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Scaffold mean pore size influences mesenchymal stem cell chondrogenic differentiation and matrix deposition

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Abstract

Recent investigations into micro-architecture of scaffolds has revealed that mean pore sizes are cell type specific and influence cellular shape, differentiation and extracellular matrix secretion. In this context, the overall goal of this study was to investigate whether scaffold mean pore sizes affect mesenchymal stem cell initial attachment, chondrogenic gene expression and cartilage-like matrix deposition. Collagen-hyaluronic acid (CHyA) scaffolds, recently developed in our laboratory for *in vitro* chondrogenesis, were fabricated with three distinct mean pore sizes (94 μm, 130 μm and 300 μm) by altering the freeze-drying technique used. It was evident that scaffolds with the largest mean pore sizes (300 μm) stimulated significantly higher cell proliferation, chondrogenic gene expression and cartilage-like matrix deposition relative to scaffolds with smaller mean pore sizes (94 μm, 130 μm). Taken together, these findings demonstrate the importance of scaffold micro-architecture in the development of advanced tissue engineering strategies for articular cartilage defect repair.

Introduction

The physico-chemical properties of biomaterials have been shown to significantly affect the behaviour of cells both *in vitro* and *in vivo*.[1-3] The micro-architecture is one such property that has been widely investigated. In particular, the effect of scaffold micro-architecture on modulating cell-scaffold interaction as well as the subsequent cell proliferation, differentiation and matrix deposition has become of interest within the field of tissue engineering. Previous work in our own laboratory has shown that the micro-architecture, specifically the mean pore size, affects initial cell attachment and migration within highly porous collagen-glycosaminoglycan (CG) scaffolds.[4-6] It is now known that a balance needs to be achieved between having scaffold pores large enough to facilitate cell migration whilst still small enough to allow an adequately high specific surface area for cell adhesion.[7] Moreover, it is clear that scaffold composition plays a deterministic role in cell behaviour through the presentation of appropriate biochemical cues.[8] However, whilst it is clear that cell adhesion may be affected by compositional and architectural characteristics, an optimal pore size range for one cell type on a particular scaffold may not necessarily be optimal for another cell type within the same scaffold.[9,10]

The limited regenerative capacity of articular cartilage has led to the development of biomaterials with intrinsic physical and biochemical characteristics that can facilitate chondrogenesis.[11] Although constructs developed hitherto have shown some promise in the repair of articular cartilage defects, there still remains an unmet need for optimal biomaterials that can support long-term regeneration with resultant hyaline-like cartilage tissue. The typically preferred cell types currently used for the investigation of chondrogenesis and cartilage tissue

engineering (TE) are chondrocytes and mesenchymal stem cells (MSCs).[12,13] MSCs are a valuable multi-potent source of cells that are easily accessible.[14] They have been used in pre-clinical models for TE bone, cartilage, muscle, marrow stroma, tendon, fat and other connective tissues.[15-21] The development of scaffolds possessing characteristics optimal for the induction of MSC-mediated chondrogenesis using a TE approach is of great interest.[22-24] Indeed, previous work in our laboratory has shown that by altering the composition and mechanical properties of CG scaffolds, the differentiation of MSCs towards a chondrogenic lineage can be significantly affected.[25,26]

A number of studies have investigated the relationship between scaffold mean pore size and cartilage-like matrix deposition by chondrocytes.[8,27] However, to date there are still conflicting theories as to which range of scaffold mean pore sizes is ideal for *in vitro* chondrogenesis. A number of studies have demonstrated that scaffolds (collagen or polyurethane urea) with small mean pore sizes (20-150 µm) facilitate greater levels of chondrocyte differentiation.[27,28] This is believed to be partly due to the high surface area for cell attachment and intercellular signalling on scaffolds with smaller mean pore sizes.[29] On the contrary, other studies have shown scaffolds (fabricated from chitosan or gelatin) with larger mean pore sizes (250-500 µm) improve chondrocyte proliferation and extracellular matrix secretion.[30,31] The effect of scaffold mean pore size on cell behaviour may be attributed to the specific cell-scaffold interaction and in particular, studies have demonstrated that the morphology of cells may play a significant role in the differentiation of cells.[27,32-34] Therefore, further investigation and understanding of mechanisms by which scaffold architecture affects cell-scaffold interaction and subsequent cartilage tissue formation remains of significant importance. Moreover, few studies have investigated the effect

4

of scaffold mean pore size on MSC differentiation towards a characteristic chondrogenic lineage. In this context, the focus of this study was to develop CG scaffolds with characteristics optimal for supporting the attachment, proliferation and differentiation of MSCs towards a chondrogenic lineage and subsequent synthesis of hyaline-like cartilage.

