Formation and characterization of collagen and hydroxyapatite composite

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Abstract. The formation of composite of collagen and hydroxyapatite (HA) was investigated by ex-situ precipitation method. Synthesis of HA was carried out by precipitation of Ca(OH)$_2$ 1 M and (NH$_4$)$_2$HPO$_4$ 0.6 M with the molar ratio of Ca/P 1.67, the mixture was exposed to microwave irradiation (450 W) for 45 min. Subsequently, the result was sintered to produce the powders of HA. Verification of HA powders was performed using X-Ray Diffraction (XRD). Chicken collagen was prepared in our laboratory (Physics Department, Universitas Indonesia). Composite was synthesized by ex-situ precipitation method with a mass fraction of collagen and HA was 2:1. The mixture was stirring continuously for 2 h and aged for 20 h at room temperature. The precipitate was dried by freeze-drying method. The composite result was examined by Scanning Electron Microscopy (SEM). XRD pattern of sintered powder shown as HA crystal. The unit cell of HA crystals was evaluated by High Score Plus Program (a=b=9.425 and c=6.884) with the accuracy 98.175% for a and b lattice and 98.406% for c lattice. The collagen had been verified by SDS PAGE as type 1 collagen and the micrograph illustrated the presence of micropores. Based on SEM examination, the composite revealed that HA had been deposited onto collagen and filled part of void space of collagen. The preliminary study will be beneficial for leading the formation of composites of collagen-HA as biomaterials.

1. Introduction
Native bone is a complex living tissue with a mineralized extracellular matrix. The bone composition generally consists of living bone cells (osteoblast, osteoclast, and osteocytes), 60% inorganic materials, 30% organic materials and water [1]. The morphology of the bone is a porous area with 50-90% porosity and has a density at average about 1.85 gr/cm$^3$. Mechanical properties of bone are reported to have 7-16 GPa for Young Modulus depending on the age of bone. Bone has a high risk of tissue damage because of the bone functions as a support of the body and enables body movements. The bone defect can be caused by trauma, infection, disease, and bone cancer. One of the healing the bone defect is using the bone graft. The natural bone grafts which derived from natural bone have several disadvantages, such as the limitation of the donor bone, autoimmune reactions and disease.
spread [2]. Therefore, a synthetic bone graft is designed as an alternative needed that can resemble the native bone.

The synthetic bone graft should be biocompatible, osteoconductivity, biodegradable and biomimetic characteristic with the surrounding the living tissue [1]. One of the synthetic bone graft approaches is based on the composition of the bone. Hydroxyapatite and collagen are bone forming biocomposites. Hydroxyapatite (HA) is a bioceramic material that has a similar chemical composition and structure to the components of the bone mineral [1,3]. Pure HA has limitations that are hard and brittle. Reducing these properties are necessary to modify by adding a polymer as a matrix. The combinations of mineral compound and polymer were called composites. Composites were potentially used as bone graft material. Furthermore, smaller crystalline sizes can produce homogeneous bone mineral resorption. HA nanocrystal is expected to have homogeneous resorption and better bioactivity than bulk crystals. Microwave synthesis of HA has several advantages including shorter synthesis time, rapid heating, fast reaction, easy reproducibility, narrow particle distribution, high yield, high purity, efficient energy transformation and throughout volume heating [4].

Collagen is an organic component of bone which is also the main fibrous protein and the main component of the extracellular matrix. Type 1 collagen is the most widely used as a matrix system for the new bone formation [5]. Moreover, the morphological characteristics of type 1 collagen are porous materials that are useful for cell migration and cell proliferation [1]. The combination of mineral and collagen makes a strong bone without brittle and can stimulate hard tissue regeneration. The use of synthetic HA and collagen composites is expected to high biocompatibility properties because the native bone is a calcium phosphate and collagen composite. The presence of collagen was a matrix for mineral growth.

The formation of HA and collagen has been carried out by in-situ precipitation with HA crystallization into the collagen matrix [1]. Mineralization process is caused by precipitation with HA crystal and collagen. This work is a preliminary study conducted on the formation of collagen-HA composite with ex-situ precipitation method. The objective of the study is to investigate the formation of composites through the precipitation reactions by combining HA crystals in the collagen solution. The composite formation was evaluated with morphology at Scanning Electron Microscopy (SEM). The preliminary study will be beneficial for leading the formation of composites of collagen-HA as biomaterials.

