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Effect of In Situ Gelatin Addition on the Synthesis and Characteristics of Limestone Based Hydroxyapatite using Sol-Gel Method

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Abstract

Hydroxyapatite (HAp) is a major component of bone and tooth tissues used to repair and rebuild malfunctioning bone. Hydroxyapatite synthesis widely utilizes materials that have a high calcium content. In this study, limestone was used as an economical calcium precursor. Gelatin was added in situ during the sol-gel process to improve biomechanical properties such as porosity and reduce the particle size. Therefore, this study aims to investigate the effect of in situ gelatin addition at different concentrations (30%, 40% and 50%) and pH variations (10, 11 and 12) during the sol–gel synthesis of limestone-based hydroxyapatite, focusing on its influence on porosity, particle size, and Ca/P ratio to evaluate its potential for biomedical applications. Highest yield was obtained when the hydroxyapatite was prepared using 30% gelatin concentration with pH 12. Fourier Transform Infrared (FTIR) spectroscopy confirmed the presence of the characteristic functional groups of hydroxyapatites, namely phosphate (PO_4^{3-}), carbonate (CO_3^{2-}), and hydroxyl (OH^-). SEM-EDX analysis revealed granular and irregular crystal morphology with particle size below 600 nm, while image analysis of SEM-EDX micrographs using ImageJ estimated the highest apparent porosity of 60.73% at pH 10. These results indicate that the in situ addition of gelatin during the sol-gel process successfully produced hydroxyapatite with biomechanical properties suitable for implant applications, especially in teeth, with optimal porosity and adequate particle size to support cell growth.

Keywords: *Hydroxyapatite; Limestone; Gelatin; Porosity; Sol-gel; in situ*

1. Introduction

Rapid developments in healthcare and biomedical technology have driven the need for economical and sustainable synthetic biomaterials, especially for bone tissue regeneration applications (Qu et al., 2019). However, the production of synthetic biomaterials is often constrained by high raw material costs and complex manufacturing processes. This results in expensive final products, limiting their accessibility and widespread use, especially in the developing countries such as Indonesia (Al-Shalawi et al., 2023). One of the key components in various biomaterials is calcium, which has a vital role in the structure and function of hard tissues such as bones and teeth. Calcium sources for biomaterial production generally come from synthetic chemicals that are relatively expensive. This situation creates an urgency to find alternative sources of calcium that are more affordable, sustainable, and easily accessible. Common synthetic calcium sources used in hydroxyapatite synthesis include calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$), calcium chloride ($CaCl_2$), calcium hydroxide ($Ca(OH)_2$), and calcium acetate ($Ca(CH_3COO)_2$) (Jeong et al., 2019). Calcium carbonate ($CaCO_3$) is also one of the synthetic precursors that is easily obtained and frequently used because it can be converted into Ca^{2+} ions through acid dissolution (Akbar et al., 2021). This study utilizes $CaCO_3$ as a calcium source for producing hydroxyapatite, while also offering a more economical alternative by employing limestone, a natural material rich in $CaCO_3$ content.

A calcium phosphate-based biomaterial, hydroxyapatite (HA) with the chemical formula $Ca_{10}(PO_4)_6(OH)_{(2)}$ has a significant role in various medical applications, especially in bone tissue regeneration. The similarity of its chemical composition to the inorganic components of natural bone makes hydroxyapatite a very promising material for bone implantation, bone defect repair, and scaffolds in tissue engineering (Jeong et al., 2019). However, hydroxyapatite has limitations in terms of mechanical

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properties, namely low tensile strength and low porosity. A spectrum of materials both ceramic and polymer have limitations that hinder their optimal performance as bone tissue scaffolds. Ceramics are often constrained by their brittle nature, while polymers sometimes do not meet the standards due to factors such as inadequate structural strength, potential toxicity to cells, and possible release of unwanted components due to their degradation process (Przekora, 2019). Recent studies have shown that modification of HAp with biopolymers such as gelatin can improve its mechanical and biological properties. Gelatin functions as a binding matrix that increases porosity, reduces particle agglomeration, and improves cell-material interactions (Budiatin et al., 2022).

