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Investigating the effect of mean pore size on cell attachment, proliferation and migration in collagen glycosaminoglycan scaffolds for 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 – 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 hours 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.

Keywords: Collagen; Scaffold; Pore size; Bone tissue engineering; Cell adhesion

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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 – 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 μm – 100 μm with an optimal range of 100 μm – 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 – 120µm] 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 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 - 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 hours 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 – 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

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 60min. 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 hr and 48 hr. The ice phase was then sublimated under vacuum (>100mTorr) at 0°C for a period of 17 hr to produce the porous CG scaffolds (Table 1).

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

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).

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 400 μ l of 3.25×10^6 cell/ml cell suspension and maintained in supplemented α -MEM at 37°C with 5% CO₂ for 24 hr, 48 hr and 7 days.

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.

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 minutes 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.

Statistical Analysis

For cell number assays two-way analysis of variance (ANOVA) followed by Holm-Sidak multiple comparisons was performed 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 $P < 0.050$.

3. Results

Effect of pore size on cell number within CG scaffolds

Fig. 1 demonstrates that pore size has an effect on MC3T3-E1 cell attachment and proliferation within CG scaffolds. A non linear relationship was found between mean pore size and cell number within scaffolds. Two-way ANOVA indicates that scaffolds with a mean pore size of 325 μm facilitate a significantly higher number of cells in comparison to scaffolds with smaller mean pore sizes ($p < 0.001$). This result was observed at each time point. However in the lower range of pore sizes (85 μm - 190 μm) an initial increase in cell number peaked in scaffolds with a mean pore size of 120 μm at 24 hr and 48 hr post seeding (Fig. 2 A). This peak declines at later time points (Fig. 2 B & C).

Initial cell adhesion to CG scaffolds

When the data above was plotted as a percentage of initial cells seeded in order to obtain seeding efficiency 24 hr and 48 hr post seeding (Fig. 3), it demonstrated a significant effect of mean pore size on MC3T3-E1 cell attachment. Scaffolds with the largest mean pore size of 325 μm facilitated the highest percentage of cell attachment with approximately 62% of cells remaining attached and viable 24 hr and 48 hr post seeding ($P < 0.007$). Within the lower range of pore sizes (85 μm - 190 μm) cell attachment increased to a peak with over 45% of MC3T3-E1 cells remaining attached to scaffolds with a mean pore size of 120 μm ($P < 0.030$). No significant difference was found in cell adhesion between intermediate scaffolds (85 μm , 164 μm and 190 μm).

Specific surface area of the scaffold was calculated for each of the five different mean pore sizes (Table 2). Fig. 4 shows cell attachment as a function of specific surface area. A non-linear effect was seen. The highest cell numbers were found in scaffolds with a specific surface area of 0.0022 μm^{-1} . This scaffold had significantly higher cell numbers than all other scaffolds. However, the scaffold with a specific surface area of 0.006 μm^{-1} showed higher cell numbers than all groups besides the 0.0022 μm^{-1} variant.

Cell Infiltration

Histological analysis provides some insight into how pore size affects cell behaviour. Fig. 5 illustrates cell infiltration and migration within the scaffolds after 7 days of incubation. Cell aggregations, as shown by arrows, were seen along the edges of scaffolds with mean pore sizes of 85 μm and 120 μm (A, B, C & D) with limited cell infiltration toward the centre of the scaffold. Cell migration in towards the centre of the scaffold improved with increasing mean pore size. Scaffolds with mean pore sizes of 164 μm and 190 μm both demonstrated similar cell migration away from the edges of the scaffold with no visible cell aggregations. However, progression was slow as cell migration did not reach the centre of the construct (E). Scaffolds with the larger pore size of 325 μm facilitated a higher rate of scaffold infiltration with even cell distribution (F & G). Cells migrated fully away from the edges into the centre of the scaffolds resulting in the absence of cell aggregation thus demonstrating that cell migration increases with increasing pore size.

