Effect of chitosan addition to characteristic and antimicrobial activity of zinc doped hydroxyapatite

A Rasyida, S T Wicaksono, N N Pradita, H Ardhyananta, A Purnomo
Department of Materials and Metallurgical Engineering, Faculty of Industrial Technology, Institut Teknologi Sepuluh Nopember (ITS) Surabaya

E-mail: amaliyarasyida@gmail.com, amaliya@mat-eng.its.ac.id

Abstract. Hydroxyapatite (HAp) doping with zinc was prepared using sol gel method; different chitosan content were further added to prepare the composite, namely 10, 15 and 20% wt. The samples were characterized using FTIR, XRD, SEM-EDX, and AAS. In vitro antimicrobial activities of the composite were evaluated against gram positive and negative bacteria. FTIR results revealed that there were no important changes in the structure of composite, while 10% wt of chitosan in composite shows the highest inhibition zone against Escherichia coli after 24 h incubation. In addition, after 7 days of immersion in simulated body fluid, there were apatite formations in the surface of the composite. These might indicate that this composite could be used as a material candidate for bone substitute applications.

1. Introduction

Biocompatibility and osteo-conductivity of hydroxyapatite [Ca₁₀(PO₄)₆(OH)₂, HAp] are well known and utilized in dentistry as well as orthopedic application due to its chemical similarity to human bone. The emerging trend of bone tissue engineering with hydroxyapatite has attracted more significant due to its bio-functional properties, such as bioactivity and biocompatibility. Recently, since HAp has high dissolvability in the body and lower mechanical properties as compare to human bone, the development HAp with various additives has been one of the primary aims in the field of biomaterials to be used for bone substitutions [1]. Zinc doped HAp has a stimulatory effect of bone formation tough both in vitro and in vivo, triggers metabolism and bone development promote bone density, and prevent bone losses [2]. In addition, it can improve dissolvability, in small concentrations serve as micronutrient for some processes in the body, but in large concentrations can be toxic to the body [3]. Concerning to the antimicrobial properties, the ability of HAp-Zn in inhibiting the bacteria proved to be limited to gram-positive bacteria such as Staphylococcus aureus, and very little observed in gram-negative bacteria one is Escherichia coli [3].

On the other hand, chitosan (CS) is one of the most abundant natural polymeric materials. Chitosan is a deacetylated derivative of chitin with amine and hydroxyl groups. Those structures bring chitosan to have tremendous ability to form metal complexes. Moreover, zinc is one of the metal ions that can easily coordinate with chitosan [4]. It is well known that both CS and Zn have the properties...
of disinfection and bactericide [5, 6]. In addition, chitosan is known to have a good anti-bacterial activity against some bacteria, both gram-positive and gram-negative.

In our previous work [7] we reported the morphology of chitosan and each composite was determined by X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and scanning electronmicroscopy (SEM). XRD and FTIR results indicate that there were interactions between chitosan and HAp presented by the decreasing of intensity and broadening of the peak, moreover SEM micrographs showed that there was the presence of particles that agglomerated HA-Zn presenting the ability of chitosan to bind HA-Zn particles.

Considering this, the aim of this work was to study if the presence of chitosan can affect considerably to biological properties of zinc doped HAp, presenting potentially interesting properties for some medical applications such as bone substitute applications.

2. Materials and Method

2.1. Materials

Chitosan derived from crab with a degree of deacetylation 84% obtained from CV. Biochitosan Indramayu, West Java. Kalsium nitrat tetrahidrat and zinc nitrat tetrahidrat were supplied by PT. Sumber Ilmiah Persada Surabaya.

2.2. Preparations

HAp doped Zn 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 with weight percent ratio of chitosan 10, 15, and 20 %wt. Chitosan was first dissolved in 100 ml of 3% acetic acid solution. Then stir with a constant temperature of 700°C for one hour. HA-Zn powder is then added and stirred for 1 h at 700°C.

2.3. Characterizations

Evaluation of antibacterial activity in vitro was measured by the amount of inhibition zone diameter on antibacterial testing. Escherichia Coli and Staphylococcus Aureus first dissolved in 2 mL of 0.85% NaCl (1.5 x 108 CFU / ml; equated to a 0.5 McFarland standard solution). 1 mL of bacterial suspension was dropped onto Mueller-Hinton Agar (MHA) and spread using sterile spatula. The characterization is performed using paper disc diffusion method by dipping a paper disc diameter of 0.6 cm into the composite mixture and using pH 7.2 phosphate buffer solutions with a concentration of 800 mg/mL. Observations were carried out after 24 and 48 h incubation.

Soaking in Simulated body fluid (SBF) was performed to evaluate in vitro bioactivity. SBF using artificial solution that has ion concentrations nearly resembles human blood plasma. Cylindrical samples soaked for 7 days in 60 mL of SBF. On third, fifth, and seventh day samples were taken from
the solution, dried and weighed to determine the mass percent lost during immersion using the formula [9]:

\[ W = \frac{(m_a-m_b)}{m_b} \times 100 \]  
………………………….(1)

ma and mb represent mass after and before immersion respectively.

