Research Article

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Biocompatibility and osteoconductivity of scaffold porous composite collagen–hydroxyapatite based coral for bone regeneration

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Abstract: The synthesis of collagen–hydroxyapatite composites has been carried out, and the biocompatibility and osteoconductivity properties have been tested. This research was conducted to determine the ability of hydroxyapatite–collagen composites to support the bone growth through the graft surface. Hydroxyapatite used in this study was synthesized from coral with a purity of 96.6%, while collagen was extracted from the chicken claw. The process of forming a scaffold of collagen–hydroxyapatite composites was carried out using the freeze-drying method at –80°C for 4 h. The biocompatibility characteristics of the sample through the cytotoxicity tests showed that the percentage of viable cells in collagen–hydroxyapatite biocomposite was 108.2%, which is higher than the percentage of viable cells of hydroxyapatite or collagen material. When the viable cell is above 100%, collagen–hydroxyapatite composites have excellent osteoconductivity as a material for bone regeneration.

Keywords: biocompatibility, osteoconductivity, composite, coral

1 Introduction

The bone defect is a disease associated with the functional disability that has a serious impact on the quality of life of patients. This can be caused by trauma, tumors, or other bone diseases [1]. The Ministry of Health of the Republic of Indonesia in 2013 stated that traffic accidents in Indonesia have increased every year by 21.8% in 5 years. There are many victims of injuries and bone fractures from the incidents that occurred. According to the data from the Hospital Information System in Indonesia in 2018, cases of fractures have increased by 105% compared to that in 2013 [2]. Under these conditions, a biomaterial is needed as a substitute for the bone (bone graft). The need for biomaterials as a substitute for the bone will increase with the increasing number of cases of bone damage due to trauma, tumors, congenital abnormalities, infections, and bone resorption due to complications in the installation of joint prostheses [3].

Several types of bone grafts are used in the treatment of bone defects – autograft, allograft, and xenograft. Autograft is the "gold standard" for bone growth in the spine because autograft has osteoconduction, osteoinduction, and osteogenesis properties [4]. Although autograft is an ideal bone graph, autograft cannot be used in large bone defects. A large size bone will cause large morbidity at the place where the bone was taken [5]. In addition, several autograft limitations that need to be considered include blood loss, wound infections and complications, prolonged pain, and local sensory loss. Allograft and xenograft are alternatives to the use of autograft. Both these procedures do not require surgery to get a bone graft from another part of the patient's body. So they do not require two surgeries similar to autograft and can reduce pain. However, an allograft can be rejected by the immune system and the transmission of diseases such as HIV and hepatitis C, while xenograft has an immunogenic risk and risk of the transmission of zoonotic diseases [6]. In addition, the sterilization process carried out in allograft and xenograft also results in the nature of osteogenesis being completely lost, and hence, all bone cells would have died [7].

The requirements for synthetic bone grafts that must be met are being acceptable to the body (biocompatible)

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and advantageous to the osteoconduction process (guiding the reparative growth of natural bone), osteoinduction (encouraging differentiated cells to active osteoblasts), and osteogenesis (living bone cells in the material bone graft that contributes to bone remodeling) [4]. Osteoconductive and osteoinductive are the most important properties of resorbable biomaterials to direct and encourage the formation of the tissue growth [8]. Osteoconductive and osteointegration of a bone graft are related to the porosity and pore size [9].

Based on the previous research, the minimum requirements for the pore size are around 100 μm [10]. This corresponds to the cell size, requirements for migration, and cell transport. However, a pore size of 300 μm is recommended for increasing new bone formation and capillary formation [11]. High macroporosity can increase bone formation, but porosity that is higher than 50% can result in the decreasing of the mechanical properties of a biomaterial [6].

A good synthetic bone graft is a bone graft that has a chemical structure and composition similar to the natural bone [12]. The collagen–hydroxyapatite (HA) composite is a synthetic bone graft that has similar properties to the natural bone. As is already known, bone's main components are hydroxyapatite (67%) and collagen (28%) and the other component is noncollagen protein [13]. Hydroxyapatite has a modulus elasticity of 73–117 GPa, a compressive strength of 600 MPa, and a density of 3.1 g/cm^3, while the modulus elasticity of collagen is 46.5 MPa [14]. Based on its mechanical properties, hydroxyapatite provides stiffness and hardness, while collagen provides tensile strength and flexibility. Collagen–HA composites have good biocompatibility and are osteoconductive, so collagen–HA composites are suitable for using as a scaffold for the bone tissue engineering [15].

