Bone Scaffold Based on Biopolymer/Carbonate Apatite by Freeze Drying Method: Synthesis, Characterization, and In Vitro Cytotoxicity

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Abstract. The global need of biomaterial products especially in bone clinical application increases every year. The gold methods like autograft and allograft have some limitations in the application such as the availability of donor sites, antigenicity issues, the high cost, etc. To solve the problems, many researches and activities in the field of biomaterial have been conducted continuously in the past decades to develop the proper synthetic materials for bone substitutes which have properties similar to bone tissue. In this research, the synthesis of biocomposite for bone scaffold application prepared by freeze drying method has been done successfully. The materials used are biopolymer (alginate and chitosan) and bioceramics (carbonate apatite) with certain mixing variations. SEM result showed that the pores obtained by freeze drying method can mimic the pores of actual bone thus they will be able to resemble cells microenvironment, enhance interface interaction, and support cell proliferation. The existence of carbonate apatite on the scaffold’s surface can be observed with particle size of 0.05 – 1 µm and has been dispersed evenly. These results are in good agreement with FT-IR analysis that indicates the presence of PO₄³⁻ functional group on the scaffold at wave numbers 569 and 1041.56 cm⁻¹ and CO₃²⁻ functional group at wave number 1411.89 cm⁻¹. The in vitro biological evaluation of HeLa cells which exposed to extract solution of scaffold (in some variations of concentration) indicated that the scaffold obtained was not cytotoxic to the HeLa cells.

Introduction

Bone loss and defects can be caused by several ways, such as: road and work accident, osteoporosis cases, born defect, etc. The methods which are commonly used in bone clinical aplication are autograft [1-5] and allograft [1,5]. Although both are claimed as the gold standard because of their ability to produce the most excellent cells regeneration, they have some limitations in the application such as the availability of donor sites [1,3-5], antigenicity issues [1], the high cost, etc. To solve the problems, many researches and activities in the field of tissue engineering have been conducted continuously in the past decades to develop the proper synthetic materials for bone substitutes which have properties similar to bone tissue. The synthetic materials are called bone scaffolds.

Bone scaffolds, which are used as a three dimensional temporary supporting media in bone tissue repairment process, should be highly porous [1,2,6,7]. Pores in scaffolds can be obtained by freeze drying, electrospinning [8-10], direct foaming methods, using template, etc. By these methods, the pores obtained will be able to resemble the cells microenvironment thus can enhance interface interaction and support cell proliferation. Scaffolds should also be biocompatible [1,6,7], bioactive [1], and biodegradable [1-2,7]. Biocompatibility is related to respons and reaction of the body due...
to the presence of foreign materials which are attached to or implanted in the body. The materials can be considered to be biocompatible if they do not lead to rejection by the body. On the contrary, they are considered as toxic. The bioactivity is related to the incorporation of new and old tissue such as cell adhesion and cell growth stimulation. Biodegradability is related to materials ability to be degraded due to chemical modification when interacting with biological tissue. Finally, scaffolds must have the appropriate mechanical abilities while supporting the growth of new tissue [6,7].

To obtain the scaffolds that meet those requirements, the constituent materials must be selected properly. Some materials that can be used are natural and synthetic polymers such as collagen [7], alginate [6-11], chitosan [1,2,12,13], gelatin, starch [2], Poly(lactic acid) (PLA) [11], Poly(glycolic acid) (PGA) [11], Poly(caprolactone) (PCL), Poly(hydroxybutyrate-co-valerate) (PHBV), etc. Other materials that can be used are bioceramics such as hydroxyapatite, α-tricalcium phosphate (α-TCP), β-tricalcium phosphate (β-TCP), carbonate apatite [4-5], etc. Alginate is synthesized from brown algae [6,8]. Alginate is biocompatible [7-10], biodegradable [7,8], can be made porous [8], and has the ability to modify the surface to generate the appropriate flexibility to suit its function. Previous studies reported that alginate has been commonly used in drug delivery applications [8,11], encapsulation [6,11], and wound dressing [6,8]. Chitosan is the most second abundant polysaccharide after cellulose which is derived from chitin by deacetylation process and synthesized from the exoskeletons of crustaceans [13]. Chitosan is biocompatible [2,3,12,13], biodegradable [3,13], anti bacterial, non toxic [2], can be made porous, and can be used to increase the strength of polymers. Carbonate apatite is hydroxyapatite which is modified by adding a certain amount of carbonate ions. Carbonate apatite is a bioceramics that has been shown to have high osteoconductivity and can be degraded. The use of carbonate apatite in bone tissue engineering applications is more appropriate than hydroxyapatite because it is more like bone apatite chemically [5] and has the higher rate of resorption. Each of those materials have advantages and disadvantages. To cover the disadvantages and get products with optimum mechanical properties and tissue interaction, it can be considered to combine polymers and ceramics to obtain composite products.

