Synthesis of Porous Carbonate Apatite/Gelatin Scaffolds Via Freeze Drying Method

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\textbf{Abstract.} Gelatin is widely used in medical field as scaffolding material. However, in order to encourage bone formation, carbonate apatite (CO$_3$Ap) which resembles the human bone composition is added into the gelatin scaffold. In this research, CO$_3$Ap/gelatin scaffold were fabricated via freeze drying method. CO$_3$Ap powder was obtained from precipitation reaction of calcium hydroxide and phosphoric acid accompanied by carbon dioxide gas as the carbonation source. The obtained powder were then analysed by X-ray diffraction (XRD), Fourier transformed infrared (FTIR) spectroscopy and carbon hydrogen nitrogen (CHN) test. The carbonate content by weight percentage is 12.8 wt. %. The mean particle size of the CO$_3$Ap powder was 7.76 \(\mu\)m. To fabricate the CO$_3$Ap/gelatin scaffold, the CO$_3$Ap powder were mixed with gelatin at various CO$_3$Ap/gelatin ratio (15/5, 10/10) wt. % followed by casting and pre-freezing temperature of -10 °C, -20 °C and -40 °C before freeze dried. The highest compressive strength achieved was 7412.36 kPa for CO$_3$Ap/gelatin with ratio (10/10) wt. %, freeze at -40 °C as the pre-freezing temperature. Field emission scanning electron microscope (FESEM) analysis shows that the CO$_3$Ap powder was distributed within the gelatin scaffold. As a conclusion, it was found that higher gelatin content and lower pre-freezing temperature increases the compressive strength of the CO$_3$Ap/gelatin scaffold.

1. Introduction

Tissue engineering field appears to be a promising approach for biological implants and prosthesis [1]. Generally, bone consists of collagen for the organic phase of bone and the inorganic phase are hydroxyapatite [2], while in some cases such as dental enamel; carbonate apatite became the main component for hard tissue [3]. Despite this, carbonate apatite is easier to dissolve compared to hydroxyapatite. The solubility will be increasing as the carbonate content increases [4] under acidic condition such as in the Howship’s lacunae of osteoclast [5], plus with its bioresorbability characteristic [6-8] and it is believed to be an ideal bone replacement.

Although collagen is naturally found in the human bone, gelatin is used as the main component in this research due to its properties. As gelatin is readily assimilated by the body with it properties such as excellent biocompatibility, ease of processing and available at a low cost [9] however, pure gelatin scaffold are not satisfactory for hard tissue application as reported by J. Sundaram et al., [10]. Therefore, a composite of gelatin and carbonate apatite are expected to be the ideal scaffold that possesses sufficient mechanical strength with good osteoconductivity and biodegradation [11].
2. Experimental study

2.1 Materials

The materials that were used in this research are calcium hydroxide (Ca(OH)$_2$); 98% purity, Fluka, UK and phosphoric acid (H$_3$PO$_4$); 15M, Merck, Germany and carbon dioxide (CO$_2$); supply by Goldengas Industrial Supply, Malaysia while the raw material used in the fabrication of the scaffold were gelatin (food grade; supplied by Halagel), polyvinyl alcohol (PVA); Sigma Aldrich, molecular weight average of 14 000 and glutaraldehyde (GA); Merck, Germany.

2.2 Method

2.2.1 Synthesis of carbonate apatite (CO$_3$Ap) powder. Precipitation method was used in the preparation of carbonate apatite powder. The process started with the reaction of Ca(OH)$_2$ and H$_3$PO$_4$ alongside carbon dioxide gas as the carbonate source that flow into the reactor with 1 l/min. Initially, a solution of 125 ml of 2 M Ca(OH)$_2$ is added into the reactor that were continuously stirred at 400 rpm with 40 ±1 °C, then 75 ml of 2 M of H$_3$PO$_4$ solution is added slowly into the reactor as carbon dioxide gas is flowing through the reactor [12].

