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Microwave-Assisted Synthesis of Nanohydroxyapatite Using a Biotemplate

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Abstract. *Geloina coaxans* shells, which are a waste material with a high calcium source, serve as an effective raw material for synthesizing hydroxyapatite biomaterials. In this study, hydroxyapatite (HA) was synthesized from Geloina coaxans shells and ammonium hydrogen phosphate $(NH_4)_2HPO_4$. The eggshell membrane (ESM) was used as a biotemplate. The synthesis process of HA-biotemplate was assisted by microwave irradiation. Analysis of X-Ray Fluorescence (XRF) shows the main chemical composition in the *Geloina coaxans shells* is CaO of 98.15%. Analysis of X-ray diffraction (XRD) of *Geloina coaxans shells* showed the presence of $CaCO_3$, and the type was aragonite minerals in accordance with JCPDS 01-075-9987, with the highest aragonite peak at 2θ = 26.18°. Nanohydroxyapatite (HA) was synthesized at a Ca/P ratio of 1.67, with variations in the mass of the eggshell membrane (ESM). Based on the results of the Fourier Transform Infra-Red (FTIR) analysis, there is a specific absorption band in the O-H, CO_3^{2-} and PO_4^{3-} groups. The absorption band of the functional group indicates the specific functional group of the hydroxyapatite synthesized. Microstructural analysis of the HA-ESM samples (prepared with 50, 100, and 150 mg of ESM) revealed spherical granules, randomly intertwined cotton-like fibers, and cotton-like fibers with stacked short rod morphology, respectively. Changing the ESM biotemplate mass effectively dictates and transforms the resulting nanohydroxyapatite morphology. ESM, with its fibrous proteins capable of molecular recognition, serves as an attractive biotemplate for developing hierarchical architectures. The variation in mass employed can stimulate the development of the HA framework, and microwave-assisted synthesis utilizing in situ heating will also accelerate the nucleation and crystal growth of hydroxyapatite.

Keywords: *nanohydroxyapatite, bio-template, Geloina coaxans shells*

Abstrak. *Geloina coaxans* merupakan salah satu limbah yang memiliki kandungan kalsium tinggi, yang dapat digunakan sebagai bahan dasar dalam sintesis biomaterial hidroksiapatit. Pada penelitian ini, sintesis hidroksiapatit (HA) dilakukan menggunakan cangkang *Geloina coaxans* dan $(NH_4)_2HPO_4$. Membran cangkang telur (MCT) digunakan sebagai *biotemplate*. Proses sintesis HA-*biotemplate* dilakukan dengan bantuan iradiasi gelombang mikro. Analisis X-Ray Fluorescence (XRF) menunjukkan bahwa komposisi kimia utama dalam cangkang Geloina coaxans adalah CaO sebesar 98,15%. Analisis difraksi sinar-X (XRD) terhadap cangkang Geloina coaxans menunjukkan adanya $CaCO_3$ dalam bentuk mineral aragonit yang sesuai dengan standar JCPDS 01-075-9987, dengan puncak aragonit tertinggi pada 2θ = 26,18°. Nanohidroksiapatit (HA) disintesis pada rasio Ca/P sebesar 1,67 dengan variasi massa membran cangkang telur (ESM). Berdasarkan hasil analisis Fourier Transform Infra-Red (FTIR), terdapat pita serapan spesifik pada gugus O-H, CO_3^{2-}, dan PO_4^{3-}. Pita serapan gugus fungsi tersebut menunjukkan karakteristik gugus fungsi dari hidroksiapatit yang disintesis. Analisis mikrostruktur morfologi sampel HA-ESM dengan massa 50, 100, dan 150 mg masing-masing menunjukkan bentuk butiran bulat, menyerupai serat kapas yang saling menjalin secara acak, serta menyerupai serat kapas dengan morfologi batangan pendek yang bertumpuk. Perubahan massa biotemplate ESM secara efektif menentukan dan mengubah morfologi nanohidroksiapatit yang dihasilkan. ESM dengan kandungan serat protein mampu melakukan pengenalan molekuler sebagai *biotemplate* untuk pengembangan struktur hierarkis; variasi massa yang digunakan dapat memicu pembentukan kerangka HA dengan sintesis bantuan gelombang mikro yang menggunakan pemanasan in situ juga akan membantu mempercepat nukleasi dan pertumbuhan kristal hidroksiapatit.

