DMMB) dye was prepared from 0.3% glycine, 0.25% sodium chloride, and 1.6% DMMB dye. A standard curve was generated from serial dilutions of chondroitin sulphate in Tris-EDTA (pH 8.0). Digested samples were diluted in Tris-EDTA buffer at a ratio of 1:10 digest to buffer. 180 µL per well of DMMB dye was subsequently added into a 96 well plate as well as 20 µL of the diluted digested samples. Readings were taken from a photometric plate reader (Wallac 1420 Victor^2 D, Perkin Elmer, MA, USA) at 530 nm, 1.0 s. The background photometric reading of the scaffolds was accounted for by subtracting the reading of cell-free scaffolds from the average reading of each cell-seeded scaffold.

2.3.7 Effect of synthesised matrix on the bulk compressive moduli of scaffolds

During the incubation period, the compressive modulus of the seeded scaffolds was investigated to determine the effect of synthesised sGAG content on the overall bulk compressive modulus. Scaffolds were seeded with rMSCs in growth medium and pre-cultured for 7 days and subsequently transferred to chondrogenic supplemented medium for up to 28 days. At days 0, 14 and 28 uni-axial, unconfined compressive testing was carried out on samples immersed in PBS using a mechanical testing machine (Zwick-Roell, Germany) as mentioned previously.

2.3.8 Statistical analysis

Differences among groups were assessed by two-way ANOVA with Tukey's post hoc analysis to identify statistical differences among three or more treatments when p was less than 0.05. Comparisons between two treatments were assessed by student's paired t-test to identify statistical differences when p was less than 0.05. A sample size of 3 per group was used throughout the study unless otherwise state. All results were reported as mean ± standard deviation.

3. Results

3.1 Effect of GAG type on scaffold morphology

Figure 1 shows representative SEM images of a CG scaffold demonstrating the pore interconnectivity. The addition of HyA significantly reduced the mean pore size of the collagen scaffolds (Table. 1). GAG-free collagen scaffolds possessed a mean pore size of 103.1 μm. The addition of CS resulted in scaffolds with the largest mean pore size of 136.2 μm which was significantly larger ($p<0.05$) than the mean pore size of the GAG-free collagen scaffolds. However, the addition of HyA resulted in scaffolds which possessed approximately 10% smaller mean pore size (93.9 μm) than GAG-free collagen scaffolds. All three scaffold variants were found to be highly porous with porosities over 98% regardless of presence or absence of GAGs.

3.2 Effect of GAG type on infrared absorption using Fourier transform infrared (FTIR) microscopy

FTIR spectra were analysed on all scaffolds and it was evident that the presence of different GAG types did not affect the cross-linking mechanism after DHT treatment (Fig. 2). Absorption bands corresponding to cross-link bonds between collagen and GAG molecules were present on the absorption spectrum. Specifically, ester bands corresponding to bonds between carboxyl and hydroxyl groups of collagen and GAG were present on the spectra of CCS and CHyA scaffolds between 970 and 1300 cm^{-1}. Intermolecular cross-links between the collagen molecules were also present on all three scaffold variants after DHT treatment between carboxyl and amino groups. In particular, amide II bands at 1550 cm^{-1} and amide I bands at 1630 cm^{-1} were present on all scaffold variants.

3.3 Effect of GAG type on scaffold compressive modulus

The compressive moduli of the scaffold variants was measured in untreated and DHT treated scaffolds and the results demonstrated that the moduli of all three scaffold variants was significantly increased ($p<0.05$) after DHT treatment (Fig. 3). The moduli of all untreated scaffolds were between 0.2 and 0.25 kPa and there was no significant difference between GAG-free collagen and CG scaffolds. After DHT treatment, the modulus of all scaffold variants significantly increased by approximately 2-fold (between 0.45 kPa and 0.55 kPa). However, no significant difference between GAG-free collagen, CCS and CHyA scaffolds was found.

3.4 Effect of GAG type on cell density

DNA quantification was assessed to determine the increase in cell density (cells per scaffold) from the point of seeding and the results demonstrated that CG scaffolds had a higher cell density than GAG-free scaffolds at day 14 (Fig. 4). The presence of both CS and HyA significantly increased cell density by day 14 (approximately 2-fold higher than GAG-free collagen scaffolds, $p<0.05$). However, by day 28, the cell density of GAG-free and CG scaffolds was comparable.

3.5 Effect of GAG type on cellular infiltration and sulphated GAG distribution within scaffolds

Histological analysis revealed that CHyA scaffolds displayed greater levels of cell migration towards the centre of scaffolds with a resulting homogeneous cellular distribution throughout the construct (Fig. 5). Consequently, there was greater distribution of sulphated GAG content on these CHyA scaffolds. GAG-free collagen scaffolds on the other hand, showed poor migration of cells towards the centre of the scaffold since the majority of cells adhered to the periphery (Fig. 5A). Similarly, CCS scaffolds displayed an encapsulation of cells on the periphery limiting the migration of cells towards the centre of the scaffold (Fig. 5B). Additionally, dense staining for sulphated GAG content was observed to be predominant on the periphery of collagen and CCS scaffolds.