4. Discussion

There are conflicting reports in the literature on the optimal pore size for bone tissue engineering. Previous fabrication procedures using CG scaffolds allowed the production of scaffolds with mean pore sizes ranging from 96 μm – 151 μm [25, 26]. Early stage experiments investigating the effect of cellular activity on this range of scaffolds, up to 48 hr post seeding, demonstrated that cell attachment increased as pore size decreased, with a pore size of 96 μm facilitating the highest percentage of cell adhesion [27]. Recent modifications to the freeze-drying process has allowed an expanded range of CG scaffolds with mean pore sizes ranging for 85 μm – 325 μm to be produced in our laboratory [28]. This study aimed to investigate the effect of this new expanded range of scaffolds on cellular activity up to 7 days and to determine whether the pattern of specific surface area affecting initial cell adhesion would continue with cell proliferation and migration. The results show that specific surface area influenced initial cell attachment in the lower pore range (85 μm – 190 μm), consistent with the previous study but a higher level of cell adhesion was demonstrated in scaffolds with larger pores than were fabricated previously. This trend continued with a mean pore size greater than 300 μm facilitating optimal proliferation and CG scaffold infiltration after 7 days. Histological analysis demonstrated that larger pores allowed for improved cellular infiltration which may overcome the advantages provided by improved surface area in scaffolds with smaller pores at the earlier time points.

A non-linear effect of pore size on cell attachment was seen over the 7 day incubation period. Cell number was highest in scaffolds with the largest mean pore size of 325 μm at all time points, with a smaller peak in cell number detected in scaffolds with a mean pore size of 120 μm , 24 hr and 48 hr post seeding. However in the later time points, this initial peak disappears and by day 7 it is clear that 325 μm pore size is the optimal pore size within this new scaffold range for cell proliferation. When cell number, 24 hr and 48 hr post seeding, was presented as a percentage of cells seeded onto CG scaffolds, it was determined that 325 μm pore size facilitated the highest cell attachment out of all the groups. However, scaffolds with 120 μm mean pore size had significantly higher cell attachment within the lower range of pore sizes (85 μm – 190 μm). This

significant increase was seen at 24 hr and 48 hr indicating that the original peak seen in cell number was a result of initial cell adhesion and subsequently disappeared by 7 days following cell proliferation in all groups.

Collagen is a natural component of bone ECM that contains binding sites (ligands) that are recognised by specific cell surface receptors (integrins) including $\alpha 1\beta 1$ and $\alpha 2\beta 1$ [2-4, 7]. Cell attachment, migration and speed are influenced by specific integrin-ligand interactions between the cell and the surrounding ECM. Therefore it is important to maintain a high surface area in scaffolds for optimal cell attachment. Previous studies by O'Brien *et al* (2005) have shown that specific surface area decreases with increasing pore size. Consistent with these findings, the specific surface area of our scaffolds decreased with increasing pore size. As a result it was hypothesised that cell attachment would decrease linearly with increasing pore size. This was demonstrated previously with a limited range of pore sizes (96 μm - 151 μm) [27]. Our results reflected this within a similar range (120 μm - 190 μm) suggesting that the specific surface area of the scaffold may influence ligand-integrin interactions. However, when this range of pore sizes was expanded (85 μm - 325 μm), the linear relationship between specific surface area and cell attachment was no longer applicable. We propose that the effect of specific surface area is overcome in larger pores by the importance of cell migration and proliferation as was seen histologically in scaffolds with 325 μm . The larger pores reduced cell aggregations along the edges of the scaffold promoting cell proliferation and migration into the centre of the scaffold. Furthermore the smallest pore size, of 85 μm , showed both reduced cell attachment and the poorest rate of cell migration.

When seeding 3-D constructs with cells it is desired that the cells will infiltrate and colonise the scaffold laying down their own ECM. An advantage of the CG scaffold is high porosity (~99%) [26] and its ability to promote cell migration and tissue growth [22, 23]. Previously it was shown that cell migration behaviour decreases with increasing pore size [7]. However, similarly to other studies [27], these results were based on limited range of mean pore sizes incubated for less than 48 hr. In this study, migration of cells was assessed histologically after 7 days incubation. Cells were observed lining the pores in all scaffolds. However, cell aggregations were seen along the edges of the scaffolds with smaller pore sizes of 85 μm - 120 μm limiting the number of cells infiltrating the scaffold. Aggregations form a skin around the outer surface of the scaffold which restricts

the diffusion of nutrients and removal of waste from the cells colonising the centre of the scaffold. As the mean pore size increased cells migrated further away from the edges and in towards the centre of the scaffold until cells were seen colonising the centre of the scaffolds with a mean pore size of 325 μm . An increase in cell number was seen in 120 μm pore size, but the aggregations seen on the surface of these scaffolds compound the hypothesis that this peak was related to initial cell adhesion and the advantages of this pore size were lost with subsequent cell proliferation and migration. The cells in scaffolds with a mean pore size of 325 μm had migrated away from the surface and had begun to colonise the centre of the scaffold after 7 days incubation. This correlates with the cell number found within this scaffold. Cell number was significantly higher in 325 μm pore size 7 days post seeding indicating that it is as a result of proliferation and migration.

