Biocompatibility of Chitosan/Collagen/PVA Nanocomposite Containing Calcium Apatite

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Abstract
Biopolymer-based nanocomposites containing bone minerals have been used intensively as bone-graft in various broken bone accidents. Main objective of this work is to prepare and characterise biocompatible chitosan/collagen/PVA nanocomposites containing calcium apatite suitable for bone-graft engineering. The calcium apatite nanofillers were prepared by calcination of cow bones at 830°C for 3 hours, followed by ball-milling and ultrasonication processes and characterization using particle size analysis (PSA) and electron scanning microscopy (SEM). It was found that particle size of the filler ranging between 100-8000 nm due partial aglomersation. The bone-graft nanocomposites specimens were prepared with various calcium apetite loading (0-10%) in constant ratio (1:1:1) of chitosan/collagen/PVA biopolymers. Compression strength of the nanocomposites containing optimum filler of 6% was found 2.01 MPa, its density was 1.19 g/cm^3 and its water absorption capacity 58.3% and biodegradation rate 2.67%/day. Degree of biocompatibillity of the bone-graft was revealed after its implementation in mice tissue which did not show any histopathological effect after 14 days.
Keywords: nanocalsium-apatite, bone-graft, biocompatibility

Introduction

Nowadays, various bone substitute materials in terms of structure and composition similar approach to natural bone have been developed. As it is well known, the bone is a composite consisting of organic and anorgnic phases. The organic phase consists of collagen and small amount of ground substance including glycosaminoglycans, proteoglycans, and glycoproteins, while the inorganic phase is composed of calcium phosphate such as hydroxyapatite and β-tricalcium phosphate (Zhao et al., 2002). In this works, bioceramic of bovine bone, chitosan, collagen and poly(vinyl alcohol) were chosen to prepare the organic-inorganic composites.

Bovine bone as biological waste is very many found in Indonesia. Its utilization as biomaterial in bone tissue engineering is still less. Bovine bone is more widely used as supplementary food for livestock. The utilization of bovine bone as raw material of manufacture of bone graft substitutes or scaffolds for bone tissue engineering has some advantages that are the porosity similar to natural bone and containing essential minerals such as hydroxyapatite, β-tricalcium phosphate and calcium carbonate that support the regeneration of bone (Laurencin et al., 2006; Ooi et al., 2007). Chitosan is a polysaccharide Obtained from the N-deacetylation of chitin, consisting of polymeric (1→4)-linked 2-amino-2-deoxy-β-D-glucopyranose units. Chitosan can accelerate the growth of new bone because it structure is similar to glycosamino glycans and hyaluronic acid found in cartilage (Risbud et al., 2005).

Collagen is a fibrous protein hollow and there are lots of joint tissues, skin, muscles, and tendons. This natural fibrous connects and supports other bodily tissues, such as bone and tendon (Hossein et al., 2007). In the field of dermatology collagen is used as a growth enhancer network. Polyvinyl alcohol is a semi-crytalline synthetic polymer that water-soluble, non-toxic, non-carcinogenic, biocompatible and elastic physical properties. In addition PVA also has a strong adhesive power so it is good as a composite matrix because it can improve the compactness and mechanical properties of the composite. These characteristics make this polymer particularly suitable for biomedical applications.

In this study, bioceramic was prepared by calcining bovine bones and crushed by ball milling and ultrasonication in aquabidest. Chitosan-collagen-poly(vinyl alcohol)/bioceramic composites were prepared by mechanical mixing and dried using freeze dryer. The morphology and physicalchemical properties of the composites such as the porosity, degradation and compressive strength were investigated. In addition, also the biocompatibility of the composites was preliminary evaluated by in vivo implantation into the femoral muscle of white mouse.

Materials and Methods

Preparation of Bioceramic and Chitosan-Collagen-Poly(Vinyl Alcohol)/Bioceramic Composites

Bioceramic was typically prepared by calcining bovine bone as follows. Firstly, bovine leg bones were cleaned and dried under sun, then boiled in boiling water for 5 hours, followed by boiling in a pot of commercial pressure for 2 hours, then washed and the process repeated three times. Furthermore, bones were dried in oven at 105 ^{o}C for 3 hours, and then pounded by mortar up into small parts, followed by immersion in n-hexane for 48 hours. After that, the particles were filtered, then the residue was dried in oven at temperature of 200 ^{o}C till dried and smokeless, and then proceed with the smoothing process using blender. After that, the bone powder was calcined at 830 ^{o}C for 3 hours in furnace, then crushed by mortar until smooth and treated by ball milling for 120 minutes (Retsch PM 100), and then sieved by 140 mesh sieve. After that, the bone powder was treated by ultrasonication (40 kHz, 250 W) for 180 minutes (Kudos). Then, the bovine bone bioceramic particles obtained were characterized by FTIR, PSA and SEM.