CG scaffolds can be fabricated with distinct mean pore sizes by using a freeze-drying process.[35] Using this technique, a suspension of the CG slurry is frozen to a particular final temperature (T_f) and subsequently producing a network of ice crystals surrounded by CG fibers. Sublimation of the ice crystals leads to formation of a highly porous CG scaffold. The formation of ice crystals in the CG suspension is influenced by the nucleation rate and the rate of heat diffusion. These are primarily controlled by T_f and accordingly, by varying this parameter, the resulting mean pore size can be altered.[7] Another method of altering the mean pore size of CG scaffolds is through introduction of an annealing step within the freeze-drying process. Annealing involves raising the temperature of the frozen suspension from the initial freezing temperature, whilst still below the melting temperature of the suspension. The frozen suspension is held at this secondary temperature for a specified period of time to allow increase in the rate of ice crystal growth by increasing diffusion of heat. It has been shown that the ice crystal size increases with increasing annealing temperature.[36] Accordingly, annealing can be used to increase scaffold mean pore size even further.[35]

Using a combination of these two approaches (altering T_f and introducing annealing), collagen-hyaluronic acid (CHyA) scaffolds were fabricated with distinct but individually homogeneous mean pore sizes. Previous work in our laboratory has shown that the addition of hyaluronic acid

to collagen scaffolds led to accelerated MSC chondrogenic differentiation and migration compared to another type of glycosaminoglycan, chondroitin sulphate.[25] We hypothesised that CHyA scaffolds with larger mean pore sizes would improve initial cell attachment with a resultant improvement in subsequent chondrogenic differentiation of MSCs. The overall goal of the study presented in this article was therefore to investigate whether scaffold mean pore size can affect the chondrogenic differentiation of MSCs. To achieve this, the effect of varying mean pore sizes on initial attachment, MSC proliferation, chondrogenic gene expression as well as the synthesis and deposition of cartilage-like matrix *in vitro* was investigated.

Materials and methods

Fabrication of collagen-glycosaminoglycan scaffolds

Collagen-hyaluronic acid (CHyA) scaffolds were fabricated using the freeze-drying method that has been previously described.[25] Briefly, 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. One method of increasing the mean pore size is by increasing the final freezing temperature above -40°C. For this study, a temperature of -10°C was used since this has been shown previously to increase the mean pore size of CG scaffolds.[35] Another method of increasing the mean pore size of the scaffolds was by introducing an additional annealing step during the fabrication process as shown previously. To achieve this, the suspensions were cooled to -20°C and subsequently heated to -10°C and held at this temperature for 24 hours before sublimating the ice phase at 0°C under vacuum (>100 mTorr). The distinct methods are summarised in Table 1. After freeze-drying, the scaffolds were cross-linked using

6

dehydrothermal treatment (DHT) under a vacuum of 50 mTorr and a temperature of 105ºC in a vacuum oven (VacuCell, MMM, Germany) for a duration of 24 hours.

Mechanical and morphological characterisation

Effect of the freezing profile on scaffold mean pore size

In order to determine whether the distinct freezing profiles altered the resultant architecture of CHyA scaffolds, pore size analysis was carried out. This was carried out using a histological technique previously described.[7,37] Briefly, 9.5 mm diameter samples were obtained from three different locations on each sheet of freeze-dried CHyA scaffolds. The samples were subsequently embedded in JB-4® glycolmethacrylate (Polysciences Europe, Eppelheim, Germany) and serially sectioned (10 µm sections) on a microtome (Leica RM 2255, Leica, Germany). The sections were stained with toluidine blue (Sigma-Aldrich, Arklow, Ireland) and microscopic images were taken at 10x magnification (Eclipse 90i, Nikon, Japan) using a digital camera (DS Ri1, Nikon, Japan). The images were exported to MATLAB (MathWorks Inc, MA, USA) and assessed for mean pore diameter using a pore topology analyser (Sigmedia Research Group, Department of Electrical Engineering, TCD, Ireland).

Effect of mean pore size on scaffold compressive modulus

The effect of scaffold mean pore size on bulk compressive modulus was investigated by uni-axial, unconfined compressive testing using a mechanical testing machine (Z050, Zwick-Roell, Germany) as described previously. Pre-hydrated samples were tested according to a protocol described previously.[38] A 5-N load cell was used for all tests and carried out at a strain rate of

10%/min and the compressive modulus was obtained from the slope of a linear fit to stress-strain curve over 2-5% strain.

In vitro analysis

Cell isolation and culture

Primary MSCs were isolated from the bone marrow of three month old wistar rats (approximately 250-300 g) after RCSI Ethics Committee approval (REC237). MSC isolation was carried out according to protocols that have been widely established.[39] Cells were suspended at a density of 5×10^6 cells per mL and a total volume of 100 µL per scaffold. 9.5 mm diameter, 4 mm height scaffolds were pre-hydrated in phosphate buffered saline (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% CO2, 37°C incubator to allow initial attachment.[6] 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 20ng/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 28 days with medium being changed at 3 day intervals.

Effect of mean pore size on initial cell attachment

Cell number was quantified using a Hoechst dye 33258 assay described previously.[6] Briefly, cell-seeded and cell-free control scaffolds were taken out of culture and 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 determined using a Hoechst dye 33258 assay. Measurements were taken using a fluorometric plate reader (Wallac 1420 Victor2 D, Perkin Elmer, MA, USA) at an emission of 460 nm and excitation of 355 nm, 1.0 s. A standard curve was created using known quantities of cells and the measurements from the samples were obtained using an equation of the curve. To determine the initial cell attachment from the cell number, a percentage of cells present in the scaffolds after 24 and 48 hours, relative to the number of cells initially seeded onto the scaffolds at day 0, was calculated.