Porosity is defined as the ratio of void space to total material volume expressed in the range 0-1 or percentage 0-100. The optimal porosity for bone applications ranges from 50-70%, depending on the type of target tissue. Specifically, implant materials with very large pore size tends to degrade faster due to a decrease in their mechanical properties (Bemis et al., 2023). Manipulating the concentration of biopolymers such as gelatin during synthesis can control pore size. However, too high gelatin concentration can actually reduce yield due to crystal growth inhibition (Milla et al., 2018). On the other hand, the synthesis pH also has a significant effect on the morphology and Ca/P ratio of HAp. At high pH (>10), HAp crystal growth is more controllable, but it can reduce porosity due to the formation of solid structures (Zhao et al., 2021).

Conventional HAp synthesis methods such as calcination and wet precipitation have been widely developed, but still face challenges in controlling particle morphology and homogeneity (Akbar et al., 2021). The sol-gel method is a superior choice because it can produce nanoparticles with high crystallinity and uniform size distribution (Hutabarat et al., 2019). In addition, the integration of organic material additions such as gelatin in situ during the sol-gel process can act as a template to direct HAp crystal growth, improve interfacial interactions, and optimize porosity (Xu et al., 2022). However, research related to the combination of the in situ sol-gel method with gelatin and limestone precursors is still very limited. The acidity (pH) in hydroxyapatite (HAp) is a measure of the concentration of hydrogen ions (H^+) in the HAP solution. At low pH, HAp will form smaller particles and coarser morphology. This is due to the higher solubility of HAp and faster growth of HAp crystals at low pH (Haruda et al., 2016). The greater the pH, the higher the crystallinity and purity produced, but at an extremly high a pH such as pH 13 will affect the purity so that the crystallinity and purity values obtained will be lower (Khoirudin et al., 2015).

Although the sol-gel method and the use of gelatin have been studied, their combination with limestone precursors has not been deeply explored. Previous studies focused more on synthetic precursors or conventional calcium sources, thus less on sustainability and production costs (Wardiana et al., 2019). In addition, the interaction between pH variation (10-12) and gelatin concentration (30-50%) in optimizing the biomechanical properties of HAp-gelatin still needs to be studied systematically. This study is very important for optimal tissue growth, as well as making the in situ sol-gel method a promising approach in scaffold development for bone and orthopedic tissue engineering applications (Ghayor et al., 2021). Based on the above description, this study aims to synthesize limestone-based HAp with an in situ addition of gelatin using the sol-gel method, and evaluate the effect of variations in gelatin concentration (30%, 40%, 50%) and pH (10, 11, 12) on the biomechanical properties of the scaffold (yield, particle size, porosity, Ca/P ratio).

2. Materials and Methods

The main material used in this research is precipitated calcium carbonate limestone (Pudak scientific, 99 wt%). In addition, supporting chemicals used include: HNO_3 (Nusa chemical, 68 wt%), $(NH_4)_2HPO_{(4)}$(SAP Chemical, 99%), and NH_4OH (Pudak Scientific, 25% v/v). The equipment used included a furnace (Thermo scientific) and a hot plate magnetic stirrer (Thermo scientific).

2.1 Synthesis of gelatin hydroxyapatite

Hydroxyapatite synthesis was carried out according to the flow diagram in figure 1, where the sol-gel method was performed by mixing PCC (precipitated calcium carbonate) limestone and $(NH_4)_2HPO_4$. A total of five grams of PCC (precipitated calcium carbonate) from limestone was dissolved in 300 mL of 0.5 M HNO_3 solution. A 0.3 M $(NH_4)_2HPO_4$ solution was prepared by dissolving 6.68 g of the compound in distilled water, with the final volume adjusted to 100 mL The $(NH_4)_2HPO_4$ solution was added dropwise to the PCC solution at a rate of 1.3 mL/minute to maintain a Ca/P molar ratio of 1.67. Gelatin was added to the mixture at varying concentrations of 30%, 40%, and 50% (m/v). The mixture was stirred at 500 rpm and 60°C for 1 hour until the achievement of homogeneous solution. Stirring of the suspension was continued at 250 rpm and 30°C for 2 hours. The pH of the solution was adjusted to 10, 11, or 12 using 33% NH_4OH according to the research variables. The suspension was aged for 24 hours to allow complete. The precipitate was filtered, thoroughly washed with aquadest until the pH of the filtrate reached neutrality, and subsequently dried in an oven at 110°C for 2 hours. The dry powder was sintered in a furnace at 900°C for 2 hours to enhance crystallinity. The final product was sieved using a 200 mesh sieve to obtain uniform particle size.

