Sonochemical Synthesis of Nano-Hydroxyapatite/Chitosan Biomaterial Composite from Shellfish and Their Characterizations

Hartatiek¹,², P Dwiasih¹, Yudyanto¹, N Hidayat¹, R Kurniawan¹, Masruroh²

¹Department of Physics, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang, Indonesia
²Department of Physics, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya Malang, Indonesia

*Corresponding author’s email: hartatiek.fmipa@um.ac.id

Abstract. In general, seashells can contain calcium up to 97.19%. Therefore, it is potentially as a calcium source for nano-HA production. In this work, nano-HA was produced by sonochemical method with a period of sonication for 2 hours. Furthermore, nano-HA was composited with chitosan by a sonochemical method with a period of sonication for 2 hrs. Some fundamental characterizations were conducted to reveal the crystal structure, particle size, lattice parameters, microstructural morphology, and particle size by means of XRD, SEM, and EDX, respectively. Our results clearly showed that this desired crystalline of the composites had been successfully formed. Nano-hydroxyapatite with particle size 25.03 nm and Ca/P ratio 1.65 have been formed. The SEM result showed that the morphology was uniform and the composite had particles with the needle-like shape. The result from the synthesis of nano-HA/chitosan is recommended for biomedical application as bone filler.

Keywords: Hydroxyapatite, chitosan, biomaterial, composite, shellfish

1. Introduction
Hydroxyapatite (HA) is the central compound of human bone, typically 70% of the human bone matrix contains nanocrystalline HA with a length of 20-80 nm and a thickness of 2-5 nm [1]. HA becomes very important to be studied, especially for bone regeneration because it has bioactive and osteoconductive characteristics [2,3]. HA has excellent biocompatibility properties because it has similarities with human bone tissue chemically and biologically [4]. In addition, HA is able to construct chemical bonds directly with neighboring tissues, which have nontoxic, noninflammatory, nonimmunogenic characteristics [5]. Here, nano-sized HA shows bioactive better than micrometer-sized [6].

The natural bone tissue generally consists of nano-hydroxyapatite (n-HA) which are distributed in the collagen [7]. HA has been a composited using a polymer matrix, such as high-density polyethylene, polymethyl methacrylate, and poly-L-lactide to emulate natural bone bio-composites [8]. Furthermore, natural biopolymers attract more attention in biomedical applications due to excellent biocompatibility and biodegradation [9]. Chitosan (CS) is one of the interesting biopolymers due to its renewable characteristic. Previous studies suggested the use of CS as a composite material in tissue engineering due to similar chemical properties, compatible with tissue, bio-resorbability, anti-bacterial activity,
hemostatic characteristics and the ability of chemical modification when interacting with HA [10-13]. In addition, the biological characters of CS make it a perfect constituent of implant materials in composites with HA [14]. These superior properties make HA/CS becomes an ideal candidate for bone reconstruction and regeneration, especially for scaffold material. This three-dimensional product has the ability to increase bone tissue formation. Several methods are used for the preparation of HA/CS composite. Among the preparation methods, simple mixing method is one of the most popular methods. Powder HA and CS solution are adjusted to get a homogeneous suspension, continued by the formation of a porous scaffold through freezing and lyophilization [15,16]. Previous work employed a mixing technique to make a biore sorbable composite of HA and CS-based bone adhesives [17]. The HA precipitate was dispersed chitosan in an acid solution for obtaining an HA/CS hybrid material.

In this study, we used a source of calcium from shellfish to make n-HA because the natural resources of marine products in Indonesia are very abundant, different with previous research that used HA from sigma Aldric and blended with CS. Addition, we make n-HA/CS composites with sonochemical methods to found out of nano-size crystal. Our result is useful to provide a good understanding of the production of the artificial bone based n-HA/CS material. In another case, our study can increase the economic value of shellfish shells, which is used to produce n-HA.

2. Methods
The synthesis of n-HA/CS was started from the synthesis of Ca(OH)\textsubscript{2} from shellfish was conducted. Shells were cleaned, dried, and ground. The powder was further sieved with 200 mesh and calcined at 1000 °C for 5 hrs. The powder was characterized by XRF and XRD. In the second stage, the synthesis of HA was performed. Powder Ca(OH)\textsubscript{2} was dissolved in aquabides to form solution and followed by sonication process at 55 °C for 2 hrs while slowly pouring with an H\textsubscript{3}PO\textsubscript{4} solution. To control the pH at about 10, the solutions were also dripped with NH\textsubscript{4}OH. HA was further dried with a furnace at 100 °C for 2 hrs. In the last stage, the synthesis of HA/CS was performed. Powder HA was dissolved in aquabides. The 2% CS solution in 3% acetic acid solution was prepared. The HA solution and CS solution was mixed in an Erlenmeyer tube, and the mixture was inserted in ultrasonic-bath and sonicated at 45 °C for 1.5 hrs. The HA/CS composites were precipitated, filtered and then dried with a furnace at 50 °C for 24 hrs. The powder was characterized using XRD, EDX, and SEM.

3. Results and Discussion
Table 1 shows the material content of shellfish. The result confirms that the shellfish has a high calcium content of 97.19%, which makes a potential calcium source for the synthesis of HA. In addition, the crystallinity of shellfish was characterized by using XRD. The calcination of shellfish at a temperature above 500 °C changed phases to calcite with hexagonal crystal structure [18]. The XRD results shellfish was analyzed by using High Score Plus. The results showed that the sample matched with AMCS 0000117, which was suitable for Ca(OH)\textsubscript{2} phase. Figure 1 presents the HA diffraction pattern analyzed by PCW software. Furthermore, the HA sample was analyzed by Rietveld refinement method using AMCS 0002297 database, as presented in Figure 2.