Scaffold must be biocompatible. In other words, it has compatibility in living tissue, not cause foreign body response, and non toxic. To determine whether a material is biocompatible or not, one of which is by cytotoxic evaluation [1-2,6-7]. Cytotoxicity refers to the toxic properties of a substance on cells in vitro. Cytotoxicity is generally characterized by the decrease in the synthesis of nucleic acids (protein), cell proliferation, or cell viability. Cell viability is generally measured using enzyme assays, such as: MTT [7], XTT, MTS [1-2], LDH, SRB assay, etc.

In this study, scaffold made of alginate, chitosan, and carbonate apatite was prepared by freeze drying method. Through this method, the pores in the scaffold will be generated so that a large surface area that can serve as the microenvironment of cells as well as a place for cells attachment and growth into a new tissue will be obtained. To determine the cytotoxicity of the scaffold, evaluation is conducted by exposing the scaffold extract solution in He-La cells to determine the effects on cells growth.

**Materials and Methods**

**Materials.** Sodium alginate (from brown algae), chitosan (low molecular weight, 75 – 85% deacetylated), and all chemical reagents used for the preparation of carbonate apatite was purchased from Sigma-Aldrich. Cells used for cytotoxic evaluation are He-La cells.

**Scaffold Preparation.** Sodium alginate solution (3% w/v), chitosan solution (1% w/v), calcium chloride solution, and carbonate apatite powder prepared by co-precipitation method were blended and stirred until well homogenized. The mixture obtained is molded and then dried by freeze drying method. In this study, four types of scaffolds were prepared, each distinguished by the mixing volume ratio of alginate to chitosan, which are 50%:50%, 60%:40%, 70%:30%, and 80%:20% for scaffolds S1, S2, S3, and S4 respectively.
**Scaffold Characterizations.** To observe the surface microstructure of the resulting scaffolds, observations were made using a Scanning Electron Microscope (SEM). The Fourier Transform Infra Red (FTIR) spectroscope is used to determine functional groups contained in the scaffold.

**Cell Culture.** He-La cells were taken from cryofile stored at a temperature of -80 °C to be adjusted to room temperature by thawing method. Cells were subsequently transferred into 15 ml tubes and given 10 ml of PBS solution in order to undergo lysis and then centrifuged. Once precipitated, the supernatant was removed and the precipitates, which is under the tube, were given 5 ml of Dulbecco's Modified Eagle's Medium (DMEM) culture media and then homogenized. The cell suspension was divided evenly into a new dish and then incubated in an incubator at 37 °C, 5% CO₂, and 20% air flow for 24 hours. After reaching confluence, cells were counted using a hemocytometer under inverted light microscope.

**Cell Viability Evaluation.** In this study, the MTT assay method was used to measure cell viability. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazolium salt which is soluble in water and will be reduced by living cells into a purple formazan crystals with the amount proportional to the number of living cells.

Before measuring the viability of cells, scaffold was sterilized by soaking in 75% ethanol solution for 30 minutes. Then, a total of 100 μl of cells (2x10⁶ cells/ml) were prepared in 96-well micro plate evenly using a pipette and incubated in an incubator at 37 °C, 5% CO₂, and 20% air flow for 24 hours. Furthermore, the cells in the micro plate were exposed to scaffold extract solution with variation of concentration of 0.01 – 100% and reincubated for 4 hours. Then, 15 μl (15 mg/ml) MTT solution was given into each well and reincubated for 3 hours. 150 μl of isopropanol was subsequently added into each well and eventually the micro plate was placed on an orbital shaker (150 rpm) for 1 hour. By comparing the amount of formazan produced by cells exposed to the scaffold extract solution to the number of formazan produced by control cells (unexposed), the absorbance value or Optical Density (OD) will be obtained. OD value of the colored solution was then quantified by measuring at certain wavelength (λ = 470 – 655 nm) using a microplate reader. The percentage of cell viability was calculated as the ratio between the value of OD treatment and control groups.