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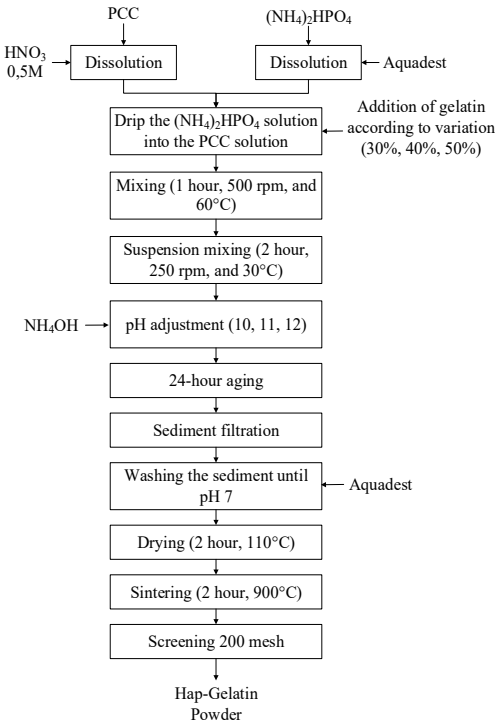

Figure 1. Flow chart of hydroxyapatite-gelatin synthesis

The determination of yield is a crucial stage in the evaluation of the entire process. Yield calculations can provide information on the conversion efficiency of raw materials into the final product. High yields indicate the effectiveness of the synthesis method used, while low yields may indicate material loss during the process or incomplete reactions. Equation 1 below shows the equation used to calculate the yield:

$$\% \text{ yield} = \frac{\text{product mass}}{\text{mass of PCC}} \text{ x } 100\% \quad (1)$$

2.2 Characterization of gelatin hydroxyapatite

The synthesized HAp-gelatin powder was analyzed using three main techniques to evaluate its physico-chemical properties and performance. Scanning Electron Microscopy (SEM) coupled with Energy Dispersive X-ray Spectroscopy (EDX) was applied to observe the surface morphology, pore distribution, and microstructure of the samples, as well as to determine the elemental composition In this method, high-energy electron beams are used to scan the sample, producing high-resolution images of the surface and structure due to the shorter wavelength of the electron beam compared to visible light. EDX analysis complements SEM by providing detailed elemental mapping, which is useful for validating the Ca/P molar ratio and detecting possible impurities or contaminants in the sample (Sahdiah & Kurniawan, 2023). Fourier Transform Infrared (FTIR) spectroscopy was performed to identify specific chemical bonds and functional groups in the synthesized powder, such as phosphate (PO_4^{3-}) and carbonate (CO_3^{2-}) groups, which are typical markers of hydroxyapatite. This technique operates by measuring the interaction between the sample and infrared radiation, where molecules absorb energy at specific wavelengths, causing their chemical bonds to vibrate and producing a spectrum rich in information about the molecular structure (Nurfitriyana et al., 2022). The synthesis efficiency was evaluated by comparing the mass of the final product to the initial mass of raw materials. The yield was calculated according to Equation. 1, where higher yield values indicated an effective synthesis process with minimal material loss.

3. Results and Discussion

3.1 Raw material analysis

The chemical composition of limestone as raw material for hydroxyapatite (HAp) synthesis was evaluated through gravimetric method. The analysis results show that the limestone sample has a calcium carbonate ($CaCO_3$) purity of 99.87%, which indicates its feasibility as an economical calcium precursor. This purity level is in accordance with the requirements of high-quality HAp synthesis, as recommended by (Wardiana et al., 2019), which states that raw materials with $CaCO_3$ content above 99% are highly recommended for the synthesis of high-quality hydroxyapatite. This near 100% $CaCO_3$ content also indicates that the limestone has few impurities, which can minimize the risk of contamination and stoichiometric variations in the final hydroxyapatite product.

