Synthesis of CPC/chitosan and its endurance test in simulated body fluid

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Abstract. Hydroxyapatite (HA) has been synthesized by solid state reaction method by using CaHPO₄ and CaCO₃ as reactants. The reaction was done at 1200°C for 7 hours in a local ceramic industry. The synthesized HA has Ca/P ratio of 1.7, crystallite size of 60 nm and morphology of rod-like shape. Then HA was used as a powder phase to produce calcium phosphate cement/chitosan (CPC/chitosan). A mixture of 2.5% Na₂HPO₄ and 1% chitosan solution was used as liquid phase. Structure and morphology of HA were slightly changed by the hardening of CPC/chitosan. Endurance test of CPC/chitosan in simulated body fluid (SBF) was performed for 36 days. CPC/chitosan was not washed out in the SBF. HA crystallinity as well as Ca/P ratio decreased by the testing time. The change of CPC/chitosan surface morphology into needle-like crystals indicated the surface growth of CPC/chitosan in SBF.

Keywords: hydroxyapatite, calcium phosphate cement, solid state reaction.

1. Introduction

Hydroxyapatite [HA, Ca₁₀(PO₄)₆(OH)₂] is the most stable calcium phosphate compound that has Ca/P molar ratio of 1.67 and theoretical density of 3.156 g.cm⁻³. The lattice crystal of HA is closed-paked hexagonal with lattice parameter of a=9.418 Å and c=6.881 Å [1]. Synthetic HA is commonly used as biomaterial for bone replacement due to its properties such as osteoconductive, biocompatible and bioactive. In vivo biocompatibility test of synthetic HA showed no systemic toxicity, inflammation and body response toward the tested animals [2]. HA also used as solid ionic, catalyst, drug delivery, fuel cell and chromatography column [3-5]. Several HA synthesis methods have been developed, such as solid state reaction [6], precipitation [7], sol-gel [8], hydrothermal [9], mechanochemical [10], electrochemistry [11] and microwave [12].

In this research, HA was synthesized by solid state reaction method by using solid phase of CaCO₃ and CaHPO₄ as reactants. The reaction was conducted in the additional furnace of Nuanza Keramik at 1200°C for 7 hours. Synthesized HA then used as powder phase in producing calcium phosphate cement (CPC). CPC has attracted biomaterial researchers due to its excellent properties as promising artificial bone material [13]. Those properties enable CPC to accelerate recovery process of damaged bones [14]. Recently, CPC has been counted as a local drug delivery to heal osteoporosis disease [15], inflammation [16] and bone cancer [17]. CPC has been considered as a carrier for chemotherapeutic agent and the result proved that the CPC morphology changed during drug release test. In this work, a small amount of chitosan was added to produce a composite of CPC/chitosan. Chitosan has been studied in an attempt to improve CPC mechanical strength [18]. The biocompatibility property of CPC/chitosan was tested by soaking hard cement in synthetic body fluid (SBF) which containing similar ion concentrations with human blood plasma. The structure of calcium phosphate was studied during testing time until 36 days.
Morphology of the composite’s surface was observed to analyse the ability of CPC/chitosan to induce a new bone.

2. Experimental Procedures

2.1. Production of HA with Solid State Reaction Method
HA was synthesized by solid state reaction of calcium hydrogen phosphate (CaHPO₄) and calcium carbonate (CaCO₃) (Merck). At first, CaHPO₄ was prepared by the slow reaction of calcium chloride (CaCl₂) (Merck) and dinatrium hydrogen phosphate (Na₂HPO₄) (Merck) with precipitation method. The synthesized CaHPO₄ and CaCO₃ with a Ca/P ratio of 1.5 were mixed by crushing them with mortar and then dispersed in ethanol (Merck) for 4 hours. The precipitate was filtered and dried at room temperature. The dried powder was shaped with pelletizer and heated in an additional furnace of a ceramic industry in Boyolali Indonesia at 1200°C for 7 hours. The product was analyzed by XRD to prove the formation of HA. XRD analysis was performed using the CuKα source operated at 30 kV and 30 mA. The diffractograms were recorded at 2θ=5–90° with a scan speed of 2°/min and a step angle of 0.02°.

