

Research Article

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Structural and Biological Enhancement of Cu-Incorporated Biphasic Calcium Phosphate Synthesized via a Sol-Gel Combustion Method

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Abstract

Biphasic Calcium Phosphate (BCP) is a critical bioceramic for bone regeneration due to its biocompatibility and osteoconductivity. This study investigates the enhancement of BCP through the incorporation of copper (Cu) ions using a sol-gel combustion method at concentrations of 0, 1, 3, and 5 mol%. XRD analysis revealed that while hydroxyapatite (HAp) remained the primary phase, Cu incorporation promoted the formation of secondary β -Tricalcium Phosphate (β -TCP) and Calcium Oxide (CaO) phases. XANES spectra confirmed that Cu exists in a +2 oxidation state and forms a separate CuO phase rather than substituting for Ca sites. Bioactivity assessments via 7-day immersion in Simulated Body Fluid (SBF) demonstrated that the 5Cu-BCP sample most effectively promoted the formation of a bone-like apatite layer with a Ca/P ratio of 1.63. Antibacterial tests showed that Cu-doped samples inhibited *S. aureus* growth, but only 5Cu-BCP was effective against the Gram-negative *P. aeruginosa*. Furthermore, while the 1Cu-BCP sample showed significant cytotoxicity, higher Cu concentrations (3Cu and 5Cu) exhibited better cell viability, likely due to more effective structural incorporation of CuO. These findings suggest that 5Cu-BCP is a promising material for biomedical applications, offering a superior balance of bioactivity, antibacterial performance, and biocompatibility.

Keywords: Biphasic Calcium Phosphate, XANES, Antibacterial Activity, Bioactivity

1. Introduction

Biphasic Calcium Phosphate (BCP) is a widely used bioceramic material in biomedical applications due to its excellent biocompatibility, osteoconductivity, and chemical similarity to the mineral phase of natural bone (1–5). BCP typically consists of a combination of Hydroxyapatite (HAp) and β -Tricalcium Phosphate (β -TCP), where the balance between these two phases allows for controlled biodegradation and bone regeneration (6, 7). Hydroxyapatite provides good structural stability and long-term support, while β -TCP exhibits higher resorbability, enabling gradual replacement by newly formed bone tissue.

Because of these properties, BCP has been extensively applied in bone graft substitutes, dental implants, and tissue engineering scaffolds (8–11).

In recent years, the incorporation of transition metal ions into BCP has attracted considerable attention as a strategy to enhance its biological and functional properties. Various transition metals, such as Zn, Fe, Mn, and Cu, have been introduced into calcium phosphate systems to improve antibacterial activity, bioactivity, and osteogenic performance (12–16). Among these elements, Copper (Cu) has been particularly interesting because of its well-known antibacterial properties and its role in angiogenesis and bone metabolism (17, 18). The

incorporation of Cu into BCP can enhance antibacterial performance and may promote tissue regeneration (19, 20). However, excessive Cu content may lead to cytotoxic effects due to the release of Cu ions. Therefore, controlling the amount and distribution of Cu within the BCP structure is essential to achieve a balance between improved biological functionality and acceptable biocompatibility (21–23).

In this work, Cu-incorporated BCP was synthesized using a sol–gel Combustion Method. The structural phase composition was analyzed using X-ray diffraction, while the local structure and chemical state of Cu were investigated by X-ray Absorption Spectroscopy. In addition, the bioactivity of the materials was evaluated through immersion in Simulated Body Fluid (SBF). The antibacterial performance was examined against representative Gram-positive and Gram-negative bacteria, and the cytotoxicity of the materials was assessed to determine their biocompatibility. These investigations provide comprehensive insights into the structural characteristics and biological performance of Cu-modified BCP materials for potential biomedical applications.

