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Fabrication of interpenetrate Chitosan: bioactive glass, using dense gas CO_2

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Abstract-**Success of bone tissue engineering relies on using bioactive scaffolds with ideal pore morphology which can mimic the properties of the natural extracellular matrix (ECM). The objective of this study was to interpenetrate bioactive glass components throughout the three dimensional (3D) structure of the chitosan scaffold to increase the average pore size of the scaffold and also the osteoconductivity and osteoinductivity of the fabricated scaffold. Scanning electron microscopy was used to observe the microsturcture of the hydrogel. The results of this study demonstrated that the average pore size in the hydrogel was increased significantly ($p<0.05$) from 97±44µm to 150±24µm by increasing the BG concentration from 0 wt% to 40 wt%. This effect might be due to the interaction between ceramic and chitosan. The composite hydrogel fabricated swell in water and has high potential to be used for bone tissue engineering applications; bioactive glass can substantially improve bioactivity of the bone tissue engineering scaffolds However, further studies are required to investigate the effect of BG on the biocompatibility of the scaffolds. In addition, *in vitro* cell studies are also required to confirm the suitability of the fabricated scaffold for bone tissue engineering.**

I. Introduction

In tissue engineering scaffolds are required as a three dimensional (3D) templates for cellular adhesion, proliferation and growth [1, 2]. Suitable mechanical properties, high degree of biocompatibility and bioactivity along with highly porous structures are the essential characteristics of scaffold for tissue engineering applications [3]. Extracellular matrix (ECM) proteins such as collagen and elastin and polysaccharides, e.g. chitosan interacts with cells via cell surface receptors and regulate or direct cell function [4].

Different techniques have been employed to create porosity throughout in scaffold including; freeze drying [5, 6], solvent casting/ salt leaching [7, 8], electrospining [9, 10], conventional gas foaming [11, 12], and supercritical/ dense gas foaming [13-16].

Carbon dioxide is the most common fluid used in gas foaming process to fabricate porosity in the polymeric matrices, due to its unique properties such as inflammablity, inertness, moderate critical temperature and pressure, In a gas foaming process, the polymer is saturated with a dense gas, a fluid at above or close to its critical temperature and pressure; the system is then depressurized and pores create in the polymer phase due to super-saturation, nucleation and growth of the gas phase [17]. Dense gas CO_2 has been successfully used for the porosity creation in hydrophobic polymers, e.g. poly (ε-caprolactone) [7, 18, 19], poly (ethylene glycol) [20]. In hydrophilic polymers, however, the application of CO_2 as the foaming agent is limited by the low solubility of CO_2 in hydrophobic polymers, leads to the creation of small pores within the polymeric structure. Ji *et al.* created pores with the average pore diameter of 30-40 µm within the 3D structure of chitosan hydrogel, using dense gas CO_2. To increase the cellular proliferation and growth within the 3D structure of the scaffold, pores with the average pore size of 100 µm is required [21].

In addition to the pore size and the mechanical properties of the scaffolds, for bone tissue engineering, osteoconductivity and osteoinductivity of the scaffolds are important [4, 22, 23]. Phosphate base glasses [24], bioglasses® [25] bioactive glasses and glass ceramics [26] have been developed that are highly bioactive for bone tissue engineering applications The composites of polymers and bioactive components are used to promote polymers bioactivity [23, 27, 28].

The aim of this study was to increase the osteoconductivity and osteoinductivity of chitosan by phosphate glass. Carbon dioxide at high pressure was used as a foaming agent to create porosity within the structure of composites. The effects of process parameter and phosphate glass composition on pore size were assessed.

A. Material and Method

Chitosan (medium molecular weight), and Genipin (GP), were purchased from Sigma. Tris((HOCH2)3CNH2) was purchased from Merck. A 0.2 M acetic acid solution was prepared using glacial acetic acid (Ajax Fine Chem) in milliQ water. Phosphate-buffered saline (PBS) was prepared by dissolving PBS tablets (Sigma) in milliQ water. Food-grade CO2 (99.99% purity) was supplied by BOC. Tetraethyl orthosilicate (TEOS) nitric acid, triethyl phosphate (TEP), calcium nitrate

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tetrahydrate were supplied by Merck.