Effect of mean pore size on chondrogenic gene expression

To determine gene expression on the cultured cells within the scaffolds real time reverse transcription polymerase chain (RT-PCR) reactions were carried out. Briefly, the total RNA was isolated using an RNeasy kit (Qiagen, Crawley, UK) using established methods.[40] 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 ($\Delta\Delta$Ct) method with a house-keeping gene, GAPDH. The target genes chosen were collagen type 1 (COL1), collagen type 2 (COL2) and sex determining region

Y-box 9 (SOX-9). These target genes were chosen since they are expressed in cells differentiating down the characteristic chondrogenic lineage.

Effect of mean pore size on sulphated GAG production

Digested scaffolds were assessed for sulphated GAG content that was synthesised by seeded cells using a solution of di-methyl-methylene blue (DMMB) assay. A standard curve using serial dilutions of chondroitin sulphate was generated. The samples and standards were placed in a solution of 0.3% glycine, 0.25% sodium chloride 1.6% DMMB within a 96 well plate and readings taken at 530nm, 1.0s. The background photometric reading of the scaffolds was accounted for by subtracting the average value obtained from cell-free scaffolds.

Statistical analysis

Statistical differences between three or more treatments were assessed by two-way ANOVA with Tukey's post hoc analysis. Statistical differences between two treatments were assessed by student's paired t-test. All results were reported as mean $\pm$ standard deviation. A probability value of 95% ($p < 0.05$) was used to determine significance. A sample size of 3 per group was used throughout the study unless otherwise stated.

Results

Effect of the freezing profile on scaffold mean pore size

The freezing profile significantly affected the mean pore size of CHyA scaffolds. Increasing the final freezing temperature significantly increased the overall mean pore size (Table 2). A final freezing temperature of -40°C resulted in a mean pore size of 93.9 µm whilst a final freezing

temperature of -10°C resulted in a significant increase in mean pore size to 130.3 μm (p<0.05). The addition of an annealing step for 24 hours further increased the mean pore size of CHyA scaffolds significantly to 300.5 μm (p<0.01).

Effect of scaffold mean pore size on scaffold compressive modulus

Although all scaffold groups were cross-linked using the same technique, in order to ensure that differences in the mean pore size did not alter scaffold mechanical properties, the compressive modulus was assessed. Increasing the mean pore size of CHyA scaffolds did not affect the bulk compressive modulus (Fig. 1). Scaffolds with a mean pore size of 94 μm had a compressive modulus of 0.49 kPa while scaffolds with a mean pore size of 130 μm had a compressive modulus of 0.48 kPa and those with the largest mean pore size (300 μm) had a compressive modulus of 0.51 kPa. There was no statistical difference between the three scaffold variants in terms of compressive modulus.

Effect of scaffold mean pore size on initial cell attachment, density and morphology

Variations in the pore architecture led to a linear correlation between mean pore size of scaffolds and initial cell attachment of MSCs over a 48 hour period as well as an increase in the overall density with mean pore size following 28 days of culture (Fig. 2 and Fig. 3). The results showed an increase in cell attachment with an increasing mean pore size, however, this was not found to be statistically significant. Cell attachment was approximately 45% on CHyA scaffolds with a mean pore size of 94 μm after 48 hours in culture whilst CHyA scaffolds with a mean pore size of 300 μm exhibited a percentage cell attachment of approximately 60% at the same time point. Although the results exhibit a linear increase in percentage cell attachment with increasing pore

size (R^2=0.98 for 24 hours, R^2=0.95 for 48 hours), there are no statistical differences between the 94 μm group and the other 2 groups.

Scaffold mean pore size did not affect the cell proliferation of rat MSCs after 14 days in culture; however, there was a significant increase between 14 and 28 days in all the groups investigated (p<0.05) (Fig. 3). There was also no statistical difference in the cell density between the 3 scaffold groups after 14 days in culture. However, at day 28, there was significantly higher cell density in the 300 μm group than the 94 μm group (p<0.05).

Using scanning electron microscopy (SEM), seeded scaffolds were imaged following 24 hours of culture to investigate whether the morphology of cells was affected by scaffold mean pore size. Interestingly, cells attached onto the scaffolds with a flat morphology on scaffolds with a mean pore size of 94 μm and 130 μm whereas cells with a rounded morphology were found on the scaffolds with a mean pore size of 300 μm (Fig. 4).

Effect of scaffold mean pore size on MSC differentiation and matrix deposition

Gene expression

Gene expression analysis showed that scaffold mean pore size significantly affected chondrogenic gene expression. Scaffolds with the largest mean pore size (300 μm) stimulated approximately 3-fold and 2-fold higher SOX9 gene expression than the 94 μm and 130 μm scaffold groups respectively after 28 days although this was found to be non-significant (p=0.07 between 94 μm and 300 μm; p=0.1 between 130 μm and 300 μm). There was an increase in SOX9 gene expression with time in culture in all three groups (Fig. 5).

There was a decrease in COL1 gene expression with increasing scaffold mean pore size as early as day 14. Although COL1 gene expression was evident in all 3 groups (Fig. 6), there was significantly higher gene expression in the scaffolds with smallest pores (94 µm) (approximately 2-fold higher, $p<0.05$) than the larger pore size group (300 µm). Whilst there was an initial increase in COL1 gene expression between 7 and 14 days, by day 28 there was a significant decrease in gene expression in all groups.

