Composite Based Chitosan/Zinc-Doped HA as a Candidate Material for Bone Substitute Applications

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Abstract. The composite based Zinc-doped in Chitosan/Hydroxyapatite was successfully prepared by wet mixing method through the addition of 10, 15, and 20wt% of chitosan. The addition of Chitosan increased the compressive strength and the modulus elasticity. However, it decreased the density and the surface hardness of HA-Zn. Mechanical characterization revealed that these composites are suitable as a candidate of a cancellous bone substitute. Composite with 10% chitosan has compressive strength and modulus elasticity of 57.03 MPa and 0.15 GPa, respectively. Hence, it has the physical and mechanical properties that meet the standards as a cancellous bone substitute material. Also, in vitro biocompatibility test against BHK-21 cells exhibited non-toxic materials.

Keywords: composite, chitosan, zinc, HA, bone

1. Introduction

Tissue engineering (TE) is a multidisciplinary field focused on the development and application of knowledge in chemistry, physics, engineering, life and clinical sciences to the solution of medical problems, such as tissue loss and organ failure. It involves the fundamental understanding of structure and function relationships in normal and pathological tissues as well as the development of biological substitutes that restore, maintain or improve tissue function [1].

Different efforts have been addressed to the development of research in the field of biomaterials namely by using various modifications in the manufacture of synthetic biomaterials. Composites comprising calcium phosphates and natural biopolymers are widely used as biomaterials for bone tissue repair and engineering [2]. Hydroxyapatite, Ca_{10}(PO_4)_{6}(OH)_{2}, has been used as a principal inorganic component of synthetic materials for orthopedic application for many years. However, this bioceramics are fragile, and the structures are unstable when mixed with body fluids or blood of patients [3,4]. Meanwhile, the addition of ions such as Zn, Mg, and Si has been performed to improve the mechanical properties of HA. Zn ion addition to the HA bioceramics can effectively improve the mechanical properties as compared to Mg and Si ions [5].

Moreover, natural polymer such as chitosan has been extensively studied and used in many tissue engineering applications as well, such as for bone substitution [1,5]. This biopolymer is widely used to modify the mechanical properties of Hydroxyapatite (HA)-based composite [5-7]. In addition, chitosan is bioresorbable, biocompatible, non-toxic, non-antigenic, biofunctional and osteoconductive which can accelerate the growth of osteoblasts.
The aim of this research was to improve the mechanical properties of HA by doping Zn ion, followed by adding different amounts of chitosan, making materials potentially interesting for tissue engineering applications.

2. Experimental Method
Chitosan derived from crab with a degree of deacetylation of 84% obtained from CV. Biochitosan Indramayu, West Java. Zn-doped HA was prepared by sol-gel method in accordance with the reference [8]. The synthesis process of composite Chitosan / HA-Zn is performed using a wet mixing method. Chitosan was first dissolved in 100 mL of 3% acetic acid solution and then stirred at a constant temperature of 700 °C for one hour. HA-Zn powder was then added and stirred for 1 hour at a temperature of 700 °C. The composites prepared with weight percent ratio of chitosan / HA-Zn WERE 10/90; 15/85; 20/80.

The functional group characterization of the composite was performed using the Thermo Scientific Nicolet iS 10 (Thermo Fisher Scientific Inc., MA, USA) with a wavelength of 4000–400 cm⁻¹. X-Ray Diffraction (XRD) were recorded using X’Pert PRO PANanalytical at room temperature by using filtered Cu Kα radiation (λ=1.54 Å). Scanning electron microscopy (SEM) was carried out using PhenomProX (Phenom-World, Eindhoven, NL) on the fracture surfaces of the specimens, previously coated by sputtering with gold and by using a SEM apparatus equipped with energy dispersive spectroscopy (EDS).

