Synthesis and Characterization of Hydroxyapatite- Collagen-Chitosan (HA/Col/Chi) Composite Coated on Ti6Al4V

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Abstract. HA-collagen-chitosan (HA/col/chi) composite is developed to increase bioactivity adhesiveness between the metal and the material composite and to improve corrosion resistance. The Ti6Al4V alloy was coated by soaking in HA/col/chi composite at room temperature and then allowed to stand for 5, 6, and 7 days. Diffraction pattern analysis of the coated Ti6Al4V alloy showed that the dominant phase were HA and Ti6Al4V alloy. Corrosion resistance test in media by using 0.9% NaCl showed the corrosion rate at the level of 0.3567 mpy, which was better than that of the uncoated Ti6Al4V alloy (0.4152 mpy). In vitro cytocompatibility assay on endothelial cell of calf pulmonary artery endothelium (CPAE) (ATCC-CCL 209) showed there was no toxicity in the cell culture with the percent inhibition of 33.33% after 72 hours of incubation.

Keywords: chitosan, collagen, cytocompatibility, hydroxyapatite, Ti6Al4V

1. Introduction
The use of titanium (Ti) and its alloys as bone plate substrates continues to evolve in accordance with the increasing need for human bone components. Titanium and its alloys are commonly used as orthopedic implants and that have high strength, light weight and high corrosive resistance compared to cobalt and stainless steel based alloys [1]. However, metal implants are susceptible to corrosion caused by body fluids, metal ions release may cause side effects on nearby tissues, bioactive properties on the surface are poorly for the formation of bonds with natural bones and tissues [2,3]. Therefore, biocompatible, fixation and life time properties of implantable materials able to enhanced by coating biomaterials on metal surfaces with a composite such as a HA/collagen composite. Collagen fibrils and HA are the main components of bone whose composites are more promising to produce preferable bone tissue reactions [4]. HA/collagen composite able to inhibit pathogenic bacteria present during the implantation process [5].

HA/collagen composite when implanted in the human body shown osteoconductive properties preferable than the monolithic hydroxyapatite and results in a similar bone matrix classification [6,7]. In this study hydroxyapatite was synthesized from the utilization of waste of snail (Bellamya javanica) shells through the wet method (precipitation). While collagen was extracted from waste white snapper (Lates calcarifer) fish [8]. The development of HA/collagen/chitosan composite to increase the mechanical properties and strength of HA coated on Ti6Al4V (Ti grade 5). The addition of chitosan to increase the quality of the material, maintaining the hydroxyapatite position remains on the metal surface. Chitosan is natural polymers that biodegradable, non-toxic, and biocompatible [3]. Ti6Al4V alloy soaking in HA/col/chi composite at room temperature for 5, 6, and 7 days. The aim of this study is to increase biocompatibility and bioactivity properties between metal material and composite from HA/col/chi composite coatings on Ti6Al4V.

2. Material and Methods
The materials are used Ti6Al4V alloy from Baoji Xinglian Titanium Metal Co.Ltd, snail (Bellamya javanica) shells obtained from Pasar Anyar (Bogor, Indonesia), CaCO3 standard from Wako, (NH4)2HPO4 from Merck, collagen fish (Lates calcarifer) scales [8], chitosan (shrimp skin) obtained...
from Department of Aquatics Products Technology, Bogor Agricultural University (Bogor, Indonesia).

2.1 Synthesis of HA

HA was synthesized from 2 types of precursors, suspension of 0.5 M Ca(OH)$_2$ as calcium precursor from extract snail (*Bellamya javanica*) shells and 0.3 M (NH$_4$)$_2$HPO$_4$ as phosphate precursor. The solution of 0.3 M (NH$_4$)$_2$HPO$_4$ was dropped on suspension of 0.5 M Ca(OH)$_2$ at a rate of 1 ml/min, heated conditions kept around 40±2 °C, and stirred for 1 hour. The pH conditions were monitored by pH meter and 1 M NH$_4$OH solution was used for pH adjustment into final pH kept around 10. The mixture was decanted for 24 hours at room temperature, sonicated for 6 hours, centrifugated for 15 minutes with a speed of 4500 rpm and filtrated. The precipitate was dried at 80 °C for 8 hours and heated at 800 °C for 2 hours.