Scaffolds with the largest mean pore size (300 µm) led to significantly higher COL2 gene expression than the 94 µm and 130 µm scaffold groups (Fig. 7). COL2 gene expression was significantly higher on the 300 µm group (approximately 5-fold higher, $p<0.01$) than the 94 µm and 130 µm groups at day 14. Moreover, at day 28 there was still significantly higher COL2 gene expression on the 300 µm group (approximately 11-fold higher, $p<0.01$) than the 94 µm and 130 µm groups.

Sulphated GAG production

Similar to COL2 gene expression, the effect of scaffold mean pore size on sulphated GAG production demonstrated that the scaffolds with largest mean pore sizes (300 µm) stimulated the highest level of sGAG production (Fig. 8). At day 14, there was an increase in sGAG content with increasing scaffold mean pore size. Consequently, there was significantly higher sGAG content in the 300 µm group (approximately 3-fold higher, $p<0.05$) than the 94 µm group. By day 28, there was still significantly higher sGAG content in the 300 µm group (approximately 2-fold higher, $p<0.05$) than the 130 µm and 300 µm groups.

Discussion

The overall goal of this study was to investigate the effects of scaffold mean pore size on mesenchymal stem cell (MSC) initial attachment, chondrogenic differentiation and cartilage-like matrix deposition. By altering the freeze-drying technique used, collagen-hyaluronic acid (CHyA) scaffolds were fabricated with three distinct mean pore sizes (94, 130 and 300 μm). The results demonstrated that although altering the mean pore size did not affect the mechanical properties of CHyA scaffolds, there were distinct differences in the response of cells to the different scaffold variants. In particular, scaffolds with the largest mean pore sizes (300 μm) stimulated the lowest level of COL1 gene expression in comparison to the scaffolds with the smallest mean pore size (94 μm) as early as day 14 while they stimulated higher COL2 gene expression and sGAG production than scaffolds with smaller mean pore sizes (94 and 130 μm). Taken together, the results demonstrate the ability of scaffold micro-architecture to elicit distinct MSC response in terms of gene expression and cartilage-specific matrix production *in vitro*, thus highlighting the significance of this scaffold property in the development of biomaterials for defect repair.

As expected, the scaffold pore size did not affect the bulk compressive modulus of CHyA scaffolds with the three scaffold groups having a compressive modulus of approximately 0.5 kPa (Fig. 1). This is likely due to the equiaxed and isotropic nature of the pore structure within the scaffolds[4] which was not altered between the three groups. This provides reassurance that the differences in MSC responses observed could be attributed solely to the changes in the mean pore sizes of the scaffolds investigated.

The *in vitro* response of MSCs to the differing mean pore sizes was assessed and it was evident that there was a strong linear trend of increasing cell attachment with increasing mean pore size. Although at day 14 there were no differences between the 3 groups, it was evident that by day 28 the scaffolds with the largest mean pore size (300 µm) had significantly higher number of cells than those with the smallest mean pore size (94 µm) (Fig. 3). A recent study that utilised porous chitosan scaffolds demonstrated that larger pores also supported greater proliferation and ultimately greater synthesised matrix accumulation following 4 weeks of culture.[30] Another study utilising porous poly-caprolactone scaffolds demonstrated using 2 mature cell types (chondrocytes and osteoblasts) that proliferation increased with increasing scaffold mean pore size.[41] Taken together, these findings suggest that scaffolds with larger mean pore sizes support greater attachment and subsequently greater proliferation which may have considerable implications for downstream effects such as differentiation and matrix deposition.

The most notable result from the study is that changes in scaffold mean pore size was seen to affect MSC chondrogenic differentiation and matrix deposition. Scaffold mean pore size affected the expression of genes specific to the chondrogenic differentiation of MSCs. Scaffolds with larger mean pore sizes (300 µm) stimulated higher SOX9 gene expression compared to scaffolds with small mean pore size (94 µm) (Fig. 5). In addition, it was evident that the scaffolds with the largest mean pore size also stimulated significantly higher COL2 gene expression (Fig. 7). To further investigate the effect of pore size on MSC differentiation, COL1 gene expression was also assessed. The results demonstrated that COL1 gene expression was inversely proportional to scaffold mean pore size, with the largest mean pore size stimulating significantly lower COL1

15

gene expression (Fig. 6). A recent study demonstrated that scaffolds with a pore size range of 300-400 μm provide an enhanced microenvironment for chondrogenic differentiation of adipose derived stem cells with significantly higher levels of SOX9 and COL2 than scaffolds with a pore range of 90-200 μm.[42] In addition, they also show significantly lower COL1 gene expression in these scaffolds with large mean pore sizes.

In vitro cartilage-like matrix production (sGAG) was consistent with gene expression with regards to the correlation between mean pore size and chondrogenic response. At day 14, the scaffolds with the largest mean pore size (300 μm) stimulated significantly higher production of sGAG content compared to the scaffolds with the smallest mean pore sizes (94 μm) (Fig. 8). By day 28, there was approximately 2-fold higher sGAG content in the scaffolds with 300 μm mean pore size than those with 94 and 130 μm mean pore size. This relationship was also observed in a previous study whereby gelatin scaffolds with mean pore sizes greater than 300 μm contained significantly higher sGAG than scaffolds with mean pore sizes less than 200 μm.[31] Other studies have also demonstrated that there exists a trend of increasing sGAG accumulation with increasing scaffold mean pore size both *in vitro* and *in vivo*.[42-44] We previously showed that the composition of CG scaffolds plays a fundamental role on chondrogenesis, with the presence of hyaluronic acid supporting significantly higher chondrocyte-specific gene expression and cartilage-like matrix production than chondroitin sulphate.[25] In the current study, hyaluronic acid was utilised and it was evident that cartilage-like matrix production increased linearly with increasing mean pore size. Collectively, these findings clearly demonstrate that whilst the composition of scaffolds is essential in directing chondrogenesis, optimising the micro-architecture of scaffolds can considerably enhance this process.

