CHARACTERIZATION OF 3D ALGINATE SCAFFOLDS USING MICRO-CT

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INTRODUCTION

In tissue engineering, scaffolds are usually three-dimensional (3D) structures made from biomaterials that can support cell growth and conduct nutrients such as growth factors so as to produce artificial organ and tissue substitutes. Alginate, naturally derived polysaccharide that is primarily derived from seaweed, is an ideal biomaterial for tissue engineering due to its properties that are biocompatible and biodegradable. Scaffolds made of alginate crosslinked with Calcium Chloride ($CaCl_2$) can support cells and provide pores for them to penetrate through.

Several requirements have been identified as crucial for the production of tissue scaffolds. These include that the scaffold: 1) be highly porous to allow cell growth and movement as well as the transport of nutrients and metabolic waste; 2) be biocompatible and biodegradable to match cell/tissue growth in vitro and/or in vivo; 3) have suitable surface chemistry for cell attachment, proliferation, and differentiation; and 4) have mechanical properties matching those of tissues at the site of implantation. Thus morphology and micro-structure of scaffolds, such as pore size, porosity, interconnectivity and wall thickness, are of crucial importance.

The most commonly used technique to characterize scaffold microstructure is Scanning Electron Microscopy (SEM). This method gives high resolution two-dimensional (2D) images of the surface of specimens down to the nanometer scale. However, in order to examine the interior features of the scaffolds, physical sectioning is required, which introduces compression and edge effects to the scaffold architecture. Samples are also required to be dry and conductive with heavy metal coating before being mounted onto sample stage. Micro-CT is an increasingly common technique applied in biomedical research, particularly in hard tissue imaging applications such as characterization of trabecular architecture in bone. In tissue engineering, it has been used to visualize the scaffold microstructure, especially in bone tissue engineering with scaffolds of high X-ray absorption contrast. However, little work has been reported on the scaffolds made from the biomaterials with absorption contrast close to that of soft tissues, such as alginate scaffolds, which have been widely used in soft tissue engineering. To the best of our knowledge, micro-CT analysis of alginate scaffolds has not yet been reported.

MATERIALS AND METHODS

Alginate Scaffolds: The scaffolds were made of sodium alginate (Sigma, USA) of different concentrations (1%, 2%, 4%, 7% w/v). Alginate was then crossed-linked with 1% calcium chloride ($CaCl_2$) at the volume ratio of 2:1. The freeze drier was a Labconco Freezone benchtop machine. Three freezing temperatures were set: -80, -50 and -20 degrees. Our original intent was to scan 10 replicates of each treatment but scan and processing time (see below) was prohibitive. Thus, one specimen from each treatment, 10 cylinder-shaped Volumes of Interest (VOI) of the same size (4000 microns in diameter, 1500 microns in height) were selected for analysis from each dataset.

X-ray Imaging: The scaffolds were scanned using a cone-beam high-resolution micro-CT Skyscan 1172 scanner (Skyscan, Belgium). The x-ray source was set at 60kV of x-ray accelerating voltage and 172A of current. No filter was used to allow more X-rays to penetrate through and ensure higher contrast. Pixel size was 3.5m and exposure time is 350ms. Approximately 2200 slices were acquired over a rotation range of 180 with a step angle of 0.2. Small camera pixel (4000*2400) was used for high resolution. Each scan took approximately 1 hour and 10 minutes. Data sets were reconstructed using standardized cone-beam reconstruction software (NRecon v1.6.1.0, SkyScan). This took approximately eight hours on a cluster of four dual-processor quad-core computers.

3D Analysis and Rendering: 3D analysis of scanning results was carried out in CTAn (Skyscan). A standardized global threshold (22 to 255 gray levels) was used to binarize the images into struts and background air. Despeckling was applied then to all of the VOIs to remove noise and undesired information. Structural parameters (pore size, porosity, wall thickness and closed porosity) were calculated in 3D from the binarized images. An automated batch analysis procedure was run on a PC.

Scanning Electron Microscopy (SEM): Philips 505 Scanning Electron Microscopy (SEM) at an operating voltage of 29.4kV was used for two-dimensional scaffold imaging. Freeze-dried specimens were sectioned and sputtered gold coating before analysis.

