The effects of collagen concentration and crosslink density on the biological, structural and mechanical properties of collagen-GAG scaffolds for bone tissue engineering.

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Abstract

In this study, we examined the effects of varying collagen concentration and crosslink density on the biological, structural and mechanical properties of collagen-GAG scaffolds for bone tissue engineering. Three different collagen contents (0.25%, 0.5% and 1% collagen) and two different dehydrothermal (DHT) crosslinking processes [1] 105°C for 24 hours and [2] 150°C for 48 hours were investigated. These scaffolds were assessed for 1) pore size, 2) permeability 3) compressive strength and 4) cell viability. The largest pore size, permeability rate, compressive modulus, cell number and cell metabolic activity was all found to occur on the 1% collagen scaffold due to its increased collagen composition and the DHT treatment at 150°C was found to significantly improve the mechanical properties and not to affect cellular number or metabolic activity. These results indicate that doubling the collagen content to 1% and dehydrothermally crosslinking the scaffold at 150°C for 48 hours has enhanced mechanical and biological properties of the scaffold making it highly attractive for use in bone tissue engineering.

Key Words: Collagen composition, DHT crosslinking, pore size, permeability

Introduction

Tissue engineering (TE) requires 3D porous scaffolds that deliver the appropriate mechanical, structural and biological environments for tissue repair and regeneration. Mechanical, material and biochemical parameters such as pore size, permeability, interconnectivity, porosity and scaffold composition all affect cellular response and the success of a tissue engineered construct (Freyman et al. 2001; O'Brien et al. 2007; O'Brien et al. 2005; Tierney et al. 2008; Zeltinger et al. 2001). In our laboratory, we utilize a collagen-glycosaminoglycan (GAG) biological scaffold that has been used in a variety of tissue engineering applications including skin (Yannas et al. 1989), peripheral nerve (Chamberlain et al. 1998), muscle (Menard et al. 2000) and cartilage (Pfeiffer et al. 2008). These scaffolds are extremely biocompatible with non-toxic degradation products, have a high porosity of ~ 99.5% with an interconnected pore architecture allowing for the infiltration of nutrients and subsequent removal of waste products.

These characteristics make the collagen-GAG scaffold extremely attractive for use in bone tissue engineering. An additional advantage of these scaffolds is that the microstructural architecture, such as the pore size, can be controlled by varying a number of parameters during the fabrication process (O'Brien et al. 2004). The scaffolds are produced through a lyophilisation or freeze-drying process whereby a suspension of collagen and GAG in acetic acid is cooled at a constant rate to a final temperature of freezing. O'Brien et al. (2005) found that by varying the final freezing temperatures (T_f = -10, -20, -30, -40°C), a range of collagen-GAG scaffolds with varying mean pore sizes (151, 121, 110, 96μm respectively) could be produced. These scaffolds were then

subjected to *in vitro* cell studies and it was found that pore size significantly affected cell attachment with the scaffolds with the smallest mean pore size demonstrating the highest levels of attachment. It was hypothesised that this was due to an increased surface area producing a higher ligand density for cells to bind to. Pore size is thus an important parameter of tissue-engineered scaffolds as it defines the specific surface area and ligand availability that is available for cells to attach to and migrate on. It has been hypothesised that pores need to be large enough for cells to migrate into the scaffold, but small enough to retain a high specific surface area and many studies have found that cell attachment and the corresponding cellular response is affected by pore size (Berry et al. 2004; Doillon et al. 1986; O'Brien et al. 2005).

Another intrinsic scaffold property that is linked to pore size and the interconnectivity of the pores is permeability. Permeability is an important characteristic of tissue-engineered constructs because it allows the diffusion of nutrients and cytokines into the scaffold and allows the diffusion of waste products out of the scaffold. It is also an important parameter in relation to the biophysical stimuli that cells experience while attached to the scaffold (Al-Munajjed et al. 2008; Maroudas et al. 1968; O'Brien et al. 2007). Studies have previously shown that a cellular solid modelling technique using a tetrakaidecahedral unit cell to represent the pores of collagen-GAG scaffolds can accurately represent and predict salient microstructural features such as permeability in the collagen-GAG scaffold (Al-Munajjed et al. 2008; Gibson and Ashby 1997; O'Brien et al. 2007). This modeling is possible because the pore structure of a variety of low-density, open-cell foams has been observed to have three consistent features: an average

of 14 faces per unit cell, 5.1 edges per face, and vertices that are nearly tetrahedral. The tetrakaidecahedron is a polyhedron that packs to fill space, approximates the structural features of many experimentally characterised low-density, open-cell foams, nearly satisfies the minimum surface energy condition, and is often used for modelling such foams. This model is used in this study to calculate permeability.

The collagen-GAG scaffold was originally designed and optimised for use in skin regeneration (Yannas and Burke 1980) and its composition had not been optimised for any other cell phenotype or tissue applications.. In our laboratory, we are interested in bone and cartilage tissue engineering and have demonstrated that the collagen-GAG scaffold can support mesenchymal stem cell (MSC) differentiation down the osteogenic and chondrogenic routes (Even-Ram and Yamada 2005; Farrell et al. 2007; Farrell et al. 2006; Hubbell 2003; Tierney et al. 2008). A recent study in this laboratory has demonstrated that osteoblastic cell behaviour is affected by varying the collagen and GAG content in scaffold indicating that scaffold composition plays an important role in maintaining cell phenotype and retaining the bioactivity of the collagen-GAG scaffold. By doubling the amount of collagen (1%) present in the scaffold relative to the proportion found in the skin regeneration template (0.5%), a significant increase in osteoblastic cell number and metabolic activity on the collagen-GAG scaffold was found (Tierney et al. 2008). This is an indicator that tissue engineering scaffolds need to be designed with specific cell and tissue applications in mind.