Kata kunci: *nanohidroksiapatit, bio-template, Geloina coaxans shells*

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INTRODUCTION

Geloina coaxans shells are a relatively abundant biowaste that can be utilized for pharmaceutical, biomedical, and environmental applications due to their main composition of $CaCO_3$ and small amounts of organic compounds. This calcium mineral can be a source for green synthesis of graft materials for bone tissue engineering, such as hydroxyapatite. The use of eggshells as a biotemplate is particularly appealing because it can produce a unique hierarchical hydroxyapatite structure.

Hydroxyapatite (HA) is a material with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$ that is frequently applied in medical fields as an adsorbent and a catalyst. It is one of the most stable calcium phosphate (Ca/P) compounds. HA shares phase similarities with bone and teeth and possesses biocompatible and non-toxic properties, making it suitable for use with hard tissues such as bone and teeth. Medical applications of HA typically include implant coatings (Hui et al., 2010), catalysis (Anau et al., 2023), heavy metal adsorption (Nunez et al., 2019; Nayak and Bhushan, 2021), and drug delivery (Qian et al., 2023).

Hydroxyapatite synthesis can be developed using biotemplates derived from natural materials or waste products. A biotemplate is a naturally occurring biological material that acts as a structure-directing agent during compound synthesis. Common biotemplates used in hydroxyapatite synthesis include sugarcane bagasse, kapok fibers, and eggshell membranes. The fibrous structure of eggshell membranes can be replicated with inorganic materials via the template, resulting in a mesh-like structure with a high surface area. Researchers have successfully utilized eggshell membranes as biotemplates in the synthesis of various materials; the membranes' unique functional groups and porous structure enable them to function as structure-directing agents for hydroxyapatite.

Previous studies successfully synthesized hydroxyapatite using eggshell membrane biotemplates, yielding a product with good crystallinity consistent with JCPDS No. 09-432 (Sabu et al., 2018). Additionally, Chen et al. (2013) successfully synthesized a biosorbent using eggshell membranes as biotemplates for arsenic adsorption. Parvin et al. (2019) also successfully used eggshell as a biotemplate. Several mollusk shells that have served as calcium precursors for hydroxyapatite (HA) synthesis include *Macoma balthica* (Felsen et al., 2015), *oyster shells* (Rujitanapanich et al., 2014), and *Geloina coaxans shells* (Yanti, 2018). Therefore, this work explores the microwave-assisted synthesis and structural characterization of nanohydroxyapatite using waste Geloina coaxans shells as a sustainable calcium precursor and eggshell membranes as a fibrous biotemplate, leveraging their unique porous architecture and functional groups for morphology-directed growth.

MATERIAL AND METHODS

Materials

The materials used for this research were *Geloina coaxans shell*, eggshells, $(NH_4)_2HPO_4$ (Merck), Hydrochloric Acid (HCl 37%) (Merck), DDW, and filter paper-Whatman 42.

Instrumentation

The equipment used in this research included glass equipment, pH (pH 2011 Pen Type), oven (Gallen kemp), hotplate (Rexim RSH-IDR As One), microwave (philips), furnace (Vulcan^{TM} seri A-130), magnetic stirrer (Spinbar),

X-Ray Fluorescence (XRF S2 Ranger Bruker), X-Ray Diffraction (XRD Gbc Emm), Fourier-Transform Infrared (FTIR Shimadzu, IR Prestige-21), and Scanning Electron Microscope (SEM)-Bruker.

Synthesis Procedures

Preparation of CaO and ESM-P

Geloina coaxans shell was collected and washed to remove its inner membrane. The sample was dried out and mashed, and the particle size of the sample was adjusted to 200 mesh. The powder of Geloina coaxans shell was analyzed by X-Ray Fluorescence to determine its chemical composition. In the next step, the sample was calcined to decompose calcium carbonate into calcium oxide at 1000°C for 12 hours.