Table 1. XRF characterization of shellfish (characterized in Central Laboratory of Mineral and advanced materials, FMIPA UM).

| Compound | S  | Ca     | Ti | Fe | Co | Ni | Cu | Sr | Mo | Ba | Er | Yb |
|----------|----|--------|----|----|----|----|----|----|----|----|----|----|
| Conc Unit (%) | 0.3 | 97.19  | 0.15 | 0.11 | 0.07 | 0.27 | 0.05 | 0.87 | 0.3 | 0.3 | 0.1 | 0.3 |
From the results of refinement, the reliability values of $R_p = 14.95$, $R_{wp} = 19.87$ and $R_{exp} = 0.93$ were obtained. HA lattice parameter values are $a = 9.4237$ Å and $c = 6.8956$ Å. This result is almost the same as the theory for HA that is $a = 9.418$ Å and $c = 6.881$ Å [19]. To calculate the crystallite size of the samples use Scherrer’s equation in Equation 1 [20].

$$D = \frac{k\lambda}{B\cos\theta}$$  \hspace{1cm} (1)

with $\lambda$ is 0.15406 nm, $B$ is full width at half maximum (FWHM) of 0.32586, obtained from peak fitting (002) with software Origin, and $k$ is 0.9. The calculation confirmed that the HA has the crystalline size of 25.03 nm. Here, the size of crystalline HA synthesized according to the HA in the original bone in a range of 20-80 nm [1]. To determine the Ca/P ratio; the HA samples were characterized by EDX instrument. Table 2 shows EDX results of HA sample. The results revealed that the sample has a percentage of Ca and P atoms of 20.58 and 12.48, respectively, with the ratio of Ca/P of 1.65. These result showed consistency with the stoichiometry value of 1.67 [21].
Table 2. EDX characterization of HA sample

| Element | Wt% | At% |
|---------|-----|-----|
| OK      | 46.92 | 66.94 |
| PK      | 16.94 | 12.48 |
| CaK     | 36.14 | 20.58 |

Matrix Correction ZAF

Figure 3. XRD pattern of n-HA and n-HA/CS composites

The XRD pattern of n-HA/CS composite is presented in Figure 3. The result shows that the XRD peak intensity decreases with increasing CS composition accompanied by widening and peak position shift of 2θ. The decrease of peaks is associated with the decrease of crystallinity of n-HA, which indicates the presence of an amorphous phase. We infer that the amorphous phase is promoted by the presence of CS in the composite system. This decrease of crystallinity of the n-HA/CS composite is consistent with the previous work [22], which produces the same system through co-precipitation method. The analysis of XRD results was performed using PCW software and the Gaussian fittings using Origin software. The obtained lattice parameter and the crystal size of the samples are presented in Table 3.

Table 3 confirms that the crystal lattice parameters increase with increasing CS composition, which is presumably due to the crosslink between NH$_2$ from CS and Ca$^{2+}$ from n-HA [23]. The crystal size of n-HA/CS composite decreases with increasing CS composition. This decrease of crystal size is presumably due to n-HA crystals grow inside the CS matrix and promotes the limitation of the n-HA crystals growth by the chitosan chain [24].

Table 3. Lattice parameter and crystal size of n-HA and n-HA/CS composite

| Parameters          | n-HA    | n-HA/CS (90-10) | n-HA/CS (80-20) | n-HA/CS (70-30) | n-HA/CS (60-40) |
|---------------------|---------|-----------------|-----------------|-----------------|-----------------|
| a (Å)               | 9.4237  | 9.4211          | 9.4254          | 9.4508          | 9.4455          |
| c (Å)               | 6.8956  | 6.8882          | 6.8884          | 6.8930          | 6.9009          |
| Crystal Volume (Å$^3$) | 612.3715  | 611.3768        | 611.9528        | 615.6664        | 616.8080        |
| Crystallite size (nm) | 25.03     | 26.59           | 15.50           | 11.36           | 12.89           |
Figure 4. SEM images of the samples: (a) n-HA and n-HA/CS composite: (b) 90/10 (c) 80/20 (d) 70/30 (e) 60/40

Table 4. List of average grain size of n-HA/CS.

| n-HA/CS composite | Average grain size (nm) |
|-------------------|-------------------------|
| 90/10             | 230.69                  |
| 80/20             | 139.8                   |
| 70/30             | 200.0                   |
| 60/40             | 226.8                   |

SEM images of n-HA and n-HA/CS composite are presented in Figure 4. SEM results show that n-HA/CS composite has uniform morphology, while the HA tends to be agglomerated. The results confirmed that n-HA/CS composite has long grain, forming needle-like shape, while HA shows spherical shape. This result has a good agreement with previous work, where n-HA particles are distributed in the chitosan matrix [25]. Zima synthesized n-HA/CS composites in the form of a hybrid with chemical methods, where HA shows a homogeneous morphology with n-HA dispersed in chitosan matrix [26]. Nano-HA fills in the chitosan matrix matches the natural bone ECM, which makes this material can be applied as a scaffold material in bone tissue engineering. Here the detail of the average grain size for each composite is presented in Table 4. The results show that n-HA/CS (80/20) composite has the smallest average grain size of 139.8 nm than that of other compositions. This Small grain size will contribute to the increase of the mechanical strength of the material [27].

4. Conclusion

The n-HA/CS composites were successfully synthesized by the sonochemical method using a calcium source from shellfish shells. n-HA has similar structure although the composition of chitosan was changed. Then-HA crystal size has decreased with increasing composition of chitosan. The Ca/P ratio of HA is 1.65, with the spherical shape, while n-HA/CS morphology has the needle-like shape. The crystal size of n-HA of 25.03 nm and n-HA/CS in the range of 11.36-26.59 nm, which can be recommended for biomedical applications as bone filler.
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