It has been reported that pores greater than 300 μm are essential for vascularisation of scaffolds and bone ingrowth [8, 16-18]. Our findings correlate with these reports as the largest pores within our range of CG scaffolds proved best by facilitating both highest cell attachment and proliferation, in addition to optimal scaffold infiltration and even cell distribution. However an early stage cellular response was seen within the lower range of pore sizes (85 μm – 190 μm) whereby cell attachment peaked in scaffolds with a pore size of 120 μm . This trend is similar to that seen in the earlier pore size study [27] which demonstrated this result to be an effect of specific surface area. It is hypothesised that the effect of specific surface area is due to the ligand density available for integrin binding after initial seeding. This result correlates with studies that report smaller pores to be optimal [1, 12, 14, 15, 27], however, the peak disappears at the later time point of 7 days due to reduced cell proliferation and poor migration. Consequently, we propose that while specific surface area may affect initial cell attachment in scaffolds with a small pore size, its importance is overcome in the larger pores as a result of cell infiltration and migration into the scaffold.

This study has a number of limitations. Due to different lyophilisation facilities, it was not possible to produce scaffolds with identical mean pore sizes to the earlier study [27]. However, we believe that the mean pore sizes in the scaffolds investigated in this study are similar enough in size at the lower range (85 μm – 190 μm) to allow comparison with the earlier study (96 - 151 μm). This study has also failed to determine the upper pore size limit for cellular activity in the CG scaffolds. Ultimately, if the pores get too

big, the surface area will reduce to a level that will limit cell adhesion and cells will simply migrate right through the matrix or the level of cell-to-cell contact will be too low to allow for proliferation and subsequent osteogenesis. Furthermore, this study has demonstrated what happens to cell in standard culture media at time points up to 7 days as the focus of the study was on initial cell adhesion and early stage proliferation prior to matrix deposition. Ultimately, in order to determine the optimal scaffold pore size for bone tissue engineering, a longer term study needs to be carried out in osteogenic culture to investigate the effect of pore size on ECM deposition and mineralisation in vitro.

In conclusion, this study has demonstrated that scaffold mean pore greatly affects cellular activity and that subtle changes in pore size can have significant effects of cellular activity. The study also provides some insight into why the literature shows conflicting reports on the optimal pore size for bone tissue engineering whereby the increased surface area provided by scaffolds with small pores may have a beneficial effect in initial cell adhesion but ultimately the improved cellular infiltration provided by scaffolds with larger pores outweighs this effect and suggests that these scaffolds might be optimal for bone tissue repair.

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Tables

Final Temperature of Freezing (T _f)	Pore size (μm)	Annealing Time (hr)	Pore size (μm)
-10°C	325	24	164
-40°C	120	48	190
-60°C	85		

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

Specific Surface Area μm ⁻¹	Pore Size (μm)
0.00845	85
0.00598	120
0.00438	164
0.00378	190
0.00221	325