The main limitation encountered with the collagen-GAG scaffolds for tissue engineering applications is that it has poor mechanical properties, particularly for use in bone tissue engineering. The mechanical properties of any scaffold are important in order to facilitate applications such as cell culture *in vitro*, *in vivo* implantation and mechanical functionality once implanted (Harley et al. 2007). Mechanical properties are also important for maintaining 3D architecture for cell attachment and cell migration, applying biophysical stimuli to cells within the scaffold and mediating adaptive cell stiffening due to cell focal adhesions attachment (Bacabac et al. 2008; Klein-Nulend et al. 2005). The mechanical stiffness of the collagen-GAG scaffold can be increased by utilising a crosslinking method such as dehydrothermal crosslinking (DHT) which binds adjacent collagen molecules together by forming crosslinks between them. DHT crosslinking is a widespread technique for stiffening and stabilising collagen composite materials (Gorham et al. 1992; Yannas et al. 1980). It involves subjecting the collagen-GAG scaffolds to temperatures above 90°C while under a vacuum, creating crosslink bonds between the chains of amino acids present in the collagen molecule. Although many other crosslinking techniques can be used, DHT crosslinking is a favoured treatment since it does not create cytotoxic products and the scaffold is sterilised by the high temperatures used. Another recent study in this laboratory has demonstrated that by subjecting the collagen-GAG scaffolds to a range of DHT temperatures (T = 105°C, 120°C, 150°C, 180°C). By varying the process, a significant increase in compressive and tensile modulus ranging from 0.5-1 kPa in compression and 1.9-7.6 kPa in tension (Haugh et al. 2008) was found in the scaffolds.

In this study, we wanted to combine the results from the two studies which demonstrated: [1] improved osteoblastic response by altering the collagen and GAG composition (Tierney et al. 2008) and [2] improved mechanical properties by DHT crosslinking (Haugh et al. 2008) to create an optimised collagen-GAG scaffold that could be used in bone tissue engineering. Specifically, we wanted to examine the effect of varying collagen concentration and crosslink density on the biological, structural and mechanical properties of collagen-GAG scaffolds for bone tissue engineering. Three different collagen contents (0.25%, 0.5% and 1% collagen) and two different crosslinking processes [1] 105°C for 24 hours and [2] 150°C for 48 hours were investigated. The former crosslinking process is used in the skin regeneration scaffolds (Yannas and Burke 1980) and the latter is the optimised process developed in the Haugh et al. study. These scaffolds were assessed for 1) pore size, 2) permeability 3) compressive strength (Young's modulus and 4) cell number and metabolic activity.

2. Materials and Methods

2.1. Fabrication of the variant collagen-glycosaminoglycan co-polymer scaffolds

The collagen-glycosaminoglycan (GAG) scaffolds were fabricated using a previously described freeze-drying technique by O'Brien et al (O'Brien et al. 2004). The collagen-GAG slurry was made by combining type I collagen isolated from bovine tendon (Integra Lifesciences, Plainsboro, NJ) with chondroitin-6-sulphate isolated from shark cartilage (Sigma-Aldrich, Germany) in a 0.05M glacial acetic acid solution. The slurry was blended together at 15,000 rpm using an overhead blender (Ultra Turrax T18, IKA Works Inc., Wilmington, NC). The suspension was maintained at 4°C using a cooling system (WKL 230, Lauda, Germany) to prevent denaturation of the collagen fibers as a result of the heat generated by mixing. The resulting suspension was degassed to remove any air bubbles and subsequently stored at 4°C. The slurry was freeze-dried using a constant cooling technique (O'Brien et al. 2004). 67.25ml of the collagen-GAG slurry was pippetted into a stainless steel pan (5 x 5, grade 304 SS). The tray was placed onto the shelf of the freeze-dryer (Advantage EL, VirTis Co., Gardiner, NY) and cooled to -40°C at 0.9°C/min. Previous work found that this freezing protocol produced scaffolds with a mean pore size of 96μm in the collagen-GAG scaffolds containing 0.5% collagen and 0.044% chondroitin-6-sulphate. A range of collagen-GAG scaffolds was fabricated by altering the quantity of collagen in solution with respect to the standard (skin regeneration) collagen composition as previously described (Tierney et al. 2008). Briefly, the standard collagen-GAG scaffold contains 3.6g of collagen (0.5% w/v solution) and 0.32g of chondroitin-6-sulphate (0.044% w/v solution). Three groups of collagen-GAG

scaffolds were fabricated containing 0.25%, 0.5% (Standard) and 1% collagen while the amount of chondroitin-6-sulphate kept constant at 0.32g.

2.2 Dehydrothermal Crosslinking Treatment

Dehydrothermal treatment (DHT) was carried out by placing the three scaffold groups in open aluminium foil packets and placing them inside a vacuum oven (VacuCell 22, MMM, Germany) under a vacuum of 0.05 bar. To determine the effect of DHT crosslinking on collagen-GAG scaffold variants, the exposure period and crosslinking temperature were varied. Two exposure periods were chosen [1] 105°C for 24 hours and [2] 150°C for 48 hours. 105°C was chosen as the control crosslinking temperature, while 150°C for 48 hours has previously been found to significantly increase the tensile and compressive moduli of the collagen-GAG scaffolds. These settings were found to give the optimal balance between increased mechanical properties and denaturation of the collagen fibres (Haugh et al. 2008).

2.3 Measurement of pore size

To determine the average pore size of the collagen scaffold variants, 9.6mm samples were removed from four corner locations of each scaffold. At each location, samples were obtained to analyze both the longitudinal and transverse planes of the scaffold. These were then embedded in JB-4 glycolmethacrylate, serially sectioned on a microtome (Leica, Laboratory Instruments and Supplies, Ireland) at a thickness of 5µm, stained using 2% toulidine blue and visualized on an optical microscope at 100x magnification for all images. These images were analysed using a linear intercept macro written for

Scion Image™ image analysis software (Scion Corp., Frederick, MD). The program constructed a best-fit ellipse representing an average pore cross-section for each image analysed. The mean intercept length was an average of the major and minor axes of the reconstructed best-fit shape of the pore. The mean pore diameter of the scaffolds was calculated by the average of the mean intercepts of the transverse and longitudinal planes.