3.6 Effect of GAG type on gene expression

Real-time RT-PCR analysis showed that overall, CHyA scaffolds resulted in significantly higher SOX-9 and collagen type II expression than CCS or GAG-free scaffolds as early as 14 days post-seeding (Fig. 6). It was evident that early stage markers of chondrogenesis appeared to be highly expressed much faster in the presence of HyA than in the presence of CS or GAG-free scaffolds. In particular, by day 14 the level of collagen type I and SOX-9 were found to be expressed significantly higher in the presence of HyA (90-fold higher collagen type I and 200-fold higher SOX-9 expression, $p<0.01$) than GAG-free collagen scaffolds. Additionally, collagen type II expression was also found to be higher in the presence of HyA than GAG-free scaffolds (40-fold higher collagen type II expression, $p<0.01$). Comparing the CG scaffolds, it was evident that the presence of HyA resulted in significantly higher SOX-9 and collagen type II expression than in the presence of CS ($p<0.01$) by day 14.

By day 28 however, SOX-9 and collagen type II gene expression were found to be significantly higher on CCS scaffolds than CHyA scaffolds ($p<0.01$). GAG-free scaffolds also showed significantly higher levels of SOX-9 expression than CHyA scaffolds by day 28 although expression of collagen type II still remained minimal. There was no evidence of significant differences in the expression of late stage marker collagen type X between the three scaffold variants by day 28.

3.7 Effect of GAG type on sulfated GAG production

The effect of GAG type on the quantity of sGAG synthesised on scaffolds demonstrated that the presence of HyA significantly accelerated sGAG production relative to CS or GAG-free scaffolds (Fig. 7). By 14 days post-seeding, the level of sGAG per cell was significantly higher ($p<0.05$) in the presence of HyA (32 µg/µg sGAG/DNA) than in the presence of CS (4 µg/µg) or absence of GAG (3 µg/µg sGAG/DNA). Between day 14 and 28 there was a significant increase in production of sGAG in CCS scaffolds (39 µg/µg sGAG/DNA) and GAG-free collagen scaffolds (37 µg/µg sGAG/DNA). CHyA scaffolds had a total sGAG content of 41 µg/µg sGAG/DNA by 28 days. There was no significant difference in the quantity of sGAG between the 3 scaffold variants by day 28.

3.8 Effect of synthesised sulphated GAG matrix on the bulk compressive moduli of scaffolds

The results showed that following culture, the modulus of all scaffold variants increased due to the newly laid down matrix when normalised to seeded scaffolds at day 0 (Fig. 8). GAG-free collagen scaffolds showed a significant increase in compressive modulus from 0.5 to 1.1 kPa from day 0 to day 28 ($p<0.05$). On the other hand, CCS scaffolds showed a significant increase in compressive modulus from 0.6 kPa to 1.62 kPa from day 0 to day 28 ($p<0.05$). CHyA scaffolds showed a significant increase in compressive modulus from 0.6 kPa to 1.67 kPa from day 0 to day 28 ($p<0.05$). By day 28, CG scaffolds had a significantly higher ($p<0.05$) bulk compressive moduli than GAG-free collagen scaffolds (approximately 0.4 kPa higher).

4. Discussion

The overall goal of this study was to investigate the effect of incorporation of two different GAG types CS and HyA on the mechanical and morphological characteristics of collagen scaffolds and subsequently the response of seeded rMSCs *in vitro*. The results demonstrated that although CHyA scaffolds possessed smaller mean pore sizes, these scaffolds were able to stimulate greater levels of rMSC infiltration in comparison to CCS scaffolds. Moreover, these CHyA scaffolds promoted significant up-regulation of early stage gene expression of SOX-9 (approximately 60-fold higher, $p<0.01$) and collagen type II (35-fold higher, $p<0.01$) in comparison to CCS scaffolds. Additionally, CHyA scaffolds promoted greater levels of cartilage matrix production (7-fold higher sGAG content, $p<0.01$) in comparison to CCS scaffolds at day 14. Taken together, the ability of the different GAG types to elicit distinct MSC response in terms of cellular migration and cartilage-specific matrix production *in vitro* demonstrates that the composition of scaffolds plays a major role in the type and quality of cartilage tissue synthesised.

Morphologically, the addition of HyA resulted in significant reduction of scaffold mean pore size (93 μm) relative to CS (136 μm). Although we produced both scaffold types using identical freeze-drying parameters, analysis of the experimental temperature profiles of both CCS and CHyA suspensions (data not shown) demonstrated that the CHyA suspension cooled to the final temperature -40°C much quicker

than CCS suspensions. The freezing characteristics of CHyA scaffolds, which determine the pore size of the fabricated scaffolds, were thus different to CCS scaffolds. The composition of a colloidal suspension clearly affects the freezing dynamics of the material during the lyophilisation process used in this study to produce the collagen-based scaffolds. The thermal conductivity of these suspensions is governed by composition and can increase or decrease the rate of freezing for a given set of freezing parameters. Consequently, a higher thermal coefficient results in a higher rate of freezing, which in turn affects the nucleation rate of ice crystals. Faster freezing produces smaller ice crystals and smaller mean pore sizes after sublimation. Based on the temperature profiles recorded, the thermal conductivity of CHyA suspension appeared to be higher than CCS suspensions, resulting in a smaller average pore size in the CHyA scaffolds.

GAG type did not affect the overall compressive modulus of CG scaffolds. However, as expected, DHT cross-linking treatment led to significant increases in the compressive moduli of all scaffold variants (2-fold increase relative to uncross-linked scaffolds) (Fig. 3). Using cross-linking treatment, the mechanical properties of collagen scaffolds can be improved by stabilising the interaction between collagen molecules. Additionally, CG scaffolds can be improved by stabilising the interaction between collagen and GAG molecules which dissociate at near neutral pH. From a TE perspective, improving the mechanical properties of scaffolds is beneficial especially for their performance *in vivo*. The mechanical properties of a substrate or scaffold have been shown to affect the fate of MSCs *in vitro* (Discher et al., 2005, Karamichos et al., 2008, Vickers et al., 2010, Tse and Engler, 2011). Given that no significant differences were observed as a result of the incorporation of the two different GAG types, it is likely that any differences in MSC response observed during the *in vitro* culture were not strongly influenced by the mechanical properties of the scaffolds but were due to differences in composition and scaffold architecture.