2.2.2 Characterization of carbonate apatite powder. The obtain powder were characterized using X-ray diffractometer (XRD) for phase identification and Fourier transform infrared spectroscopy (FTIR) for identification of the functional group presents. Elemental analyser; Carbon, Nitrogen, Oxygen (CHN) test was used to identify the carbonate content of the obtained powder, while the particle size of the powder were determined by Helos particle size analyser.

2.2.3 Preparation of porous carbonate apatite/gelatin scaffold via freeze drying method. Porous CO$_3$Ap/gelatin scaffold was fabricated by freeze drying method. Initially, 5 wt. % of gelatin were dissolved in 77.75 wt. % distilled water at the temperature of 60 °C. The gelatin solution is then stirred using mechanical stirrer at 300 rpm. Next, 15 wt. % of CO$_3$Ap powder and 2 wt. % of PVA is added slowly into the gelatin solution (to avoid bubble and coagulation) until homogenous mixture is form. Next, the mixture was put in the vacuum desiccator for 2 minutes to reduce the formation of air bubble. Then, the mixture is stirred again as 0.25 wt. % of GA is added. The mixture is then quickly poured into mold and allowed to freeze for 24 hours at different pre-freezing temperature; -10 °C, -20 °C and -40 °C. The solid specimen is then placed in freeze dryer for 24 hours. Similar steps are then repeated for the ratio (10/10) wt. % of CO$_3$Ap/gelatin. The microstructure of the specimen was observed using field emission scanning electron microscope (FESEM SUPRA 35VP ZEISS). The compressive strength of the scaffolds was measured using an Instron 3369 according to ASTM-F-451-95 standard at testing speed of 2 mm/min.

3. Results and discussion

3.1 Characterization of the synthesized CO$_3$Ap powder

Figure 1 shows the XRD pattern of hydroxyapatite (HA) as reference pattern and synthesized CO$_3$Ap powder. From these figure it can be observed that the peaks of CO$_3$Ap powder matches to the peaks of HA. However, the intensity of the peaks in CO$_3$Ap pattern are broader indicating low crystallinity apatite was obtained by precipitation method.

Carbonate content (wt. %) of the CO$_3$Ap powder calculated from the CHN analysis was determined to be about 12.8 wt. %. While, the mean particle size of the CO$_3$Ap powder was 7.76 μm; the particle size was measured by Helos particle size analyser.
Figure 1. XRD pattern of the synthesized CO$_3$Ap powder and HA pattern as reference

Figure 2 illustrate the FTIR spectra of the obtained apatite powder proving that obtained powder is type-B carbonate apatite powder similar to the type of carbonate apatite that found in human bone. From this figure it can be observed an intense band at 1308.98 cm$^{-1}$, 1035.05 cm$^{-1}$, 602.95 - 565.03 cm$^{-1}$ similarly as reported by Mohamed K. R. et al. which corresponds to the PO$_4$ functional groups [13].

Absorption band assigned to C-O in CO$_3^{2-}$ group were detected at 1636.96 - 1584.9 cm$^{-1}$, 1427.98 cm$^{-1}$ and 874.81 cm$^{-1}$. This result is parallel with previous report by dos Santos et al., where stretching vibration band observed at 876 cm$^{-1}$ and 1470 cm$^{-1}$ [14]. In addition to this, Daitou, F. et al., also stated that carbonate ions can be identified at wave number of 1450 cm$^{-1}$, 1414 cm$^{-1}$ and 874 cm$^{-1}$ [15].