The measurement of bulk density was performed according to DIN 2738 for testing in determining the density of the porous material. Compression mechanical properties of samples were evaluated using a traction-compressive device. Compression molded cylindrical samples were used and positioned between parallel plates. Hardness testing was conducted in accordance with ASTM C 1327 – 08, using Vickers indenter with a diameter of 2.5 mm, a load of 30 kg and indentation time for 10 seconds.

Biocompatibility of composite chitosan/HA-Zn was evaluated through an in vitro cytotoxicity test in accordance with reference [1] using BHK-21 Cells. The samples were incubated at a temperature of 37 °C for 24 hours.

3. Results and discussion

3.1. X-Ray Diffraction

Figure 1 shows the XRD pattern of chitosan and HA-Zn and its composites, it can be identified that there were peaks belong to HA-Zn and Chitosan. Chitosan is characterized by a typical peak at 2θ 9-
10° and 19-20° [9]. It can be evaluated that its composite has a shifted peak and lower intensity. Likewise, the HA-Zn peaks show more ramps along with the increased addition of chitosan added. It indicates the occurrence of hydroxyapatite and chitosan molecular interactions [5,10].

3.2. Fourier transform infrared spectroscopy (FTIR)
The functional group characterizations are shown in Figure 2. It can be seen that HA-Zn and Chitosan have the same spectral pattern with their composite. The uptake in the waves that indicated a chitosan identified by OH peak at around 3400 cm⁻¹ and NH peak at around 1600 cm⁻¹. In addition to chitosan, phosphates, hydrogen phosphate, and carbonate, which indicated HA appear in the range of 1000, 900, and 1400 cm⁻¹, respectively. Moreover, the OH peak of the composite in the range of 3400 cm⁻¹ increased intensively along with the increasing addition of chitosan. In addition, the amide peak at around 1600 cm⁻¹ showed the same behavior. These results are in agreement with previous XRD data proved that the synthesis of K/HA-Zn composite via sol-gel method was successful without any formation of a new compound. The decreasing intensity and broadening of the peak indicated the interactions between the constituent elements of the composite, such as hydrogen bonding between chitosan and hydroxyapatite [11].

![Figure 2. FTIR patterns of chitosan, and HA-Zn and its composite](image)

3.3. Scanning Electron Microscopy (SEM)
The Scanning Electron Microscopy of Figure 3a revealed that the HA-Zn particles have morphology as a rock. The results of the elemental analysis performed on the HA-Zn elements showed the presence of the elements of Ca, P, O and Zn as ions doped. The Ca / P ratio of HA-Zn was calculated to be 1.81, which was greater than the Ca / P pure hydroxyapatite which was 1.67. It was likely to occur because the carbonate groups replaced phosphate groups in the structure of hydroxyapatite. Although there were no materials having carbonate groups in the process of synthesis, it can form inside the apatite [12].

Figure 3b shows that there is a chitosan compound characterized by the presence of fibers connecting the HA-Zn particles to one another. In Chitosan-HA composites, a HA particle will act as a filler which is dispersed evenly when there is a high amount of chitosan in the composites. However, along with the decrease of chitosan, it may not cover the HA particles and tend to act as a binder [12].

Figure 4 show that chitosan was able to bind HA-Zn particles. Figure 4B illustrates the presence of particles that agglomerated HA-Zn and spread evenly in a relatively small form. This form seemed bigger in Figure 4C and 4D when the amount of chitosan addition increased to 15% and 20%. From these, it can be assumed that the greater the amount of Chitosan added, the higher its ability to bind HA-Zn particles.
Figure 3. SEM patterns of HA-Zn (a) and 20% K/HA-Zn (b)

Figure 4. SEM patterns of HA-Zn powder distribution, 10% K / HA-Zn (B), 15% K / HA-Zn (C) and 20% K / HA-Zn (D) composites