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

Figures

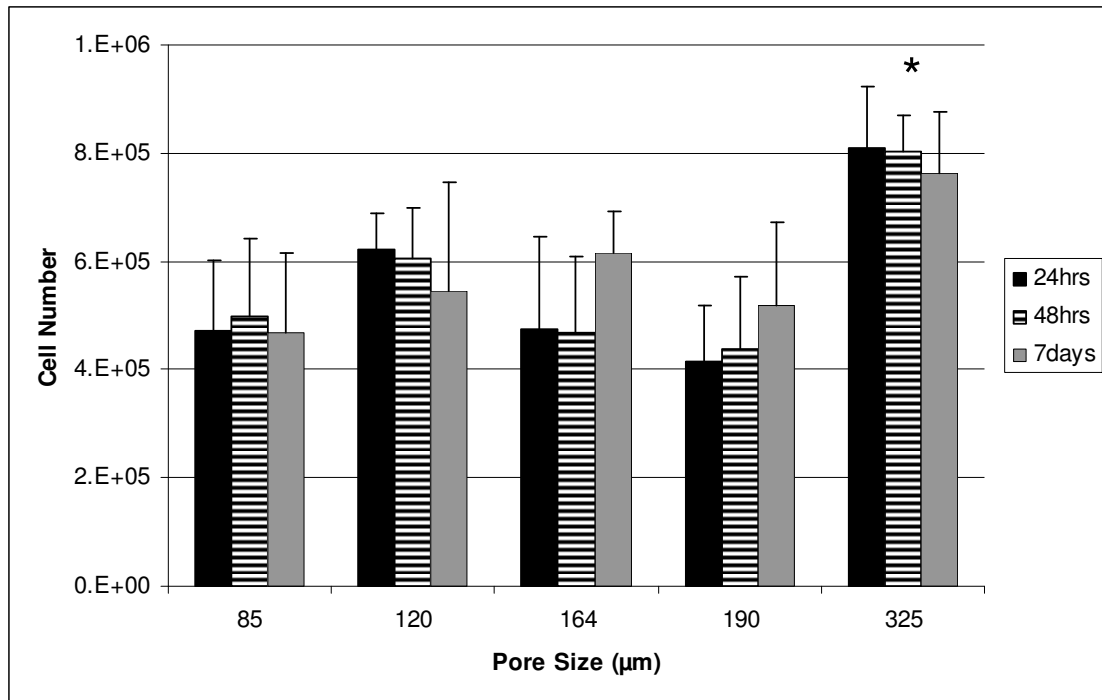
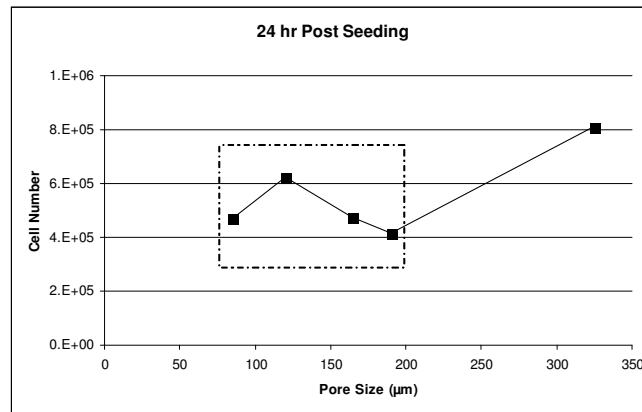
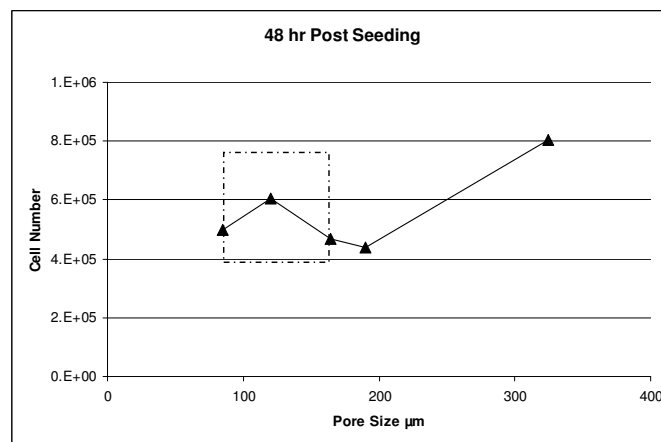


Fig. 1: Effect of mean pore size on cell number within scaffolds 24 hr, 48 hr and 7 days after incubation. A non-linear relationship is seen between mean pore size and cell number within scaffolds. Two-way ANOVA determined the scaffold with a mean pore size of 325μm to have the highest cell number ($P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

A)



B)



C)