Bioactive glasses (BG) were fabricated, using the procedure described elsewhere [29]. Briefly, Glass materials, SiO_2–P_2O_5–CaO (molar 64% SiO_2, 31% CaO, and 5% P_2O_5), were synthesized by the sol–gel method and characterized by X-ray diffraction (XRD).

1) BG synthesis

Initially, 13.33 g (0.064 mol) of tetraethyl orthosilicate was added into 30 ml of 0.1 M nitric acid for 30 minutes to achieve the acid hydrolysis of TEOS. Afterwards, 0.91 g (0.005 mol) of TEP, and 7.32 g (0.031 mol) of calcium nitrate tetrahydrate were added to the solution with 1 hour interval between each addition. The final solution was stirred for 1 hour after the addition of calcium nitrate tetrahydrate. The solution was cast in a sealed cylindrical Teflon container and aged for 10 days at room temperature. Afterwards, the gel was kept in a sealed container and heated at 70 °C for 3 days to remove the residues of water. The dried gel was then heated for 24 h at 700 °C to stabilize the formed glass and also to remove the nitrate residues.

2) Composite scaffold fabrication

Chitosan was dissolved in 0.2M acetic acid (1.5 % wt/v) and stirred for 24 hours to achieve a homogenous solution. Specific amount of BG particles were added to the chitosan solution every six hours, till achieve the final concentration of BG:chitosan (0-80 wt%). The final solution was stirred for 12 hours and sonicated for 15 minutes to achieve a uniform solution. GP solution (0.5 wt/v %) was subsequently added to the chitosan/BG and mixed for 1 minute. Subsequently, the final solution was moved into the high pressure vessel. The crosslinking procedure of chitosan with GP under high pressure was previously described [30]. Briefly, the final solution (GP and chitosan: BG) was placed in vessel and pressurized with dense gas CO_2 to the pressure of 60 bar at 37°C for three hours and depressurized at the rate of 12 bar/min. The fabricated scaffolds were then PBS washed and Tris treated to inhibit further crosslinking. After this, the scaffolds were washed with PBS for three times, to remove the Tris residuals, and stored in PBS for further usage.

3) Scanning Electron Microscopy

The SEM images of samples were obtained using a Zeiss Qemscan at 15 KV to determine the pore characteristics of the fabricated PCL scaffold and to examine the penetration of elastin into the 3D structures of PCL. Lyophilized constructs were mounted on Aluminum stubs using conductive carbon paint then gold coated prior to SEM analysis. Image J software was used as a numerical tool to determine the morphology of the fabricated samples by evaluating average pore diameter of samples. 100 pores for each group of samples were analyzed. The average pore diameter was calculated by the Image J software by considering the hypotheses of spherical shape and the factor of $4/\pi$, according to the ASTM D 3576.

4) Degradation Behavior of the composite

The composite scaffolds were place in SBF and collected after 4, 6, and 8 days. The samples were then weighted and the degradation ratio of the sample was calculated using Equation 1.

$$DR = \frac{W_i - W_a}{W_i} \quad (1)$$

Where, W_i is the weight of the scaffold after 24 hours of submerging in PBS, W_a is the weight of the scaffold after soaking in SBF for the defined period of time (4, 6, and 8) and DR is the degradation ratio.

5) Statistical Analysis

Data is reported as mean ± STD. One way analysis of variance (ANOVA) for single comparisons and Bonferroni post hoc tests for multiple comparisons were performed, using SPSS software for Windows, version 18.0.1. Statistical significance was accepted at $p < 0.05$ and indicated in the figures as *($p<0.05$), ** ($p<0.01$) and ***($p<0.001$).

II. Results and Discussion

The aim of this study was to fabricate composite scaffold of PLDL and BG, suitable for tissue engineering applications, using dense gas CO_2. In this study, chitosan and BG were mixed, GP used as the crosslink agent. The effects of BG concentration on the degradation behavior of the composite, morphology and the swelling behavior of the scaffold were investigated. The concentration of the BG was varied in the range of 0-80 % (wt:wt chitosan).