2.4 Mathematical (calculated) permeability

A low-density, open-cell foam cellular solids model utilising a tetrakaidecahedral unit according to O'Brien et al (2005, 2007) was used to calculate the mathematical and predicated permeability of the collagen-GAG variants. This modeling is possible because the pore structure of a variety of low-density, open-cell foams has been observed to have three consistent features: an average of 14 faces per unit cell, 5.1 edges per face, and vertices that are nearly tetrahedral. These studies have shown that cellular solids modelling techniques using the tetrakaidecahedral unit cell to represent the pores of the collagen-GAG scaffold can accurately represent and predict salient microstructural features such as surface area and permeability. The tetrakaidecahedron is a polyhedron that packs to fill space, approximates the structural features of many experimentally characterised low-density. Permeability (K) of the collagen scaffolds is described in terms of mean pore diameter (d), percentage compression (ε), scaffold relative density (ρ^*/ρ_s) and a dimensionless system constant (A') taken from O'Brien et al (2007). No compression of the scaffold was performed ($\varepsilon = 0$), therefore $(1 - \varepsilon)^2$ was set as 1. The density ρ^* was measured for each collagen variant and the relative density (ρ^*/ ρ_s) and

porosity of each of the collagen variants calculated (Table 2.2: data from Tierney et al. 2008). These values where then imputed into Equation (1) and permeability calculated.

$$K = A'' \cdot \left(\frac{d}{2.785} \right)^2 \cdot (1-\varepsilon)^2 \cdot \left(1 - \frac{\rho^*}{\rho_s} \right)^{3/2} \quad (1)$$

2.5 Mechanical Testing

Unconfined compression testing was used to determine the effect of crosslinking and scaffold composition on the mechanical properties of the scaffolds. Testing was carried out using a mechanical testing machine (Z050, Zwick/Roell, Germany) fitted with a 5-N load cell. Samples of 9.6 mm diameter were cut from the scaffolds using a punch and pre-hydrated in phosphate buffered saline (PBS) for one hour prior to testing. All testing was carried out in a bath of PBS. Testing was conducted at a strain rate of 10%/min. The modulus was defined as the slope of a linear fit to the stress-strain curve over 2-5% strain.

2.6 Cell Culture

The scaffolds were subjected to cell culture to assess the effects of collagen content and crosslinking density and compared to a control scaffold of 0.5% collagen crosslinked at 105°C for 24 hours. MC3T3-E1 mouse clonal pre-osteoblastic cells (sourced ATCC, LGC, Europe) were cultured in standard tissue culture flasks using α-MEM supplemented with 10% fetal bovine serum, 1% L-glutamine and 2% penicillin/streptomycin solution (Sigma-Aldrich, Germany). The media was replaced every 3-4 days. Cells were removed

from the flasks using a trypsin-EDTA solution (Sigma) and cell number calculated using a hemacytometer. The resulting cell solution was centrifuged for 5 min at 20°C and re-suspended in supplemented media to obtain a cell suspension of 1 x 10^6 cells per 400µl. 1 x 2 cm (~ 4 mm in height) were cut from each of the collagen-GAG scaffold variants crosslinked at 150°C for 2 days and placed into a 6-well tissue culture plate (Sarstedt Ltd, Ireland). Each well was pre-coated with 2% agarose gel (Promega, AGB Scientific Ltd, Ireland) to prevent cells from adhering to the tissue culture plate. Each scaffold sample was then seeded with 2 x 10^6 cells. Constructs were maintained at 37°C with 5% CO_2 with 4mls of supplemented media in each well. Constructs were evaluated at 24, 48 hours and 7 days post-seeding.

Cell Number Assessment

Cell number was evaluated by culturing the collagen-GAG scaffolds as described above for 24, 48 hours and 7 days. After cell culture, samples were flash frozen in liquid nitrogen and stored at -80°C till analysis. Samples were then thawed and the matrices digested in papain. Cell number was evaluated using Hoechst 33258, which labels double stranded DNA fluorescently (Sigma-Aldrich, Germany), according to a previously published protocol (Kim et al. 1988). Measurements were taken in triplicate and read at 355 nm excitation and 460 nm emission using a fluorescence spectrophotometer (Wallac Victor^2, PerkinElmer Life Sciences). Readings were converted to cell number using a standard curve. These experiments were repeated to provide a sample size of n = 4 per scaffold variant group for 24 and 48 hours and a sample size of n =7 for 7 days, combining the scaffolds used from the alamarBlue assay metabolic assay.

Cell Metabolic Activity

Cell metabolic activity was evaluated using alamarBlue™ (Invitrogen, BioSciences, Dublin) at 24, 48 hours and 7 days. This assay is a non-endpoint, non-toxic assay that measures mitochondrial metabolic activity (O'Brien et al. 2000). Scaffolds were transferred to 6-well plates and placed in 4 ml of supplemented media α-MEM containing 10% alamarBlue™. Plates were placed on an orbital shaker (Biosan, Riga, Latvia) in the incubator at 37°C with 5% CO_2. After 2.5 hours, 100µl of the supernatant was plated out in triplicate into a 96-well plate and the absorbance read on a spectrometer (Titertrek MultiScan, MSC, Ireland) at 540nm and 620nm. Percentage reduction of the alamarBlue solution was determined according to the manufacturers' specifications. These experiments were repeated to provide a sample size of 7 scaffolds per scaffold variant group. Scaffolds were flash frozen with liquid nitrogen and stored at -80°C and used for cell number analysis.