3.2 Gelatin hydroxyapatite product

The hydroxyapatite products obtained have varying masses. The results in Table 1 show that gelatin concentration and pH have a significant effect on product yield. The highest yield was achieved under conditions of 30% gelatin concentration with pH 12 (98.64%), followed by pH 10 (93.95%) and pH 11 (77.62%). Alkaline conditions (pH 12) are optimal for HAp crystal growth as it facilitates stable hydrolysis and nucleation, while the decrease in yield at pH 11 is due to particle agglomeration. Increasing the gelatin concentration above 30% decreased the yield gradually. At 40% gelatin, the highest yield occurred at pH 10 (98.96%), but dropped dramatically at pH 11 (72.69%) and pH 12 (86.09%). The 50% gelatin produced the lowest yield on average (79.51%), with a decreasing trend as the pH increased (90.95% at pH 10 to 70.96% at pH 12). This phenomenon indicates that excess gelatin (>30%) may inhibit the growth of HAp crystals, possibly due to the formation of an overly dense gelatin matrix that interferes with the diffusion of calcium and phosphate ions. This finding confirms that strict control of pH and gelatin concentration is key in the optimization of limestone-based HAp synthesis, especially for biomedical applications that require precision in material composition and morphology.

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Table 1. Yield data of hydroxyapatite gelatin from limestone

Concentration Gelatin	pH	Mass PCC (gram)	Mass product (gram)	Yield	average yield
30%	10	5	4.6977	93.95%	90.07%
	11	5	3.8809	77.62%	
	12	5	4.9319	98.64%	
40%	10	5	4.9479	98.96%	85.91%
	11	5	3.6346	72.69%	
	12	5	4.3047	86.09%	
50%	10	5	4.5477	90.95%	79.51%
	11	5	3.8304	76.61%	
	12	5	3.5479	70.96%	