2. Materials and Methods

The chemicals that were used for the HA synthesis were Ca(OH)\(_2\) and (NH\(_4\))\(_2\)HPO\(_4\). All chemicals were analytical grade and are from Merck-Germany. Collagen was originated from chicken and was prepared in our Laboratory (Physics Department, Universitas Indonesia). Synthesis of HA was carried out by precipitation of Ca(OH)\(_2\) 1 M and (NH\(_4\))\(_2\)HPO\(_4\) 0.6 M with the ratio of Ca to P to be 1.67 (a ratio stoichiometric of hydroxyapatite). Phosphate solution was titrated to the calcium solution at an average flow rate of 5 mL/min with continuously stirring for 2 h. The mixture was exposed to microwave irradiation (Sharp, R-728(W)-IN 900 W) with 450 W for 45 min. Subsequently, the result was sintered at 900 °C for 5 h to produce the powders of HA.

Verification of HA powders was performed using X-Ray Diffractometer (PANalytical X’Pert Pro MPD) for the crystal phase and crystallite size. The data were collected in the 20 range of 10\(^0\) – 90\(^0\) with step size 0.02\(^0\) and time per step 0.05 s using 0.02 mm Ni-filtered with a monochromator Cu K\(\alpha\) (\(\lambda\text{Cu K}\alpha = 1.549508 \text{ Å, setting the radiation at 40 kV and 30 mA}\)). High Score Plus Program was used to evaluate the crystal phase, crystal size and unit cell of HA.

Composite was synthesized by ex-situ precipitation method with a mass fraction of collagen and HA was 2:1. Collagen powders were added into HA solution spoon by spoon. The mixture was stirring continuously for 2 h and aged for 20 h at room temperature. The precipitate was filtered with Whatman paper no. 42 and lyophilized at -45.3 °C with vacuum gauge 31.2 Pa (Eyela FD 1000 Freeze Dryer) for 24 h.
The composite result was examined by Scanning Electron Microscopy (Quanta 650). The morphology of composite formation was evaluated with low magnification at 2000X, and 5000X. Compositional determination of the composite was performed by energy dispersive X-Ray analysis (EDAX) fitted with a dual beam SEM system.

3. Results and discussion

X-Ray pattern of sintered powder shown as HA crystal on Figure 1. The unit cell of HA crystals was evaluated by High Score Plus Program (a=b=9.425 and c= 6.884) with the accuracy 98.175 % for a and b lattice and 98.406 % for c lattice. All the patterns showed the sharp and narrow characteristic peaks indicated high crystallinity with the crystallite size was 57.32 nm. Sintered powder has similar apatite to the hexagonal HA based on the JCPDS (Joint Committee on Powder Diffraction) card no 09-432.

![Figure 1. XRD spectra of sintered powder HA](image)

Chicken collagen had been verified by SDS PAGE (Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis) as a type 1 collagen based on the molecular weight was more than 120 kDa which had α1 chain [6,7]. Furthermore, the micrograph of the collagen on Figure 2 illustrated the presence of macropores at low magnification (200X and 500X). The average of diameter sample was 35.701 ±14.822 (µm).

![Figure 2. SEM micrograph of chicken collagen at a) 200X and b) 500X magnification.](image)
Figure 3 recorded the micrograph of samples at different magnifications as a function of the morphology and the size of the deposited HA particles. After the mineralization of an isolated collagen matrix, a homogenous HA deposition was observed at 2000X magnification. In mineralized hydrolysate – enriched matrices, the morphology was dependent on the hydrolysate content. An increase in the amount of hydrolysate induced a higher content of fine acicular HA, which is unusual in pure collagen matrix mineralization [8]. Based on SEM examination the composite revealed that HA had been deposited onto collagen and filled part of void space of collagen.

![Figure 3](image_url)

**Figure 3.** Micrograph of composite HA-collagen at: a) 2000X of sample 1, b) 2000X of sample 2, c) 5000X of sample 1, and d) 5000X of sample 2.
Elements composition of the composite were examined by Energy Dispersive X-Ray analysis (EDAX) for the sample 1 and 2. Figure 4 recorded EDAX spectra of these two samples. The results indicated that the molar ratio of Ca to P was 1.67 in both samples. Moreover, this method conducted that ex-situ precipitation method could produce pure crystal HA with the ratio Ca to P was similar to stoichiometric HA.

4. Conclusions
In this work, formation of composite collagen and HA was produced by ex-situ precipitation method. The obtained HA crystals were deposited onto collagen matrix and the morphology was dependent on the hydrolysate content. This work is a preliminary study and will be continued with investigating the formation of composite HA – collagen with several methods. Further information, this composite will be a candidate of biomaterials.
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