2.2 Preparation of HA/Col/Chi Composite Coating On Ti6Al4V

As much as 2 ml of collagen 2%, 2 ml of chitosan 2% mixed with 2 gram of HA powder was dissolved in 20 ml of the ethanol solution and stirred for 1 hour at room temperature. Ti6Al4V alloy with a thickness of 5 mm and a diameter of 12 mm smoothed with paper grinding (1200 grit) and immersed in 96% ethanol for 24 hours, sonicated for 1 hour in 0.1 M NaOH solution and dried for 24 hours at room temperature. Ti6Al4V immersed in a composite HA/col/chi solution at room temperature with variation of immersion time (5, 6, and 7 days) and then dried at room temperature. HA/col/chi coatings was characterized by SEM, XRD, corrosion resistance test (galvanostatic/potentiostatic), in vitro assay in NaCl 0.9% solution and calf pulmonary artery endothelium (CPAE) (ATCC-CCL 209).

2.3 In vitro Test

2.3.1 Release of Ca$^{2+}$ in NaCl 0.9%

HA/col/chi coatings immersed in NaCl 0.9% solution for 10 days. Immersion results were taken daily into 0, 1, 3, 5, 7, and 10 each 10 ml. Then that measured calcium content by using AAS ($\lambda = 422.7$ nm).

2.3.2 Cytotoxicity in calf pulmonary artery endothelium (CPAE) (ATCC-CCL 209) Cells [9]

The cells were grown using 6 well plates with $2 \times 10^5$ cells/wall and incubated for 24 hours at 37 °C with 5% CO$_2$. Meanwhile, samples of Ti6Al4V coated sterilized with gamma-ray irradiation doses of 15 kGy. Then sample was placed on a cultured CPAE cell (24 hours of cell culture). Endothelial cell culture without treatment was used as a negative control. Samples in the culture medium were then incubated for 72 hours at 37 °C with 5% CO$_2$. The number of cells is determined using a hemocytometer. Cell viability was examined with tripan blue dye and determined percent of its inhibition.

3. Results and Discussion

3.1 Phase Structure Analysis

Phase structure of HA/col/chi coatings was characterized by X-ray diffraction (XRD) shown in Figure 1. XRD results compared with JCPDS No. 00-009-0432 (HA) and JCPDS No. 00-044-1294 (Ti6Al4V). The diffraction peak of HA occurs shifts after coating. As shown in Figure 1b, a sharp peak and indicating the presence of HA at 2θ = 25.65$^\circ$ (002), 31.79$^\circ$ (211), 32.89$^\circ$ (300), 33.88$^\circ$ (202), and 49.34$^\circ$ (213). While the diffraction peak of Ti6Al4V appeared at 2θ = 35.18$^\circ$ (100), 38.29$^\circ$ (002), 40.20$^\circ$ (101), 53.01$^\circ$ (102), 76.82$^\circ$ (112), and 77.88$^\circ$ (201). The diffraction peak not show any peak diffraction of chitosan, collagen or other compounds. The diffraction peak in chitosan will appear when chitosan contained in the composite more than 30% [10]. The degree of crystallinity of HA/col/chi coatings was obtained 73.35 %.
3.2 Surface Morphologies

The surface morphology of HA/col/chi coatings observed using by SEM shown in Figure 2. The image shows the presence of granules showing of HA and its distribution less homogeneous. Also shows the shape of the chunks that distributed less evenly. In surface morphology, the presence of fibers, ie; collagen fibers (Figure 2c).