There are two types of carbonate ions exists in CO$_3$Ap structure [16]; at wavelength 1650 cm$^{-1}$ to 1300 cm$^{-1}$ are assigned to surface carbonate ions whereby the carbonate ions exist in the lattice of phosphate ions while the peak at 873 cm$^{-1}$ is responsible for complete replacement the OH$^-$ ions at the interface of growing crystal, as the hydroxyl ions are present at 3428.14 cm$^{-1}$.
3.2 Morphological analysis of porous CO$_3$Ap/gelatin scaffold

The morphology of the composite scaffold was examined by FESEM. The effect of CO$_3$Ap/gelatin ratio and pre-freezing temperature on the morphology of the CO$_3$Ap/gelatin scaffold are shown in Table 1. It can be observed that pores are presence throughout the specimens with different arrangement and size regardless of ratio and pre-freezing temperature. A good interconnectivity between the pores is achieved along with homogenous mixture between the gelatin and carbonate apatite with no coagulation present.

Having different pre-freezing temperature, i.e. -10 °C, -20 °C, -40 °C produced different pore size. For ratio of (15/5) wt. % the size obtained is 100-150 μm, 45-55 μm, and 200-400 μm. While for the ratio (10/10) wt. %, the size obtained is 80-270 μm, 125-150 μm, and 75-200 μm. These data correspond to the theory of pore size. As lower temperature is used, the smaller the pore size that will produced [17] but for the temperature -40 °C for the ratio (15/5) wt. % the pores appear to be largest among all this is due to the formation air bubble that trapped in the scaffold during casting process.

Table 1. Morphology of porous CO$_3$Ap/gelatin with ratio of gelation to CO$_3$Ap powder (15/5) and (10/10) at pre-freezing temperature of -10 °C, -20 °C, and -40 °C

| Ratio of carbonate apatite powder to gelatin (wt. %) | Temperature (°C) |
|----------------------------------------------------|------------------|
|                                                    | -10              | -20              | -40              |
| 15/5                                               | ![Image]         | ![Image]         | ![Image]         |
| 10/10                                              | ![Image]         | ![Image]         | ![Image]         |

3.3 Compressive strength of porous CO$_3$Ap/gelatin scaffold

Figure 3 represent the maximum compressive strength of the scaffolds with different ratio of carbonate apatite to gelatin and different pre-freezing temperature used. From this figure it can be observed that, the higher the weight percentage of gelatin with lower pre-freezing temperature resulting higher compressive strength; as the highest compressive strength achieved is 7412.36 kPa for the ratio of (10/10) wt. %. Incorporation of ductile polymeric material will enhance the strength of the scaffold [18]. In this case, the stiffness of the carbonate apatite powders incorporated into the gelatin will increase the strength and toughness of the scaffold.

Theoretically, by controlling factors such as the freezing rate, freezing temperature and concentration will affect the porosity, pore size and pore morphology of the scaffolds [19] as freezing temperature is the most important steps in the microstructure formation as the formation of ice particles in the specimen.
will represent the final microstructure of the end product. Rapid cooling will results in small ice crystal and vice versa [20] as in can be seen in the pore formation in the scaffold in Table 1.

Smaller ice crystals formed as lower freezing temperature were used thus increasing the compressive strength. The ratio of (10:10) wt. % with -40 °C as pre-freezing temperature has the highest maximum strength due to more energy can be absorbed and distributed along the scaffold structure before failure occurred. However, for the temperature -40 °C for ratio of (15/5) wt. % the maximum compressive strength decreases dramatically due to the formation of non-uniform large size pores compared to the others.

![Figure 3. Compressive strength of porous CO\textsubscript{3}Ap/gelatin scaffold containing different ratio of carbonate apatite to gelatin with different freezing temperature](image)

### 4. Conclusion

As a conclusion, B-type CO\textsubscript{3}Ap powder was obtained by the precipitation method of calcium hydroxide and phosphoric acid along with carbon dioxide gas as the carbonation source. The incorporation of CO\textsubscript{3}Ap powder in the gelatin scaffold increases the compressive strength of the CO\textsubscript{3}Ap/gelatin composites as the carbonate apatite powder induces stiffness while the gelatin introduced the ductility into the scaffold.

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