2.7 Statistical Analysis

Results are expressed as mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) followed by multiple comparisons procedures (Holm-Sidak) was used to evaluate the effects of collagen concentration and DHT temperature on mean pore size and compressive modulus. Two-way repeated measures analysis of variance (RM-ANOVA) followed by multiple comparisons procedures (Holm-Sidak) was used to evaluate the effect of collagen concentration and culture duration on cell number and metabolic activity. Statistical significance was declared at $p \leq 0.05$.

3. Results

3.1 Pore Structure

The smallest pore size was found both on the 0.25% collagen variants crosslinked at the two different temperatures. A mean pore size of 58µm was found on the 105ºC DHT with a slight increase to a mean of 81µm found on the 150ºC DHT as shown in Table 1. The 0.5% collagen pore sizes correlated well with existing data with the mean being measured at 93µm and 95µm for the 105ºC and 150ºC DHT respectively. The largest pore size was found on the 1% collagen scaffold with a mean 112µm on the 105ºC DHT and a mean of 106µm on the 150ºC DHT (Fig 2). These 1% collagen scaffolds had a significantly larger pore diameter compared to the other two scaffold groups ($p = 0.009$) (Fig.1).

3.2 Calculated Permeability

The mathematically calculated permeability values are shown in Table 2. Scaffolds with larger pore sizes displayed increased permeability. The 1% collagen scaffold DHT at 105°C experiences the highest permeability value at 0.758×10^{-10} m^4/Ns with lowest permeability experienced by the 0.25% collagen variant at 0.203×10^{-10} m^4/Ns (mean pore size 58 µm).

3.3 Compressive Modulus

Compressive modulus increased with increasing collagen content and with increased extent of crosslinking (Fig 3). The highest compressive modulus measured was on the 1% collagen scaffold at 150ºC DHT which was measured as 3kPa $\pm$ 0.94 ($p< 0.001$). This is a

3.8-fold increase compared to the modulus of the 1% collagen at a DHT temperature of 105ºC.

3.4 Cell Number

Statistical analysis indicated that collagen concentration had an effect on cell number ($p < 0.001$) but culture duration did not ($p = 0.118$) (Fig 4). The 1% collagen scaffold DHT at 150°C had a higher cell number across all time points when compared to the other scaffold groups including the control ($p < 0.001$). Crosslinking temperature was found not to have an effect on cell number ($p = 0.349$).

3.5 Cell Metabolic Activity

For the collagen scaffold variants, the RM two-way ANOVA indicated that both collagen concentration ($p < 0.001$) and culture duration ($p < 0.001$) had an effect on cell metabolic activity. The 1% collagen scaffold experienced the highest metabolic activity over all time points when compared to the other scaffold groups ($p < 0.001$) and there is a significantly higher metabolic activity on the scaffolds at day 7 when compared to 24 hours ($p = 0.025$) (Fig 5). Crosslinking temperature was found not to have an effect on cellular behaviour ($p = 0.723$)

4. Discussion

Scaffolds for tissue engineering such as the collagen-GAG scaffold must encompass a set of parameters that facilitate cellular adhesion, migration and tissue formation. These include a sufficient, interconnected pore structure to allow cell migration into the scaffold, cell attachment, adequate diffusion of nutrients in and waste products out of the scaffold and sufficient mechanical stability to provide functionality of the scaffold *in vitro* and *in vivo* (Even-Ram and Yamada 2005; Harley and Gibson 2007; O'Brien et al. 2005; Zeltinger et al. 2001). The aim of this study was to create an optimal collagen-GAG scaffold for bone tissue engineering by combining the optimum composition for osteoblast attachment (Tierney et al. 2008) with the optimised mechanical properties (Haugh et al. 2008). The collagen-GAG scaffolds were fabricated into groups containing 0.25%, 0.5% and 1% collagen with the amount of chondroitin-6-sulphate kept constant. These were then dehydrothermally crosslinked at 105°C and 150°C to increase mechanical integrity and the resultant mechanical, structural and biological properties were then measured.

The size of the pores in the collagen-GAG scaffold is controlled by the rate of ice nucleation during the cooling process and the consequent sublimation of ice crystals (O'Brien et al. 2004). The 1% collagen scaffold had a significantly larger mean pore diameter compared to the other two scaffold groups ($p = 0.009$) with pore size measured at 112μm and 106μm for the 105°C and 150°C DHT groups respectively (Fig 1 and Fig 2). The smallest pore size measured was found on the scaffold containing the least amount of collagen, the 0.25% scaffold measuring at 58μm with a slight increase in pore

diameter to 81μm on the 0.25% scaffold DHT at 150°C. This small diameter of pore size can be attributed to the weak mechanical properties of the 0.25% collagen scaffold which undergoes a loss of 3D structural integrity due to pore collapse. The pore size of the 0.5% collagen scaffold corroborated well with the currently reported pore size of 96μm (O'Brien et al. 2004), with our scaffolds measuring 93μm and 95μm for the 105°C and 150°C DHT groups respectively. In general, pore size was found to increase with collagen content. Increased collagen content will promote ice crystal nucleation and raise the freezing temperature of the collagen slurry. Ice crystal size has previously been shown to be proportional to the freezing temperature, explaining the increase in pore size (which mirrors ice crystal structure) with increasing collagen content (Searles et al. 2000).