3.3 Functional Groups of Hydroxyapatite-Gelatin

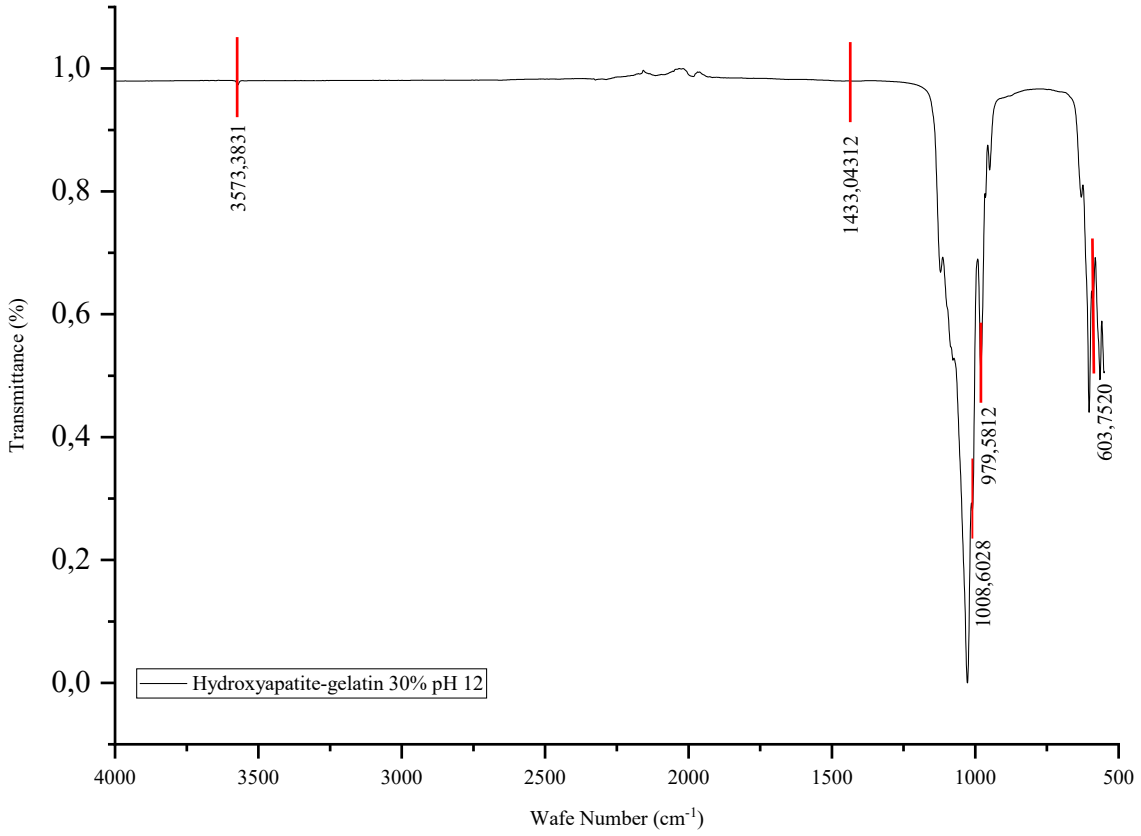

Figure 2. FTIR spectrum of hydroxyapatite with 30% gelatin addition concentration at pH 12

The FTIR spectrum of hydroxyapatite-gelatin presented in Figure 2 shows major peaks in the wave number range of 4000-500 cm-¹. Analysis of these peaks is important to identify the functional groups and chemical bonds present in the sample. In the hydroxyapatite-gelatin sample, there are several distinctive peaks that can be identified. The phosphate functional group (PO_4^{3-}) showed the highest intensity compared to the other groups. At pH 12, the PO_4^{3-} group was clearly detected at wave numbers 603.75 cm-¹, 979.58 cm-¹, and 1008.60 cm-¹. These peaks correspond to the bending and stretching vibrational modes of phosphate ions, which are the main characteristics of hydroxyapatite. In addition, additional peaks appearing around 1400-1450 cm-¹ generally correspond to the vibrations of carbonate groups (CO_3^{2-}). However, in this sample at pH 12, the carbonate peak was detected with very low intensity, barely even detectable. This indicates that carbonate substitution in the hydroxyapatite structure is minimal or almost non-existent. This is important, as the presence of carbonate can affect the mechanical

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properties and biocompatibility of hydroxyapatite. The broad peak around 3573.38 cm-¹ corresponds to the OH^- stretching vibration. The intensity of this peak is relatively weak, which indicates the presence of a limited number of hydroxyl groups. This weakness in intensity could be due to the low water content in the system, as water is often involved in the formation of hydroxyl networks through hydrogen bonding. In hydroxyapatite, water plays an important role in maintaining the stability of the crystal structure and contributes to the physicochemical properties of the material. Based on this analysis, it can be concluded that hydroxyapatite-gelatin synthesized at pH 12 exhibits a phosphate-dominant structure, with detectable but limited amounts of hydroxyl, and very little or no presence of carbonate. These findings are in agreement with the characteristics of pure synthetic hydroxyapatite, which is important for biomedical applications such as bone and tooth regeneration (Tekege et al., 2023).

3.4 Morphological analysis of gelatin hydroxyapatite

Observations on the morphology, microstructure, and porosity of the HAP-Gelatin samples were made through SEM analysis. This analysis was applied to hydroxyapatite-gelatin samples with the highest yield of gelatin concentration of 30%. Based on Figure 3, it can be seen that the morphology of HAP-Gelatin shows a granular and irregular crystal form which results in a smaller size and porous. The small size of HAP-Gelatin occurs because the sol-gel process involves the transformation of sol (colloidal solution) into a gel, which can then produce nanoparticle-sized particles with a large surface area (Akbar et al., 2021). Through this experiment, pH 12 has a particle size smaller than 600 nm. The average particle size of hydroxyapatite in hydroxyapatite-gelatin synthesis with pH 12 setting is 445.6667 nm. The results of particle size measurements in this study are in line with the findings of (Anggresani et al., 2020) who observed variations in hydroxyapatite particle size based on different Ca/P molar ratios and the smallest was at a Ca/P stoichiometric ratio of 1.67, namely particle size between 0.39-0.479 μm or equal to 390 nm-479 nm.