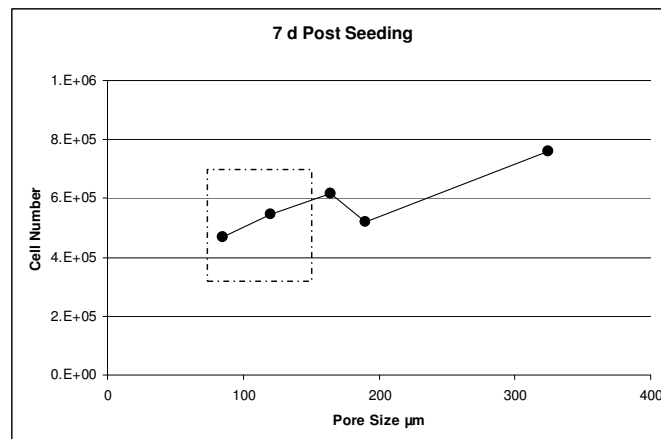


Fig. 2: Effect of mean pore size on cell number at each time point. Cell number increases to a small peak 24 hr post seeding in scaffolds with a pore size of 120 μm. This peak declines at later time points. Cell number significantly peaks in scaffolds with a mean pore size of 325 μm.

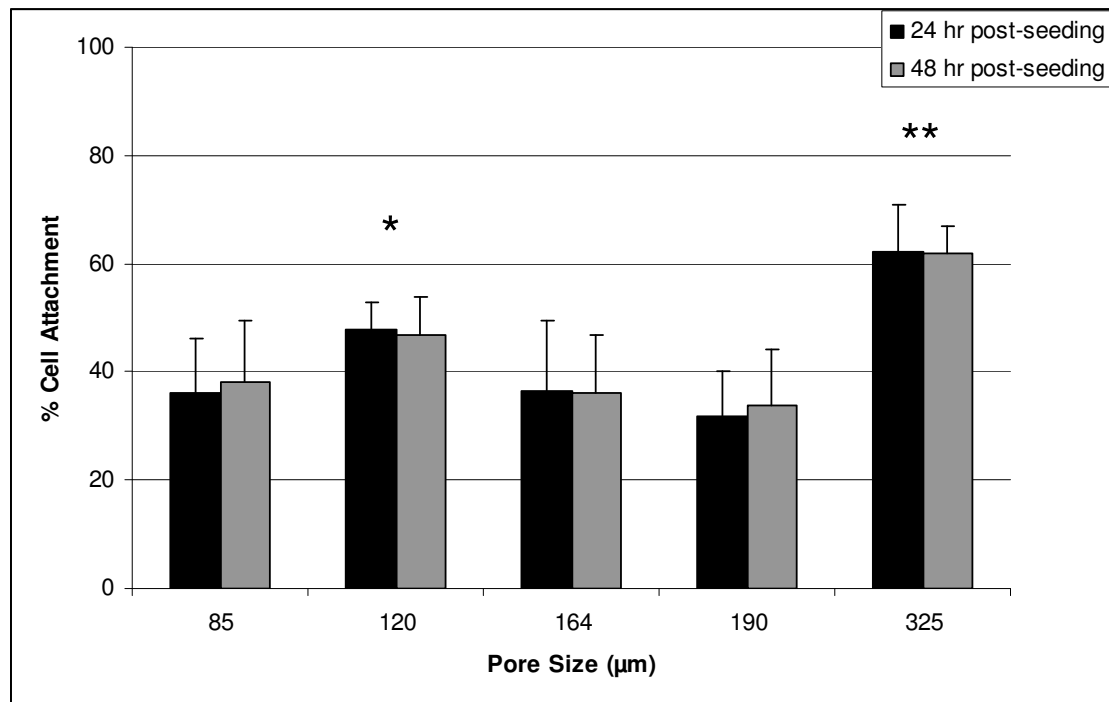


Fig. 3: Cell attachment as a percentage of seeding efficiency. Scaffolds with the largest pore size of 325 μm have the highest percentage of cells attached 24 hr and 48 hr post seeding. Cell attachment increases to a peak in the lower range of pore sizes at 120 μm pore size.