Table 2 shows that the addition of chitosan against HA-Zn will reduce the density of the composites since chitosan is a polymer which has a specific gravity that is lower than the HA-Zn which is a ceramic material. This result is in agreement with the previous results [13]. Moreover, bone is a composite material consisting of calcium phosphate and biopolymers. Therefore, the bone has a lower density equivalent to 1.8 g/cm$^3$ [14]. Based on this theory, it is known that the densities of all composites were in the range of bone density values.

| Materials          | Density (g/cm$^3$) |
|--------------------|--------------------|
| HA-Zn              | 2.74               |
| Composite 10% K / HA-Zn | 1.94               |
| Composite 15% K / HA-Zn | 1.86               |
| Composite 20% K / HA-Zn | 1.85               |
3.4. Compression test
The addition of chitosan increased the compressive strength value of the composite (see Table 2). The compressive force of the cortical and cancellous bone was 137.8 MPa and 41.4 MPa, respectively. Thus, the values of the compressive strength of the composite are in the range of cancellous bone compressive strength. Moreover, the modulus of elasticity of the cortical bone was 7-30 GPa, while the elastic modulus of the cancellous bone was approximately 10 - 1500 MPa [14]. Thus, we may say that a composite with 10% chitosan meets the standard value of cancellous bone mechanical properties.

| Material     | Modulus Elasticity (GPa) | Compressive strength (MPa) |
|--------------|--------------------------|----------------------------|
| HA-Zn        | 0.29                     | 14.05                      |
| Composite 10% K/HA-Zn | 0.15                  | 57.03                      |
| Composite 15% K/HA-Zn | 0.18                  | 61.09                      |
| Composite 20% K/HA-Zn | 2.25                  | 66.93                      |

3.5. Hardness test
The highest hardness value was obtained from the HA-Zn composite of 29.2 VHN. 20% K / HA-Zn composite had the lowest hardness value of 20.6 VHN. It can be concluded that the composite hardness values decreased together with the increasing amount of chitosan. It was due to the addition of chitosan that lowered the surface hardness of the composite material since chitosan is a polymeric material which has lower mechanical properties as compared to the ceramic material.

| Material     | Hardness (VHN) |
|--------------|----------------|
| HA-Zn        | 29.2           |
| Composite 10% K/HA-Zn | 25.3            |
| Composite 15% K/HA-Zn | 21.3            |
| Composite 20% K/HA-Zn | 20.6            |

3.6. In vitro biocompatibility test
The most important requirement for a material to be used in medical applications is its compatibility, not only regarding physical and mechanical properties but also in those that define its behavior at the time it is in contact with the body. MTT assays were conducted to the composites to test their cytotoxicity. BHK-21 fibroblast cell lines were selected in the MTT assay. The viability of cells that were exposed to composites is summarized in Table 4. MTT results reveal no visible reduction in viability after incubation for 24 hours, presenting that these materials had good biological safety and displayed as almost non-cytotoxic. Also, the cells incubated in the composite suspension can be clearly observed using an inverted microscope (Fig. 5). It is possible to observe that the cell viability is in a similar rate against time of incubation, which was in agreement with the MTT assay. In accordance with the references [5], these composites can be categorized as non-toxic materials.

| Samples     | Cell Viability (%) |
|-------------|--------------------|
| HA-Zn       | 33.8               |
| 10% K/HA-Zn | 48.7               |
| 15% K/HA-Zn | 46.2               |
| 20% K/HA-Zn | 48.4               |
Figure 5. Cell phase contrast micrographs, control (a) 10% K/HA-Zn (b) 15% K/HA-Zn (c) 20% K/HA-Zn (d)

4. Conclusion
The addition of chitosan resulted in a more evenly distributed bond between chitosan and HA-Zn particles. The density and hardness values of chitosan/HA-Zn composites decreased together with the increasing compressive strength. The composite with 10% Chitosan has physical and mechanical properties that meet the standards of material as a candidate for substitute material for cancellous bone. In addition, biocompatibility test showed that these composites are non-toxic materials.

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