Although the presence of the different GAG types resulted in scaffolds with comparable mechanical properties, differences in cell response were observed between scaffolds containing different GAG types. After culture for 28 days in chondrogenic media with rMSCs, there was no difference in the cell density of

CCS and CHyA scaffolds (Fig. 4). However, CHyA scaffolds showed greater levels of cell infiltration than CCS scaffolds as shown by histological analysis (Fig. 5). In addition, CHyA scaffolds exhibited more homogeneous cell distribution than CCS scaffolds which were encapsulated by a layer of cells. This encapsulation on CCS scaffolds was observed as cells aggregating on the edge of scaffolds with limited cell infiltration towards the centre of scaffolds. There is growing evidence that the characteristic micro-architecture of scaffolds plays a role in the migration of cells. A recent study showed that scaffolds with larger pore sizes (mean pore size range of 160-325 µm) resulted in greater levels of cell infiltration than scaffolds with smaller pore sizes (mean pore size range of 85-120 µm) (Murphy et al., 2010). In our study, it was evident that CCS scaffolds which possessed a mean pore size of 136 µm exhibited poor cell infiltration but surprisingly, CHyA scaffold with a smaller mean pore size of 93 µm exhibited greater levels of cell infiltration. This finding suggested that cell infiltration in CHyA scaffolds was governed by a mechanism distinct from pore size and architecture. Since the physical influence of the scaffold was minor, due to the similar mechanical properties of CCS and CHYA scaffolds, chemical properties of HyA, within the scaffold over the 28 days may also play a major role in this behaviour. The high anionic charge of HyA may alter the visco-elastic properties of the pericellular matrix surrounding cells and as a result, preventing cells from adhering firmly onto scaffolds struts possibly leading to increased migration (Toole, 2001, Chen and Abatangelo, 1999). Another mechanism by which cell migration could be occurring may be a result of intracellular signalling pathways initiated by the specific interaction of MSCs and HyA through cell receptors such as integrins, CD44 and RHAMM (Receptor for Hyaluronic Acid Mediated Motility) (Toole, 2001, Chen and Abatangelo, 1999, Friedl and Bröcker, 2000, Knudson, 2003). Indeed it has been shown that inhibition of CD44 receptor resulted in MSCs with a reduced motility on HyA coated surfaces (Zhu et al. 2006). Therefore, there is a possibility that one of the stimuli for the greater levels of cell infiltration on CHyA scaffolds may be mediated by the interaction of MSCs and HyA.

While the presence of TGF-β resulted in MSC differentiation down a chondrogenic lineage in all scaffold groups, the level of gene expression was seen to be largely influenced by the composition of the scaffolds. The presence of HyA markedly affected the level of early-stage chondrogenic gene expression within the scaffolds at a number of culture time points (Fig. 6). In particular, SOX-9 and collagen type II

genes were found to be highly expressed on the CHyA group (60-fold and 35-fold increase respectively) relative to CCS scaffolds at day 14. Previous studies investigating MSC differentiation down a chondrocytic lineage within porous scaffolds in the presence of TGF-β have been shown to induce MSC differentiation down a chondrogenic lineage (Jenner et al., 2007, Li et al., 2005, Chung and Burdick, 2008). In particular, a number of studies have shown that at 14 days, MSC differentiation and subsequent collagen type II gene expression was evident in the presence of TGF-β (Lee et al., 2008, Lisignoli et al., 2005, Bosnakovski et al., 2006, Park et al., 2009). Pellet culture systems are believed to be ideal culture environments to induce chondrogenesis due to the cell-cell contact which mimics the high cell density of embryonic cartilage development and maintains a chondrocytic phenotype (Fan et al., 2008, Chang et al., 2008, Zhang et al., 2010). A study comparing pellet culture and 3-dimensional culture showed that there was a significant increase in collagen type II expression on pellet cultures than on alginate and chitosan beads at day 14 (Bernstein et al., 2009). However, our results demonstrated that the use of CHyA scaffolds, with a relatively low cell density, exhibited chondrocyte-specific gene expression with high levels of SOX-9 and collagen type II as early as 14 days. Due to the highly porous nature of the scaffolds used in this study, we believe that the cell density was sufficient to investigate the interaction of cells and scaffolds. This cell seeding density was based on previous work carried out in our laboratory and has been used both *in vitro* and *in vivo* (Farrell et al., 2006, Byrne et al., 2008, Lyons et al., 2010b, Alhag et al., 2011). A higher cell density would create a phenomenon whereby cell-cell interaction out-weighed cell-matrix interaction resulting in an imprecise interpretation of the response of MSCs to the differing compositions. The exact mechanism as to how HyA mediated earlier expression of collagen type II than CS is not fully understood. Nevertheless, we would suggest that the presence of HyA on the pericellular matrix of cells may facilitate the association of soluble growth factors available through immobilisation. Thus, HyA may provide co-receptors for sequestration and presentation of the necessary biochemical factors for commitment towards a chondrocytic lineage leading to gene expression and protein synthesis.