2. Materials and Experiment

2.1 Synthesis

Cu-modified CaP was synthesized using a sol–gel method. The precursor materials were accurately weighed to obtain Cu/Ca ratios of 0.0, 1, 3, and 5 mol%, designated as CaP, 1Cu–CaP, 3Cu–CaP, and 5Cu–CaP, respectively. Calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 99%, Sigma–Aldrich) and copper nitrate (CuNO_3 , 99.8%, Sigma–Aldrich) were dissolved in 100 mL of 2 M ethanol and stirred at room temperature for 24 h. Triethyl phosphate ($(\text{C}_2\text{H}_5)_3\text{PO}_4$, TEP; Sigma–Aldrich, Singapore) was measured according to the stoichiometric Ca/P ratio of 1.67 and hydrolyzed in an ethanol–water mixed solvent (6:1 v/v), followed by stirring at room temperature for 24 h. The two prepared solutions were then mixed and continuously stirred for 30 min. The resulting sol was aged at room temperature for 20 h and subsequently heated at 50 °C for 55 h before being dried at 80 °C. The obtained dried powder was first combusted with a heating rate of 20 °C min^{-1} up to 400 °C and maintained for 2 h. Finally, the powder was calcined at 780 °C for 2 h with a heating rate of 5 °C min^{-1} (24–26). BCP powder was uniaxially pressed into 10 mm

diameter pellets using a stainless steel die, followed by sintering in air at 1250 °C in an electric furnace with heating and cooling rates of 5 °C/min and a dwell time of 5 h.

2.2 Structural Characterization

The phase composition and structural properties of the samples were analyzed using X-ray diffraction (XRD). The XRD patterns were recorded over a 2θ range of 20–70° with a step size of 0.02° using Cu K α radiation on a Bruker D2 PHASER diffractometer. The detailed XRD results and refinement analysis are discussed in the following sections.

To investigate the local structure and oxidation states of Cu, X-ray absorption spectroscopy (XAS) measurements were performed at Beamline 5 of the Synchrotron Light Research Institute at the Siam Photon Laboratory. The XAS experiments were carried out in fluorescence mode using a four-channel Si drift detector.

2.3 Simulated Body Fluid (SBF) immersion

Bone-like apatite formation on the sample surfaces was induced by immersion in simulated body fluid (SBF), prepared according to the Kokubo method. Each porous sample was immersed in SBF at 37 °C for 2 weeks. After immersion, the pellets were retrieved, allowed to dry naturally, and subsequently gold-coated via sputtering. The formation and surface morphology of the apatite layer were then examined using SEM (27).

2.4 Antibacterial activity

Antibacterial activity was assessed against Gram-positive *Staphylococcus aureus* (ATCC 25923) and Gram-negative *Pseudomonas aeruginosa* (ATCC 27803) via the disc diffusion method. Bacterial cultures were prepared in nutrient broth at 37 °C for 24 h, and agar plates were inoculated with fresh suspensions (1×10^8 CFU/mL). The pellets were incubated at 37 °C for 24 h, and inhibition zone diameters were measured using a Vernier caliper and expressed in mm (28).

2.5 Cytotoxicity

L-929 cells (ATCC CCL-1) were cultured in EMEM supplemented with 10% FBS and seeded into 96-well plates at 3×10^3 cells per well. After 6 h incubation at 37 °C with 5% CO_2 for cell attachment, the medium was replaced

with fresh EMEM containing 10% FBS, and sterile samples (0.1 mg/well) were added. Following 18 h incubation, cytotoxicity was assessed using an MTT assay kit (ab211091). The cells were washed with PBS (pH 7.4), then incubated with 50 μ L serum-free EMEM and 50 μ L MTT reagent for 3 h. After adding 150 μ L MTT solvent and shaking for 15 min, absorbance was measured at 590 nm relative to control cells.

3. Results and Discussion

3.1 XRD analysis

The XRD patterns of BCP, 1Cu-BCP, 2Cu-BCP, 3Cu-BCP, and 5Cu-BCP are presented in Figure 1. By comparing the experimental patterns with the calculated standard XRD patterns of hydroxyapatite (HAp; JCPDS No.09-0432), β -Tricalcium Phosphate (β -TCP; JCPDS No.09-0169), and Calcium Oxide (CaO; JCPDS No.00-004-077), it is evident that the primary crystalline phase in all samples is HAp. In addition, β -TCP and CaO are detected as secondary phases. As the Cu content increases, the relative amounts of the β -TCP and CaO phases also increase. In particular, the formation of the β -TCP phase becomes more pronounced with higher Cu incorporation. This result suggests that the presence of Cu may influence the phase balance of the biphasic calcium phosphate system, promoting the formation of the β -TCP phase. Furthermore, no diffraction peaks corresponding to metallic Cu or any Cu-containing compounds are observed in the XRD patterns. This absence is likely due to the very low concentration of Cu in the samples, which may be below the detection limit of the XRD technique.

