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The effect of mean pore size on cell attachment, proliferation and migration in collagen–glycosaminoglycan scaffolds for bone tissue engineering

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ABSTRACT

In the literature there are conflicting reports on the optimal scaffold mean pore size required for successful bone tissue engineering. This study set out to investigate the effect of mean pore size, in a series of collagen–glycosaminoglycan (CG) scaffolds with mean pore sizes ranging from 85 μm to 325 μm, on osteoblast adhesion and early stage proliferation up to 7 days post-seeding. The results show that cell number was highest in scaffolds with the largest pore size of 325 μm. However, an early additional peak in cell number was also seen in scaffolds with a mean pore size of 120 μm at time points up to 48 h post-seeding. This is consistent with previous studies from our laboratory which suggest that scaffold specific surface area plays an important role on initial cell adhesion. This early peak disappears following cell proliferation indicating that while specific surface area may be important for initial cell adhesion, improved cell migration provided by scaffolds with pores above 300 μm overcomes this effect. An added advantage of the larger pores is a reduction in cell aggregations that develop along the edges of the scaffolds. Ultimately scaffolds with a mean pore size of 325 μm were deemed optimal for bone tissue engineering.

1. Introduction

Pore structure is an essential consideration in the development of scaffolds for tissue engineering. Pores must be interconnected to allow for cell growth, migration and nutrient flow. If pores are too small cell migration is limited, resulting in the formation of a cellular capsule around the edges of the scaffold. This in turn can limit diffusion of nutrients and removal of waste resulting in necrotic regions within the construct. Conversely if pores are too large there is a decrease in surface area limiting cell adhesion [1]. Extracellular matrix (ECM) provides cues for cellular behaviour. Cellular activity is influenced by specific integrin–ligand interactions between cells and the surrounding ECM [2–4]. However, it is initial cell adhesion that mediates all subsequent events such as proliferation, migration and differentiation within the scaffold [5]. Cells can discriminate subtle changes in the ECM that may affect their behaviour; consequently pore size can influence certain criteria such as cell attachment, infiltration and vascularisation within biological scaffolds [6,7]. Therefore maintaining a balance between the optimal pore size for cell migration and specific surface area for cell attachment is essential [8].

The relationship between scaffold pore size and osteoblast activity within tissue engineered constructs is not fully understood as is evident from the conflicting reports on the optimal pore size found within the literature. Scaffolds with mean pore sizes ranging from 20 μm to 1500 μm have been used in bone tissue engineering applications [9–13]. A study into porous implants demonstrated that the minimum pore size for significant bone growth is 75–100 μm with an optimal range of 100–135 μm [14,15]. Since this early work many studies have suggested a need for pores exceeding 300 μm for bone formation and vascularisation within constructs. By facilitating capillary formation, pores greater than ~300 μm lead to direct osteogenesis while pores smaller than ~300 μm can encourage osteochondral ossification [8,16–18]. However, it is important to identify the upper limits in pore size as large pores may compromise the mechanical properties of the scaffolds by increasing void volume [8].

Collagen–glycosaminoglycan (CG) scaffolds, originally developed for skin regeneration, have demonstrated great potential for bone tissue engineering due to their ability to promote cell growth and tissue development [19–24]. When investigated for skin regeneration and wound healing it was hypothesised that there is a critical range of pore size [20–120 μm] for optimal cellular activity and viability [1]. The CG scaffolds are fabricated using a freeze-drying (lyophilisation) process whereby a constant cooling rate technique is used to produce scaffolds with a homogenous pore

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structure. A recent study has demonstrated that by modifying this lyophilisation process, it is possible to control the scaffold pore architecture and using the refined lyophilisation process, CG scaffolds with mean pore sizes ranging from 96 to 151 μm [25,26] were produced. The authors devised a cellular solids approach to estimate the specific surface area for scaffolds with different pore sizes. When these scaffolds were seeded with osteoblasts and cellular adhesion monitored up to 48 h post-seeding, it was demonstrated that cell attachment decreased with increasing pore size. The authors suggested that the rationale for this result was the effect of specific surface area on cell adhesion [27] i.e. scaffolds with smaller pores have a greater surface area which provides increased sites for initial cellular attachment post-seeding. Since this study, further modifications to the lyophilisation process in our laboratory and improvements in technical capability allow for the production of an expanded range of CG scaffolds with mean pore sizes ranging from 85 μm to 325 μm [28].

Using this expanded range of CG scaffolds, this study set out to identify the optimal CG scaffold pore size for bone tissue engineering and to determine how scaffold mean pore size affects initial cell adhesion and subsequent proliferation at time points up to 7 days post-seeding. The earlier study demonstrated that initial cell adhesion decreased with increasing pore size [27]. This study investigated whether this trend will follow through in scaffolds with a bigger range of pore sizes with an additional later time point to allow for proliferation in order to investigate whether scaffold surface area is important for cell proliferation in addition to cell adhesion.

2. Materials and methods

2.1. Fabrication of CG scaffolds

A CG suspension was produced by blending micro-fibrillar bovine tendon collagen (Integra Life sciences, Plainsboro, NJ) with chondroitin-6-sulphate, isolated from shark cartilage (Sigma–Aldrich, Germany) in 0.05 M acetic acid. This suspension was maintained at 4 °C during blending to prevent denaturation of the collagen.