Chicken eggshell waste was collected, washed with water, and dried. The eggshells were soaked in hydrochloric acid (HCl) solution to separate the eggshell membrane from the shell. The eggshell membrane was then rinsed with double-distilled water and dried to obtain the eggshell membrane (ESM). This sample was analyzed using FTIR to identify functional groups.

Eggshell membrane (ESM) produced was impregnated with phosphate ions by immersion in a diammonium hydrogen phosphate $(NH_4)_2HPO_4$ solution for 24 hours, yielding ESM-P.

Synthesis of HA and HA-ESM

Synthesis of HA was conducted at a Ca/P concentration ratio of 1.67. CaO was dissolved with HNO_3 to produce $Ca(NO_3)_2$. The $Ca(NO_3)_2$ solution was added to the ESM-P samples under constant stirring until the entire volume of the $Ca(NO_3)_2$ solution had been added. The samples were aged at room temperature for 12 hours. The samples were placed in a microwave oven operated at 700 W for 20 minutes at a microwave frequency of 2.45 GHz. Finally, they were dried at 105°C for approximately 2 hours. The same procedure was applied to the samples of 50, 100, and 150 mg.

Characterization of the sample

The X-Ray diffractometer was used to study the phase composition and crystallinity of the sample, verifying its purity and stability. The scanning was carried out at the 2θ angle range from 10 to 70° with a step size of 0.1°. The X-ray diffractometer was operated using Cu Kα radiation (λ = 1.540 Å) with an operating voltage and current of 40 kV and 30 mA. FTIR spectroscopy was conducted to analyze functional groups and chemical structure of the samples with a frequency range set from 4000 to 400 cm^{-1} to capture crucial hydroxyl (OH^-) stretching vibrations at 3300-3500 cm^{-1} and amide bands from the eggshell membrane proteins. SEM was used to learn the morphology of the nanohydroxyapatite obtained.

RESULTS AND DISCUSSION

Chemical Composition Analysis of *GCS*

The chemical composition of *Geloina coaxans Shell* can be determined through analysis using X-Ray Fluorescence (XRF). The results are presented in **Table 1**.

Table 1. Chemical composition of *GCS*

No.	Chemical composition	(%)
1	CaO	98.15
2	Al_2O_3	0.43
3	MgO	0.18
4	SO_3	0.09
5	Na_2O	0.07

The XRF analysis of *Geloina coaxans shell* revealed that the primary component of the shell is CaO (98.15%), and other compounds: Al_2O_3 (0.43%), MgO (0.18%), SO_3 (0.09%), and Na_2O (0.07%).

Functional Groups Analysis of GCC

The functional groups present in the *Geloina coaxans* shell before and after calcination can be identified through FTIR analysis. The results of the FTIR analysis are shown in Figure 1. FTIR analysis of $CaCO_3$ revealed that a specific carbonate phase appeared at wavenumbers 1480 and 712 cm^{-1}, corresponding to the carbonate groups within $CaCO_3$ (Figure 1). Previous research by Hoque et al. (2013) also identified carbonate phases at wavenumbers 1421 cm^{-1} and 705 cm^{-1}. Absorption bands for H_2O and CO_3^{2-} were observed at wavenumbers 2522 and 3261 cm^{-1}.

FTIR analysis of *Geloina coaxans* shell calcined (CaO) at 1000°C for 12 hours showed the presence of C-O groups at wavenumbers 1472 and 1137 cm^{-1}. Research by Yanti (2018) identified C-O group absorption bands at wavenumbers 1425 and 1049 cm^{-1}, while an O-H absorption band appeared at 3641 cm^{-1}. Similarly, Yanti (2018) reported an O-H absorption band at 3641 cm^{-1} and a Ca-O absorption band at 438 cm^{-1}, although also noted a Ca-O absorption band at 354.9 cm^{-1}. The appearance of weak peaks for the functional groups C-O, O-H, and Ca-O is attributed to possible surface reactions with ambient air after calcination at 1000 °C.