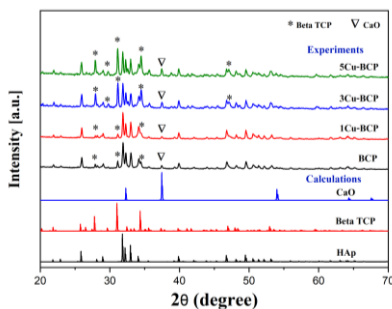


Figure 1 XRD pattern for all samples.

3.2 XAS analysis

The normalized Cu K-edge XANES spectra of 1Cu-BCP, 2Cu-BCP, 3Cu-BCP, and 5Cu-BCP are presented in Figure 2. X-ray Absorption Spectroscopy (XAS) is a powerful technique that can provide information about the local environment and atomic coordination surrounding Cu atoms in the samples. The spectra were compared with standard references of Copper(II) Oxide (CuO) and Copper(I) Oxide (Cu₂O). All samples exhibit an absorption edge at approximately 8985 eV, which is consistent with the absorption edge position of the CuO standard. This result indicates that the oxidation state of Cu in all samples is +2. Furthermore, the overall spectral shapes of all samples closely resemble that of the CuO reference. This observation suggests that Cu atoms do not substitute for Ca sites in the BCP structure. Instead, Cu segregates and forms a separate CuO phase within the samples.

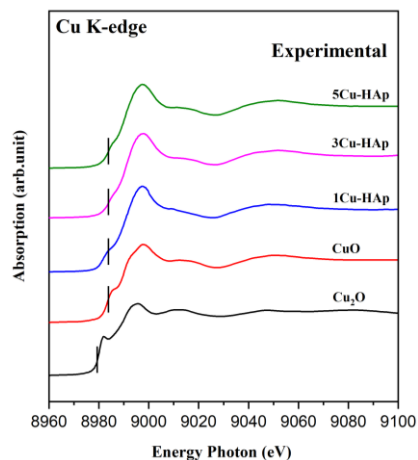


Figure 2 Normalized Cu K-edge XANES spectra for all samples and the Cu standards.

3.3 SBF immersion

Figure 3 shows the SEM images of the surface morphology of ceramic granules of **Biphasic Calcium Phosphate (BCP)**, 1Cu-BCP, 2Cu-BCP, 3Cu-BCP, and 5Cu-BCP after immersion in **Simulated Body Fluid (SBF)** for 7 days. After immersion, particle aggregates were observed to deposit on the surfaces of the samples, indicating the formation of new surface layers associated with bioactive reactions in the SBF environment.

For the BCP sample (Figure 3A), the surface shows aggregated particles forming clusters on the ceramic granules. When compared with Cu-modified samples, the deposition behavior becomes more pronounced and exhibits distinct morphological characteristics depending on the Cu content. In the 1Cu-BCP sample (Figure 3B), the deposited particles exhibit a pineapple-like morphology on the surface. In the 3Cu-BCP sample (Figure 3C), small granular particles are observed to be uniformly distributed across the surface. Meanwhile, in the 5Cu-BCP sample (Figure 3D), the deposited layer appears as rod-like crystalline structures covering the sample surface.

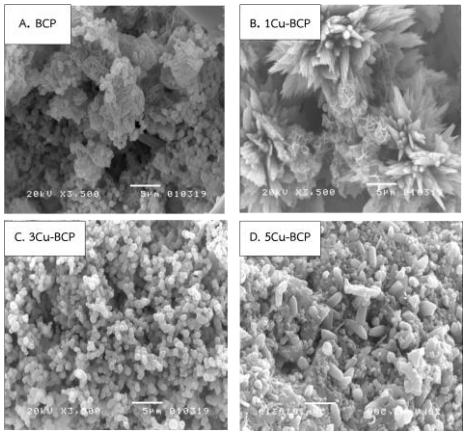


Figure 3 SEM image after immersion in SBF for 7 days.

Table 1 The elemental composition in atomic percentages of the formation layers after immersion in SBF for 7 days.

Atomic%	Sample			
	BCP	1Cu-BCP	3Cu-BCP	5Cu-BCP
Ca	12.65	21.73	21.19	17.69
P	2.92	N/A	8.48	10.88
O	73.66	77.87	64.53	71.43
Mg	7.55	0.02	0.35	N/A
Na	0.59	0.38	1.51	N/A
Cl	2.63	N/A	3.94	N/A
Ca/P	4.33	N/A	2.5	1.63

Energy-dispersive X-ray spectroscopy (EDS) was used to analyze the elemental composition of the deposited layers, and the

corresponding EDS spectra are shown in Figure 4. The atomic percentages derived from the EDS analysis are summarized in Table 1.