* $P < 0.030$ relative to 85 μm, 164 μm and 190 μm. ** $P < 0.007$ relative to 85 μm, 120 μm, 164 μm and 190 μm

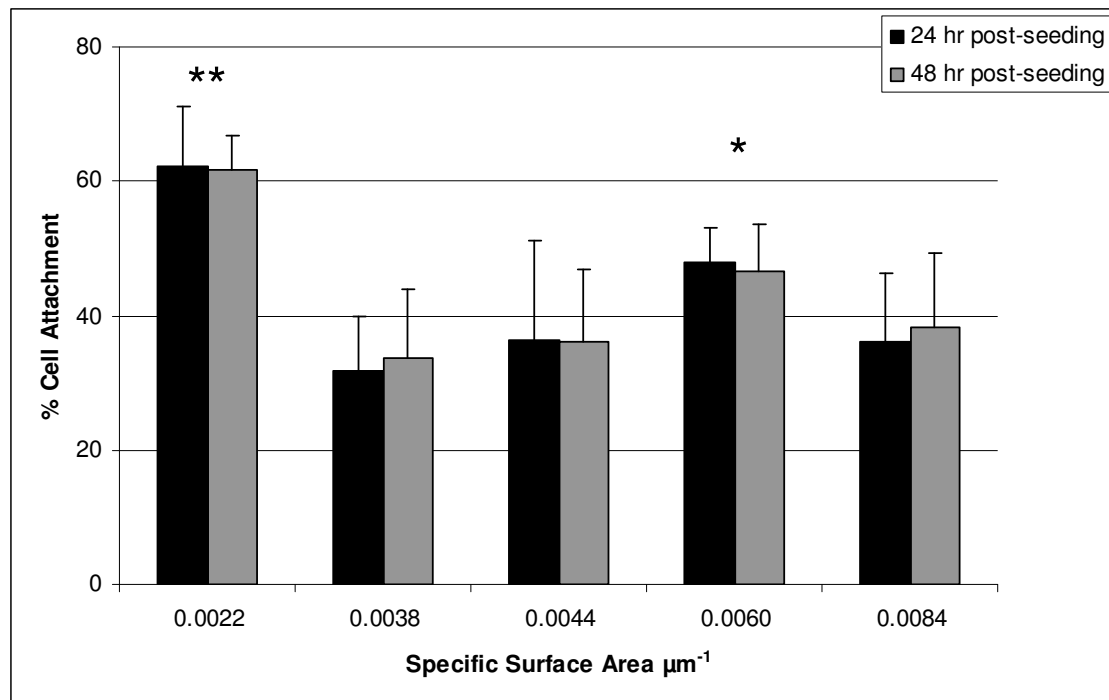