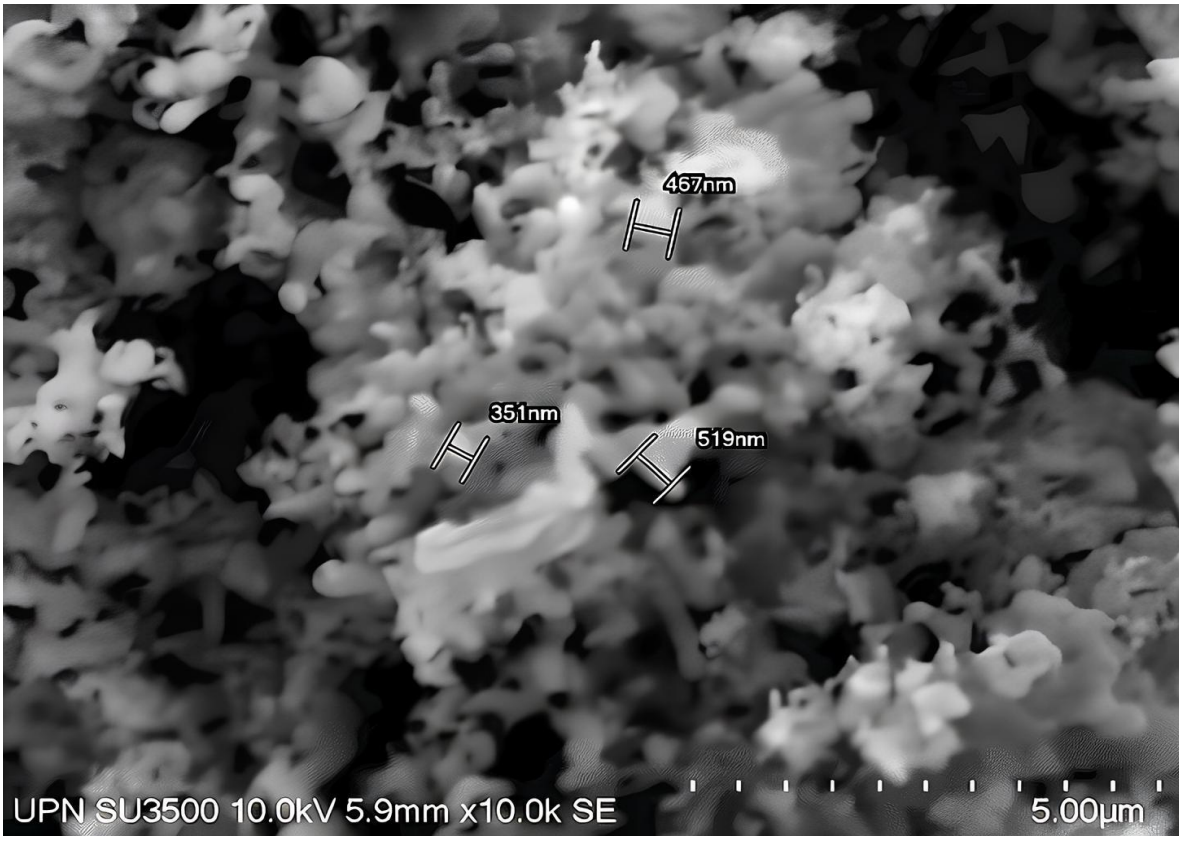

Figure 3. Morphology of hydroxyapatite with 30% gelatin concentration on SEM at 10,000x magnification pH 12