RESULTS

Alginate Concentration Effects on Scaffolds

a. Qualitative Observations

We firstly investigated the effects of sodium alginate concentrations (7%, 4%, 2% and 1% w/v) on microstructure of scaffolds, when keeping the freezing temperature the same (-80 degree). Micro-CT cross-sectional images (Figure 1a, c, e, g) and 3D volume rendering results (Figure 1b, d, f, h) provide a qualitative appreciation of scaffold structure. Pore size and porosity becomes smaller with higher alginate concentration. Also, at lower concentration, an isotropic cellular structure is more obvious, while at higher concentration, an anisotropic lamellar plate-like structure predominates. The 3D models in Figure 1 show one of the VOIs from the corresponding samples. The 2D Micro-CT cross-sectional images are the first layer of from the 3D models.

SEM images of sectioned surface of alginate scaffolds provide similar results (Figure 2). Pore size decreased with higher alginate concentrations. Also, with higher alginate concentrations, such as 4% and 7%, preferentially oriented structure indicating the ice growth direction in horizontal cross-section can be observed. On the other hand, with lower alginate concentrations, such as 1% and 2%, isotropic structure is shown. These features are in accordance with those observed from Figure 1.

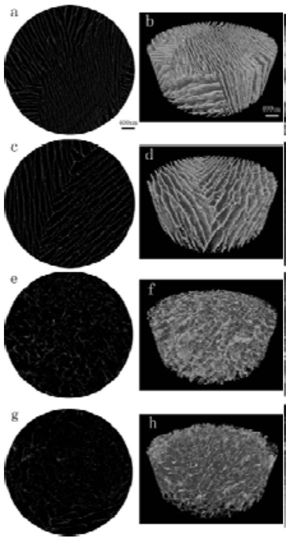

Figure 1: Micro-CT cross-sectional images of 7% (a), 4% (c), 2% (e) and 1% (g) alginate solutions. Figure 1(b), (d), (f) and (h) show the three-dimensional rendering of the corresponding 7%, 4%, 2% and 1% alginate scaffolds.

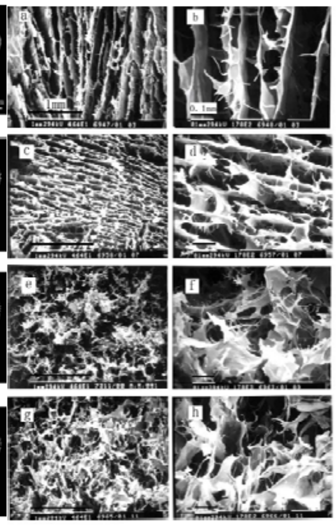

Figure 2: SEM of alginate scaffolds at 7% (a), 4%(c), 2%(e) and 1%(g) of solution concentration (*46.4). Figure 2(b), (d), (f) and (h) are corresponding magnified images (*178).

b. 3D Quantitative Analysis of Porosity and Pore Size

From 3D analysis results of micro-CT data using CTAn, it can be seen that under same freezing temperature, lower alginate concentration tends to have higher porosity (Figure 3). In statistics, P-value is 0.000, which means the relationship between porosity and alginate concentration is statistically significant. Moreover, the change of porosity as a function of alginate concentration is linear. On the other hand, with higher alginate concentrations at same freezing temperature, smaller pore size was observed ($p=0.00$) (Figure 4). The 3D quantitative analysis results are a strong support of what is observed qualitatively above.

Freezing temperature effects on scaffolds

a. Qualitative Observations

When keeping alginate concentration unchanged (7% w/v), we evaluated the effects of changing of freezing temperature on scaffolds. Micro-CT cross-sectional images and 3D volume rendering results of alginate scaffolds frozen to -80, -50 and -20 degrees are shown in Figure 5. Pore size gets smaller with lower freezing temperature and also plate-like structures are more obvious.

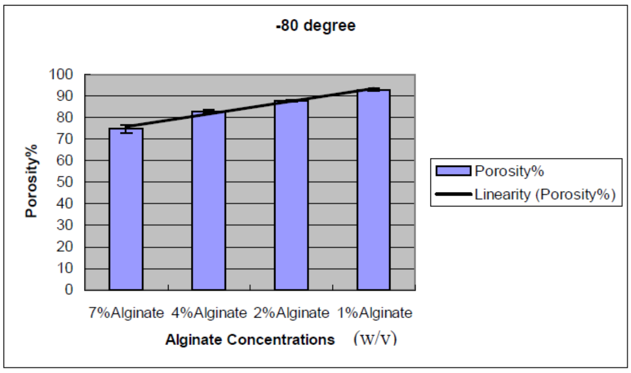

Figure 3: Changes of porosity with different alginate concentrations (1%, 2%, 4%, 7%) at -80 degree. The approximate line shows the linearity of porosity as a function of alginate concentration.