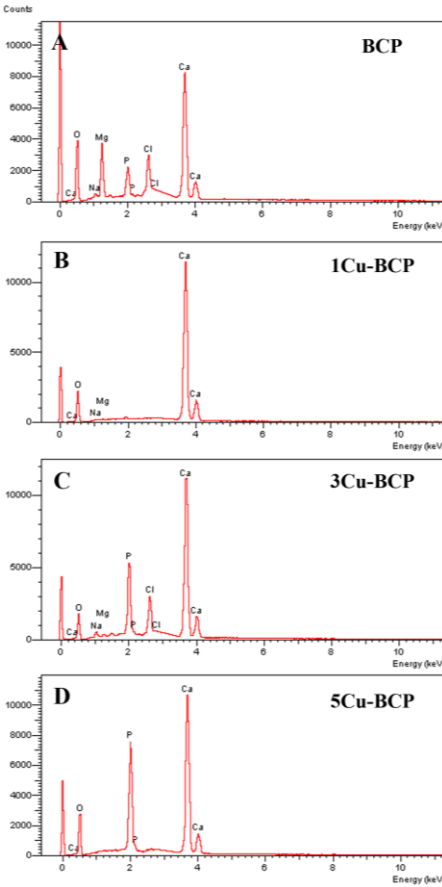


Figure 4 EDS spectra for all samples.

For the BCP sample, the calculated Ca/P molar ratio is 4.33, which deviates significantly from the typical apatite ratio. This deviation is attributed to the precipitation of calcium oxide (CaO) on the surface during immersion in SBF, which increases the Ca content and alters the Ca/P ratio. In the 1Cu-BCP sample, the detected elements include Ca, O, Mg, and Na, while P is not observed, indicating that CaO is predominantly deposited on the sample surface. For the 3Cu-BCP sample, the detected elements are Ca, P, O, Mg, Na, and Cl, suggesting the initial formation of an apatite-like layer on the surface, with a Ca/P ratio of 2.5, indicating that some CaO is still present. In

contrast, the 5Cu–BCP sample shows the presence of Ca, P, and O, with a Ca/P molar ratio of 1.63, which is very close to that of hydroxyapatite (Ca/P = 1.67). These results indicate that the 5Cu–BCP sample promotes the formation of an apatite-like layer most effectively and therefore exhibits the best bioactivity among all investigated samples.

3.4 Antibacterial activity

The antibacterial activity of the samples was evaluated against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *S. aureus* is a Gram-positive bacterium with a spherical shape that typically forms grape-like clusters. This bacterium is commonly found on human skin and in the nasal cavity. It is also recognized as one of the major causes of infectious arthritis, which most frequently affects the knee joint. In contrast, *P. aeruginosa* is a Gram-negative, rod-shaped bacterium. Infections caused by this pathogen can spread in hospital environments and may be associated with contaminated medical equipment, skin contact, disinfectants, and food.

The antibacterial performance of Cu-incorporated BCP samples was evaluated using an inhibition zone test against both bacterial strains. The inhibition zones appeared as clear circular regions surrounding the samples, as shown in Figure 5. The results indicate that most Cu-doped BCP samples were able to inhibit the growth of *S. aureus*, with inhibition zone diameters summarized in Table 2.

In contrast, for *P. aeruginosa*, an inhibition zone was observed only in the 5Cu–BCP sample. This finding suggests that a higher Cu content significantly enhances the antibacterial effectiveness against Gram-negative bacteria. Therefore, among all samples, 5Cu–BCP exhibits the best antibacterial performance, demonstrating effective inhibition against both *S. aureus* and *P. aeruginosa*.

Table 2 The antibacterial activity tested by the disk diffusion technique.

