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Synthesis And Study of Antimicrobial Properties of Beta-Tricalcium Phosphate/Silver Nanoparticles

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Abstract

Beta-tricalcium phosphate (β-TCP) is a compound used as a basic material for making bio-scaffolds and applied for controlled drug delivery and bone regeneration. This material has the advantages of osteoconductivity and biodegradability. However, this material has weaknesses in its antimicrobial properties, so it requires adding other ingredients to increase its ability, such as NpAg. This research aims to obtain β-TCP/NpAg, which has antimicrobial properties. This research started with NpAg, made β-TCP from a combination of Ca(OH)2 and H3PO4 synthesized β-TCP/NpAg, and tested antimicrobial activity using the disc method. The results of this study show that β-TCP has XRD characteristics in the diffraction pattern, such as 21,541, 25,339, 27,619, 30,881, 32,179, and 34,231. In addition, β-TCP/NpAg has XRD characteristics in the diffraction pattern, such as 25.90, 26.67, 27.95, 29.65, 31.17, 32.61, and 34.47, with the highest peak at 31.17, which is the diffraction pattern of NpAg. The antimicrobial β-TCP/NpAg results showed the ability to inhibit the test microbes by 84.37% on S. aureus, 95.17% on E. coli, and 38.83% on C. albicans.

Keyword: *β-Tricalciumfosfat, Silver Nanoparticles, Antimicrobial*

Introduction

Beta-tricalcium phosphate (β-TCP) is a cornerstone compound in the production of bioscaffolds for controlled drug delivery and bone regeneration applications (Hossain et al., 2023). Derived from natural and synthetic sources, β-TCP boasts biocompatibility, osteoconductivity, and low toxicity, resembling the natural apatite phase in hard tissues (Shim et al., 2018). However, its insufficient antibacterial capability poses a challenge in medical applications (Chen et al., 2015).

Regarding this matter, researchers have explored the incorporation of metal ions into β-TCP, leveraging its unique chemical structure for modification (Porsani et al., 2020). Silver (Ag) emerges as a promising candidate due to its well-known antibacterial properties (Singh et al., 2016). Studies have shown that β-TCP augmented with silver ions exhibits enhanced antibacterial efficacy against common pathogens such as *S. aureus* and *E. coli* (Gokcekaya et al., 2021).

Traditionally, Beta-TCP synthesis involves calcium nitrate tetrahydrate and diammonium hydrogen orthophosphate or calcium nitrate tetrahydrate with ammonium phosphate (Singh et al., 2016; Gokcekaya et al., 2021). However, this study proposes an alternative synthesis method utilizing $Ca(OH)_2$ and H_3PO_4, which have not been explored in β-TCP synthesis. $Ca(OH)_2$ is a calcium precursor, while H_3PO_4 is the phosphate source, both essential for β-TCP formation (Sembiring & Sirait, 2023; Urrohman et al., 2023).

Incorporating silver nanoparticles (NpAg) into β-TCP involves a chemical reduction process using p-hydroxybenzoate as a reducer. The resulting β-TCP/NpAg composite aims to enhance antimicrobial properties against *S. aureus*, *E. coli*, and *Candida albicans*, with potential applications in dentistry and orthopedics.

This research commences with NpAg synthesis and characterization using UV-Vis spectrophotometry and particle size analysis. X-ray diffraction (XRD) is employed to assess the crystallinity of β-TCP and β-TCP/NpAg composites. Antimicrobial assays against *S. aureus*, *E. coli*, and *Candida albicans* provide insights into the efficacy of the synthesized materials.

Methodology

Material

The tools used in this research include a set of glassware, hotpiece, exchange rate, magnetic stirrer, pH meter (Hanna-HI110), a set of instruments for characterization of UV-Vis spectrophotometer (UV-1800 Shimadzu), XRD (X-Ray Diffraction) (XPert MPD), Particle Size Analysis (PSA) (Microtac), shakers, furnaces, scales and ovens.

The materials used in this research include distilled water (H_2O), silver nitrate ($AgNO_3$) (Merck), p-hydroxybenzoic acid (Sigma-Aldrich), calcium hydroxide ($Ca(OH)_2$) (Merck), phosphoric acid (H_3PO_4) (Merck), sodium hydroxide (NaOH) (Merck), streptomycin ($C_{12}H_{39}N_7O_{12}$), nystatin ($C_{47}H_{75}NO_{17}$), dimethyl sulfoxide 1% (DMSO) (Merck), *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*.