![Figure 2](image-url)

**Figure 2** The surface morphology of Ti6Al4V (a); HA/col/chi coatings magnification 200x (b); 500x (c); 1000x (d).
3.3 Measurement of Ca\(^{2+}\) Release in 0.9% NaCl Solution

HA/col/chi coatings was carried out measurement of release of Ca\(^{2+}\) by soaked sample in 0.9% NaCl solution for 10 days. The average concentration release of Ca\(^{2+}\) of shown in Figure 3 using by AAS was obtained 624.46 ppm and shown an increase Ca2+ concentration from day 1 to day 10. According Sharma, et al. in [11], calcium concentration increased due to ion exchange in SBF solution between the surface of the HA-coated and due to differences in chemical potential between HA with the metal release and increased Ca\(^{2+}\) concentration. According Gu, et al. in [12], reported the concentration of Ca\(^{2+}\) release in SBF, HA-coated for 56 days soaking obtained 54.4% and increased concentration on day 0 until day 14, and decreased concentration of Ca\(^{2+}\) after day 14 to day 56. The longer the immersion time causes the concentration of calcium ions to decrease exponentially and indicate continuous precipitation [13].

![Figure 3](image3.png)

**Figure 3** Concentration of Ca\(^{2+}\) release for 10 days

3.4 Corrosion test

The corrosion rate on uncoated Ti6Al4V obtained 0.4152 mpy (0.0105 mmpy) with \(I_{\text{corr}}\) 0.61 (\(\mu\text{A/CM}^2\)) and coated Ti6Al4V decreased obtained 0.3567 mpy (0.0090 mmpy) with \(I_{\text{corr}}\) 0.52 (\(\mu\text{A/CM}^2\)). The resultant corrosion rate under the permissible corrosion rate for implanted material and based on European standards of 0.457 Mpy (0.0116 mmpy). Inspection curves (Figure 4) of Ti6Al4V coated indicates a general tendency for potential values to be more stable and indicates excellent value. This indicates the formation of a major passive layer comprising TiO\(_2\) and an additional oxide such as Al\(_2\)O\(_3\) and V-oxide. Aluminum is capable of forming the Al\(_2\)O\(_3\) passive oxide layer on Ti6Al4V alloys.

![Figure 4](image4.png)

**Figure 4** Polarization curve of Ti6Al4V coated
3.5 *In vitro cytocompatibility assay on endothelial cell*

The cytocompatibility test aims to determine the viability of cells when there is direct contact with the sample and was performed in vitro on endothelial cell culture media. Endothelial cells are the main cells involved in the formation of blood vessels. HA/col/chi coatings as implants that accelerate the healing process should not cause cytotoxicity in endothelial cells. The morphology of endothelial cells in HA/col/chi coatings was observed for 24 hours (Figure 5c) and 72 hours after incubation (Figure 5d), that shown endothelial cells able to alive and adapted in the presence of samples. The results of the cytocompatibility test obtained percent inhibition which showed inhibition of cell growth due to exposure by the sample. According Matsuura, et al. in [14], the samples with percentage inhibitions exceeds 50% is toxic. The percent inhibition of HA/col/chi coatings obtained 33.33% that classified as non-toxic and qualifies as a biocompatible implant.

![Figure 5](image)

**Figure 5** Morphology of endothelial cells of 80x magnification on 24 h control (a); 72 h control (b); HA/col/chi coated Ti6Al4V for 24 h (c); HA/col/chi coated Ti6Al4V for 72 h (d)

4. **Conclusion**

Ti6Al4V alloy with HA/col/chi composite coatings was successfully performed with the best coated at immersion for 7 days. The corrosion resistance properties Ti6Al4V coated has better than Ti6Al4V
uncoated. Based on the corrosion resistance and in vitro test of in vitro endothelial cells, Ti6Al4V coatings is biocompatible and non-toxic for bone implant applications.

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