The obvious question from this study is why scaffold pore size influences MSC differentiation. One hypothesis is that scaffold with larger mean pore sizes stimulate chondrogenic differentiation simply as a result of more efficient transport of chondrogenic factors, nutrients and oxygen. However, analysis of the morphology of cells on the different scaffold variants revealed another interesting insight. SEM imaging demonstrated that the differences in cell response due to scaffold mean pore size may be partly attributed to their morphology which could be driven by the cell-scaffold interactions with scaffold with larger pores more likely to facilitate rounded morphology. The process of differentiation is partly dependent on the cell morphology and shape.[27,45,46] It is known that the density of ligands in CG scaffolds is proportional to the specific surface area.[7] Furthermore, scaffold specific surface area is inversely proportional to mean pore size. This was calculated in a study which demonstrated that scaffold specific surface area decreases with increase mean pore size.[4] Therefore, it is plausible that scaffolds with large mean pore sizes have lower ligand density. The mechanism of initial adhesion may affect the shape of cells within the scaffolds, with large mean pore sizes possibly presenting fewer ligands for cell adhesion thereby inhibiting flat morphologies but rather permitting rounded morphologies; characteristic of a chondrocytic phenotype. On the contrary, scaffolds with small mean pore sizes may permit flat cell attachment (Fig. 4). Flat, fibroblast-like cell shape is known to initiate de-differentiation and expression of COL1.[32,33] Following analysis of cell morphology using SEM, it was evident that the CHyA scaffolds with smaller pores were more likely to facilitate cells to spread flatly across the pore structures within the initial 24 hours post-seeding. However, scaffolds with larger pores were more likely to impede flat morphologies, but rather cells with rounded morphologies were found to adhere to the struts

17

within the initial 24 hours post-seeding. Therefore, this finding demonstrates that scaffold micro-architecture alters the overall morphology of seeded cells and may thus be one of the governing factors that drive the differentiation of MSCs towards a chondrogenic lineage.

Conclusions

In this study we have shown that the micro-architecture of scaffolds plays a significant role in MSC differentiation and matrix synthesis. By increasing the mean pore size of CHyA scaffolds, it was evident that the scaffolds with the largest mean pore size (300 µm) stimulated higher cell proliferation, chondrogenic gene expression and cartilage-like matrix production relative to scaffolds with the smaller mean pore size (94 and 130 µm). The superiority of scaffolds with large mean pore sizes can be attributed to improved initial cellular attachment and proliferation. Moreover, variations in mean pore size of scaffolds led to changes in the morphology of seeded cells with larger mean pore sizes more likely to support rounded cell morphology and this may have been one of the driving factors for the chondrogenic differentiation. Therefore, these findings demonstrate the importance of physical properties of biomaterials in influencing stem cell mediated chondrogenesis and may have implications in the development of advanced tissue engineering strategies for cartilage defect repair.

Acknowledgements

18

Disclosures

No competing financial interests exist.

References

1. Milella E, Brescia E, Massaro C, et al. Physico-chemical properties and degradability of non-woven hyaluronan benzylic esters as tissue engineering scaffolds. Biomaterials **23**, 1053, 2002.
2. Angele P, Abke J, Kujat R, et al. Influence of different collagen species on physico-chemical properties of crosslinked collagen matrices. Biomaterials **25**, 2831, 2004.
3. Mastrogiacomo M, Scaglione S, Martinetti R, et al. Role of scaffold internal structure on in vivo bone formation in macroporous calcium phosphate bioceramics. Biomaterials **27**, 3230, 2006.
4. O'Brien FJ, Harley BA, Waller MA, Yannas IV, Gibson LJ, Prendergast PJ. The effect of pore size on permeability and cell attachment in collagen scaffolds for tissue engineering. Technol Health Care **15**, 3, 2007.
5. Byrne E, Farrell E, McMahon L, et al. Gene expression by marrow stromal cells in a porous collagen–glycosaminoglycan scaffold is affected by pore size and mechanical stimulation. J Mater Sci Mater Med **19**, 3455, 2008.
6. Murphy CM, Haugh MG, O'Brien FJ. The effect of mean pore size on cell attachment, proliferation and migration in collagen-glycosaminoglycan scaffolds for bone tissue engineering. Biomaterials **31**, 461, 2010.
7. O'Brien FJ, Harley BA, Yannas IV, Gibson LJ. The effect of pore size on cell adhesion in collagen-GAG scaffolds. Biomaterials **26**, 433, 2005.
8. Miot S, Woodfield T, Daniels AU, et al. Effects of scaffold composition and architecture on human nasal chondrocyte redifferentiation and cartilaginous matrix deposition. Biomaterials **26**, 2479, 2005.
9. Zeltinger J, Sherwood JK, Graham DA, Müeller R, Griffith LG. Effect of pore size and void fraction on cellular adhesion, proliferation, and matrix deposition. Tissue Eng **7**, 6, 2001.
10. Salem AK, Stevens R, Pearson RG, et al. Interactions of 3T3 fibroblasts and endothelial cells with defined pore features. J Biomed Mater Res **61**, 212, 2002.
11. Temenoff JS, Mikos AG. Review: Tissue engineering for regeneration of articular cartilage. Biomaterials **21**, 431, 2000.
12. Bhardwaj N, Kundu SC. Chondrogenic differentiation of rat MSCs on porous scaffolds of silk fibroin/chitosan blends. Biomaterials **33**, 2848, 2012.
13. Bhardwaj N, Nguyen QT, Chen AC, Kaplan DL, Sah RL, Kundu SC. Potential of 3-D tissue constructs engineered from bovine chondrocytes/silk fibroin-chitosan for in vitro cartilage tissue engineering. Biomaterials **32**, 5773, 2011.
14. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol **213**, 341, 2007.