Consistent with the gene expression results, cartilage matrix production occurred earlier in the presence of HyA relative to GAG-free and CCS scaffolds. By day 14, significantly higher levels of sGAG production in CHyA scaffolds in comparison to GAG-free and CCS scaffolds were observed (Fig. 7). GAG-free

collagen and CCS scaffolds exhibited low sGAG accumulation by day 14 and it was only by day 28 that the total sGAG content was significantly increased and comparable to CHyA scaffolds. While the quantities of sGAG were comparable between CCS and CHyA scaffolds at day 28, it was only after comparing sGAG production with histological analysis that the advantage of CHyA was realised. It was evident that sGAG content was more evenly distributed in the CHyA scaffolds than the GAG-free collagen scaffolds and CCS scaffolds. Absence of HyA resulted in accumulation of sGAG on the periphery of scaffolds. CS is the main type of GAG in cartilage ECM and has been shown to be chondro-inductive *in vitro* (van Susante et al., 2001). From our results, it was evident that the presence of CS led to MSC differentiation and chondrogenesis. However, the restricted cell and matrix distribution on CCS scaffolds may have inhibited early retention of sGAG content within the scaffold and may explain the low sGAG content at day 14. HyA is secreted prior to condensation during cartilage growth and is quickly followed by formation of aggregates of proteoglycans held together by the HyA molecule (Toole, 2001, Knudson and Knudson, 2004). Due to the proteoglycan-binding role of HyA in the native cartilage ECM, we hypothesise that the presence of HyA in a scaffold may aid in the aggregation of synthesised sGAG within the scaffold and thus may explain the better distribution and higher level as early as day 14.

As matrix deposition commences, it is believed that there is a switch to expression of late stage genes such as aggrecan (Goldring et al., 2006). Therefore we believe that the decrease in SOX9 and collagen type II from 14 to 28 days in CHyA scaffolds may indicate that cells are maturing and initiating expression of later stage markers of chondrogenesis. A similar trend was observed in sGAG content whereby production was higher in CHyA scaffolds at day 14 in comparison to CCS and GAG-free scaffolds but the rate of production decreased beyond day 14. At day 28 CHyA scaffolds may have been saturated with the synthesised sGAG owing to the slowed rate of production between 14 and 28 days. It is not certain whether other scaffolds would show a similar decline at later time points based on our results. However, this down regulation may simply point to a faster rate of differentiation and maturation in the presence of HyA in comparison to CS.

The CG scaffolds in this study showed an increase in compressive modulus from 0.6 kPa to approximately 1.6 kPa after 28 days in culture due to matrix deposition. For comparison, native bovine articular cartilage has a compressive modulus of approximately 300 kPa (Pfeiffer et al., 2008). This is mainly due to the complex organisation of collagen, proteoglycans and water in native articular cartilage. We are not attempting to engineer tissue *in vitro* that is comparable to native articular tissue as we would expect that if these constructs were implanted *in vivo*, they would be remodeled and replaced with new tissue and that the most important mechanical properties are that of the regenerated tissue. However, we observe an increase in bulk compressive modulus of the scaffolds (by approximately 1 kPa) as the cells synthesise new matrix after only 28 days in culture. In addition, the ability of MSCs to differentiate to a chondrocyte-specific phenotype and synthesise matrix increasing the overall modulus of the scaffolds demonstrates both the effectiveness of using MSCs as an alternative to chondrocytes for *in vitro* investigation and the potential of these scaffolds for cartilage repair.

5. Conclusion

In this study, we have shown that the composition of scaffolds for tissue engineering plays a major role in the behaviour of MSCs. With the addition of GAGs, the biofunctionality of collagen-based scaffolds for chondrogenesis was significantly enhanced. Furthermore, we speculate that HyA may stimulate migration of MSCs into scaffolds by a mechanism distinct from micro-architecture, conceivably through a chemotaxic or perhaps through the high anionic charge of HyA in altering the interaction of cells and matrices. These CHyA scaffolds demonstrated the ability to facilitate MSC differentiation down a chondrogenic lineage and promote cartilage-specific matrix production *in vitro*. The quality of cartilage matrix produced within CHyA scaffolds, as assessed by collagen type II gene expression, was superior to that of CCS or GAG-free collagen scaffolds as early as 14 days. These findings make CHyA an excellent scaffold for further investigation of cartilage defect repair.

Acknowledgements

Funding for this study was provided by Enterprise Ireland, Commercialisation Fund Technology Development Phase CFTD/2009/0104.

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Table and figure legends

Table 1. Effect of GAG type on mean scaffold pore size and percentage porosity. Mean pore size was measured as an average diameter (μm) of scaffold pores. Percentage porosity was calculated as a relative ratio of scaffold density to the density of CG. The presence of CS significantly increased the mean pore size of collagen scaffolds whereas HyA significantly decreased the mean pore size. Values are expressed as mean ± standard deviation, n=6. (*) denotes $p<0.04$ statistical significant difference in comparison to collagen.

Figure 1 Representative SEM images of a CG scaffold showing the pore interconnectivity. The images were taken at 15 kV and a magnification of x10 (A) and x100 (B). The scale bars represent 1mm (A) and 100 μm (B).

Figure 2. FTIR spectroscopy of collagen and CG scaffolds cross-linked using DHT treatment showing characteristic features of collagen and GAG. Amide bands were found to be present in the scaffold structure of all scaffold variants after DHT treatment. Ester bands which correspond to the presence of GAGs were also found to be present on the structure of CCS and CHyA scaffolds after cross-linking.