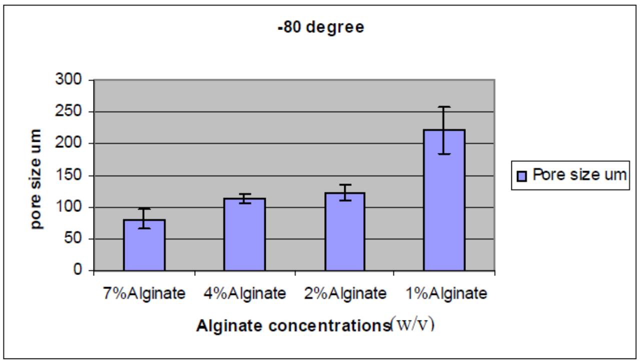

Figure 4: Changes of pore size with different alginate concentrations (1%, 2%, 4%, 7%) at -80 degree.

SEM images (Figure 6) give geometrical observations of the scaffolds. Figure 6 indicates that with higher freezing temperature, isotropic structure is more obvious, which is another support to what is observed in Figure 5.

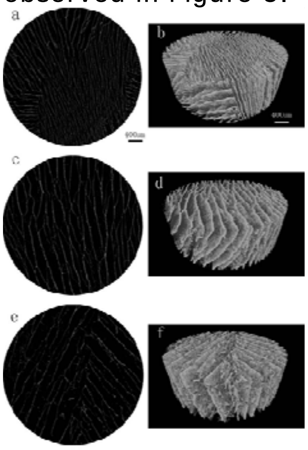

Figure 5: Micro-CT cross-sectional images of scaffolds made with 7% alginate solution and frozen to -80 (a), -50 (c) and -20 (e) degrees. Figure 5(b), (d) and (f) are 3D volume rendering of alginate scaffolds frozen to -80, -50 and -20 degrees respectively.

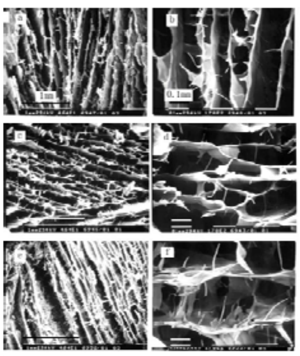

Figure 6: SEM of alginate scaffolds at -80 (a), -50 (c) and -20 (e) of freezing temperature (* 46.4). Figure 6(b), (d) and (f) are corresponding higher magnification images (*178).

b. 3D Quantitative Analysis of Porosity and Pore Size

From 3D analysis results of Micro-CT data, it can be concluded that at same alginate concentration, lower freezing temperature resulted in smaller pore size, as shown in (p=0.000) Figure 7. As to the porosity, lower freezing temperature tends to result in smaller porosity (p=0.000) (Figure 8). These quantitative results are in accordance with morphological observations given from micro-CT and SEM above.

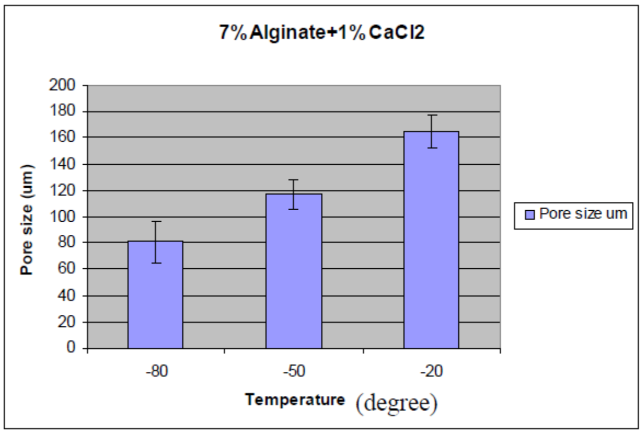

Figure 7: Changes of pore size with different freezing temperature of 7% alginate concentration.

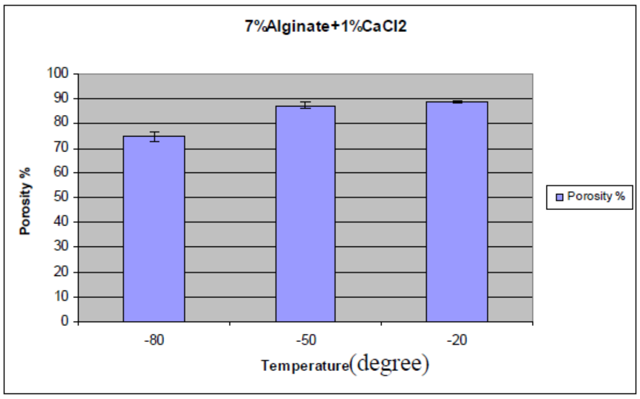

Figure 8: Changes of different porosity of scaffolds made with 7% alginate solution.