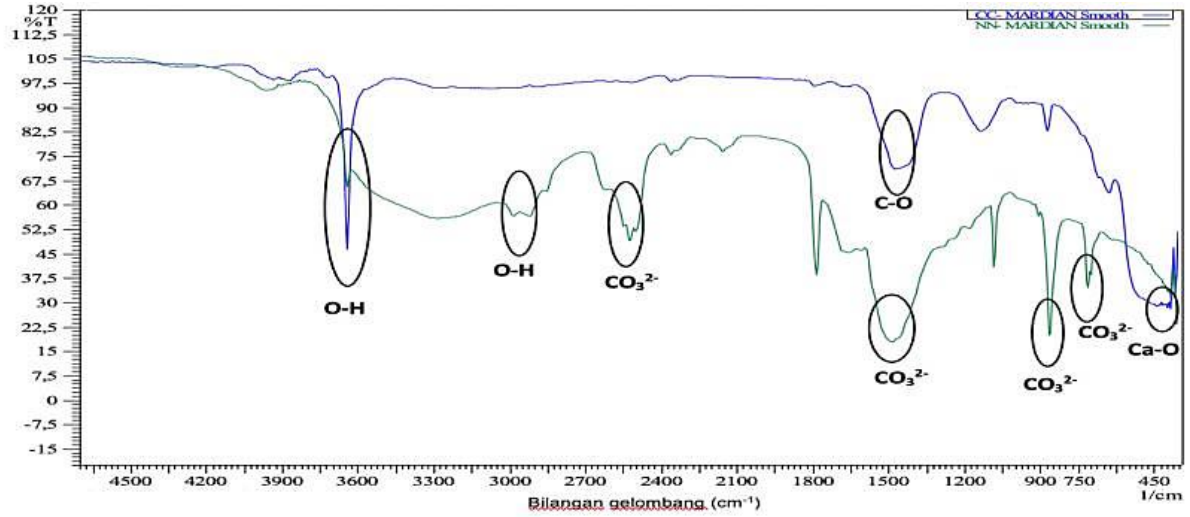

Figure 1. FTIR spectra of $CaCO_3$ and CaO

Mineral Phase Analysis of GCC

The mineral phase of the sample *Geloina coaxans* shell can be seen in Figure 2. The mineral phase of the *Geloina coaxans* shell can be determined through X-Ray Diffraction (XRD) analysis. Analysis of *Geloina coaxans shell* (CaCO3) revealed several major peaks at 2θ = 33.0861°, 26.1797°, 37.8424°, 45.8403°, and 27.1953°. These peaks indicate that the $CaCO_3$ compound present is the aragonite polymorph, consistent with JCPDS 01-075-9987, with the highest aragonite peak occurring at 2θ = 26.18°. 2A previous study by Hoque et al. (2013) also identified the highest aragonite peak at 2θ = 26.23°.

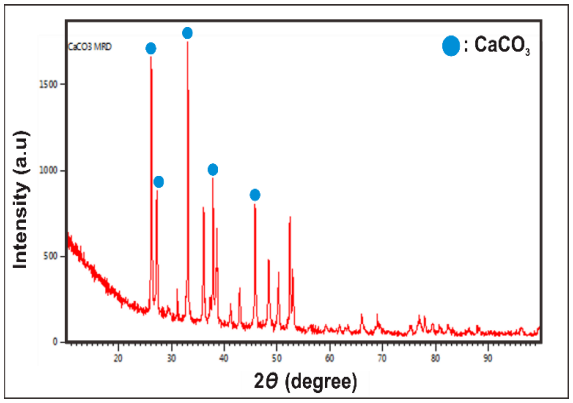

Figure 2. Diffractogram of $CaCO_3$

Marin Marin et al. (2012) state that the minerals most abundant in mollusk shells are aragonite and calcite. Mollusc shells are made of calcium carbonate, and aragonite is one of the primary mineral forms they use, alongside calcite. This aragonite form of calcium carbonate ($CaCO_3$) is metastable and suitable for tissue engineering applications (Hoque et al., 2013). This is due to its suitability for mechanical applications when bound with biopolymers.