2.2. Preparation of CPC and CPC/Chitosan
CPC was synthesized by mixing powder phase (synthesized HA) and liquid phase (solution of 2.5% wt of Na₂HPO₄, Merck) with a liquid/powder (l/p) ratio of 1. HA was crushed with mortar and sieved with 230 mesh sieve before used. CPC/chitosan was synthesized by adding 1% wt of chitosan (medium molecular weight, Aldrich) into HA and followed with the same procedure to synthesized CPC. The mixing of powder phase and liquid phase was conducted in a petri dish until it formed a paste. The paste was dried at room temperature to form a hard cement of CPC and CPC/chitosan. Structural transformation of CP, CPC and CPC/chitosan were studied by FTIR (Shimadzu) and XRD (Shimadzu XRD-7000). The mean crystallite size was calculated by Scherer equation.

2.3. Endurance test of CPC/chitosan in SBF
SBF was used to evaluate the behaviour of CPC/chitosan in human body environment. SBF has similar ion concentrations with human blood plasma [19]. Hard cements of CPC/chitosan were soaked in SBF for 1, 2, 3, 4, 8, 20 and 36 days. After soaking, CPC/chitosan was analysed with XRD (Shimadzu XRD-7000) to confirm the structural change and the CPC/chitosan surface was examined by SEM (JSM-6510LA) to see the calcium phosphate formation. Calcium phosphate compound was predicted by measuring Ca/P ratio using EDS (JSM-6510LA).

3. Results and Discussion

3.1. Study of CP, CPC and CPC/chitosan structure
Sudden contact of CaCO₃ and CaHPO₄ mixture with high temperature in furnace can change the lattice structure of CaCO₃ and CaHPO₄. At first CO₂ gas and H₂O was released from the reactant mixture and produced crystal defects in a form of hole. The heat energy has triggered vibrational exitation of CaCO₃ and CaHPO₄ lattice. The calcium and phosphorous ions moved in between oxygen ions and formed a stabel structure of calcium phosphate. The XRD data (figure 1) confirmed that the product is pure HA according to JCPDS no 09-0432.
Production of CPC was conducted by mixing of synthesized HA and Na$_2$HPO$_4$ solution with spatula until a stable paste was formed. While for CPC/chitosan, 1% wt of chitosan was added in HA and continued with same preparation step for CPC production. The encounter of powder and liquid phase led to the partly dissolution of powder phase then the pasta was formed. The required time to produce pasta was no longer than 1 minute. Then the paste transformed into hard cement by releasing H$_2$O molecules in an evaporation process that took 2 and 2.3 hours for CPC and CPC/chitosan, respectively.

Figure 2 shows the FTIR spectra for HA, CPC and CPC/chitosan. The three spectras show identical patterns of specific peaks of HA according to previous research Vijayalakshmi and Rajeswari [20]. Peak at 956.69 cm$^{-1}$ is the vibration adsorption $\nu_1$ of PO$_4$. Vibration $\nu_3$ and $\nu_4$ appear at wave number of 1041.56 and 570.93 cm$^{-1}$, respectively. A broadband at 3448.72 cm$^{-1}$ and a band at 1635.64 cm$^{-1}$ indicate the presence of water in the HA and CPC. The spectra of HA and CPC show the appearance of CO$_3$ at wave number of 1519.91 and 1543.05 cm$^{-1}$. The present of CO$_3$ in product of HA synthesized by the solid state method was very commanding, due to the presence of CO$_2$ in the atmosphere. The peaks CO$_3$ didn't appear at CPC/chitosan spectra.
XRD diffractogram of HA, CPC and CPC/chitosan indicated that high peaks were occurred at $2\theta=25$-$35^\circ$ (figure 3). The pattern of HA and CPC diffractogram were similar. This signifies that the HA crystal structure was not altered by dissolution and evaporation process. The strong peaks at $2\theta=31.74$, $32.18$ and $32.85^\circ$ indicated that HA and CPC contain of pure HA base on JCPDS card no. 09-0432. Those angles derived from x-ray diffraction of (211), (112), and (300) HA crystal planes. The diffractogram of CPC/chitosan was quite different from HA’s and CPC’s. Diffraction angles were shifted about $0.2^\circ$. The peaks were appearing at $2\theta=31.95$, $32.38$ and $33.07^\circ$. This fact indicates that compositing HA and chitosan cause the distance of the HA’s crystal plane becomes closer. The mean HA’s crystallite size for HA and CPC were measured as 60 nm and 61 nm, respectively. This value decreased to 57 nm for CPC/chitosan.

