Analysis of structurally variable lyophilized collagen scaffolds for cell sieving using micro-CT

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Aims

Dual-layer, structurally variable, collagen based lyophilized scaffolds for tissue engineering have been produced through application of a multi-stage freezing process. A structurally variable scaffold structure not only better mimics native tissue, but may also provide cell sieving capabilities. The partitioning of specific cell types in certain regions of a scaffold template, thus mimicking the cell distribution in the native tissue is hypothesized to optimize the regeneration and repair process.

Clearly, in order to predict cell-sieving capabilities, it is essential to have a detailed understanding of the scaffold pore structure. This work considers micro-CT as a tool for this characterization and discusses the influence of a key scan parameter, pixel size on the analyses. A preliminary consideration of the influence of measurements in a hydrated state through the use of phosphotungstic acid contrast agent is also considered.

Method

Scaffolds were produced from 1 wt% suspensions of insoluble collagen from Bovine Dermis. Suspensions were pipetted into standard 24 well culture plates for the freezing and lyophilisation process with a schematic of the scaffold production process demonstrated in *Figure 1*.

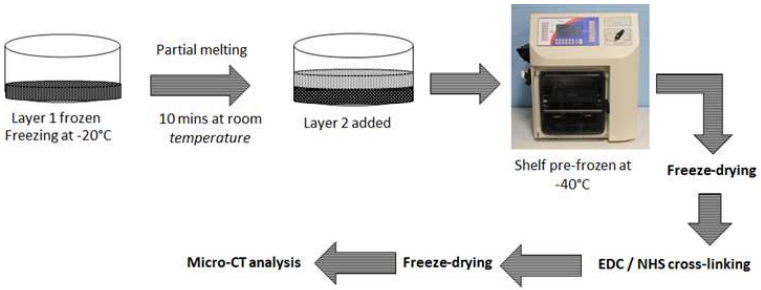


Figure 1: Schematic of the production flow chart for dual layer graduated collagen scaffolds. Collagen slurry was pipetted into standard 24 well plates prior to freezing in -20°C freezer. Once the second layer of slurry was added, further freezing and sublimation was carried out using a VirTis AdVantage freeze-dryer with a shelf temperature of -40°C. A carbodiimide EDC/NHS cross-linking was applied prior to a final freeze-drying step.

The initial collagen slurry layer was frozen at -20°C in a standard freezer for 1 hour, before the plate was held for 10 minutes at room temperature to allow for partial melting and subsequent interfacial mixing. The second layer was then pipetted on before further freezing and sublimation using a VirTis AdVantage freeze dryer with a shelf temperature of -40°C. The scaffold was thus dried and the ice-templated structure retained. EDC/NHS cross-linking was carried out at a

concentration of 100% as defined previously [1]. Cross-linking reagents were dissolved in 95% ethanol and samples soaked for 2 hours within the well plate prior to washing (5*5 minutes deionised water). A further freeze-drying cycle was then carried out.

Samples were extensively analysed using a Skyscan 1272 Micro-CT. 5 mm diameter samples were punched and scanned initially with a pixel size of 1.5 μm (no camera binning applied), operating voltage of 25kV, 0.2 step size with frame averaging of 2 and 180° rotation. Scanning was also repeated with 2* and 4* camera binning applied (3 μm and 6 μm pixel size respectively) in order to investigate the influence of pixel size on porosity analysis.

In order to analyse the influence of hydration on pore structure, a phosphotungstic acid staining protocol based upon that routinely used in soft tissue staining was applied [2]. Whole scaffolds were submerged in an aqueous 0.3 wt% solution of phosphotungstic acid and degassed in order to ensure full penetration of the stain. After soaking for 48 hours, samples were thoroughly washed with deionized water with a final degassing step prior to scanning. 8mm diameter samples were scanned in 2ml Eppendorf tubes with sponge sections above and below to eliminate movement. Scans were carried out with a pixel size of 3 μm (no camera binning), 0.25 mm Al filter, 60 kV operating volatage and frame averaging of 3.