15. Bruder SP, Kurth AA, Shea M, Hayes WC, Jaiswal N, Kadiyala S. Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. J Orth Res **16**, 155, 1998.
16. Bai L, Caplan A, Lennon D, Miller R. Human Mesenchymal Stem Cells Signals Regulate Neural Stem Cell Fate. Neurochem Res **32**, 353, 2007.
17. Askari AT, Unzek S, Popovic ZB, et al. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet **362**, 697, 2003.
18. Farrington-Rock C, Crofts NJ, Doherty MJ, Ashton BA, Griffin-Jones C, Canfield AE. Chondrogenic and adipogenic potential of microvascular pericytes. Circulation **110**, 2226, 2004.
19. Lange C, Tögel F, Ittrich H, et al. Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion-induced acute renal failure in rats. Kidney Int **68**, 1613, 2005.
20. Qu-Petersen Z, Deasy B, Jankowski R, et al. Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. J Cell Biol **157**, 851, 2002.
21. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng **7**, 211, 2001.
22. Cancedda R, Dozin B, Giannoni P, Quarto R. Tissue engineering and cell therapy of cartilage and bone. Matrix Biol **22**, 81, 2003.
23. Cascio BM, Sharma B. The Future of Cartilage Repair. Oper Tech Sports Med **16**, 221, 2008.
24. Getgood A, Bhullar TPS, Rushton N. Current concepts in articular cartilage repair. Orthop Trauma **23**, 189, 2009.
25. Matsiko A, Levingstone TJ, O'Brien FJ, Gleeson JP. Addition of hyaluronic acid improves cellular infiltration and promotes early-stage chondrogenesis in a collagen-based scaffold for cartilage tissue engineering. J Mech Behav Biomed Mater **11**, 41, 2012.
26. Murphy CM, Matsiko A, Haugh MG, Gleeson JP, O'Brien FJ. Mesenchymal stem cell fate is regulated by the composition and mechanical properties of collagen-glycosaminoglycan scaffolds. J Mech Behav Biomed Mater **11**, 53, 2012.
27. Nehrer S, Breinan HA, Ramappa A, et al. Matrix collagen type and pore size influence behaviour of seeded canine chondrocytes. Biomaterials **18**, 769, 1997.
28. Stenhamre H, Nannmark U, Lindahl A, Gatenholm P, Brittberg M. Influence of pore size on the redifferentiation potential of human articular chondrocytes in poly(urethane urea) scaffolds. J Tissue Eng Regen Med **5**, 578, 2011.
29. van Tienen TG, Heijkants RGJC, Buma P, de Groot JH, Pennings AJ, Veth RPH. Tissue ingrowth and degradation of two biodegradable porous polymers with different porosities and pore sizes. Biomaterials **23**, 1731, 2002.
30. Griffon DJ, Sedighi MR, Schaeffer DV, Eurell JA, Johnson AL. Chitosan scaffolds: Interconnective pore size and cartilage engineering. Acta Biomater **2**, 313, 2006.
31. Lien S-M, Ko L-Y, Huang T-J. Effect of pore size on ECM secretion and cell growth in gelatin scaffold for articular cartilage tissue engineering. Acta Biomater **5**, 670, 2009.
32. Glowacki J, Trepman E, Folkman J. Cell Shape and Phenotypic Expression in Chondrocytes. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY) **172**, 93, 1983.
33. Li W-J, Tuli R, Okafor C, et al. A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. Biomaterials **26**, 599, 2005.
34. Nehrer S, Breinan HA, Ramappa A, et al. Canine chondrocytes seeded in type I and type II collagen implants investigated In Vitro. J Biomed Mater Res **38**, 95, 1997.
35. Haugh MG, Murphy CM, O'Brien FJ. Novel Freeze-Drying Methods to Produce a Range of Collagen–Glycosaminoglycan Scaffolds with Tailored Mean Pore Sizes. Tissue Eng Part C Methods **16**, 887, 2010.