Analysis of Functional Groups of HA-ESM

The functional groups present in the synthesized HA-ESM were identified through FTIR analysis. The FTIR analysis results for HA-ESM revealed O-H stretching vibrations at wavenumbers of 3620 cm^{-1} (for 50 mg ESM), 3593 cm^{-1} (for 100 mg ESM), and 3620 cm^{-1} (for 150 mg ESM) (Figure 3).

A previous study by Al-Ghouti (2018) also observed O-H stretching vibrations at a wavenumber of 3316 cm^{-1}. O-H bending vibrations were observed at wavenumbers of 1604 cm^{-1} (50 mg ESM), 1592 cm^{-1} (100 mg ESM), and 1763 cm^{-1} (150 mg ESM); significant results regarding O-H bending.

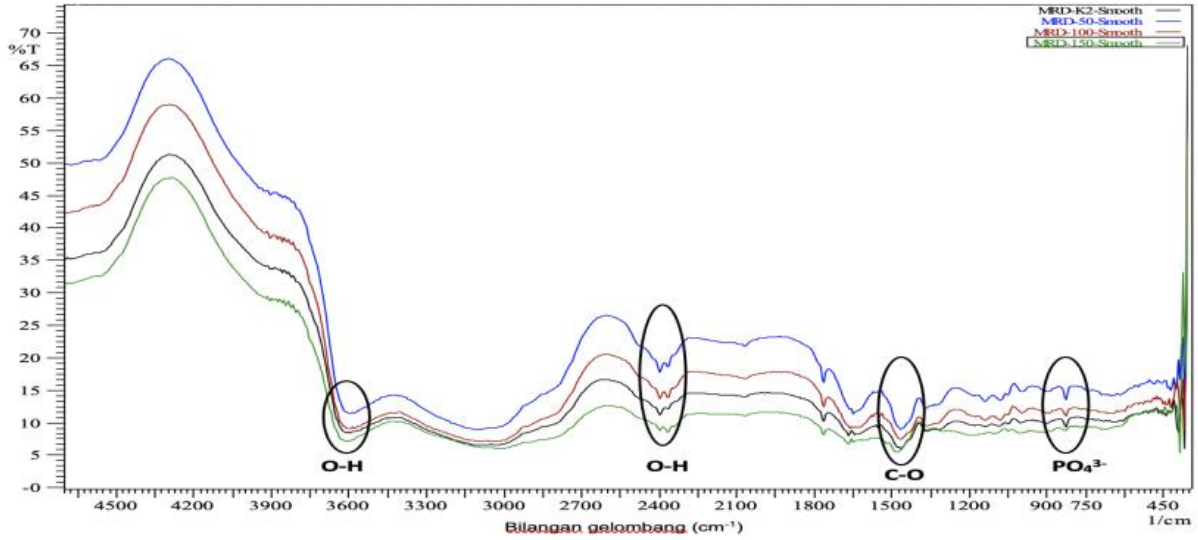

Figure 3. FTIR Spectra of HA/HA-ESM

Absorption bands corresponding to C-O vibrations appeared at wavenumbers of 1416 cm^{-1} (50 mg ESM), 1420 cm^{-1} (100 mg ESM), and 1417 cm^{-1} (150 mg ESM); according to Al-Ghouti (2018), C-O vibration absorption bands also appear at 1413 cm^{-1}. Meanwhile, absorption bands for PO_4^{3-} vibrations appeared at wavenumbers of 1047 cm^{-1} (50 mg ESM), 1048 cm^{-1} (100 mg ESM), and 1019 cm^{-1} (150 mg ESM); significant results were also shown in the previous study. The observed shift in wavenumbers within the HA-biotemplate system, as biotemplate mass varies, is attributed to interactions between the biotemplate and hydroxyapatite (HA). Increasing the biotemplate mass enhances interactions and hydrogen

bonding between proteins and HA ions, including Ca^{2+}, PO_4^{3-}, and OH^-. This intensification modifies bond strengths and molecular vibrational energies, resulting in the observed wavenumber shifts.

Microstructural Analysis of the HA-ESM

The scanning electron microscopy analysis was conducted to examine the microstructure and surface morphology of the synthesized hydroxyapatite using biotemplate ESM.