Cellular solids modelling allows the calculation of permeability of the collagen-GAG scaffolds (Gibson and Ashby 1997; O'Brien et al. 2007). Pore size, porosity, interconnectivity as well chemical composition of scaffolds can all influence permeability. The results show that permeability of the collagen scaffolds increases with increasing pore size and increasing collagen content, with the 1% collagen scaffold exhibiting the highest permeability rate of 0.758×10^{-10} m^4/Ns (Table 2). This rate of permeability is a reasonable value for a TE scaffold when you account for the increased collagen composition of the scaffold and when compared against lower permeability rates of tissues such as cartilage (10^{-15} m^4/Ns) but which is still several orders below that of trabecular bone (10^{-6} m^4/Ns) (O'Brien et al. 2007). This value correlates well with published literature of permeability of collagen-GAG scaffolds where the experimentally

derived permeability value of a collagen-GAG scaffold with a pore size of 110μm is 0.714×10^{-10} m^4/Ns for the 0.5% collagen scaffold at 0% compression (O'Brien et al. 2007). This is important for nutrient diffusion and waste removal, biophysical stimuli due to fluid flow exchanges during mechanical loading and permeability can also affect the degradation rate of biological scaffolds. The 0.25% collagen scaffold experienced the lowest permeability of 0.203×10^{-10} m^4/Ns due its smaller mean pore diameter of 58μm.

The compressive moduli of the collagen scaffolds were considerably altered with both collagen content and crosslinking temperature (Fig 3). The 1% collagen scaffold crosslinked at 150°C exhibited the highest compressive modulus of 3kPa $\pm$ 0.94 and this was significantly higher compared to all other groups at both DHT temperatures ($p < 0.001$). This is a 3.8-fold increase in stiffness compared to the 1% collagen scaffold crosslinked at 105°C, indicating that DHT crosslinking temperature noticeably alters the mechanical integrity of collagen-GAG scaffolds. These results indicate that DHT is a feasible method for improving the mechanical properties of the scaffolds and can allow the production of collagen-GAG scaffolds with a range of mechanical properties and compositions depending on the tissue being engineered. The 0.25% collagen scaffold had the weakest compressive modulus at 0.317 kPa for both 105°C and 150°C DHT temperatures, indicating the weak 3D mechanical integrity of the scaffold due to the limited amount of crosslink sites available. Crosslinks are formed between carboxyl and amino groups of the adjacent collagen molecules and the weak modulus can be explained by the limited amount of functional crosslinks available in the 0.25% collagen scaffold.

The changes in composition, architecture and mechanical properties were found to significantly affect cellular behaviour. Increasing the DHT temperature and exposure periods of crosslinking leads to a change in the triple helical structure of the collagen molecule to a more random chain structure potentially affecting cellular activity (Haugh et al. 2008; Pieper et al. 1999; Yunoki et al. 2003) Cell culture results show that collagen composition had an effect on cell number over time ($p < 0.001$), with the 1% collagen scaffold having the highest cell number compared to all other groups (Fig 4), emulating the results from our previous study (Tierney et al. 2008). It is hypothesised that the combined effects of the increased specific surface area provided by the increased amounts of collagen, combined with increased mean pore size, and improved permeability allows a greater number of cells to attach and proliferate on the 1% collagen scaffold accounting for the high cell number measured. Statistical analysis of the 0.5% collagen scaffold crosslinked at 150°C and the control 0.5% collagen scaffold DHT at 105°C, indicates that DHT crosslinking does not have an effect on cell number ($p = 0.349$). As increasing the DHT crosslinking temperature to 150°C causes an increase in denaturation, from 25% to 41%, these results suggest that the increased level of collagen denaturation at 150°C does not affect cell attachment rates or proliferation (Haugh et al. 2008). Cell metabolic activity was also found to be affected by collagen composition ($p< 0.001$), with the 1% collagen scaffold having the highest metabolic activity compared to all other groups over time ($p< 0.001$) (Fig 5). A significant advantage of the collagen-GAG scaffold is that it is natural polymer and therefore bioactive, allowing osteoblasts to maintain high metabolic rates that increase over time and there was a significant increase in cell metabolic activity on all collagen scaffolds at 7 days compared to 24 hours ($p =$

0.025). Again, metabolic activity was not affected by the DHT crosslinking temperature, with no statistical difference between the control 0.5% collagen and the 0.5% collagen DHT crosslinked at 150°C ($p = 723$).

In conclusion, these results indicate that doubling the collagen content to 1% and dehydrothermally crosslinking the scaffold at 150°C for 48 hours has enhanced mechanical and biological properties making it highly attractive for use in bone tissue engineering. The new optimised scaffold has a larger pore size for cell infiltration and attachment and increased stiffness for mechanical functionality *in vitro* and *in vivo*. The increased collagen content to 1% increases both cell number and metabolic activity without sacrificing bioactivity of the scaffold as might have been expected due to potential denaturation of the collagen molecule at the higher DHT temperatures.

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Figure Captions

Table 1: Mean Pore Sizes of Collagen-GAG Scaffolds at 105°C and 150°C DHT

Mean pore sizes of the collagen scaffold variants as measured by the linear intercept method with the 1% collagen scaffold displaying the highest mean pore size compared to the other two groups ($p = 0.009$). Results expressed as microns (μm) ± S.D.

Table 2: Calculated permeability of the collagen-GAG scaffolds

Permeability (K) of the collagen-GAG scaffolds as a function of their mean pore sizes and relative density. Units for K are 1×10^{-10} m^4/Ns

Table 3: Relative densities and porosity values for collagen-GAG scaffolds

The measured relative densities of the collagen-GAG scaffolds and the porosity values.

Figure 1: Mean pore diameters of the collagen-GAG scaffolds DHT crosslinked at 105°C and 150°C. Pore diameter expressed in microns (μm). The 1% collagen scaffold has the highest mean pore size at both DHT temperatures compared to the other 2 collagen groups. Error bars represent S.D. * $p = 0.009$ relative to all other groups.

Figure 2: Micrographs of the collagen-GAG scaffolds to evaluate pore size and distribution, visualised using a 100x magnification: (A) 0.25% Collagen DHT 105°C, (B) 0.5% Collagen DHT 105°C, (C) 1% Collagen DHT 105°C, (D) 0.25% Collagen DHT 150°C, (E) 0.5% Collagen DHT 150°C, (F) 1% Collagen DHT 150°C

Figure 3: Compressive moduli of the collagen-GAG scaffolds crosslinked at 105°C and 150°C DHT ($n = 10$ per scaffold variant and temperature). Results are expressed in kPa with the error bars representing S.D. The 1% collagen scaffold showing a 3.8-fold increase in compressive modulus compared to all other collagen groups. *$p < 0.001$ relative to all other groups, including the 1% collagen DHT crosslinked at 105°C.