The composites were prepared as follows. 3% chitosan solution was prepared in 1% acetic acid. 3% collagen solution and 10% PVA were prepared in distilled water. Then, all of the solutions with a weight ratio 1:1:1 were mixed and stirred homogeneously for 1 hour at room temperature. After that, added 4 g bioceramics. Then the mixture was stirred to form a homogeneous paste. After that, the paste was put into a cylindrical mold with a diameter of ± 2 cm and height of ± 1.5 cm. Furthermore, the samples were frozen and then dried by freeze dryer. Do the same thing for the mass variation of bioceramics 5, 6 and 7 g which the composites were named as C_4, C_5, C_6, and C_7.

Biocompatibility
The biocompatibility was tested for the optimal composite by implanting the composite in form of granules into thigh muscles of eighteen 8-weeks-old male *Sprague Dawley rats* which were obtained from Physiology and Anatomy of Animal Laboratory, Departement of Biology, University of Sumatera Utara. Before implantation, the granules were sterilized by immersion into 70% ethanol solution, followed by drying in UV-radition box. Besides, 12 experiment animals were adapted in a cage for 7 days. After that, the experiment animals were treated by hair removal ,and then anesthetized by ketamine at dose of 1 mg/kg.

After implantation at 7 and 14 days, the experiment animals were treated by dislocation and then dissected their thigh muscles, next the granules and surrounding tissue were taken. The granule edhering in the tissue were fixed in bouin solution and treated by embedding in paraffin. It was sectioned into 5 μm thick slices perpendicular to its surface. The histological sections were stained with hematoxylin and eosin (HE) reagen and observed under an optical microscope.

Results and Discussion
Preparation of Bioceramic and Chitosan-Collagen-Poly(Vinyl Alcohol)/Bioceramic Composites
In the bioceramic preparation, 121alcinations process on bovine bone aims to obtain pure calcium-apatite minerals without there are organic compounds. The process caused the bovine bone undergoing physical changes, which the bovine bone color before heating process at 200 ^{o}C and 121alcinations was white-yellow with 1000 g of weight. After undergoing the processes , the color of bovine bone changed becoming pure white with 516,8 g of weight or 51,68% of yield. The color and weight changes of the bovine bone were caused by releasing of organic compounds contained in the bone (Ooi et al., 2007).
Milling process using ball mill and ultrasonication process produced finer particles of bioceramic than before being processed. The composites are obtained seem homogeneous and also have pores.

Characterizations
Particle Size Analyzer
PSA analysis result shows that the bioceramics are obtained have particle size distribution around 100-8000 nm. Size take effect to bioactive properties of a biomaterial, which the smaller particle or nano material has larger surface area so that has better bioactive properties than coarse particle (Dorozhkin, 2010).

Degradability
Degradation of a scaffold is a very important parameter for bone graft substitute application. It should degrade slowly to allow bone regeneration (Vacanti and Langer, 1999; Hollinger et al., 2005). As shown in Figure 1, the all composites can biodegrade slowly. The degradation of the composites may be due to the solubility of collagen and beta-elimination or the protonation of amino/imine groups and the mechanical relaxation of coiled chitosan chains in SBF. The ions in SBF can accelerate the hydrolysis of chitosan (Zhou et al., 2012). Thus chitosan-collagen-poly(vinyl alcohol)/bioceramic

composite possesses controlled biodegradation, which is suitable for a biomaterial for bone graft substitute.

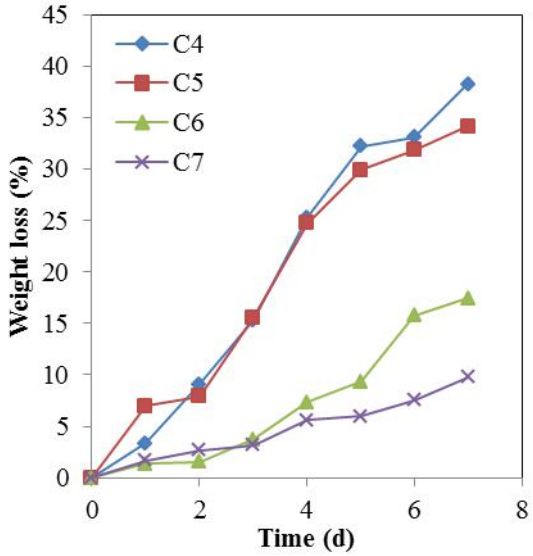


Figure 1. Biodegradation of chitosan-collagen-poly (vinyl alcohol)/bioceramic composites.