The method to obtain composites that have the same structure and the composition as a natural bone is by collaborating several methods of synthesis. One of the keys to the synthesis of macroporous composites can be done through variations in the freezing rates [2]. The most appropriate synthesis method for fabricating porous biomaterials is the freeze-drying method. In this method, controlling the growth of ice crystals is very important to obtain the appropriate pore diameter and shape because the pore structure is a replication of the ice crystal dendrite entrapment.

2 Materials and methods

2.1 Synthesis of hydroxyapatite from marine coral

In this study, hydroxyapatite was synthesized from marine coral. Fossilized coral is used because this coral has a higher calcium content than that of a marine coral. The coral is taken from the southern coast of Java Island (Popoh Tulungagung beach) as shown in Figure 1. Then, the coral is cleaned and dried in an open space. Corals are manually crushed to become smaller particles. The crushed corals are calcined at 900°C to remove some unnecessary elements or compounds and form CaO compounds. Then, the calcined coral was mashed using a mortar so that the size of the coral became powdered and filtered using a 200 mesh or 74 m sieves. After that, it is smoothed using high-energy milling (HEM type E3D) for 2 h with dry milling, so that the particles are reduced to a smaller size and formed as $Ca(OH)_2$. The synthesis of hydroxyapatite is carried out by reacting calcium hydroxide and phosphoric acid (H_3PO_4, 85% Aldrich-Sigma) as stated in equation (3).

2.2 Collagen extraction from chicken claws

In this study, collagen was extracted from broiler chicken paws obtained at the Wonokromo Surabaya

Figure 1: The process of scouring the coral becomes powder.

market, East Java, Indonesia. Chicken claws were separated from the bones by being cut using a knife to facilitate the destruction process. The claw pieces were crushed with a blender. Then, the crushed chicken claws were soaked in HCl solution for 24 h with 5 wt/vol% ratio. Soaking process was carried out in the cold environment of the refrigerator and then filtered. Liquid filtrate was mixed with 0.1 N NaOH solution until it reached a neutral pH and was then allowed to stand until the collagen agglomerated. When approaching neutral pH, collagen clots can be observed and the fibers began to form and coalesce, and hence, clots appeared more clearly. Collagen clots formed perfectly at neutral pH (pH 7). The chemicals used were Sigma–Aldrich HCl 37% and NaOH with 97% purity.

2.3 Synthesis of collagen–hydroxyapatite composite

The synthesis of collagen–hydroxyapatite composite begins by forming hydroxyapatite solution and collagen solution. Collagen solution is a dissolved collagen in acetic acid, while the hydroxyapatite solution is hydroxyapatite, which was dissolved in the phosphoric acid. The hydroxyapatite solution and the neutral collagen solution are mixed while manually slowly stirring them. The mixture was put into a cylindrical tube and then frozen at −80°C in freeze-drying with a variation of freezing time of 2, 4, and 6 h. Dried collagen–hydroxyapatite composites were removed from the mold for characterization.

2.4 Biocompatibility test

The biocompatibility of biomaterials was tested by the MTT assay method. The MTT method tests (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) to measure the number of cells that still survive without the need for calculations. Cells that live with active metabolism can convert MTT into purple formazan products with maximum absorption at a wavelength of 570 nm, while dead cells lose the ability to convert MTT to formazan. Thus, the formation of color serves as a marker for viability cells [16]. The results of the MTT assay are shown in Figure 2. Biomaterials are considered toxic if the cell death is more than 50% and the number of viable cells is less than 50%. The increase or decrease in the number of viable cells indicates the level of toxicity of a biomaterial. Biomaterials have better biocompatibility if the number of proliferating cells is higher or the inhibitory value is lower [17,18].