Procedure

Synthesis of β-Tricalisum Phosphate (β-TCP)

Fifty grams of $Ca(OH)_2$ was dissolved in 500 mL of distilled water. The $Ca(OH)_2$ solution was dropped gradually into the 80% H_3PO_4 solution while stirred at a speed of 400 rpm and a temperature of 40^{O}C until pH 9. The solution was stirred using a magnetic stirrer for 24 hours. After stirring, the solution was dried at a temperature of 105^{O}C for 5 hours and then calcined at a temperature of 800^{O}C for 2 hours. The calcination results were analyzed using XRD.

Synthesis of Silver Nanoparticles (NpAg)

Synthesis of silver nanoparticles (NpAg) was carried out by reacting 5 mL of silver nitrate with a concentration of 4.0 x 10^{-4} M with 5 mL of p-hydroxybenzoic acid with a concentration of 8.0 x 10^{-3} M in a test tube. P-hydroxybenzoic acid is first conditioned to a pH of 11 by adding sodium hydroxide solution. The silver nitrate and p-hydroxybenzoic acid mixture was shaken and then heated in boiled water for 1 hour until NpAg formed, as indicated by the change in color of the solution from colorless to brownish yellow. The nanoparticles formed are then cooled. Measurement of silver nanoparticles with a UV-Vis spectrophotometer uses a cuvette with a thickness of 1 cm and is measured in the wavelength range of 300-800 nm. Particle size and distribution measurements were carried out using PSA. 3 mL of the silver nanoparticle solution was measured at a scattered angle of 90° (Gusrizal et al., 2020).

Synthesis and Characterization of β-TCP/NpAg

Four grams of β-TCP were dissolved in 60 mL of silver nanoparticles with a concentration of 2x10-4M. The mixture was stirred using a hotplate for 15 minutes. Then, when two phases had formed, the filtrate and residue were separated. The residue was heated in an oven at 110°C for 3 hours. The results were weighed, and composite analysis was carried out using XRD (Holguin and Reyes, 2020).

Antimicrobial Activity Test of β-TCP Embedded with NpAg

The antimicrobial activity test was carried out using the disc diffusion method. Antibacterial activity involves Nutrient Agar (NA) media. Bacterial cultures were prepared by transferring one full loop of bacterial cells from the culture stock to a test tube containing Nutrient Broth (NB) medium and incubating at 37^{O}C for 24 hours. This strain was used to determine its antibacterial activity against Gram-positive S. aureus and Gram-negative E. coli and antifungal activity against *C. albicans*.

Antibacterial activity was carried out by smearing the test bacterial suspension into NA solid media. Then, the disk was dripped with 20 µL of each sample (5 mg/mL β-TCP, NpAg, and 5 mg/mL β-TCP/NpAg), dimethyl sulfoxide (DMSO 1%) as a negative control, and standard streptomycin (1 mg /mL) as a positive control. The medium was incubated for another 24 hours at 37°C, and microbial growth was checked by measuring the diameter of the inhibition zone.

Antifungal activity and test procedures are almost the same. Potato Dextrose Agar (PDA) for the medium fungi test was smeared with the test fungal suspension. Then 20 µL of the disc was dropped on each test sample (5 mg/mL β-TCP, NpAg, and 5 mg/mL β-TCP/NpAg), negative control (1% DMSO), and positive control (1 mg/ mL Nystatin). The level of antifungal activity was measured by incubating the sampled medium at 37^{O}C for 24 hours, and the diameter of the inhibition zone was recorded.

Result and Discussion

Synthesis of Silver Nanoparticles (NpAg)

NpAg was synthesized using a chemical reduction method or bottom-up approach. This method was employed to create silver nanoparticles from silver nitrate with p-hydroxybenzoic acid due to the chemical properties of p-hydroxybenzoic acid, which enable it to act as a complexing agent and a precipitant.

The formation of silver nanoparticles was observed through the color change of the colorless solution to yellowish-brown. The results indicated the formation of NpAg, as evidenced by the appearance of yellowish-brown coloration. This was observable through the absorption peak at a 410-425 nm wavelength. As the particle size increases, the Uv-Vis test results will shift towards longer wavelengths in the red spectrum. The results obtained in this study showed that silver nanoparticles were formed at a wavelength of 425 nm (Figure 1). This is consistent with the findings of Yanti et al. (2021) and Suryadi et al. (2022), where the formation of NpAg occurred at a wavelength of 425 nm. Subsequently, particle size analysis (PSA) was conducted on the results.