3.2. Surface morphology and Ca/P ratio of HA, CPC and CPC/chitosan
The surface morphology of HA, CPC and CPC/chitosan can be seen in figure 4. The SEM photograph indicates that HA’s grain was partially melted due to high temperature treatment at the solid state reaction (figure 4a). Adding liquid phase to HA caused partial dissolution of HA’s grain and produced a supersaturated solution. When water molecules evaporated from the supersaturated solution, the recrystallization process of HA occurred. Figure 4b shows the formation of irregular small grain of HA as paste transformed to hard CPC. The basic shape of CPC then looked clearly as irregular cuboid. The present of chitosan in CPC/chitosan changed the morphology. The grain distance became closer (figure 4c). From EDS analysis, it was known that Ca/P ratio of synthesized HA, CPC and CPC/chitosan were 1.7, 1.57 and 1.65, respectively. However, those values are close to the Ca/P molar ratio of HA 1.67.
3.3. Endurance test of CPC/chitosan in SBF

The endurance test of CPC/chitosan in SBF was done for 36 days. The purpose of this test was to identify the ability of CPC/chitosan to stand in aqueous environment. It is shown that clarity of SBF did not shift during the test and CPC/chitosan was not dissolved in SBF (figure 5). CPC/chitosan still intact and have not washed out after 36 days of biocompatibility test.

![Figure 5](image)
(a) (b) (c)

**Figure 5.** Endurance test of CPC/chitosan in SBF for (a) 6, (b) 20 and (c) 36 days.

XRD diffractogram of CPC/chitosan after soaking in SBF for 1–36 days can be seen in figure 6. By observing peaks at 2θ=25-35° it is known that the HA structure has not changed, but its crystallinity was decreasing with the increase of soaking time. At 36 days, HA lost its crystallinity or transformed into amorphous phase. The crystallite size of HA tends to decrease by testing time. The crystallite size fluctuates from 24.47 to 57.08 nm during 20 days of testing (figure 7). This result was in accordance with the HA crystallite size in human bones which is about 40 nm. The lowest crystallite size was happened on 8 days of testing.

![Figure 6](image)

**Figure 6.** XRD diffractogram of CPC/chitosan after endurance test in SBF.
Figure 7. The crystallite size of HA after endurance test.

Figure 8. SEM Image of CPC/chitosan surface after (a) 4, (b) 8, (c) 20 and (d) 36 days of biocompatibility test.

CPC/chitosan surface morphology was investigated by SEM. On four day endurance test (figure 8a), the CPC/chitosan surface looked clean without small particles which previously covered the surface of the CPC/chitosan (figure 4c). The small particles of calcium phosphate were dissolved in SBF. After 8 days, calcium phosphate in a form of needle-like crystal grew on CPC/chitosan surface. The growth crystals were in submicron dimensions as indicated in figure 8b. This crystal was getting thicker and denser on 20 day test (figure 8c). On 36 day test, the crystals were continuing to spring up and merged with CPC/chitosan. As the result, CPC/chitosan surface become denser (figure 8d).

Table 1 shows Ca/P ratio of CPC/chitosan after endurance test. The Ca/P ratio tended to decrease by the increase of testing time. The encounter of calcium and phosphate ions in SBF with CPC/chitosan
surface induces the deposition of calcium phosphate compound. The attraction of CPC/chitosan surface to phosphate ions was greater than calcium ions. Increasing the number of P lowered the Ca/P ratio. The EDS data for 36 days of testing showed the emergence of atoms that were previously not found on the 1-20 days. The atoms were Na (1.18%), Mg (0.05), and K (0.04%) which were possible to balance the ions on the surface of the CPC/chitosan and in the SBF solution.

Table 1. The Ca/P ratio of CPC/chitosan during endurance test.

| Days | Ca/P Ratio |
|------|------------|
| 0    | 1.65       |
| 4    | 1.33       |
| 8    | 1.32       |
| 20   | 1.28       |
| 36   | 1.22       |

4. Conclusions
Structure and morphology of HA that syntesized by solid state method at ceramic industry were slightly changed when used as powder phase of CPC. Endurance test of CPC/chitosan in simulated body fluid (SBF) showed that CPC/chitosan was not washed out in aqueous environment. HA crystallinity as well as Ca/P ratio decreased by the testing time. The change of CPC/chitosan surface morphology into needle-like crystals indicated the surface growth of CPC/chitosan in human body fluid. It can be concluded that the CPC/chitosan can be used as an artificial bone material.

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