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and discussion

3.1 Results of hydroxyapatite made from coral

The preparation of coral-based hydroxyapatite is carried out using the precipitation method. Coral is used because this material contains $CaCO_3$ of 95.5%, $MgSiO_3$

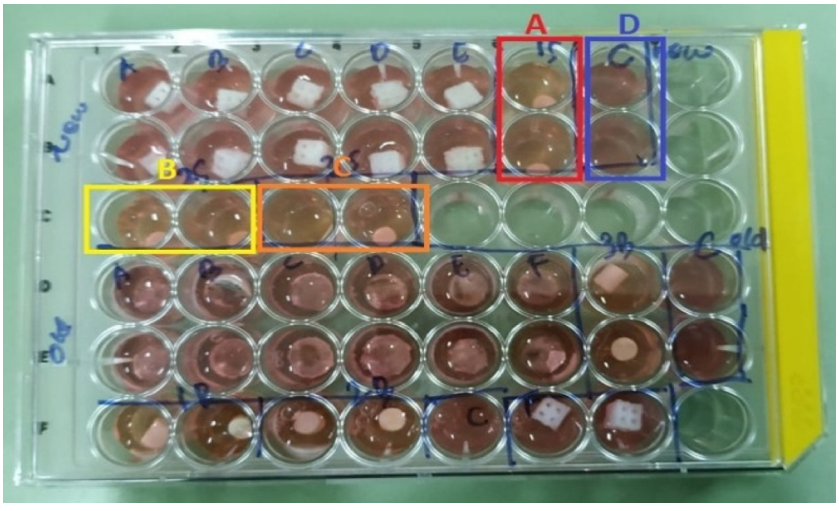

Figure 2: Incubation of collagen-composite biomaterials.

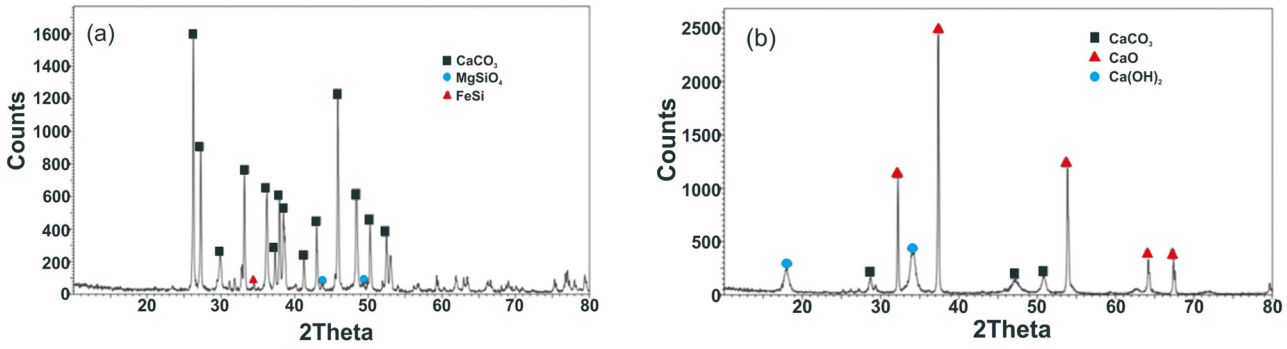

Figure 3: XRD of (a) coral and (b) heated coral at 900°C for 3 h.

of 3.8%, and FeSi of 0.7%, as indicated by the X-ray diffraction (XRD) spectrum in Figure 3a.

Coral that is heated at 900°C for 3 h produces new compounds, namely, calcium oxide (Lime, syn) CaO and calcium hydroxide $Ca(OH)_2$. The presence of $Ca(OH)_2$ in this process is thought to be due to the furnace not in a vacuum state, and hence, when the temperature is reduced to the ambient temperature, a reaction between water vapor and CaO occurs. The remaining $CaCO_3$ (aragonite) compound in this heating is 8.9%. Silicate compounds are not detected in XRD peaks as in the results of the pure coral spectrum; however, they have good thermostability. The invisible height of the silicate compound is assumed to be of an insignificant quantity and the amount of calcium phosphate which is very dominant. To improve the efficiency of the reaction of hydroxyapatite formation, milling for 20 h is carried out on coral powder, so the powder size becomes smaller. During the milling process, CaO reacts with H_2O to form $Ca(OH)_2$ as shown in Figure 4a.