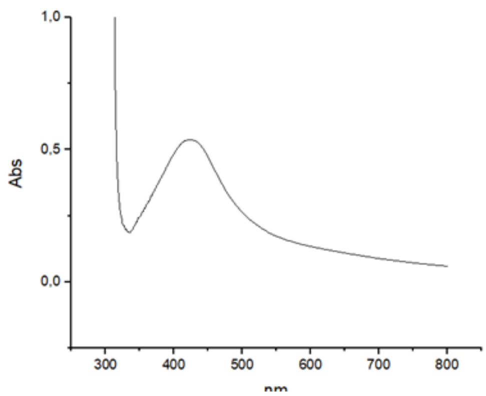

Figure 1. Spectrofotometer Uv-Vis NpAg

PSA analysis revealed that the particle size of the obtained NpAg ranged up to 92.2 nm (Figure 2). This finding aligns with existing literature, where NpAg typically has a size below 100 nm (Ridwan et al., 2019). However, based on the results (Figure 2), particle sizes exceeding 100 nm were still observed. This indicates the presence of size variation in the synthesized NpAg. The concentration of AgNO3 can influence such size discrepancies used in the synthesis of NpAg, affecting the uniformity of nano-sized particles (Ridwan et al., 2019). Moreover, the solution's Brownian motion effect and Van der Waals forces impact particle size. This is supported by the study of Nalawati (2021), which obtained NpAg with a size of 116.3 nm.

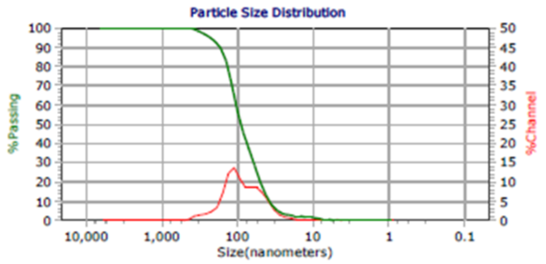

Figure 2. PSA analysis of NpAg

Synthesis and Characterization of β-TCP/NpAg

The XRD data were utilized to identify the formation of crystalline phases, diffraction patterns, and diffraction patterns using the JCPDS (Joint Committee for Powder Diffraction Standard) database. The obtained XRD results revealed the characteristic diffraction peaks (2θ) indicative of the formation of β-tricalcium phosphate (β-TCP) (Figure 3A). These peaks appeared at 21.541 (024), 25.339 (1010), 27.619 (214), 30.881 (0210), 32.179 (128), and 34.231 (220) (JCPDS No.9-169). These results signify the characteristic peaks of β-TCP formation (Figure 3). This finding is corroborated by the study of Padmanabhan et al. (2022), which reported nearly identical diffraction patterns at 21.8 (024), 25.8 (1010), 27.8 (214), 31.0 (0210), 32.4 (128), and 34.3 (220) (JCPDS No.9-169). Additionally, a similar XRD diffraction pattern of β-TCP was observed in the research by Selvia (2012), where the characteristic β-TCP phase exhibited three prominent peaks at 2θ angles of 27.74°, 31.04°, and 34.4°.

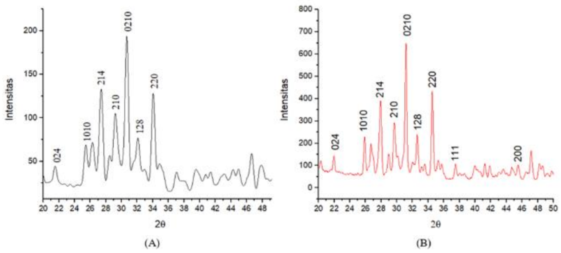

Figure 3. Characteristic (A) β-TCP and (B) β-TCP/NpAg in XRD

Based on the XRD results, the diffraction pattern of NpAg in the synthesis of β-TCP/NpAg was found at 31.1703°. This observation aligns with the literature. According to the study by Padmanabhan et al. (2022), the diffraction pattern of NpAg in the XRD spectrum appeared at 31.76° (211). The XRD results further revealed the presence of NpAg peaks at 37.51° (111) and 45.48° (200) (Figure 3B), consistent with literature findings. According to Gurunathan et al. (2015), NpAg exhibits characteristic peaks in the XRD pattern at 31.9° (111) and 45.31° (200). Similarly, Vanaja and Annadurai (2013) reported NpAg XRD peaks at 38.19° (111) and 46.18° (200). Sahayaraj et al. (2012) also identified NpAg XRD peaks at 38.03° (111) and 46.18° (200).