Mechanical Properties

Mechanical properties, especially compressive strength, are important and usually imperative criterion in selecting the bone graft substitute materials (Yin et al., 2003; Panzavolta et al., 2009). Hence, the compressive strength of these materials were shown in Figure 2. From the records, it could be found that the compressive strength of the composites had a relation to the bioceramic content in composites which the mechanical properties increased by rise of mass of bioceramics from 4 until 6 g in the composites. However, the mechanical properties decreased by adding the bioceramics more than 6 g or when the mass of the bioceramics exceeded 7 g. it means that presence of small amount of the bioceramics could improve the mechanical properties of the composites but the large amount of those could reduce the degree of the mechanical properties. Zhou et al.. (2012) reported that compressive strength of gelatin/CM-chitosan/β-TCP declined with the addition of fraction of β-TCP more than 5%. In this study, the composite with 6 g mass of bioceramics is the composite that has the compressive strength in range of the degree of cancellous's compressive strength which is 2-12 MPa (Ficai, *et al*., 2011). It was potential used as bone graft substitute materials.

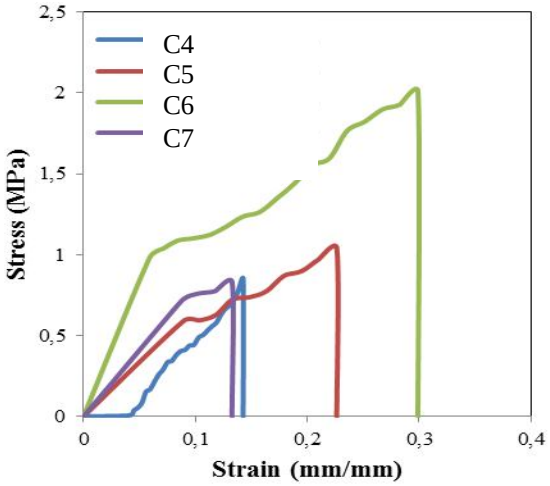


Figure 2. Stress-Strain curve of chitosan-collagen-poly (vinyl alcohol)/bioceramic composites.

Fourier Transform Infrared Spectroscopy

FTIR spectra were recorde to a better understanding of the interactions between the components in the composites. The FTIR spectra of chitosan, collagen, poly(vinyl alcohol), bioceramic, and the composites before and after degraded were shown in Figure 3.

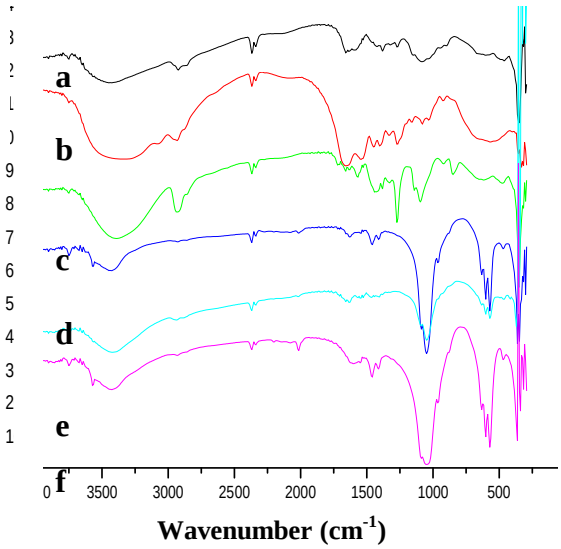


Figure 3. FTIR spectra of (a) chitosan, (b) collagen, (c) poly(vinyl alcohol), (d) bioceramic, (e) chitosan-collagen-poly(vinyl alcohol)/bioceramic composite with 6 g bioceramic content before and (f) after degraded.

The FTIR spectra of chitosan-collagen-poly(vinyl alcohol)/bioceramic composite with 6 g bioceramic content showed the absorption at 3425.58cm^{-1} was assigned to –OH stretching vibration from hydroxyapatite, chitosan, and poly(vinyl alcohol). The peak of v_3 mode of PO_4^{3-}, v_4 mode of PO_4^{3-}, and v_2 mode of PO_4^{3-} are also present at 1049.28 cm^{-1}, 570.93 cm^{-1}, and 470.63 cm^{-1}, respectively. It seems the magnitude of these bands becomes weaker. The absorption bands of chitosan and collagen such as amide A, amide B, amide I, amide II, amide III and COO-, and also v_1 mode of PO_4^{3-} and v_5 mode of PO_4^{3-} are absent in the composites (Figure 3**e**). these spectral changes and absents indicate there have been interactions between the components.

The composite after degraded for 7 days has similar FTIR spectra (Figure 3**f**) with FTIR spectra of the bioceramic (Figure 3**d**). The peak of the all vibration mode of PO_4^{3-} become stronger and the absorption of v_1 and v_5 mode of PO_4^{3-}, and also carbonate are present again in the composite. It indicate that may be due to the solubility of collagen, hydrolysis, beta-elimination and the mechanical relaxation of coiled chitosan chains in SBF.