Figure 4: Cell number on the collagen-GAG scaffolds. Error bars represent S.D. (n = 4 for all time points). The 1% collagen scaffold has the highest cell number over all time points compared to the other collagen groups DHT at 150°C and the control DHT at 105°C . *p <0.001 relative to all other groups

Figure 5: Cell metabolic activity on the collagen-GAG scaffolds. Results are expressed as % reduction of the alamarBlue^{TM} solution. Error bars represent S.D. Again, the 1% collagen scaffold has the highest metabolic activity over all time points compared to all other groups. *p <0.001 relative to all other groups. All collagen scaffolds also experienced an increase in metabolic activity at 7 days compared to 24 hours (p = 0.025).

Table 1

	105°C	150°C
0.25% Collagen	58.38 µm ± 10	81.31 µm ± 17
0.5% Collagen	92.95 µm ± 17	94.56 µm ± 11
1% Collagen	112.02 µm ± 33	106.92 µm ± 8

Table 2

Scaffold	Mean Pore Size 105°C, μm	K_{calc} $\varepsilon = 0\%$	Mean Pore Size 150°C, μm	K_{calc} $\varepsilon = 0\%$
0.25% Collagen	58	0.203×10^{-10}	81	0.397×10^{-10}
0.5% Collagen	93	0.525×10^{-10}	96	0.559×10^{-10}
1% Collagen	112	0.758×10^{-10}	107	0.692×10^{-10}

Table 3

Scaffold DHT 105°C	Relative Density	% Porosity
0.25% Collagen	0.010	98.9 %
0.5% Collagen	0.007	99.3 %
1% Collagen	0.011	98.8 %