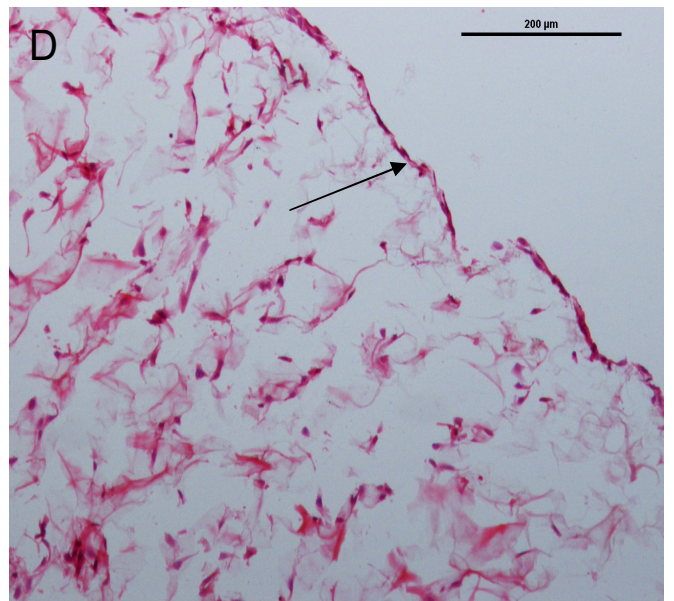
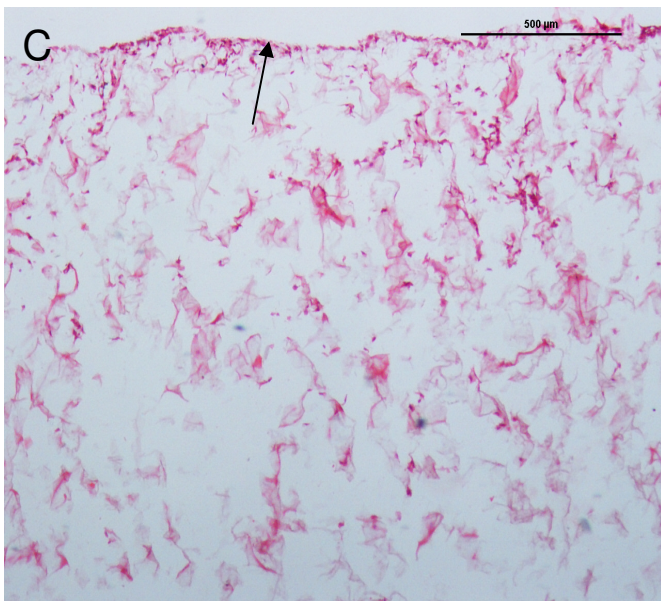
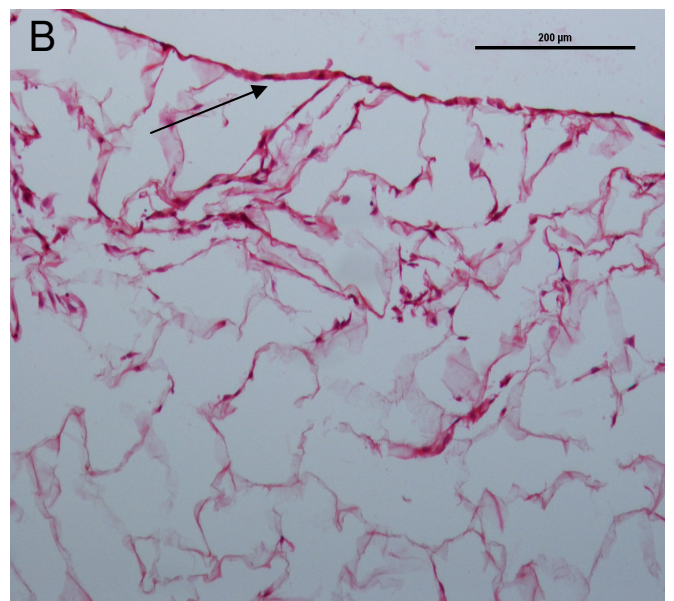
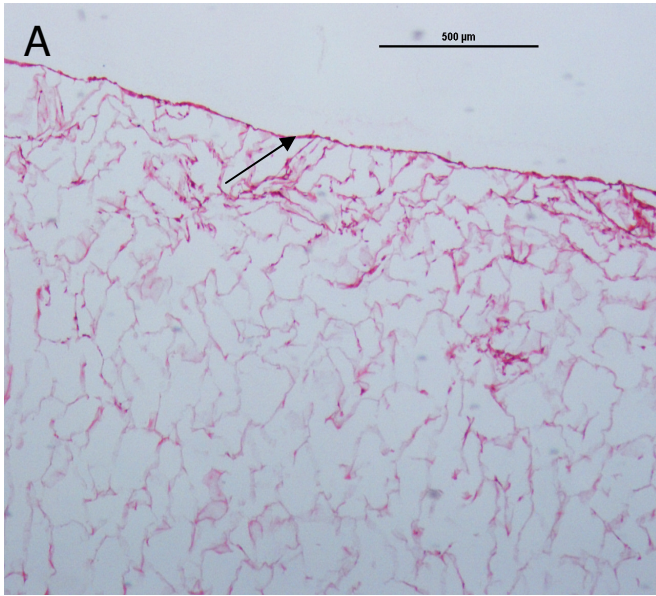