Resulting projections were reconstructed using NRecon, and systematic volumes of interest (VOIs) selected in top and bottom sections of the scaffold. A three dimensional analysis was carried in CTAn after automatic Otsu thresholding and sweep despeckling. Volumes of interest were modified to allow penetration only from the top x-y plane and an interconnectivity analysis carried out using the ROI shrink wrap feature with increasing voxel size. Interconnectivity was thus defined as:

$$Interconnectivity = \frac{V - V_S}{V - V_M} \times 100 \qquad \textit{Equation 1}$$

Where V = total VOI
Vs = inaccessible scaffold volume after shrink wrap
V_M=volume of solid material within VOI

Using the same shrink-wrap process a percolation analysis was also carried out. The percolation diameter is the diameter of the largest sphere able to penetrate through an infinitely large scaffold and unlike percentage interconnectivity is a scalable measure. By increasing the voxel diameter ‘d’, the corresponding length of accessible pore volume ‘l’ in the z direction can be measured and this data plotted using the relationship from percolation theory in order to calculate the percolation diameter.

$$L = L_o(d - d_c)^{-v} \qquad \textit{Equation 2}$$

where v is 0.88 for a 3D system.[3]

Volume rendered models were created in CTVox.

Results

Collagen scaffolds were successfully created with through-thickness structural variability and a continuous, highly interconnected interface between the two layers. The false coloured, volume rendered model of scaffold structure superimposed with pore size distribution of Figure 2, clearly demonstrates this structural variability.

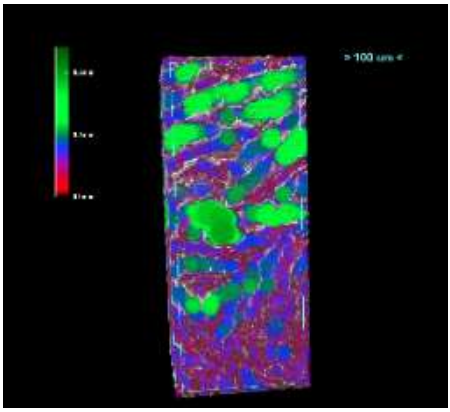
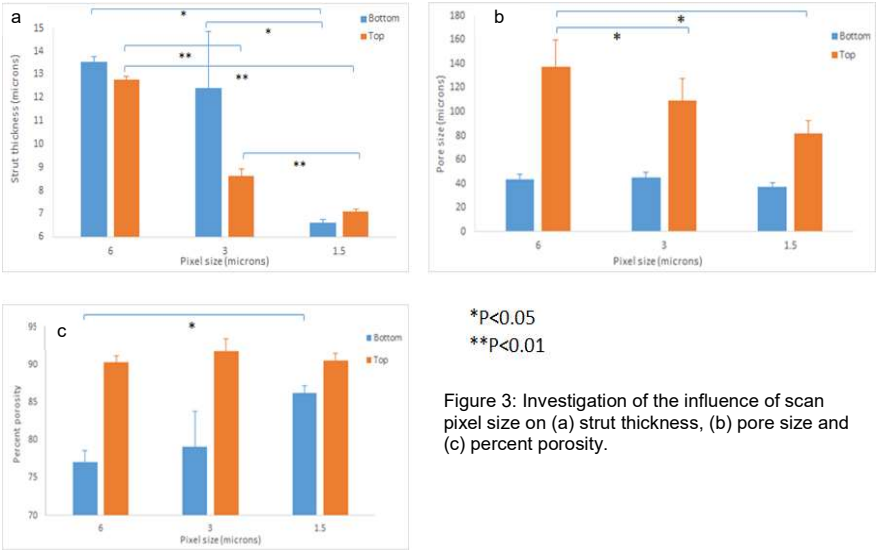


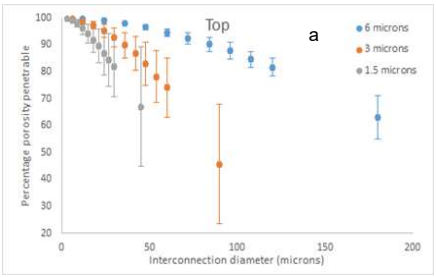
Figure 2: Composite volume rendered model of scaffold structure and false-coloured pore size distribution. Volume represents a through-thickness VOI taken from a scan at 1.5 μm resolution.