Figure 3. The compressive modulus of collagen and CG scaffolds in kPa. Unconfined uni-axial testing was carried out on uncrosslinked and DHT-treated (105ºC for 24 hours) scaffolds. The graph shows an increase in compressive modulus due to DHT treatment. Values are expressed as mean ± standard deviation, n=3. * denotes $p<0.05$ statistical significant difference in comparison to uncrosslinked scaffolds.

Figure 4. Cell density of collagen and CG scaffolds after 14 and 28 days in culture with rMSCs in chondrogenic medium. Cell density was analysed by Hoechst dye DNA quantification. GAG-free collagen scaffolds appear to have significantly lower cell density than CCS and CHyA scaffolds after 14 days. Values are expressed as mean ± standard deviation, n=3. * denotes $p<0.05$ statistical significant difference in comparison to collagen at day 14.

Figure 5. Histological sections of collagen (A), CCS (B) and CHyA (C) scaffolds after 28 days in culture with rMSCs in chondrogenic medium. The sections were stained with haematoxylin (red) which stains cellular nuclei, safranin-O (pink) which stains synthesised sulphated GAG, and fast green a light counter stain. Scale bar represents 200 μm length. CHyA scaffolds have a homogeneous cell distribution and more positive staining for synthesised sGAG. GAG-free collagen scaffolds and CCS scaffolds demonstrate an encapsulation of cells and synthesised sGAG

Figure 6. Gene expression of SOX-9 (A); collagen type I (B); collagen type II (C); and collagen type X (D), assessed after day 14 and 28 in culture with rMSCs in chondrogenic medium. The expression was normalised to a housekeeping gene GAPDH. The values are expressed as fold difference in comparison to expression by cells in collagen scaffolds at day 0. a denotes $p<0.01$ statistical significant difference in comparison to collagen at day 14, b denotes $p<0.01$ statistical significant difference in comparison to CHyA at day 14, c denotes $p<0.01$ statistical significant difference in comparison to CHyA at day 28. Early up-regulation of SOX-9 and collagen type II observed on CHyA scaffolds in comparison to GAG-free collagen and CCS scaffolds at day 14.

Figure 7. The total content of synthesised sulphated GAG (sGAG) in scaffolds assessed after 14 and 28 days in culture using a DMMB assay, normalised to DNA content. Graph shows mean $\pm$ standard deviation, n=3 (b denotes $p<0.01$ statistical significant difference in comparison to CHyA at day 14). The graph shows significant increase in sGAG content on the CHyA scaffold in comparison to GAG-free and CCS scaffolds as early as day 14.

Figure 8. The bulk compressive modulus of rMSC-seeded scaffolds cultured up to 28 days with chondrogenic medium. The graph shows an increase in compressive modulus with time in culture from day 0. CCS and CHyA scaffolds possessed significantly higher compressive modulus than GAG-free collagen scaffolds after 28 days in culture. Values are expressed as mean $\pm$ standard deviation, n=3. *

denotes $p<0.05$ statistical significant difference in comparison to collagen at day 28. # denotes $p<0.05$ statistical significant difference in comparison to day 14.

Table 1.

Scaffold type	Mean pore size (μm)	Porosity (%)
Collagen	103.15 ± 11.17	99.49 ± 0.014
CCS	136.17 ± 18.98 (*)	99.51 ± 0.023
CHyA	93.88 + 8.78	98.79 + 0.017