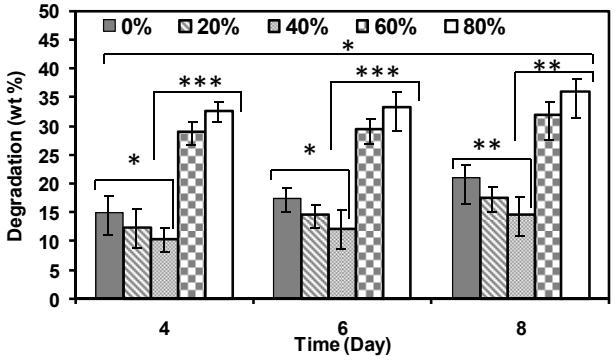

Figure 1. Degradation ratio of the scaffold fabricated with different BG concentration (*=p<0.05, **=p<0.01, ***=p<0.001).

The degradation rate of the scaffolds was decreased from 14.9 wt% to 11.2 wt%, after four days of soaking in SBF, by increasing the BG concentration from 0 wt% to 40 wt%. However, further increase of the BG concentration to 60 wt% and 80 wt% increased the degradation rate of the scaffold threefold. This indicated that the structure of the composite fabricated with high concentration of BG was unstable and excessive addition of BG might inhabit the cross linking of the

chitosan. Therefore, for further analysis, the BG concentration was varied in the range of 0-40 wt%.

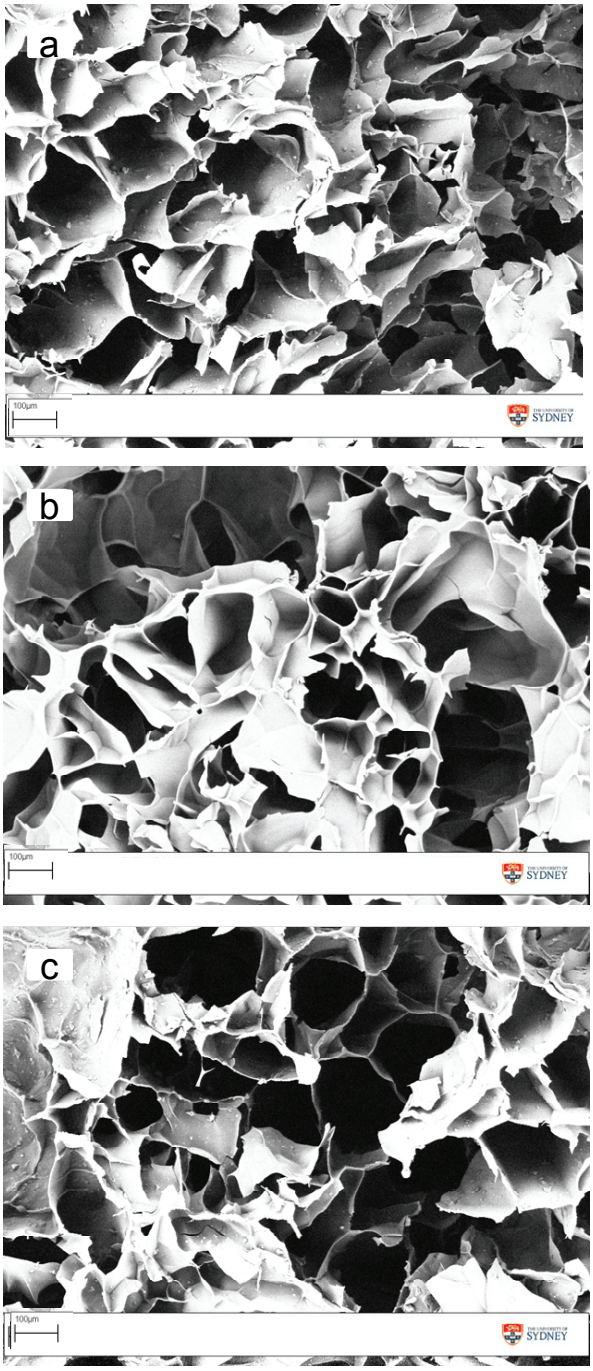

Figure 2. SEM micrographs of the composite scaffolds of chitosan and BG, fabricated with 0 wt% (a), 20 wt% (b) and 40 wt% (c) BG.

SEM micrographs of the scaffolds are shown in Figure2. By increasing the BG concentration, pore size in the scaffolds also increased.