36. Abdelwahed W, Degobert G, Fessi H. Freeze-drying of nanocapsules: Impact of annealing on the drying process. Int J Pharm **324**, 74, 2006.
37. O'Brien FJ, Harley BA, Yannas IV, Gibson L. Influence of freezing rate on pore structure in freeze-dried collagen-GAG scaffolds. Biomaterials **25**, 1077, 2004.
38. Haugh MG, Murphy CM, McKiernan RC, Altenbuchner C, O'Brien FJ. Crosslinking and mechanical properties significantly influence cell attachment, proliferation, and migration within collagen glycosaminoglycan scaffolds. Tissue Eng Part A **17**, 1201, 2011.
39. Farrell E, O'Brien FJ, Doyle P, et al. A collagen-glycosaminoglycan scaffold supports adult rat mesenchymal stem cell differentiation along osteogenic and chondrogenic routes. Tissue Eng **12**, 459, 2006.
40. Duffy GP, McFadden TM, Byrne EM, Gill SL, Farrell E, O'Brien FJ. Towards in vitro vascularisation of collagen-GAG scaffolds. Eur Cells Mater **21**, 15, 2011.
41. Oh SH, Park IK, Kim JM, Lee JH. In vitro and in vivo characteristics of PCL scaffolds with pore size gradient fabricated by a centrifugation method. Biomaterials **28**, 1664, 2007.
42. Oh SH, Kim TH, Im GI, Lee JH. Investigation of Pore Size Effect on Chondrogenic Differentiation of Adipose Stem Cells Using a Pore Size Gradient Scaffold. Biomacromolecules **11**, 1948, 2010.
43. Yamane S, Iwasaki N, Kasahara Y, et al. Effect of pore size on in vitro cartilage formation using chitosan-based hyaluronic acid hybrid polymer fibers. J Biomed Mater Res A **81A**, 586, 2007.
44. Malda J, Woodfield TBF, van der Vloodt F, et al. The effect of PEGT/PBT scaffold architecture on the composition of tissue engineered cartilage. Biomaterials **26**, 63, 2005.
45. Spiteri CG, Pilliar RM, Kandel RA. Substrate porosity enhances chondrocyte attachment, spreading, and cartilage tissue formation in vitro. J Biomed Mater Res A **78A**, 676, 2006.
46. Stoppato M, Carletti E, Sidarovich V, et al. Influence of scaffold pore size on collagen I development: A new in vitro evaluation perspective. J Bioact Compatible Polym **28**, 16, 2013.

Figure legends

Table 1: Summary of the different methods used to alter the mean pore size of CHyA scaffolds

Table 2: The effect of final freezing temperature on the mean pore size of CHyA scaffolds measured as average diameter (μm) of scaffold pores. Increasing the final freezing temperature significantly increased the mean pore size of scaffolds. Values are expressed as mean $\pm$ standard deviation, n=6, * denotes p<0.05 statistical differences relative to -40°C, ** denotes p<0.01 statistical differences relative to -40°C.

Figure 1: The effect of scaffold mean pore size on bulk compressive modulus. Alterations in scaffold mean pore size did not alter scaffold mechanical properties. The values are expressed as mean $\pm$ standard deviation, n=6.

Figure 2: The effect of scaffold mean pore on percentage cell attachment after 24 and 48 hours in culture with rat derived MSCs. There is no statistical difference in percentage cell attachment between the scaffolds with differing mean pore sizes at 24 and 48 hours post-seeding. The values are expressed as mean $\pm$ standard deviation, n=3.

Figure 3: The effect of scaffold mean pore size on cell density after 14 and 28 days in culture with rat MSCs. There was a statistically significant increase in cell density in all 3 groups between 14 and 28 days in culture. The values are expressed as mean $\pm$ standard deviation, n=3, * denotes $p<0.05$ statistical significant differences in comparison to day 14 groups, ** denotes $p<0.05$ statistical significant differences relative to the 94 µm group at day 28.

Figure 4: Scanning electron micrograph image of a CHyA scaffold with varying mean pore sizes: 94 µm (A & B), 130 µm (C & D) and 300 µm (E & F) seeded with rat derived MSCs for 24 hours. Cells appear flatly attached on the 94 and 130 µm mean pore size scaffolds whereas cells on 300 µm mean pore size scaffolds attached with a rounded morphology. Scale bar represents 20 µm. Cells have been falsely coloured in bronze to aid visualisation of the cells within the porous scaffolds.

Figure 5: The effect of scaffold mean pore size on SOX9 gene expression assessed after 7, 14 and 28 days relative to day 0. Rat MSCs were cultured on CHyA scaffolds of varying mean pore sizes. Increase in SOX9 gene expression observed with time in culture; however, with no statistical significant differences (p=0.07 between 94 μm and 300 μm; p=0.1 between 130 μm and 300 μm). The values are expressed as mean $\pm$ standard deviation, n=3.

Figure 6: The effect of scaffold mean pore size on COL1 gene expression assessed after 7, 14 and 28 days relative to day 0. Rat MSCs were cultured on CHyA scaffolds of varying mean pore sizes. Increase in COL1 gene expression observed with time in culture in all groups between day 7 and 14, followed by a significant decrease at day 28. COL1 gene expression was significantly higher at day 14 on the 94 μm group than the 300 μm group. The values are expressed as mean $\pm$ standard deviation, n=3, * denotes p<0.05 statistical significant differences relative to the 94 μm group at day 14.