Figure 1

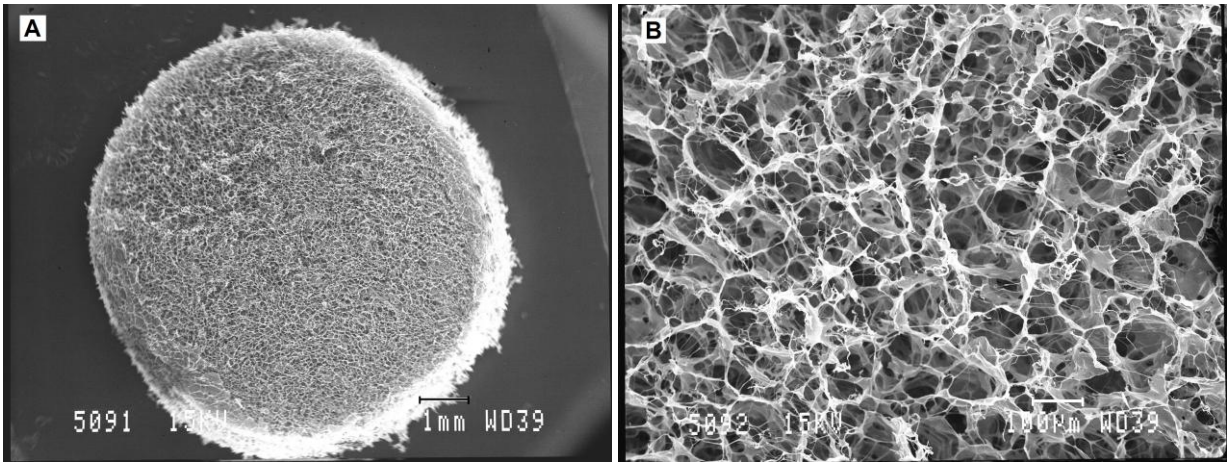


Figure 2

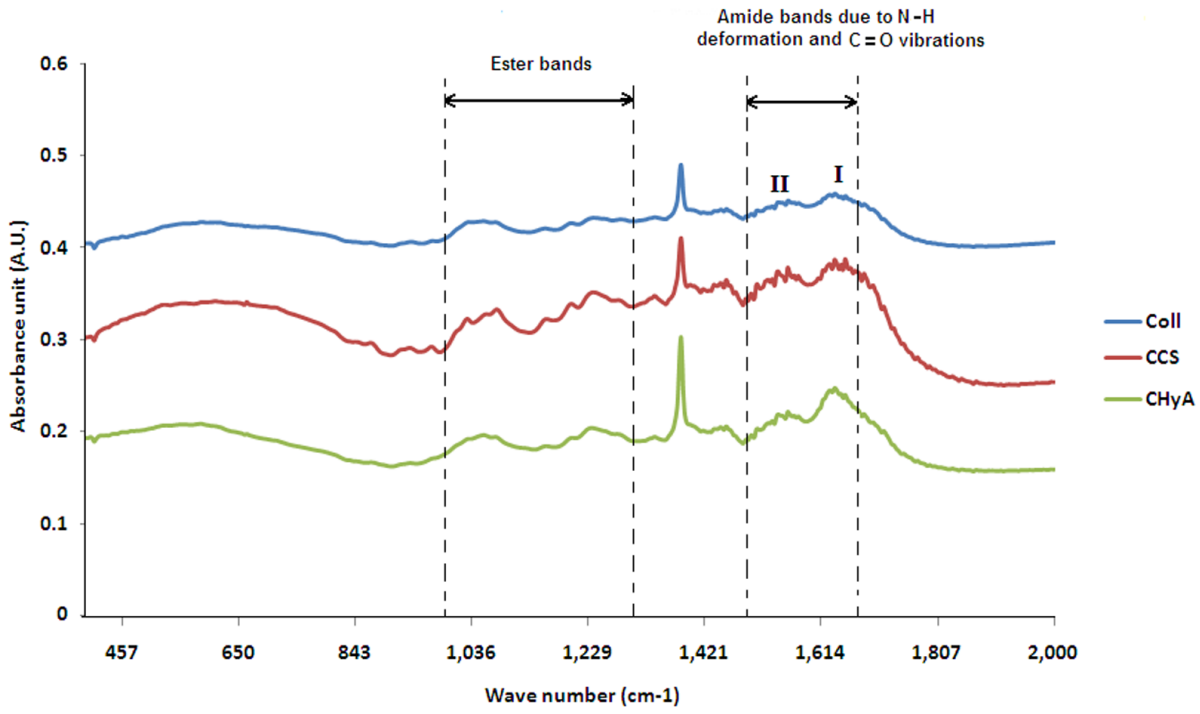

Amide bands due to N-H deformation and C=O vibrations
Ester bands
II
I
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance unit (A.U.)
457
650
843
1,036
1,229
1,421
1,614
1,807
2,000
Wave number (cm-1)
ColI
CCS
CHyA

Figure 3

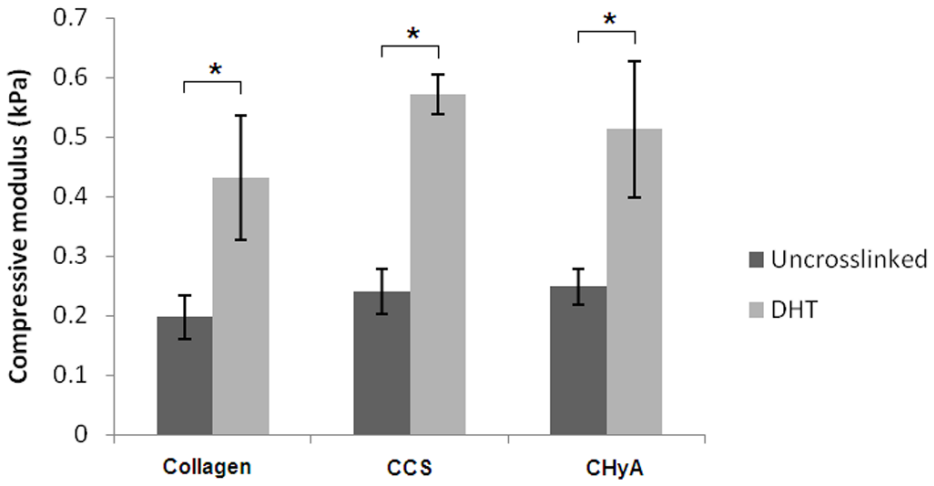

Compressive modulus (kPa)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
*
Collagen
CCS
CHyA
Uncrosslinked
DHT

Figure 4

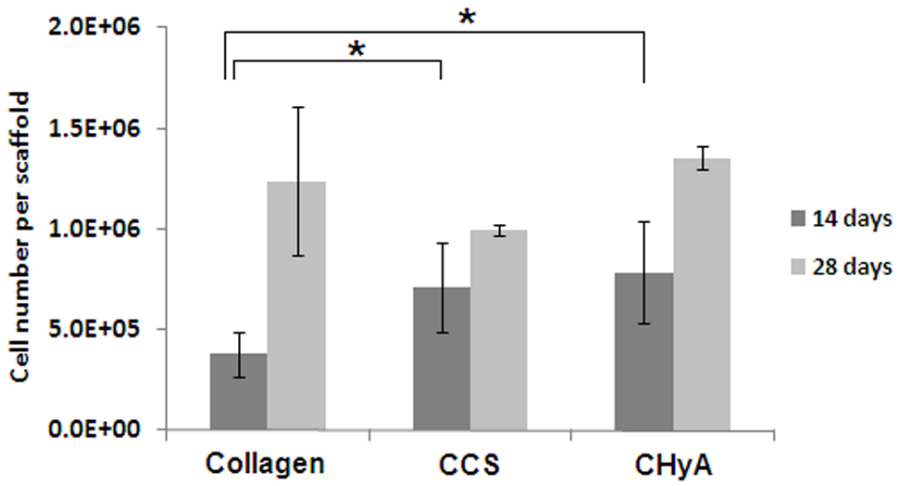

2.0E+06
1.5E+06
1.0E+06
5.0E+05
0.0E+00
Cell number per scaffold
*
*
Collagen
CCS
CHyA
14 days
28 days