Fig. 4: Initial cell attachment plotted against specific surface area demonstrating a non linear increase at 24 hr and 48 hr with cell attachment peaking in scaffolds with a mean pore size of 120 μm and 325 μm .

* $P < 0.030$ relative to 0.0038 μm^{-1} and 0.0084 μm^{-1} . ** $P < 0.007$ relative to 0.0038 μm^{-1} , 0.0044 μm^{-1} , 0.0060 μm^{-1} and 0.0084 μm^{-1}



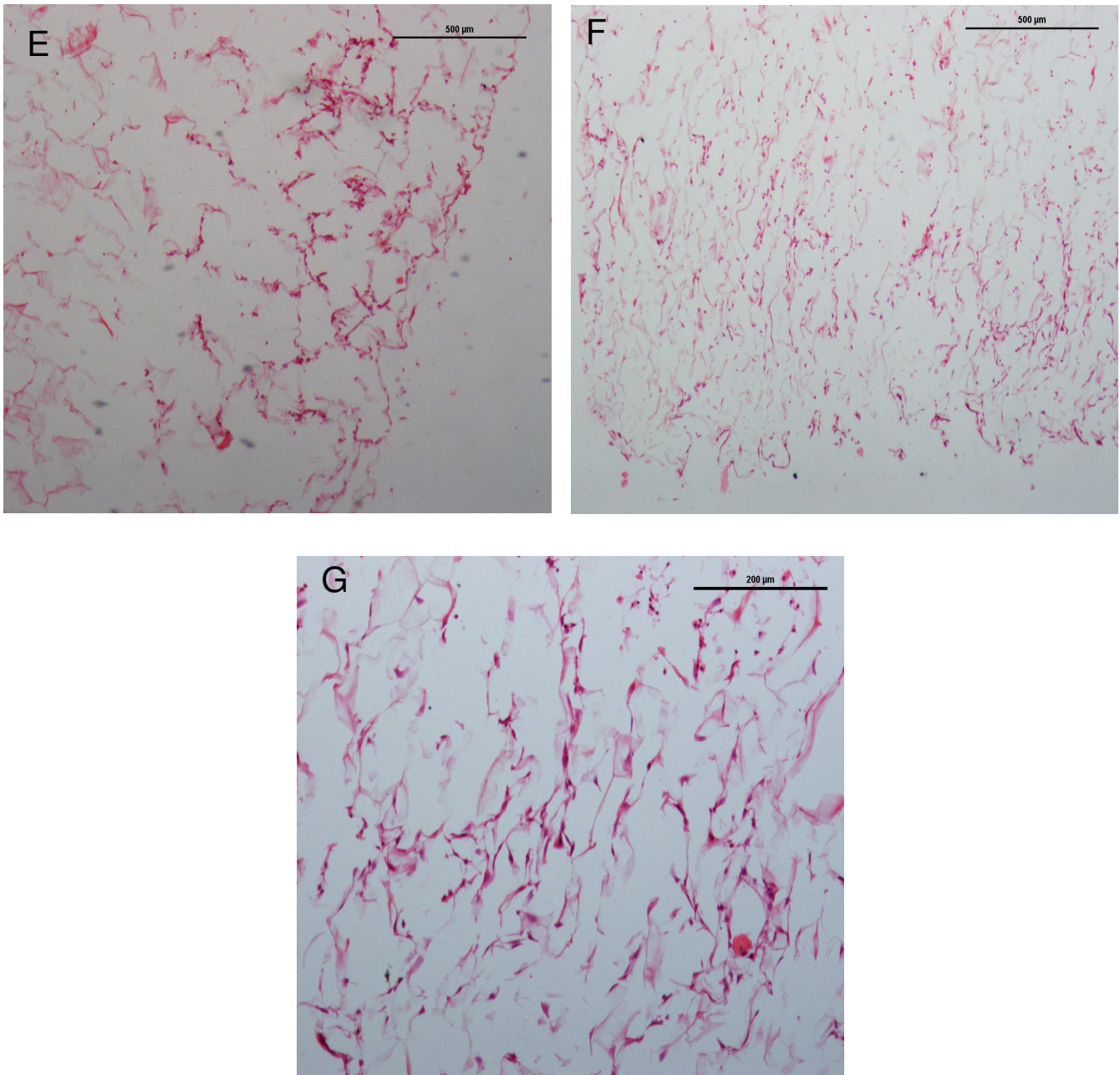


Fig. 5: Effect of mean pore size on cell infiltration and distribution CG scaffolds after 7 days. Scaffolds were stained with H&E:

- (a) 85μm pore size at x 40 magnification, (b) 85μm pore size at x 100 magnification,
- (c) 120μm pore size at x 40 magnification, (d) 120μm pore size at x 100 magnification,
- (e) 190μm pore size at x 40 magnification, (f) 325μm pore size at x 40 magnification

and (g) 325 μ m pore size at x 100 magnification. Collagen scaffold is stained pink and cell nuclei a deep purple. The arrows indicate cell aggregations along the edges of the scaffold. Aggregations disappeared and cell migration increased with increasing pore size.