Figure 7: The effect of scaffold mean pore size on COL2 gene expression assessed after 7, 14 and 28 days relative to day 0. Rat MSCs were cultured on CHyA scaffolds of varying mean pore sizes. Increase in COL2 gene expression observed with time in culture in all groups between day 7 and 14, followed by a significant decrease at day 28. COL2 gene expression was significantly higher at day 14 and 28 on the 300 μm group than the 94 and 130 μm groups. The values are expressed as mean $\pm$ standard deviation, n=3, * denotes p<0.01 statistical significant differences relative to the 300 μm group at day 28, ** denotes p<0.01 statistical significant differences relative to the 300 μm group at day 14.
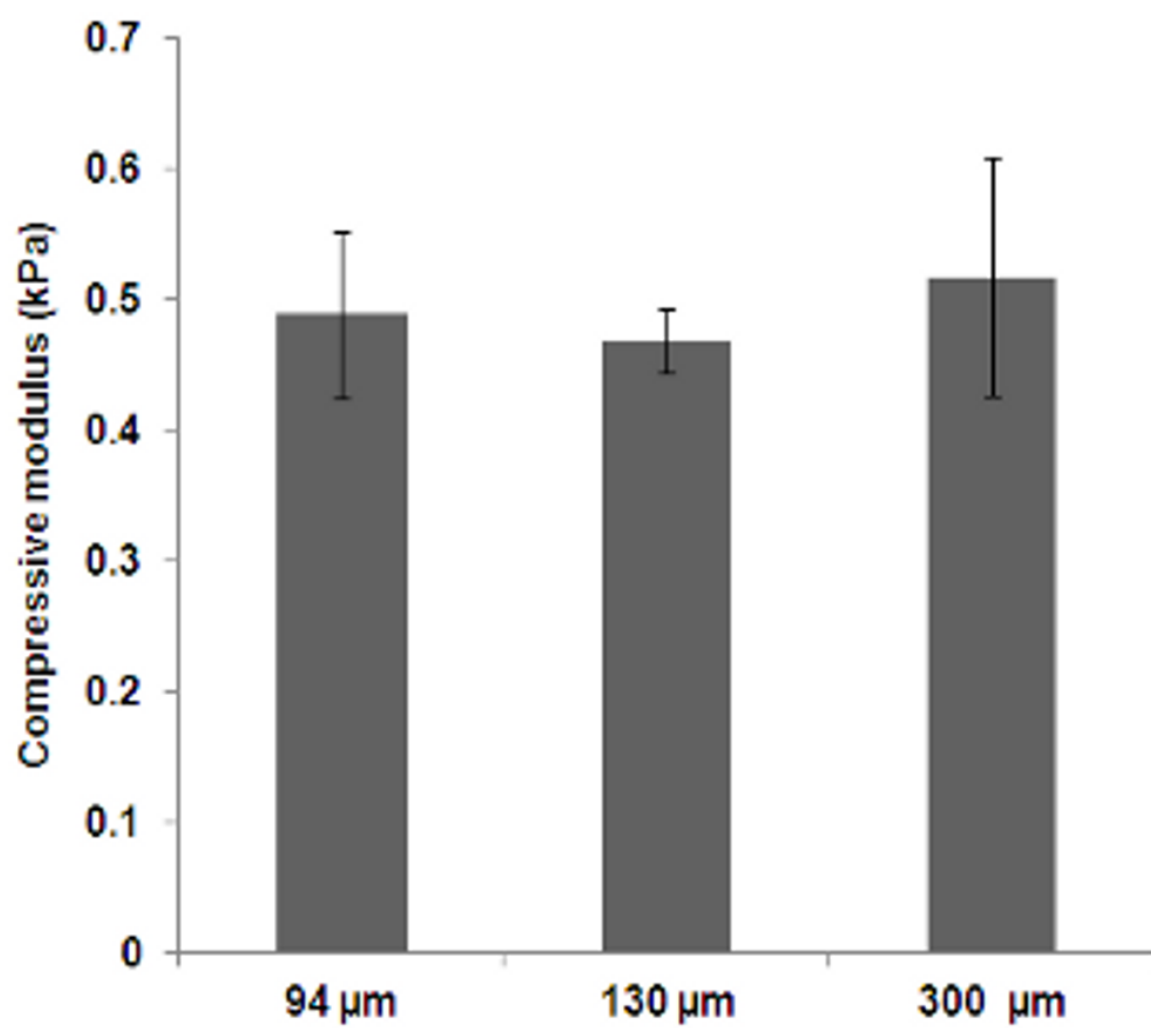
Figure 8: The total content of synthesised sulphated GAG (sGAG) in CHyA scaffolds of varying mean pore sizes assessed after 14 and 28 days in culture using a DMMB assay, normalised to DNA content. The graph shows significantly higher sGAG content in the 300 µm scaffolds in comparison to the 94 and 130 µm scaffolds at day 28 in culture. The values are expressed as mean $\pm$ standard deviation, n=3, * denotes p<0.05 statistical significant differences relative to the 300 µm group at day 14, ** denotes p<0.05 statistical significant differences relative to the 300 µm at day 28.

Matsiko. Table 1

Method used	Final freezing temperature (T_f)
Standard freeze-drying	**-40°C**
Increased T_f	**-10°C**
Annealing	**-20°C, -10°C Annealed (24 hours)**

Matsiko. Table 2

Freezing profile	Mean pore size (µm)
-40°C	93.9 $\pm$ 8.8
-10°C	130.3 $\pm$ 7.2 (*)
-10°C Annealed (24 hours)	300.5 $\pm$ 8.3 (**)

Matsiko.Fig.2

SOX9

COL1