The CG suspension was lyophilised using a previously developed protocol [25]. The suspension was degassed and placed in the chamber of the freeze-dryer at room temperature (20 °C). Both the chamber and the shelf of the freeze-dryer were cooled at a constant rate to a final temperature of freezing (T_f) and held constant for 60 min. T_f of −10 °C, −40 °C and −60 °C were used. Additionally, an annealing step was introduced whereby the suspension was initially cooled to T_f −20 °C. The temperature was then raised to −10 °C and held there for a specific annealing time to produce scaffolds with different mean pore sizes. The annealing times used were 24 h and 48 h. The ice phase was then sublimated under vacuum (>100 mTorr) at 0 °C for a period of 17 h to produce the porous CG scaffolds (Table 1).

After freeze-drying the scaffolds were dehydrothermally crosslinked at 105 °C for 24 h in a vacuum oven at 50 mTorr (VacuCell, MMM, Germany).

2.2. Determination of specific surface area

Using a cellular solids model utilising a polyhedral unit cell, a previous study calculated the specific surface area per unit volume (SA/V) in CG scaffolds to be inversely related to the pore diameter by the function [27]:

$$\frac{SA}{V} = \frac{0.718}{d}$$

The authors hypothesised that this measurement of specific surface area was representative of ligand density in CG scaffolds with different mean pore sizes. A similar methodology was used in the current study to calculate the specific surface area in the new range of scaffolds (Table 2).

Table 1
Final temperatures of freezing and annealing times used and the resulting pore sizes.

Final temperature of freezing (T_f)	Pore size (μm)	Annealing time (h)	Pore size (μm)
−10 °C	325	24	164
−40 °C	120	48	190
−60 °C	85		

Table 2
Mean pore sizes and resulting specific surface area. Specific surface area decreases with increasing mean pore size.

Specific surface area (μm^{-1})	Pore size (μm)
0.00845	85
0.00598	120
0.00438	164
0.00378	190
0.00221	325

2.3. Cell culture

MC3T3-E1 cells, a pre-osteoblastic cell line, were cultured in standard tissue culture flasks using α-MEM supplemented with 10% foetal bovine serum, 1% L-glutamine and 2% penicillin/streptomycin. Media was changed every 4 days and cells were removed from flasks using trypsin–EDTA solution. Cell number was calculated using a haemocytometer.

Prior to seeding, sections were cut from the range of scaffolds fabricated as above and seeded with 200 μl of 3.25×10^6 cell/ml cell suspension and maintained in supplemented α-MEM at 37 °C with 5% CO_2 for 24 h, 48 h and 7 days.

2.4. Quantification of cell adhesion within CG scaffolds

The constructs were digested in papain to expose the DNA. Cell number was quantified using a Hoechst 33258 DNA assay which fluorescently labels double-stranded DNA, as previously described [29]. Measurements were obtained using a fluorescence spectrophotometer (Wallac Victor, PerkinElmer Life Sciences) and the readings were converted to cell number using a standard curve.

Cell attachment was determined based on the results from the Hoechst 33258 DNA assay as a percentage of the cells seeded onto the scaffolds.

2.5. Determining cell infiltration

Histological analysis was used to investigate cell infiltration into the scaffolds. At each time point the cell seeded scaffolds were fixed with 10% formalin for 30 min and processed using an automatic tissue processor (ASP300, Leica, Germany). The constructs were embedded in paraffin wax before sectioning and staining with haematoxylin and eosin (H&E) to determine cell infiltration.

2.6. Statistical analysis

For both cellular activity and cell number assays two-way analysis of variance (ANOVA) followed by Holm-Sidak multiple comparisons was preformed to compare data. A total of $n = 6$ scaffolds for each mean pore size was analysed for cell number at each time point. One-way ANOVA followed by Holm-Sidak multiple comparisons were used to analyse cell attachment data. Error is reported in figures as the standard deviation (SD) and significance was determined using a probability value of $P < 0.050$.

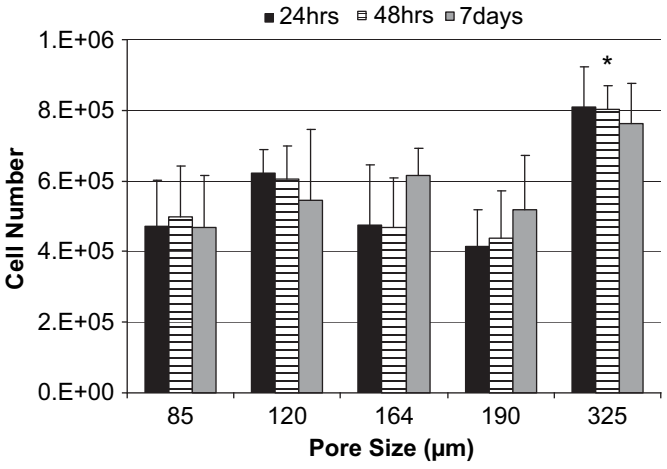

Fig. 1. Effect of mean pore size on cell number within scaffolds 24 h, 48 h and 7 days after incubation. A non-linear relationship is seen between mean pore size and cell number within scaffolds. Two-way ANOVA determined the scaffold with a mean pore size of 325 μm to have the highest cell number ($P < 0.001$).