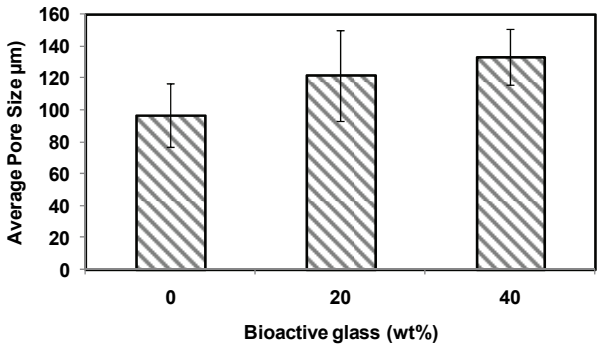

Figure 3. Average pore diameter of the composite scaffolds fabricated with different BG ($P<0.05$)

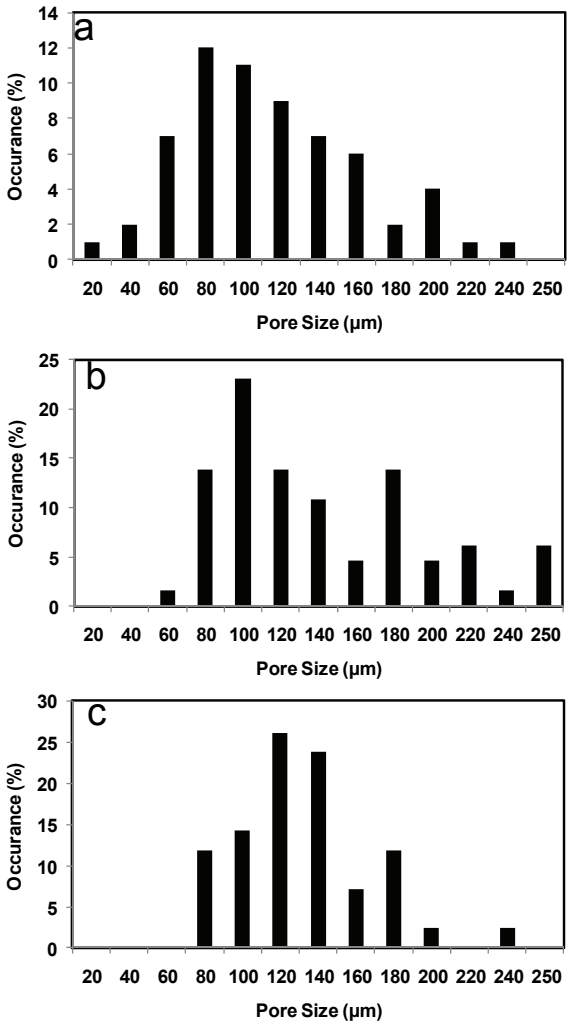

Figure 4. Pore size distribution of the chitosan/ BG scaffolds fabricated with 0 wt% (a), 20 wt% (b), and 40 wt % (c) BG.

The average pore size of the scaffolds fabricated was increased from 97±44µm to 150±24µm by increasing the concentration of BG from 0 wt% to 40wt%, shown in Figure

3. Ji *et al* [30] reported the average pore size of 30-40 μm for the chitosan hydrogel fabricated under high pressure CO_2 using GP as the cross linking agent. The significant difference between the measured value for the average pore size of the neat chitosan scaffold and of that reported in the literature, might be due to the different drying process, used in these two studies.

The pore size distributions for the scaffolds fabricated with different BG concentration are shown in Figure 4. Wide range of pores sizes for all the scaffolds fabricated with different BG concentration indicated that pores in all the scaffolds were interconnected and seeded cells would be proliferated throughout the 3D structure of the scaffold.

III. Conclusion

Dense gas CO_2 was successfully used in this study to create porosity within the 3D structure of the chitosan scaffold. The degradation rate of the scaffolds was manipulated, by changing the concentration of the BG in the interpenetrated network of the chitosan/ BG hydrogel. Moreover, by addition of BG, the average pore size of the fabricated scaffolds significantly ($p<0.05$)was increased. Due to the high average pore and also the interpenetration of BG throughout the 3D structure of the composite scaffold, it can be concluded that fabricated scaffold might be a suitable candidate for bone tissue engineering. However, further cell culturing studies are required to confirm this hypothesis.

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