The formation of the coral hydroxyapatite by the precipitation method can be described as follows.

$$CaCO_3(s) \rightarrow CaO(s) + CO_2\ (g) \quad (1)$$

$$CaO(s) + H_2O(l) \rightarrow Ca(OH)_2(s) \quad (2)$$

$$10Ca(OH)_2(s) + 6H_3PO_4(l) \rightarrow Ca_{10}(PO_4)_6(OH)_2(s) + 18H_2O(l) \quad (3)$$

Sintering of precipitation results is carried out at 900°C. The identification of the XRD spectrum in Figure 4b produces hydroxyapatite compounds of 96.6% and tri-calcium phosphate compounds of 3.4%.

3.2 Results of collagen extraction

Collagen in this study was obtained by extracting chicken claws. Fourier-transform infrared spectroscopy (FTIR) collagen extraction from chicken paws is shown in Figure 5.

The main markers used for the collagen identification are amide A, amide I, amide II, and amide III. The peaks of 3369.64, 3439.08, 3126.61, and 2445.74 cm^{-1} are the amide A absorption area at the wavenumber of 3,600–2,300 cm^{-1}, which indicates the presence of N–H bond. The peak at the wave number 1649.14 cm^{-1} indicates the presence of C=O bonds, which is the amide I absorption area. The amide I absorption area is in

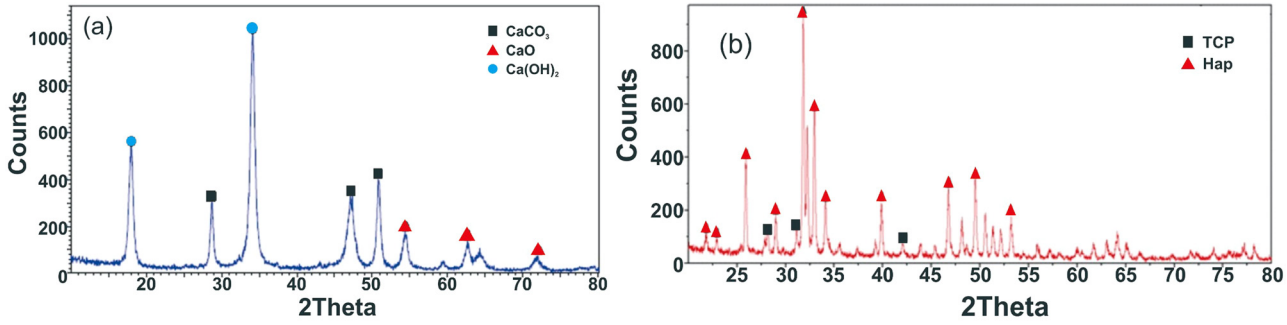

Figure 4: XRD of (a) coral milling for 20 h and (b) synthesized hydroxyapatite.

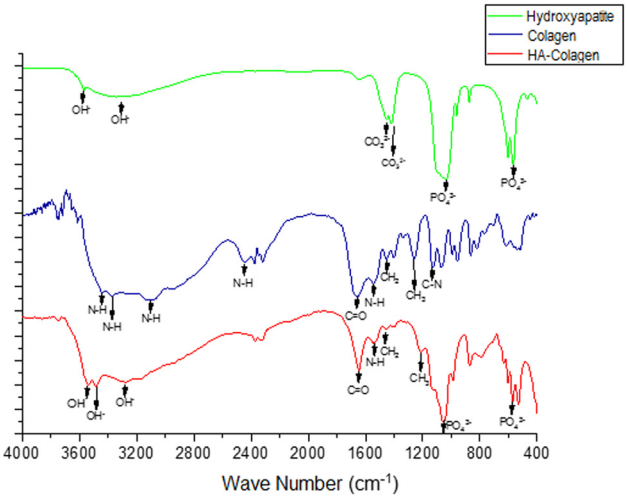

Figure 5: FTIR of HA, collagen, and collagen–HA composite.

the wavenumber 1,636–1,661 cm^{-1}. Meanwhile, the peak at the numbers 1544.98, 1454.33, and 1402.25 cm^{-1} is the amide II absorption area. The wave number of 1544.98 cm^{-1} indicates the presence of N–H bonds, while wavenumbers of 1454.33 and 1402.25 cm^{-1} also indicate the amide II uptake regions that have CH_2 and CH_3 bonds. The four markers are also found at the peak of absorption of pure collagen, especially collagen type I, as shown in Figure 6.