Antimicrobial Activity Test of β-TCP Embedded with NpAg

The test results indicate that β-TCP/NpAg exhibits the highest antimicrobial activity, with inhibition percentages of 84.37% against *S. aureus*, 95.17% against *E. coli*, and 38.83% against *C. albicans*. Furthermore, when compared with the diameter of the clear zone, β-TCP/NpAg demonstrates the most robust antimicrobial activity against *S. aureus* and *E. coli* but weaker activity against *C. albicans* (Table 4).

Table 4. The Result of Antimicrobial Activity β-TCP Embedded with NpAg

Mikroba uji	Zona Hambat (mm)				
	β-TCP	β-TCP/NpAg	DMSO 1%	Standar	
				Streptomycin	Nystatin
SA	0	17.01 ± 0.52	0	20.16 ± 1.42	-
EC	0	13.99 ± 3.06	0	14.7 ± 1.86	-
CA	0	2.14 ± 0.04	0	-	5.51 ± 0.06

The release of silver ions from nanoparticles contributes to antimicrobial activity (Qing et al., 2018). The combination of Ca-P and Ag+ demonstrates a synergistic effect. Ca-P provides a stable platform for silver incorporation, allowing better control over silver release profiles and minimizing cytotoxicity issues (Kose et al., 2013). Ca-P enhances silver stability, prevents aggregation, and maintains optimal antimicrobial effectiveness (Mokabber et al., 2020). Thus, the combination of calcium phosphate and silver ions exhibits antimicrobial solid properties, utilizing each component, with Ca-P serving as a stable silver base, ensuring slow silver ion release while silver enhances antimicrobial properties.

Furthermore, the absorption of NpAg into β-TCP during the process, where initially the NpAg solution turns from yellow to colorless, suggests that NpAg is fully absorbed into β-TCP. The mass of NpAg absorbed in β-TCP/NpAg is 0.0015 grams. The amount of NpAg absorbed will affect the resulting antimicrobial activity (Table 4). The amount of Ag used for antimicrobial activity in β-TCP/NpAg is 0.0003 mg in 1 mL of the sample.

Combining silver nanoparticles as fillers into the β-TCP matrix has been shown to inhibit the growth of *C. albicans* and *S. aureus* (Spirandeli et al., 2023). Additionally, β-TCP is considered a suitable substrate for antimicrobial metal ions (Spirandeli et al., 2023). The combination of β-TCP with silver nanoparticles enhances antimicrobial activity. Therefore, the synergistic effect of silver nanoparticles and β-TCP leads to the effective inhibition of various microorganisms, making it a potential candidate for various antimicrobial applications (Spirandeli et al., 2023).

Conclusion

The beta-tricalcium phosphate (β-TCP) synthesized from $Ca(OH)_2$ and H_3PO_4 in this study exhibits a hexagonal crystal structure characterized by pure crystal planes with diffraction pattern peaks at 2θ angles of 21.541, 25.339, 27.619, 30.881, 32.179, and 34.231. Additionally, the synthesized silver nanoparticles (NpAg) have an average particle diameter of 92nm and a polydispersity index value of approximately 0.2788. Remarkably, the β-tricalcium phosphate (β-TCP) silver nanoparticle composite demonstrates distinctive XRD characteristics, featuring peak positions for NpAg at 2θ angles of 31.17, 37.51 (111), and 47.10 (200). Moreover, the combined formulation of β-TCP/NpAg showcases promising antimicrobial properties against various test microbes, including S. aureus, E. coli, and Candida albicans, with inhibition values of 84.37%, 95.17%, and 38.83%, respectively. Further research is suggested to explore the variations in furnace temperature during β-TCP formation. Additionally, investigations into the effects of stirring duration on the formation of β-TCP/NpAg and its antimicrobial activity are warranted.

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