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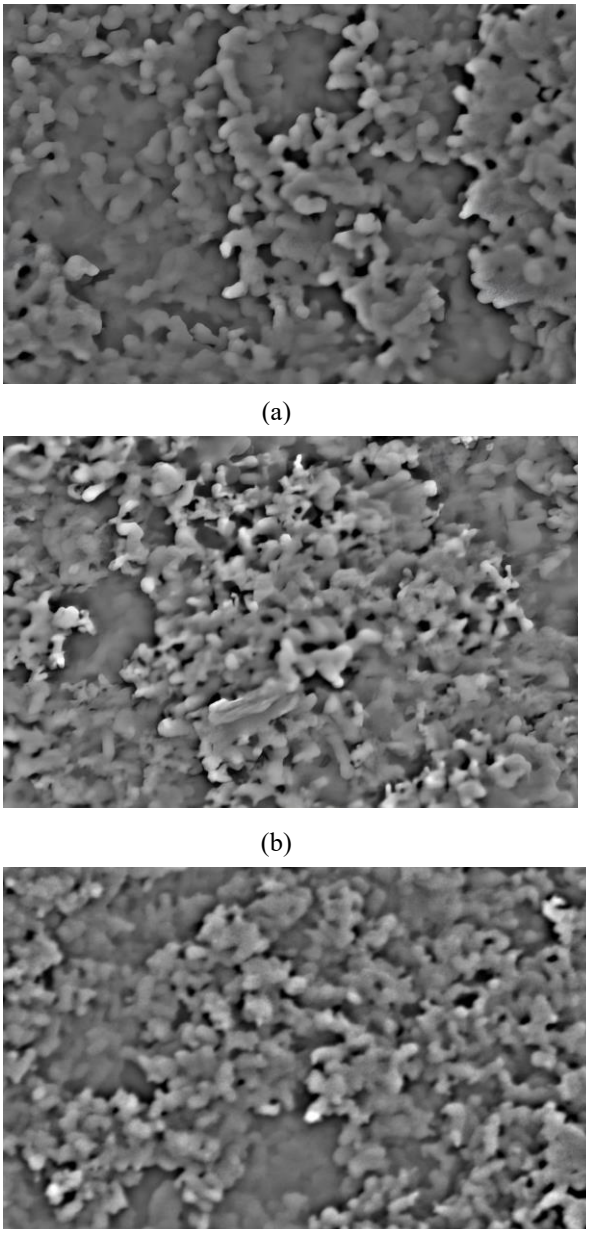

(a) (b) (c)

Figure 4. Porosity of gelatin hydroxyapatite (a) pH 10 30% concentration; (b) pH 11 30% concentration; (c) pH 12 30% concentration

3.5 Porosity analysis of gelatin hydroxyapatite

Scanning Electron Microscopy (SEM) analysis combined with ImageJ image processing was utilized to evaluate the surface porosity characteristics of the HAp-gelatin composites. In this approach, the SEM micrographs were first calibrated according to the scale bar, converted to 8-bit grayscale, and processed using an automatic thresholding technique to distinguish pore areas from solid regions. The surface pore area fraction was then quantified as the percentage of pore pixels relative to the total analyzed area, providing a semi-quantitative estimation of surface porosity.

The results of the porosity analysis of HAp-gelatin in Figure 4 with a gelatin concentration of 30% show a decreasing trend in porosity values with increasing synthesis pH. At pH 10, the highest porosity reached 60.73%, decreasing to 53.02% at pH 11, and 50.85% at pH 12. This decrease can be explained by the increased solubility and reaction rate at stronger alkaline conditions, thus facilitating faster hydroxyapatite crystal growth and larger crystal size. Enlarged crystals tend to fill the inter-particle space more efficiently, thus reducing the pore volume in the gelatin matrix (Xu et al., 2022). Based on research Bemis et al. (2023), states that an important requirement for implant materials is to have porosity that is compatible with body tissue. Panseolus bone has a porosity of 70-95%, in teeth 40-60% and in compact bones 5-30%. Therefore, HAP-gelatin samples with pH 10, 11 and 12 at 30% concentration can be used as dental implants.

3.6 Hydroxyapatite-gelatin constituent composition as Ca/P analysis

SEM-EDX analysis is used to identify and characterize the components contained in hydroxyapatite, providing a detailed description of the microstructure and elemental composition of the material. This experiment used a concentration of 30% gelatin added during the hydroxyapatite synthesis process and the pH was set according to the variables of 10, 11 and 12. The results of the hydroxyapatite-gelatin component analysis can be seen in Table 2. Based on the data displayed in the table, it can be seen that the elemental composition of hydroxyapatite-gelatin varies according to the pH used in the synthesis. The main elements detected are Carbon (C), Oxygen (O), Sodium (Na), Magnesium (Mg), Aluminum (Al), Phosphorus (P), and Calcium (Ca).