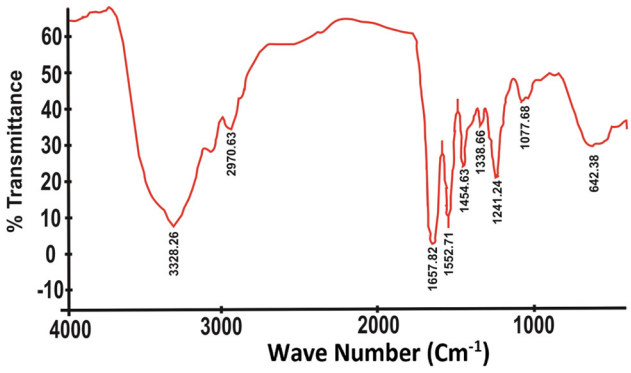

Figure 6: FTIR of pure collagen [17].

3.3 Results of the collagen–hydroxyapatite composite

Collagen–hydroxyapatite composites were synthesized using the freeze-drying method with a freezing time of 4 h. The FTIR results of this composite are stated in Figure 5. The characteristic peak of hydroxyapatite is at a wavelength of 500–600 cm^{-1}. The FTIR of the collagen–hydroxyapatite composite was found at a wavelength of 553 cm^{-1}. The characteristic peak of collagen was found at 2,873 cm^{-1} for CH stretching, 1,716 cm^{-1} for group C═O, and above 3,000 cm^{-1} for N–H. Amide I bending occurs between a wavelength of 1,600–1,700 cm^{-1}, and $(PO4)^{3-}$ bending occurs between 900 cm^{-1}–1,200 cm^{-1}.

The possible bond between collagen and hydroxyapatite is a hydrogen bond because collagen including protein and hydroxyapatite is ceramic. Hydrogen bonds occur between the hydrogen atom in collagen with the oxygen atom in hydroxyapatite as shown in Figure 7.

Mixing hydroxyapatite and collagen results in mineralized collagen by hydroxyapatite. Mineralized collagen occurs because of interactions that arise between the structure of collagen and hydroxyapatite crystals. This interaction occurs between the carboxylic group and the Ca^{2+} cation, which is illustrated in Figure 8. This hypothesis is supported by the FTIR data, which is explained based on the C–O and C═O band spectra of pure collagen and collagen–hydroxyapatite composites [19].

The synthesis of the collagen–hydroxyapatite scaffold was carried out using the freeze-drying method. The freezing time during freeze-drying affects the porosity of the scaffold formed. In this research, the freezing time is carried out in 2, 4, and 6 h. The measurement data and the calculation of porosity with freezing time are presented in Table 1. Composite porosity formed by freezing for 2 h has the highest porosity compared to porosity formed by freezing for 4 and 6 h. The greater the percentage of porosity, the easier the cell proliferation, but it is recommended not to exceed 60% because it will

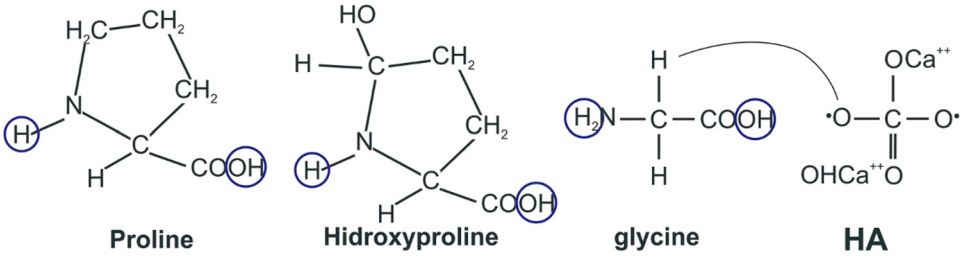

Figure 7: Bonding of hydrogen atoms in collagen.