Scaning Electron Microscope

Morphology of the bioceramic and chitosan-collagen-poly(vinyl alcohol)/bioceramic composite with 6 g bioceramic content are shown in Figure 4. The morphology of the bioceramic (Figure 4**a**) indicates serious aggregation of its. It may be coused by ultrasonic treatment which can promote the distribution of the bioceramic particles. However, extensive ultrasonic treatment will lead to the aggregation of the bioceramic (Zhou et al., 2012). The morphology of the composite significantly displays homogenous distribution of the bioceramic particles within the chitosan-collagen-poly(vinyl alcohol) matrix and open pore structure with both micropores (3.65 μm) and macropores (81.5 μm). an open pore structure can promote the formation of internal mineralized bone and serve for nutrient delivery. In addition, it is also good for blood supply and cell attachment (Laurenchin et al., 2008; Zhou et al., 2012).

Although, the diameter pores of the composite are not in range of 100-800 μm that will benefit bone regeneration (Karageorgiou and Kaplan, 2005), but there is no definite pore size for each bone tissue in a body , because every bone part has different characteristics that are suitable to perform the cell migration, proliferation, adhesion, and differentiation.

As shown in Figures 5 healing process of the mice wound were not affected by implantation of the nanocomposite after day-7 (1) and day-14 (2). Implantaton of the bone graft as biopolymers and its components did not show any toxicity effects. The hydroxyapatite, filler which was on nanosized eased the dissolution process into the mice tissue. Chitosan and collagen matrices were also polyionic biopolymers which may induced formation of new cell and therefore revealed biocompatibility of the nanocomposite into the live mice tissue.

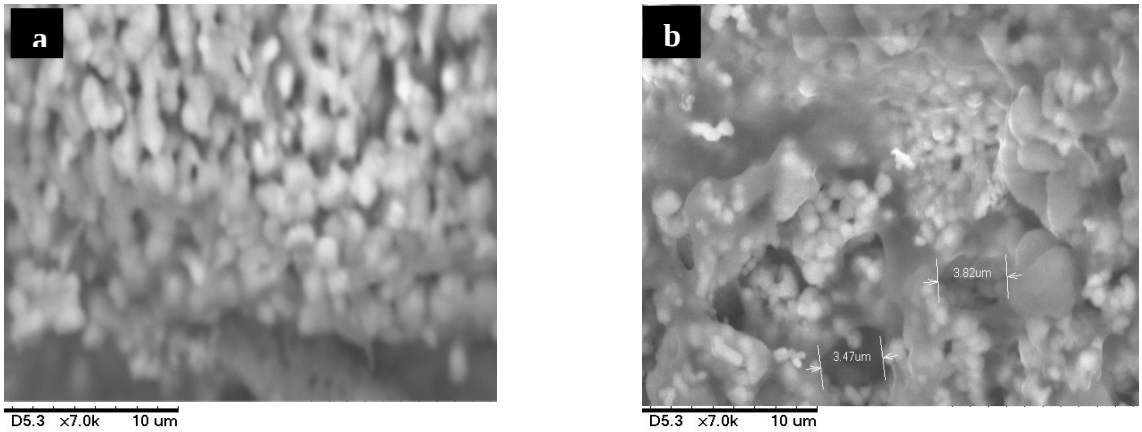
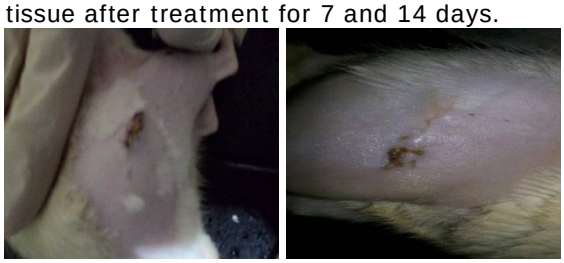


Figure 4. SEM images of (a) bioceramic and (b) chitosan-collagen-poly(vinyl alcohol)/bioceramic composite with 6 g bioceramic content.

Biocompatibility of chitosan-collagen-poly(vinyl alcohol)/bioceramic composite

Biocompatibility tests of the nanocomposites as *bone graft* were carried out using live mice wounded tissue after treatment for 7 and 14 days.



(1) (2)

Figure 5. Mice tissue wound healing on day-7 (1) and day-14 (2) after implantation of the nanocomposite as *bone graft*

Conclusions

Mineral of calcium apetite has been successfully isolated from bovine bone waste by calcination at 830^{o}C and after ballmilling and characterization using particle size analyser (PSA) it was found that the particle size was ranging 100 - 8000 nm. Nanocomposites of the calcium apetite-filled chitosan-collagen showed good mechanicl properties (compression strength: 2.01 MPa and more importantly were biocompatible with live mice tissue without indiction of toxicity.

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