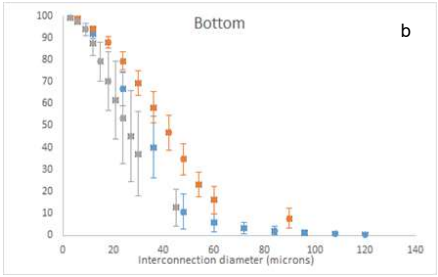
Porosity analysis demonstrated statistically significant variation between pore size and percentage porosity in top and bottom layers of the scaffold (Figure 3). Perhaps more importantly in the instances highlighted, scan pixel size was also found to have a significant effect particularly in the case of pore size. With a decrease in pixel size from 6 to 1.5 μm, mean pore size decreased from approximately 130 μm to around 85 μm. Investigation of pixel size influence was further extended to a consideration of interconnectivity (Figure 4) and percolation analysis. Calculations of interconnectivity demonstrated decreased accessibility with increased voxel size, with the influence on percolation analysis being less straightforward. Whilst percolation theory applied well in the low resolution scans, at smaller pixel size the relationship given in (equation 2) appeared non-linear. It is hypothesized that the increased features resolved in higher resolution scans resulted in a structure more complex than can be described by current percolation theory.

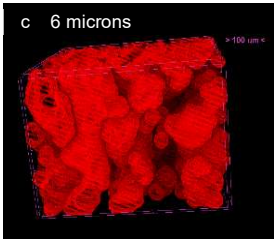


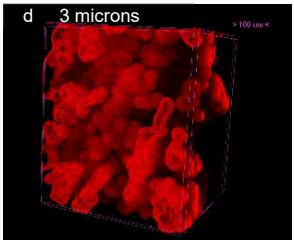
*P<0.05
**P<0.01

Figure 3: Investigation of the influence of scan pixel size on (a) strut thickness, (b) pore size and (c) percent porosity.









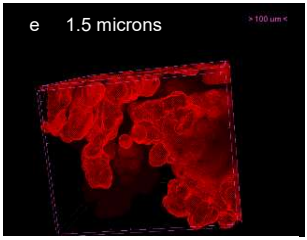


Figure 4: Influence of scan pixel size on interconnectivity in top (a) and bottom (b) regions of the scaffold; (c) - (e) volume rendered models of accessible space for a sphere of 60 μm diameter with 6 μm, 3 μm and 1.5 μm pixel size.

Preliminary scanning of hydrated scaffold with phosphotungstic acid contrast agent appeared to show significant promise as demonstrated by the volume rendered representation in Figure 5. Noise was significantly greater in the case of hydrated imaging and thresholding may thus require some optimization prior to thorough quantitative analysis. Gross scaffold structure did however appear to be largely unaffected by hydration although strut thickening was significant.

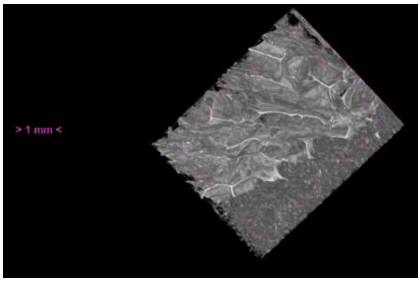


Figure 5: Volume rendered representation of hydrated scaffold structure. Preliminary quantitative analysis suggests significant strut swelling.

	Bottom	Top
Percent porosity	69.1 (4.9)	85.6 (1.7)
strut thickness (microns)	15.81 (0.3)	18.8 (0.5)
pore size (microns)	32.25 (8.0)	137.1 (15.7)

Conclusion

- Micro-CT is a valuable tool for the analysis of the pore structure of complex scaffolds, a wealth of both qualitative and quantitative information can be obtained
- It is important to note that scan pixel size has a significant influence on porosity data and this should be taken into account with any comparatitive analysis.
- An aqueous suspension of phosphotungstic acid appears to hold significant promise as a means for imaging hydrated collagen scaffolds and thus getting a better impression of the environment experienced by cells *in vivo*.

A complete understanding of pore structure is the first step in the optimization of complex scaffold structures for cell sieving applications.

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