Figure 5

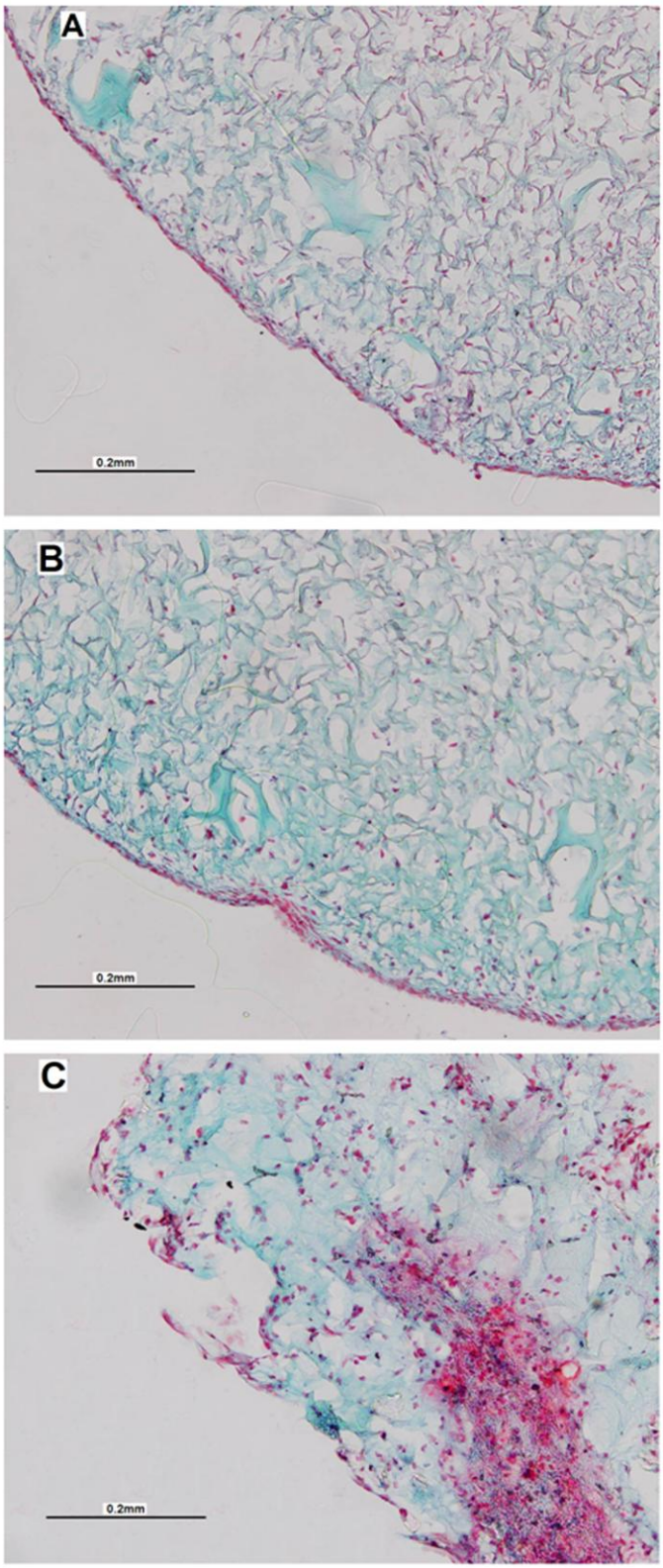


Figure 6

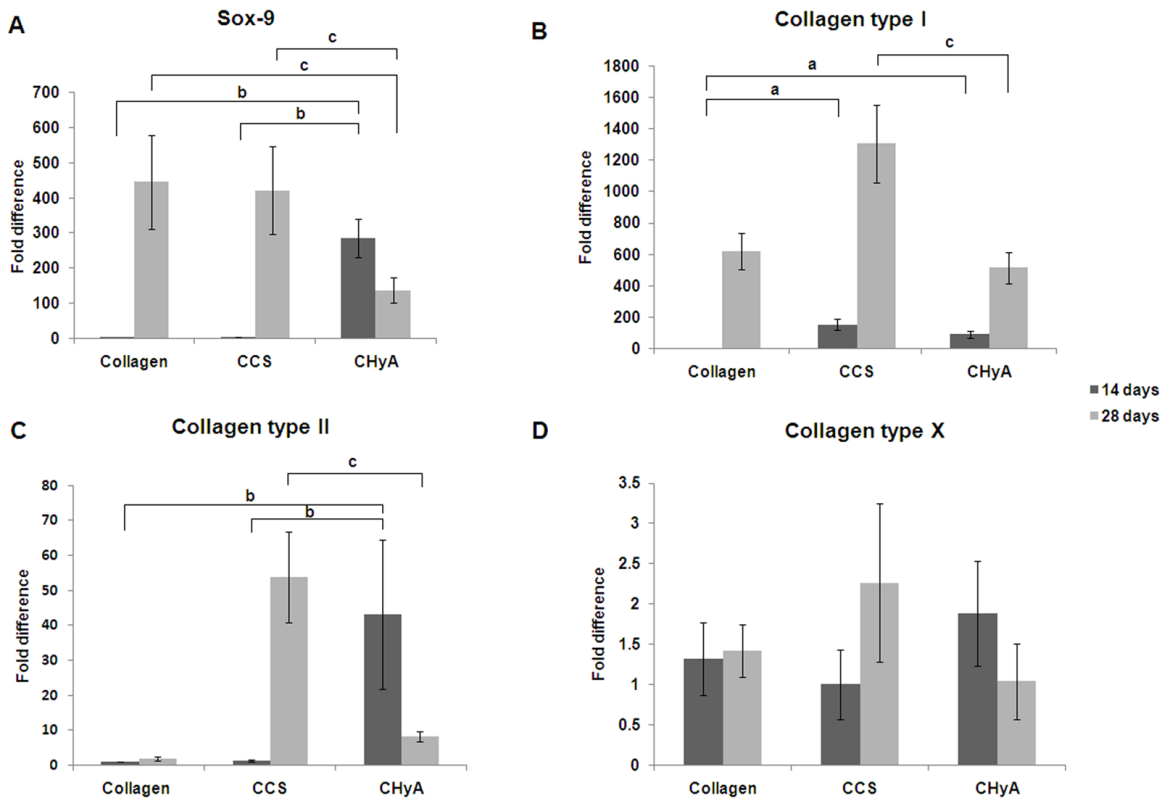

A
Sox-9
Fold difference
Collagen
CCS
CHyA
B
Collagen type I
C
Collagen type II
D
Collagen type X
14 days
28 days

Figure 7

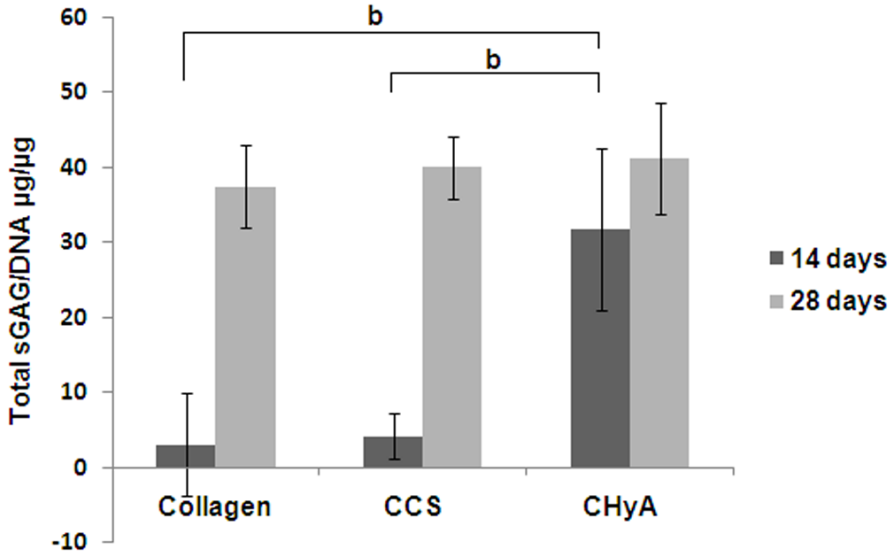