Sample	The diameter of the zone of inhibition (cm)	
	<i>S. aureus</i>	<i>P. aeruginosa</i>
BCP	1.5	1.3
1Cu-BCP	1.7	N/A
3Cu-BCP	1.75	N/A
5Cu-BCP	1.82	1.4

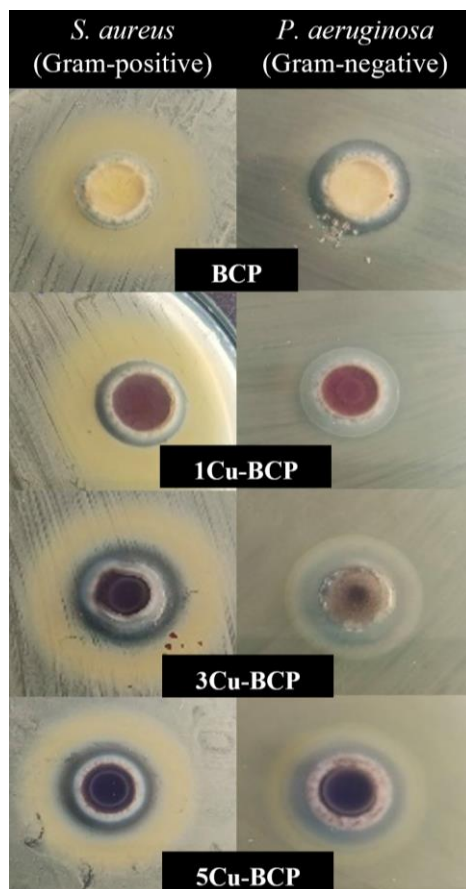


Figure 5 Photographs of antibacterial test results against *S. aureus* and *P. aeruginosa* of all samples.

3.5 Cytotoxicity

The cytotoxicity of the samples was evaluated using the MTT assay. The percentage of cell viability for BCP, 1Cu–BCP, 2Cu–BCP, 3Cu–BCP, and 5Cu–BCP was found to be 80.82 ± 2.27 , 47.40 ± 3.78 , 76.21 ± 5.12 , and 79.37 ± 3.81 , respectively, as shown in Figure 6.

Although XRD analysis reveals the presence of a CaO secondary phase, it does not exhibit a significant effect on cytotoxicity. The results indicate that the 1Cu–BCP sample exhibits the highest cytotoxicity among all samples. This behavior may be attributed to the more pronounced phase separation of Copper(II) Oxide (CuO) from the BCP matrix in the 1Cu–BCP sample, which could facilitate the release of Cu ions into the surrounding environment. In contrast, for the 3Cu–BCP and 5Cu–BCP

samples, CuO may be more effectively embedded or incorporated within the BCP structure. As a result, the release of Cu ions is likely more limited, leading to lower cytotoxic effects and higher cell viability. These findings suggest that the distribution and structural incorporation of Cu species play an important role in controlling the cytotoxic behavior of Cu-modified BCP materials.

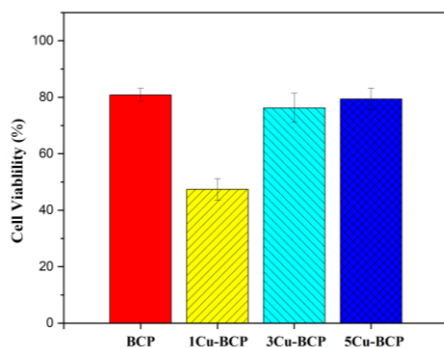


Figure 6 The cytotoxicity test of all samples.

4. Conclusions

The synthesis of Cu-incorporated BCP via the sol-gel combustion method successfully produced materials with enhanced biological properties. Structural analysis through XRD and XAS indicates that Cu exists as CuO and influences the phase balance by promoting β -TCP formation, which is beneficial for controlled biodegradation. The biological performance of these materials is heavily dependent on the Cu concentration; specifically, the 5Cu-BCP sample demonstrated the highest bioactivity by forming an apatite-like layer closest to the stoichiometric ratio of natural bone. This same concentration also provided the most robust antibacterial defense, successfully inhibiting both Gram-positive (*S. aureus*) and Gram-negative (*P. aeruginosa*) bacteria. Crucially, the study revealed that higher Cu content does not necessarily equate to higher toxicity; the 5Cu-BCP sample showed significantly higher cell viability than the 1Cu-BCP sample, suggesting that the structural distribution of Cu within the BCP matrix is vital for controlling ion release and ensuring biocompatibility. Consequently, Cu-modified BCP, particularly at a 5 mol% concentration, represents a multifunctional bioceramic capable of supporting bone regeneration while

simultaneously preventing post-surgical infections.

Declaration of Conflicting Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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