Interconnectivity and Wall thickness

Table 1 demonstrates that there is no closed porosity of the alginate scaffolds regardless of alginate concentration or freezing temperature. In other words, the scaffolds made with alginate have excellent interconnectivity.

Also for wall thickness, no significant differences are found related to alginate concentrations or freezing temperatures. (Table 1)

Table 1: Changes of closed porosity and wall thickness of alginate scaffolds.

	Closed Porosity %:	Strut Thickness um:
7% Alginate+1% CaCl2 -80	0.004176±5.14E-06	26.57±4.26
4% Alginate+1% CaCl2 -80	0.002237±7.4E-06	24.60±0.66
2% Alginate+1% CaCl2 -80	0.001024±3.35E-07	26.09±2.65
1% Alginate+1% CaCl2 -80	0.001158±1.19E-07	25.41±1.02
7% Alginate+1% CaCl2 -50	0.000806±6.44E-08	21.00±0.65
7% Alginate+1% CaCl2 -20	0.000838±1.06E-06	24.91±0.43

From linear regression test (SPSS V.17.0), p-value for two observations, different freezing temperatures (-80, -50, -20) under same alginate concentration (7%) and wall thickness, is 0.174, larger than 0.05. Therefore, freezing temperature has no statistically significant relationship with wall thickness. P-value for different alginate concentrations (7%, 4%, 2%, 1%) under same freezing temperature (-80) and wall thickness is 0.259. So alginate concentrations have no statistically significant relationship with thickness. That is to say, wall thickness is independent of alginate concentrations and freezing temperatures.

DISCUSSION

Hydroxyapatite (HA), an osteophilic ceramic related to the inorganic component of bone, is a good substitute material in bone tissue engineering. It has good X-ray absorption contrast and is easy for micro-CT imaging. Turco et. al. [1] have done a micro-CT analysis of alginate/hydroxyapatite scaffolds in bone tissue engineering. However, in nerve tissue engineering, biomaterials used to build scaffolds are required to have excellent biodegradability. Alginate is ideal for nerve tissue engineering but presents a big challenge for micro-CT imaging as it has poor x-ray absorption contrast. Our current results suggest that micro-CT does have the potential to characterize low-contrast biomaterials with X-ray imaging.

The microstructure of scaffolds, such as pore size and porosity, is of great importance, as it affects not only cell survival, signaling, growth, propagation and reorganization, but also plays a major role in influencing cell shape modeling. Scaffolds must possess an open-pore (interconnected) geometry with a highly porous surface and microstructure that allows cell in-growth and reorganization in vitro and provides the necessary space for neovascularization from surrounding tissues in vivo. The optimal porosities are greater than 90%. The required pore sizes vary from one cell type to another. For example, pore sizes ranging from 200 to 400 um are optimal for bone tissue engineering, 20 to 125 um for adult mammalian skin and 45-150 um for regenerating liver tissues. In this study, we have the pore size of alginate scaffold ranging from 80 to 220 um and porosity from 75% to 93%. These data show that alginate scaffolds made with freeze-drying technique are suitable for cell in-growth.

In the future, Schwann cell culture can be carried out inside the cylinder-shaped alginate scaffolds. By also using micro-CT technique, cell adhesion and proliferation can be measured in the scaffolds after cultured for several days.

CONCLUSION

This paper reports a novel study on the use of micro-CT imaging to characterize, both qualitatively and quantitatively, low-contrast alginate scaffolds. The scaffold microstructure was characterized in terms of pore size, porosity, interconnectivity and wall thickness and then related to the fabrication process parameters including the freezing temperature and alginate concentration. The results obtained suggest that the micro-CT technique, as a non-destructive method, is promising to be used in the characterization of microstructure of the scaffolds in soft tissue engineering. Also, the results show that lower freezing temperature and higher alginate concentration lead to smaller pore size, while higher freezing temperature and lower alginate concentration result in higher porosity.

Acknowledgements

The present study was supported by the Saskatchewan Health Research Foundation (SHRF) and the Natural Science and Engineering Research Council (NSERC) of Canada.

References

[1] Gianluca Turco et. al., “Alginate/Hydroxyapatite Biocomposite For Bone Ingrowth: A trabecular Structure With High And Isotropic Connectivity.” Biomacromolecules 2009, 10, 1575-1583.