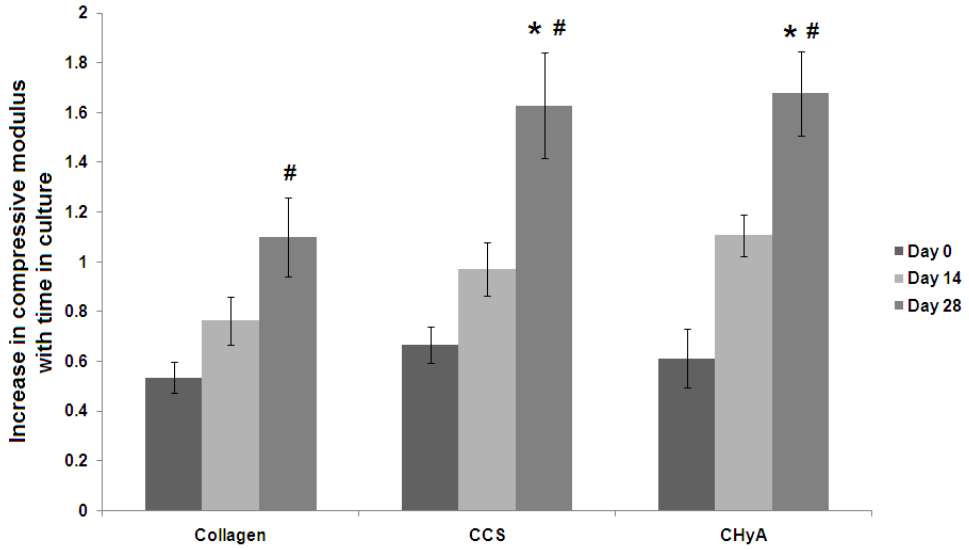

b
b
60
50
40
30
20
10
0
-10
Total sGAG/DNA µg/µg
Collagen
CCS
CHyA
14 days
28 days

Figure 8


Increase in compressive modulus with time in culture
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
* #
* #
Collagen
CCS
CHyA
Day 0
Day 14
Day 28