Figure 1

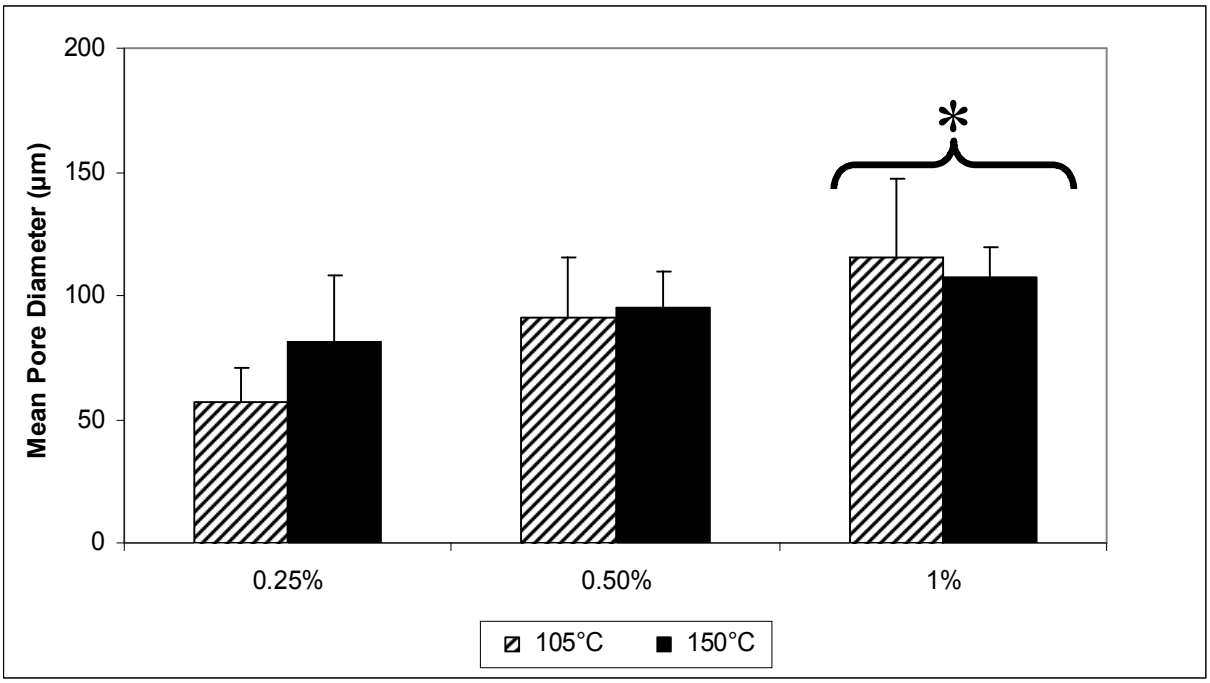


Figure 2

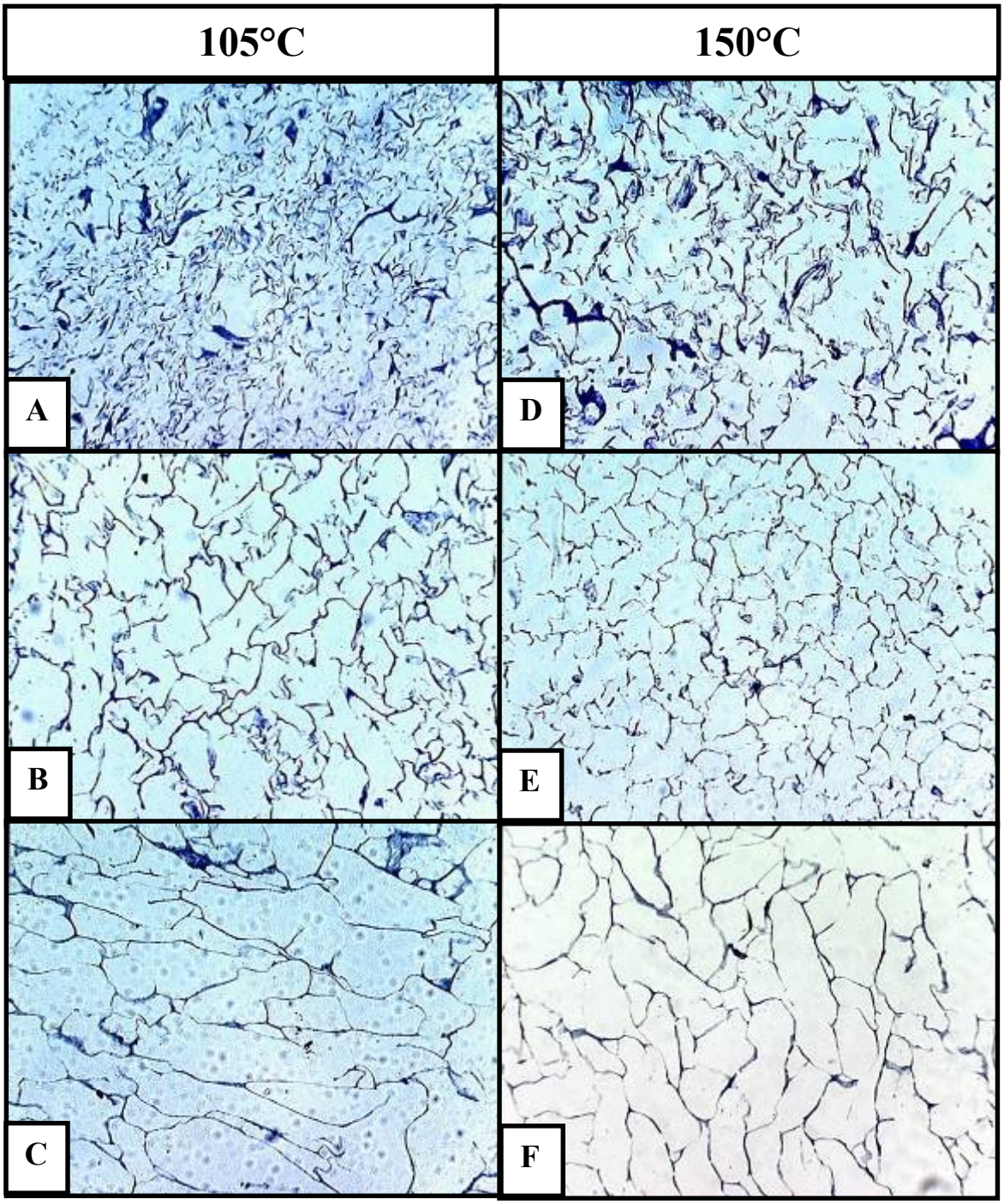


Figure 3

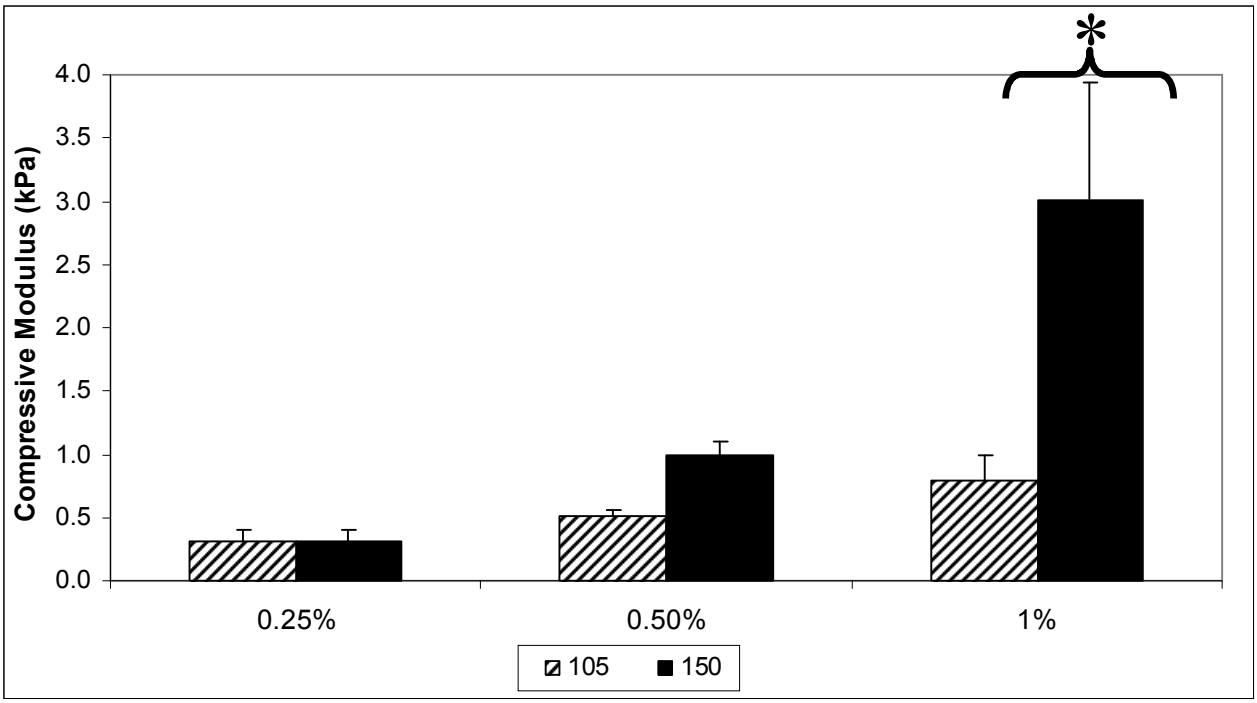
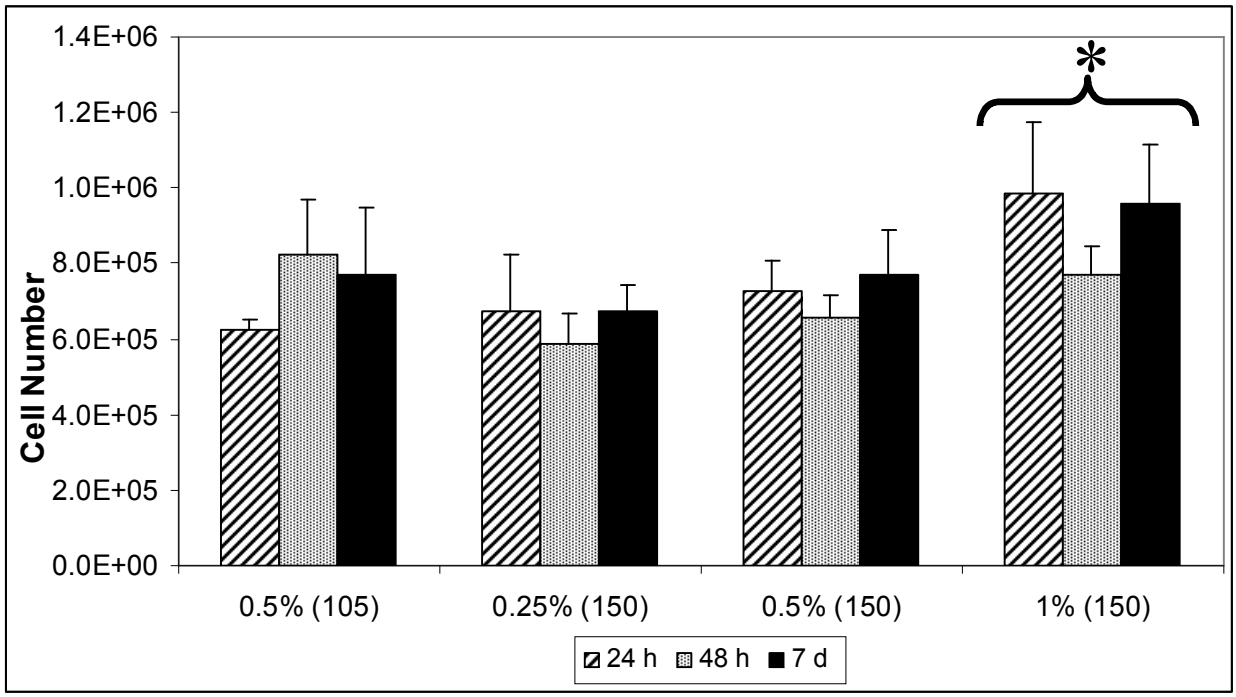