The percentage of phosphorus (P) was relatively stable across the three pHs, ranging from 17.65% to 18.41% by weight. Other elements such as Na, Mg, and Al were present in small but consistent amounts at all pH variations. Analysis of the Ca/P ratio of the hydroxyapatite-gelatin samples showed interesting results. At pH 10 and 12, the Ca/P ratio was recorded as 1.76, while at pH 11 it was slightly higher at 1.81. These values deviate from the ideal ratio of 1.67 generally found in pure hydroxyapatite. The Ca/P molar ratio values obtained are still within the acceptable range and do not raise significant concerns regarding material safety or quality. This is in line with research by (Amalia et al., 2022) which states that hard and rigid tissues, such as bones and teeth, show high levels of toxicity when the Ca/P molar ratio approaches 0.3. Meanwhile, the body's soft tissues, such as internal organs and muscles, tend to experience toxic effects when the Ca/P molar ratio is around 0.5.

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Table 2. Hydroxyapatite-Gelatin constituent composition 30% concentration

pH 10			pH 11			pH 12		
Element	Weight %	Atomic %	Element	Weight %	Atomic %	Element	Weight %	Atomic %
C	0.07	0.15	C	0.96	1.93	C	0	0
O	37.64	57.73	O	38.07	57.46	O	37.8	57.84
Na	0.49	0.52	Na	0.63	0.66	Na	1.18	1.26
Mg	0.44	0.45	Mg	0.48	0.48	Mg	0.47	0.47
Al	0.94	0.86	Al	0.94	0.84	Al	0.57	0.52
P	18.41	14.58	P	17.65	13.76	P	18.32	14.48
Ca	42	25.71	Ca	41.28	24.87	Ca	41.65	25.44

Previous studies have indicated that states hard and rigid tissues, such as bones and teeth, show high levels of toxicity when the Ca/P molar ratio approaches 0.3. Meanwhile, the body's soft tissues, such as internal organs and muscles, tend to experience toxic effects when the Ca/P molar ratio is around 0.5 (Hutabarat et al., 2019). In their research, it was mentioned that the limitations of energy diffraction X-ray (EDX) spectroscopy in identification can cause errors in elemental interpretation because the energy levels produced by each element can be similar. In addition, there may be inhomogeneity in the composite sample, where elements are not evenly distributed in each area examined. The results of EDX analysis showed that the Ca/P ratios of all composites were within the appropriate range ($1.65 \leq Ca/P \leq 1.82$). According to research Khoirudin et al. (2015), when the Ca/P ratio exceeds the standard value of 1.67, it is recommended to modify the reaction temperature parameters and extend the process time. This strategy aims to increase the purity of hydroxyapatite produced. By optimizing these reaction conditions, it is expected to produce hydroxyapatite with a higher purity level and a composition that is closer to the desired stoichiometric value and can eliminate impurities or unwanted intermediate phases.

4. Conclusion

The results showed that the in situ addition of gelatin in the synthesis of limestone-based hydroxyapatite (HAp) through the sol-gel method provides good control of yield, crystal structure, and micro and macro properties of the material. The highest yield was obtained at 30% gelatin concentration with pH 12 (98.64%), while the addition of gelatin above 30% tended to decrease the yield due to the formation of gelatin matrix. FTIR analysis showed typical peaks of phosphate (PO_4^{3-}), hydroxyl (OH-) and carbonate (CO_3^{2-}). SEM morphology confirmed the shape of crystals with an average size below 600 nm, indicating successful control of particle size at the nanoscale through the in situ process with gelatin as a crystal growth template. The porosity of HAp-gelatin with 30% gelatin decreased as the pH increased where the increased solubility and crystal growth rate under stronger alkaline conditions caused the voids between particles to be filled more tightly. SEM-EDX analysis showed that the Ca/P ratio is still within the acceptable range of 1.65-1.82. In terms of application, the condition of 30% gelatin at pH 12 is recommended for dental scaffolds because the porosity is close to the optimal range of 40-60%.

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