Figure 4 displays the morphology of HA-ESM samples with several masses of ESM. The resulting SEM was influenced by temperature, particle size, and the mass of the eggshell membrane (ESM). At 3500x magnification, the HA-ESM sample with 50 mg of ESM exhibited a surface morphology characterized by randomly distributed, spherical granules of varying sizes (Figure 4.b). At the same magnification (3500x), the sample with 100 mg of ESM displayed a surface morphology resembling randomly intertwined cotton fibers (Figure 4.c). Meanwhile, the sample with 150 mg of mass of ESM observed at 5500x magnification showed a rough surface morphology resembling cotton fibers interspersed with stacks of short, rod-like structures of varying sizes (Figure 4.d). The HA-ESM morphology features intertwined fibers with surfaces covered by hydroxyapatite crystals (Figure 4.e). In contrast, the HA-ESM surface morphology observed in the study by Wu et al. (2013) consisted of small, convoluted needle-like structures with a large surface area (Figure 4.f).

SEM results for the hydroxyapatite in the study by Wu et al. (2013) demonstrated a favorable aspect ratio, with a physical form resembling the crystalline structure of natural bone. In the study by Liao et al. (2010), the surface morphology of HA-ESM was found to consist of solid platelets with a large surface area. The variation in mass employed can stimulate the development of the HA framework; microwave-assisted synthesis utilizing in situ heating will also accelerate the nucleation and crystal growth of hydroxyapatite

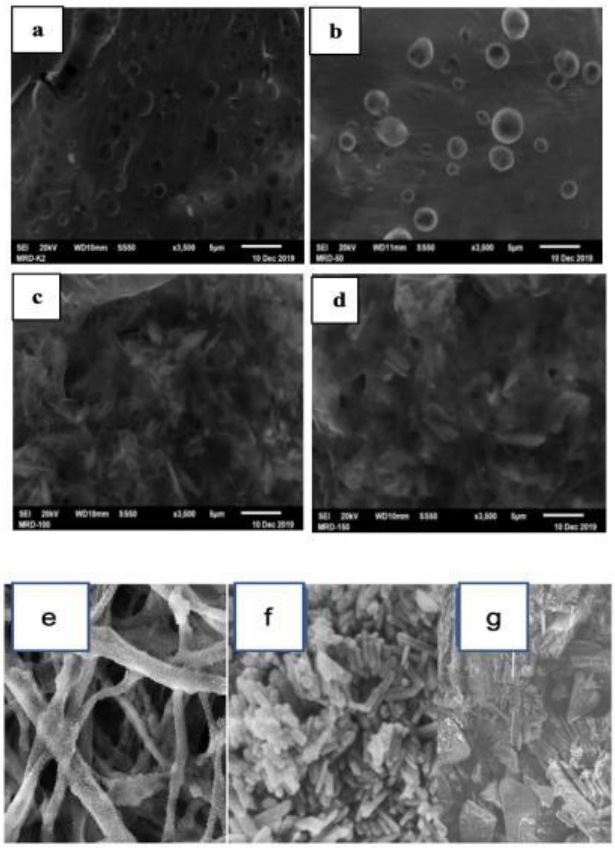

Figure 4. The morphology of HA/ESM sample (a) control, (b) ESM 50 mg, (c) ESM 100 mg, (d) 150 mg,(e) Sabu et al. (2019), (f) Wu et al. (2013), (g) Liao et al. (2010)

CONCLUSION

The synthesis of nanohydroxyapatite (HA) eggshell membrane (ESM) as a bio-template demonstrated the presence of O-H, C-O, and PO_4^{3-} vibrations via FTIR analysis. Microstructural analysis of the HA-ESM samples (50, 100, and 150 mg) showed spherical granules, randomly intertwined cotton-like fibers, and cotton-like fibers with stacked short rod morphology, respectively. The result proved that

a morphological transformation of Eggshell membrane (ESM) as a biotemplate significantly influences the morphology of the resulting nanohydroxyapatite samples. Morphology is a physical characteristic that can influence the application of the resulting HA or HA-biotemplate. Spherical and rod-like morphologies can usually be used to enhance osteoblast function, whereas hollow spheroids have potential for drug delivery applications.

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