Compressive Modulus (kPa)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
0.25%
0.50%
1%
105
150

Figure 4


1.4E+06
1.2E+06
1.0E+06
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4.0E+05
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*
0.5% (105)
0.25% (150)
0.5% (150)
1% (150)
24 h
48 h
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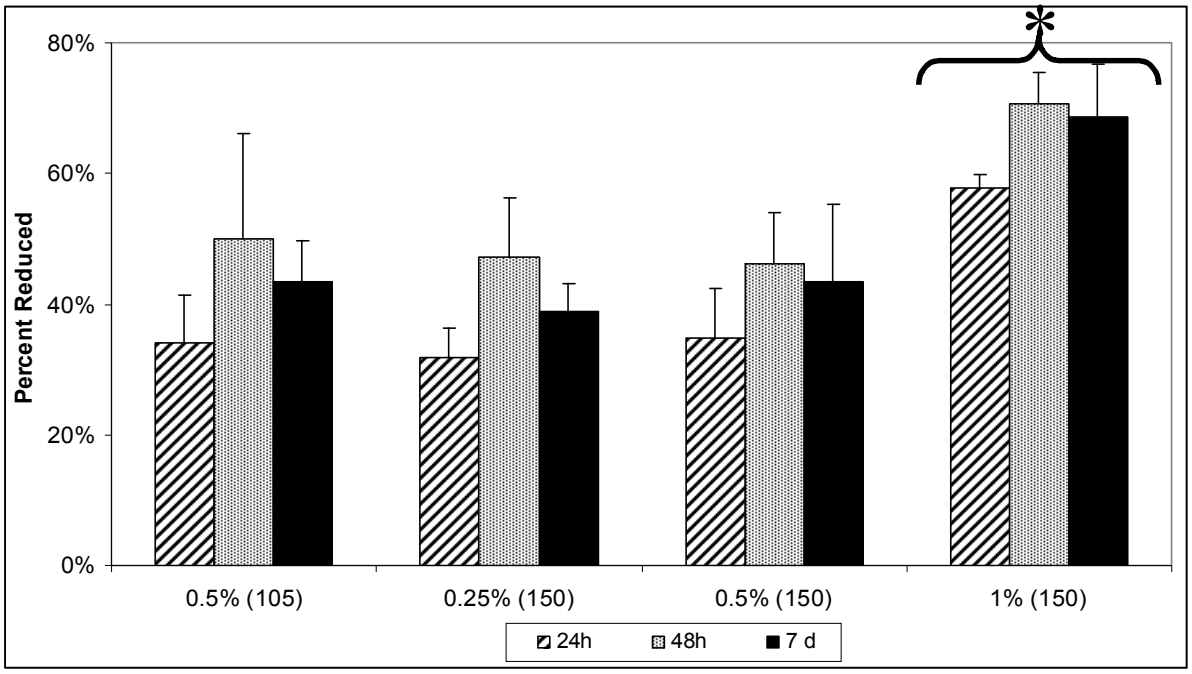
Figure 5


80%
60%
40%
20%
0%
Percent Reduced
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0.5% (105)
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24h
48h
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