**Results and Discussion**

SEM results in Fig. 1 showed the microstructure of scaffold surface which were prepared by freeze drying method with the mixing volume ratio of alginate to chitosan by 50%:50%, 60%:40%, 70%:30%, and 80%:20% for scaffolds S1, S2, S3, and S4 respectively. The use of freeze drying method can produce pores in the four scaffolds. The pores vary in size in the range of 60 – 300 μm, which is in accordance with the commercial pore size. The presence of carbonate apatite in the form of white granules which are spread evenly on the surface of the scaffold can be seen clearly. On scaffold S4, which is a sample that contains chitosan in the least amount, carbonate apatite is seen most clearly. This is probably caused by the flocculant properties owned by the chitosan. when the chitosan content is less than the alginate, the carbonate apatite coating layer is only a thin layer. For scaffold S1, which was prepared with the same amount of carbonate apatite, because the content of chitosan was more than other samples, the carbonate apatite on the surface of the scaffold is coated with a thicker layer, so it does not look as clear as other scaffold samples. The presence of carbonate apatite plays an important role in improving the osteoconductive properties of the scaffold. High osteoconductivity is directly proportional to the ability of the scaffold to attach and to actively stimulate the growth of new bone tissue.
Figure 1. Microstructure of scaffolds which is prepared by freeze drying method. (a) Scaffold S1, with the mixing volume ratio of alginate to chitosan by 50%:50%; (b) Scaffold S2, 60%:40%; (c) Scaffold S3, 70%:30%; and (d) Scaffold S4, 80%:20%.

The presence of carbonate apatite is indicated also by the FTIR spectra in Fig. 2. Based on the figure, there are some active functional groups owned by the scaffold. Phosphate functional groups at wave numbers 569 and 1041.56 cm\(^{-1}\) and carbonate functional groups at wave number 1411.89 cm\(^{-1}\) indicate the presence of carbonate apatite on the scaffold. Other functional groups, i.e. carboxyl (from alginate), hydroxyl (from alginate and chitosan), and amine (from chitosan) are shown at wave numbers 1629.85, 3425.58, and 3448.72 cm\(^{-1}\) respectively. Hydroxyl and amine functional groups showed the highest absorbance values, this can be understood as alginate and chitosan are the major constituent of the scaffold.

Figure 2. FTIR spectra of scaffold which is prepared by freeze drying method.

Measurement of He-La cell viability by MTT assay has been performed on scaffold S4 with three times repetitions. The amount of formazan formed as a result of mitochondrial response to tetrazolium salts in the treatment group was quantified into the OD value of the treatment group and then compared to the OD of the control group to obtain the percentage of cell viability. Fig. 3
showed the viability percentage of cells exposed to the scaffold extract solution on the concentration of 0.01 to 100%.

![Graph showing cell viability percentage vs. concentration of scaffold extract solution.](image)

**Figure 3.** The viability percentage of He-La cells exposed to the scaffold S4 extract solution on the concentration of 0.01 to 100%.

Based on Fig. 3, while cells exposed to the scaffold extract solution with concentration of 100%, almost all cells undergo inhibition and leaving only less than 4% of living cells. This is quite different from the situation when the concentration of the extract solution exposed at 10, 1, 0.1, and 0.01%. In average, cell viability for the scaffold in those concentrations range are not less than 71%. Furthermore, the LC$_{50}$ value was calculated. LC$_{50}$ is defined as the concentration of the sample solution required to cause the death of cells by 50%. A sample is considered cytotoxic if the LC$_{50}$ is less than 1000 ppm or 0.1%. By interpolation technique, the LC$_{50}$ value is obtained around 43%. Because the LC$_{50}$ is more than 1000 ppm or 0.1%, it can be stated that the scaffold is not cytotoxic against He-La cells.

**Conclusion**

The scaffolds based on alginate, chitosan, and carbonate apatite have been successfully prepared by freeze drying method with the mixing volume ratio of alginate to chitosan by 50%: 50%, 60%: 40%, 70%: 30%, and 80%: 20%. SEM micrographs showed that all of the resulting scaffolds have pores that resemble the commercial pore size, i.e. in the range of 60 – 300 µm. Carbonate apatite was spread evenly on the surface of the scaffold. These results are in good agreement with FTIR analysis that indicates the presence of phosphate and carbonate functional groups on the scaffold, which also indicates the presence of carbonate apatite. MTT assay gives results that despite the presence of the scaffold affect the growth of He-La cells, the scaffold was not toxic to the cells.

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References