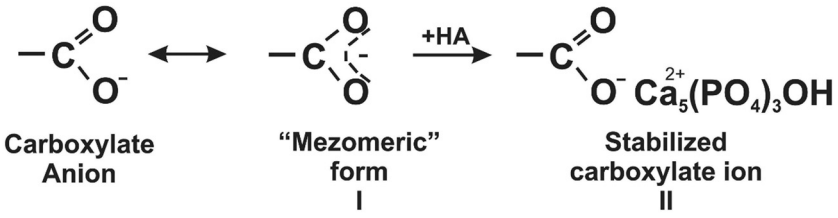

Figure 8: The mezomeric form of the stable carboxylic group during mineralization.

Table 1: Porosity scaffold with various freezing time

Quantity	Freezing time, 2 h	Freezing time, 4 h	Freezing time, 6 h
Porosity	52%	35%	27%

affect the physical and mechanical properties. The presence of pores in the scaffold is useful for facilitating the transport of oxygen and cell nutrition [20].

The results of cytotoxicity tests on samples of collagen, hydroxyapatite, and collagen–hydroxyapatite composites are shown in Figure 9. The graph of the MTT test results shows that collagen and hydroxyapatite are not toxic because the percentage of living cells is above 100%. The percentage of living cells above 100% also indicate cell proliferation in the MTT process. The occurrence of cell proliferation in collagen and hydroxyapatite is thought to be caused by two components that have osteoinductive factors, namely, bone morphogenetic proteins (BMPs) [21]. Type 1 collagen has BMP7 and hydroxyapatite has BMP2 [22].

Collagen–hydroxyapatite composites increase the percentage of living cells [22]. This proves that using collagen and hydroxyapatite together has benefits in terms of the cell growth. These MTT data also show that hydroxyapatite, collagen, or combined collagen–hydroxyapatite has an excellent biocompatible property. In addition, the cell growth above 100% can be interpreted that the collagen–hydroxyapatite composite is a very good medium for the growth and the development (osteoconductive) of bone cells. To strengthen this suspicion, currently, researchers are still conducting in vitro collagen–hydroxyapatite composites using the alkaline phosphatase test.

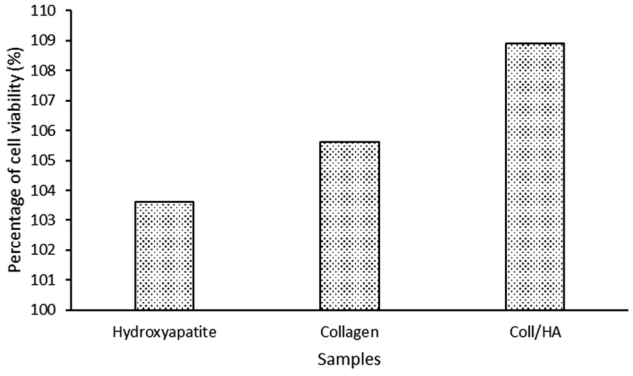

Figure 9: MTT assay of samples.

4 Conclusion

From the results of the preparation of hydroxyapatite-based coral and its composites with collagen, the following conclusions can be made. First, coral can be used as a bone replacement biomaterial because it can produce calcium phosphate in the form of 96.6% hydroxyapatite and 3.4% tricalcium phosphate. These two compounds are essential parts of the compact bone and the cancellous bone. Second, the MTT assay test results showed that cell viability in hydroxyapatite, collagen, and collagen–hydroxyapatite composites were 103.6%, 105.6%, and 108.9%, respectively. This shows that collagen and hydroxyapatite and its composites are nontoxic and biocompatible. Cell viability percentage values above 100% also indicate the cell proliferation in the MTT process. Cell proliferation in collagen and hydroxyapatite is thought to be caused by the two ingredients having osteoconductive and osteoinductive factors, namely, BMPs.

Conflict of interest: The authors declare no conflict of interest.

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