Atomic Absorption Spectroscopy (AAS) was carried out to determine the concentration zinc and calcium dissolved. Characterization of functional groups of composite were performed using the Thermo Scientific Nicolet iS 10 (Thermo Fisher Scientific, Inc, MA, USA) with a wavelength of 4000-400 cm\(^{-1}\) to evaluate the structure after SBF immersion. Scanning electron microscopy with Energy-dispersive X-ray spectroscopy (SEM-EDAX) was performed to analyze the surface morphology and the constituent elements contained in the composite using machine type PhenomProX (Phenom-World, Eindhoven, NL). This characterization was carried out on both sample, before and after immersion of SBF.

3. Results and Discussions

3.1. Characteristics of Product

In vitro evaluation using Simulated Body Fluid (SBF) presented in Figure 1, it shows that the compositied has a higher solubility as compared to pristine HAp-Zn. It can be due to chitosan is not able to envelop HAp particles. Chitosan serves as the glue which holds the particles of HAp [15]. In addition, the presences of porosity facilitate SBF solution to go into and effects on chitosan to be more easily degraded. This is strengthened by the results of FTIR after 7 days of immersion in which the OH peak at 3000-3400 cm\(^{-1}\) and amide peak at around 1600 to 1650 cm\(^{-1}\) in Figure 2 decreased intensity [9].

![Figure 1. Weight loss curve](image1.png)

![Figure 2. FTIR pattern after SBF immersion for 7 days](image2.png)

Moreover, SEM micrographs, before and after 7 days of immersion, support the previous results. In Figure 3, marked with a red circle, the chitosan was no longer visible. Before soaking
structure exists as the glue which holds the grains of HAp-Zn. However, after soaking the adhesive is lost, leaving the porosity between the grains HAp-Zn one another, so that a similar structure as the adhesive can be assumed as chitosan.

![Figure 3. SEM micrographs of composite containing 15% before (a) and after (b) soaking in SBF for 7 days](image)

Although chitosan is expected to be first degraded, it does not rule out the possibility of calcium and zinc in the composite subsequently degraded after chitosan which serves as an adhesive merged in SBF. From the test results AAS, in Table 1, Zinc dissolved in SBF increase in line with the addition of chitosan, but still within safe levels of Zinc contain in blood. The concentration of zinc in normal human red blood cells is in the range of 3.6 - 25.4 ppm, while the blood plasma ranging from 0.49-7.70 ppm. We have to keep in mind that the range Zinc concentration levels in the blood are different for each population [10]. This difference can be due to geographical factors, environment and diet.

| Sample          | Zinc dissolved (ppm) |
|-----------------|----------------------|
| HAp85-Zn15      | 3.25±2               |
| 10%CS/HAp-Zn90  | 3.45±2               |
| 15%CS/HAp-Zn85  | 3.44±3               |
| 20%CS/HAp-Zn80  | 3.25±2               |

Bone substitutes material must have good bioactivity and the ability to trigger the growth of bone cells, as in vitro it can be showed through the formation of apatite layer on the surface of the sample. Table 2 present the concentration (ppm) of calcium dissolved in the SBF solution after soaking the samples for 7 days. It can be observed that the addition of chitosan lead to enhancement of Ca ions. Calcium ions are used in the formation of apatite layer, resulting in the decrease in Ca concentration in solution, thus the possibility of the apatite layer found on the surface is greater [9].
Table 2. The results from AAS for Calcium dissolved after 7 days of immersion SBF

| Sample              | Calcium dissolved |
|---------------------|-------------------|
|                     | 0 day  | After 7 days |
| HAp85-Zn15          | 0.69   | 0.28         |
| 10%CS/HAp-Zn90      | 8.55   | 12.41        |
| 15%CS/HAp-Zn85      | 47.72  | 12.83        |
| 20%CS/HAp-Zn80      | 42.01  | 5.46         |

Figure 4. SEM micrographs of composite with 10%wt (a), 15%wt (b) and 20% (c) of chitosan after 7 days immersion in SBF

Those results are in accordance with SEM micrographs on the surface of the composite shown in Figure 4. The white layer on the surface of the composite prior to soaking was not detected. However, after immersion, many similar layers appear. Thus, from the weight gain started on the fifth day and reducing the concentration of calcium in SBF solution, this white coating is assumed to be the apatite layer. Similar results were found in agreement with previous research by Machou et al., (2008) [9].

