INTRODUCTION: Porous scaffolds used for tissue engineering applications serve as an analog of the extracellular matrix, acting as a physical support structure that affects cellular processes such as migration, contraction, and division. The scaffold pore structure and mean pore size have been observed to significantly affect biological activity. For maximal bioactivity, the mean pore size must lie in a critical range: large enough to allow cell migration and nutrient diffusion into the scaffold, but small enough so that the specific surface area is sufficiently large for cell proliferation and viability. If the mean pore size lies outside of this critical range, the number of cells bound to the scaffold may be inadequate for the desired level of cell attachment or activity. Cell attachment is mediated by an interaction between cell receptor molecules on the cell surface, termed integrins, and binding sites on the extracellular matrix which serve as targets for integrin binding, termed ligands [1]. The scaffold biological activity depends sensitively on the density of available ligands, a criterion defined by the composition of the extracellular matrix, which determines the identity and surface density of the ligands, and by the specific surface area of the scaffold [1,2]. In cellular solids with a defined porosity, the specific surface area is inversely proportional to the pore size of the structure [4]. The objectives of this study were to fabricate collagen-GAG (CG) scaffolds with different mean pore sizes and to investigate the effect of the mean pore size and specific surface area on cell adhesion and viability.

METHODS:

Scaffold Fabrication: CG scaffolds were fabricated from a CG suspension using an established lyophilization technique [3]; different final temperatures of freezing (Tf) were utilized in order to produce scaffolds with different mean pore sizes. The CG suspension was produced by mixing bovine, microfibrillar collagen with chondroitin-6-sulfate from shark cartilage; To produce the porous structure, the CG suspension was frozen and then the ice content sublimated under a vacuum (100mTorr) at 0ºC for 17 hours. The final temperature of freezing was varied (Tf = -10ºC, -20ºC, -30ºC, -40ºC) to produce a homologous series of scaffolds with a constant composition and solid volume fraction (0.005) but with four different mean pore sizes and consequently, four different specific surface areas. The scaffolds were dehydrothermally crosslinked for 24 hours at 105ºC to stiffen the collagen network [1].

Pore Structure Analysis: The mean pore size each scaffold was determined by analyzing 10 samples from random locations throughout each CG scaffold. The samples were embedded in glycolmethacrylate, serial sectioned at a 5µm thickness, stained with aniline blue, digitized, and analyzed using a linear intercept macro written in Scion Image™ that reconstructed a best-fit shape to fit all pores in each image. The specific surface area of each CG scaffold was calculated by modeling the internal volume of a tetrakaidecahedral cell as similar to that of a sphere: 

\[ \text{SA} = \frac{4}{3} \pi \times 0.155 \]

Cell Seeding: MC3T3-E1 mouse clonal osteogenic cells were maintained in α-MEM supplemented with L-glutamine, 10% fetal bovine serum (Intergen, Purchase, NY, USA), penicillin (100 IU/ml; Sigma), and streptomycin (100 µg/ml; Sigma). Prior to seeding the cells into the scaffolds, the number of viable cells was determined by staining live cells with 0.4% Trypan Blue and counting with a standard hemacytometer, 6 x 10⁶ cells were seeded onto each scaffold. The seeded scaffolds were placed into 6-well tissue culture plates that had been pre-coated with agarose gel (JT Baker, Philipsburg, NJ) to prevent cell migration out of the scaffolds and onto the tissue culture plastic. The samples were then covered with 2 ml of α-MEM, placed into a cell culture incubator (37ºC, 5.0% CO2), and maintained in culture for either 24 or 48 hours. At the end of that time period, each sample was rinsed with 37ºC Dulbecco's phosphate buffered saline (DPBS; Gibco, Grand Island, NY) to remove unattached cells and then placed in a 2.0 U/ml solution of dispase (Gibco) for 30 minutes to digest the collagen scaffold. The number of remaining viable cells was then counted to determine the percentage cell attachment at the two time-points.

RESULTS SECTION:

Fig. 1 shows the effect of freezing temperature on the mean pore size of the CG scaffolds. Scaffolds fabricated at a final temperature of freezing of -40ºC, -30ºC, -20ºC and -10ºC were found to have a mean pore size of 96µm, 110µm, 121µm and 151µm, respectively. Paired t-tests carried out between each of the four groups indicated that there was a significant difference in mean pore size (p<0.05) between all groups.

Fig. 2 shows the percentage of cells attached to the CG scaffolds at 24 (dark gray) and 48 hours (light gray) post seeding. In the scaffolds with the smallest mean pore size (96 µm) over 40% of cells seeded attached to the scaffolds in both 24 and 48 hours groups compared to approximately 20% scaffolds with the largest mean indicated that the mean pore diameter of the scaffolds had a significant effect on cell attachment at both 24 (p<0.01) and 48 hours (p<0.001). Fig. 3 shows a graph of percent cell attachment plotted against specific surface area showing a strong linear relationship (R² = 0.95, 0.91) between specific surface area and the percentage of attached cells at 24 (solid line) and 48 hours (dashed line) post-seeing. No difference in percent cell attachment was found between the two groups (p>0.05).

DISCUSSION: This study has developed a technique to fabricate CG scaffolds with different mean pore sizes. The cell seeding experiments showed there was a significant difference in cell attachment in the different scaffolds (P<0.05), but there was no significant change in cell attachment between 24 and 48 hours for any of the different scaffolds (P>0.05). The fraction of cells attached to the CG scaffold decreases with increasing average pore diameter, and increases linearly with specific surface area. The fraction of MC3T3-E1 cells attached to the CG scaffold decreases with increasing average pore diameter and increases linearly with specific surface area, consistent with the increase in ligand binding site density being responsible for the increased cell attachment.


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