3.2 Antimicrobial Activities

The bacteria used were Escherichia coli (gram negative) and Staphylococcus aureus (gram positive) which is a common cause of bacterial pathogens Orthopedic Implant Infections [11, 12].
Observations were carried out on *E. coli* during the first 24 h showed that the addition of chitosan on HAp-Zn affect the antibacterial activity of composite. However, the presences of chitosan narrow the inhibition zone of bacteria. HAp-Zn have better inhibition zone of 0.25 mm, while for the composition 10% K, 15% K, and 20% of K has 3 mm, 1.16 mm and 0.5 mm respectively. Meanwhile, antimicrobial activity against S. aureus present reducing the ability of HAp-Zn. It was measured 6.65 mm, while the addition of 10% K, 15% K, and 20% K is 0.5; 0.25; and 0.4 mm respectively (see Table 3). From the picture it can be seen that the inhibition zone of HA-Zn found to be greater against *E. coli* then *S. aureus*. This result is in agreement with previous research [3,13] showed that S. aureus are more susceptible to HA-Zn as compared to *E. Coli* supported by the greater percentage reduction in bacterial colonies on S.aureus. This difference occurs due to the gram-positive bacteria have membrane cells structure composed of peptidoglycan and sour Teihoic (TA) which is easier to be penetrated by zinc causing damage to DNA and RNA and inhibit the reproduction of bacteria that can lead to death [13].

**Table 3.** Antibacterial activity measured by inhibition zone of Hap-Zn and its composite

| Composition     | Inhibition zone (mm) |   |   |
|-----------------|----------------------|--|--|
|                 |                      | S. Aureus | E. Coli |
|                 | 24 h | 48 h | 24 h | 48 h |
| HAp85-Zn15      | 1.43 | 1.23 | 0.65 | 0.00 |
| 10%CS/HAp-Zn90  | 0.63 | 0.61 | 0.70 | 0.77 |
| 15%CS/HAp-Zn85  | 0.64 | 0.61 | 0.70 | 0.73 |
| 20%CS/HAp-Zn80  | 0.61 | 0.00 | 0.67 | 0.00 |

Meanwhile, the addition of chitosan gives significant effect on the inhibitory activity of *E. coli* but not so with *S. Aureus* [14]. However, the decrease can be due to lower amount of HAp-Zn with the addition of chitosan. The presences of Zn strengthen the ability of chitosan in inhibiting bacteria. Zn binds to the active site of chitosan, the amide group at C2 position resulting from protonation;thus can facilitate interaction with the anionic components on the surface of cells such as LPS and proteins. This interaction alter the structure of the outer membrane protein causes the release of a number of cells [15].

Observations for 48 h present the antibacterial activity of composite decrease. Those results confirmed that the chitosan has a bacteriostatic effect which is inhibiting for a certain span of time in this case the first 24 h post-incubation. It can be due to subpopulation emergence of resistant bacteria as a result of physiological adaptation to exposure chitosan cells [9].
4. Conclusions

Zinc doped HAp and its composite based on different weight of chitosan namely 10, 15, and 20 %wt were prepared in order to evaluate if the presence of chitosan could affect the antimicrobial activity as well as in vitro bioactivity properties of HAp-Zn. The additions of chitosan in the composite affect in antibacterial properties. Greater inhibitory activity of bacteria found against E. coli than S. aureus with inhibition zone diameter of 3 mm. In addition, after 7 days of immersion in SBF it was observed a white apatite layer on the surface of the composite, pointed by the reducing of calcium ions in SBF solution. From the test results, the optimal composition is obtained when the chitosan addition of 10% by weight in terms of antibacterial activity and apatite layer formed.

Acknowledgements

The author would like to acknowledge PENELITIAN PEMULA PNBP ITS 2016 for funding.

References

[1] Deepa C et al 2013 Nanosystems: Physics, Chemistry, Mathematics. 4 (3) 370–377.
[2] Fuzeng R et al 2009 Acta Biomaterialia 5 3141–3149.
[3] Radovanovic S et al 2012 J The Serbian Chemical Society 77 (2012) 1787–1798.
[4] Wang X et al 2004 Carbohydr. Polym. 56 21–26.
[5] Jeon Y J, Kim S K 2000 Carbohydr. Polym. 41 133–141.
[6] Takai K et al 2002 Microbiol. and Immunol. 46 75–81.
[7] S T Wicaksono et al 2017 IOP Conf. Ser.: Mater. Sci. Eng. 202 012080 doi:10.1088/1757-899X/202/1/01208.
[8] Norhidayu D 2008 Development of Zinc Doped Hydroxyapatite for Bone Implant Application ICCBT - F 24 257-270.
[9] Maachou H et al 2008 Trends Biomater. Artif. Organs 22 (1) 16-27.
[10] Lisa C et al 2012 J. Infections 64 169-175.
[11] Byrne F M dan Wilcox M H 2011 Int. J. Care Injured S3-S6.
[12] Kolmas et al 2014 Bio. Med Research Int. 1-15.
[13] Xiaohui W et al 2004 Carbohydr. Polym. 56 (1) 21-26.
[14] Raafat, Dina 2008 Chitosan as an antimicrobial compound: Modes of action and resistance mechanisms. Dr.rer.nat Dissertation, Bonn: Universität Bonn.
[15] Zhang L et al 2005 J. Mater Sci. : Mater. in Medicine 16 213-219.