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Alginate-Whitlockite Composite Scaffolds for Bone Tissue Engineering: Synthesis, Characterization, and Biocompatibility Evaluation

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Abstract

Whitlockite (WH), a calcium phosphate mineral, has emerged as a promising material in bone tissue engineering due to its excellent biocompatibility and osteogenic potential. This study aimed to biologically characterize an alginate-whitlockite (Wh-Al) composite scaffold to evaluate its potential in promoting bone regeneration. The scaffold was synthesized using an ultrafast hydrothermal method to produce WH nanoparticles, which were subsequently incorporated into an alginate matrix. The scaffold was extensively characterized to assess its morphology and composition. Scanning electron microscopy (SEM) revealed a porous microstructure favorable for cell attachment and proliferation, while X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) confirmed the successful integration of WH nanoparticles into the alginate matrix. Biological evaluation was conducted using the MTT assay with human osteoblast-like MG-63 cells under ISO 10993-6:2016 standards. The results demonstrated excellent biocompatibility, with cell viability exceeding 90% across all tested conditions, indicating a high potential for osteogenesis. These findings underscore the ability of the alginate-whitlockite composite scaffold to support cellular activity and promote bone tissue regeneration. The porous structure and bioactive composition of the scaffold make it a suitable candidate for bone tissue engineering applications. Further in vivo studies are recommended to investigate the scaffold's osteoinductive properties and its clinical efficacy in repairing bone defects, ultimately advancing the development of regenerative therapies for orthopedic and dental applications.

Keywords: *Alginate, Bone Regeneration, Osteogenesis, Scaffold, Whitlockite.*

Introduction

Whitlockite (WH; Ca18Mg2(HPO4)2(PO4)12) is a calcium phosphate ceramic that is notably enriched with magnesium ions. Constituting approximately 25–35% of bone mineral by weight, WH is recognized as the second most abundant mineral in human bone, following hydroxyapatite (HAp). [1, 2]. While hydroxyapatite is the primary component of bone mineral, whitlockite's distinct magnesium-rich composition is particularly noteworthy, as magnesium plays a critical role in bone metabolism and overall health. The incorporation of magnesium in whitlockite's structure makes it an attractive candidate for applications in bone tissue engineering, due to its potential to enhance bone regeneration and improve mechanical properties when used in scaffolding materials. [3, 4].

Several studies have highlighted WH nanoparticles for their substantial anti-inflammatory effects, which are mediated through the suppression of key inflammatory

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cytokines and mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and nitric oxide (NO). These inflammatory markers are pivotal in the body's response to injury and infection, and their overproduction can impede the bone regeneration process [5]. WH's ability to downregulate these markers provides a dual benefit: promoting tissue healing while simultaneously reducing inflammation, which is essential for maintaining the delicate balance between bone resorption and formation during the healing process [6, 7]. WH has demonstrated the ability to promote osteogenic differentiation, a process where mesenchymal stem cells (MSCs) are stimulated to develop into osteoblasts, the cells responsible for bone formation. This property of WH, combined with its capacity to inhibit osteoclastic activity—responsible for bone resorption—makes it a promising candidate for therapeutic applications in bone repair and regeneration [8, 9]. Notably, WH can uniquely convert into hydroxyapatite (HAp) during the early stages of bone regeneration, forming mechanically enhanced HAp-neo bone tissue. This conversion process contributes to the mechanical stability and strength of the newly formed bone, a key factor in the successful integration of bone scaffolds in clinical applications [7, 10, 11].

In tissue engineering, alginate, a naturally derived polysaccharide obtained from brown seaweed, has garnered considerable interest due to its excellent biocompatibility, biodegradability, and versatile applications. Alginate has been extensively used in the development of various three-dimensional (3D) scaffold materials, including hydrogels, microspheres, microcapsules, sponges, and fibers. Its ease of gelation, combined with its non-toxic nature, makes it an ideal candidate for use in biomedical applications, particularly in tissue engineering. Alginate-based biomaterials are frequently employed as cell carriers and drug delivery systems due to their ability to encapsulate cells and bioactive molecules within their structure, thereby protecting them and ensuring sustained release [12–15]. The biocompatibility of alginate is especially important in tissue engineering, where scaffolds must support cell adhesion, proliferation, and differentiation without inducing adverse immune responses. Alginate's versatility has made it a staple in regenerative medicine, particularly in bone regeneration, where it can be combined with other biomaterials to enhance the mechanical and biological properties of scaffolds [13, 14, 16].

Bone regeneration is a highly orchestrated physiological process that is vital for the natural healing of fractures and the continuous remodeling of bone tissue throughout life. This process, however, can be compromised in certain clinical situations, such as trauma, infections, tumor excision, and congenital skeletal abnormalities, where large-scale bone regeneration is necessary. Conditions such as osteoporosis, atrophic non-unions, and avascular necrosis further complicate bone healing, often necessitating the use of advanced bone grafts or scaffolding materials. A major challenge in such clinical scenarios is the risk of bacterial contamination, which can lead to treatment failure despite the use of antibiotics. One of the primary reasons for this failure is the formation of resistant microbial biofilms on implant surfaces, which not only impede the healing process but also alter the inflammatory response that is critical for successful bone regeneration. Bacterial biofilms are highly resistant to antibiotics and immune responses, making the prevention of infection a key consideration in the design of bone scaffolds [17].

Bacterial infections are a leading cause of implant failure, particularly in the context of bone grafts. [18]. An ideal bone scaffold must possess both antibacterial properties and biocompatibility to address this issue. Treating implant-associated infections is challenging

and costly, often requiring prolonged antibiotic use or implant removal. This has prompted a growing interest in developing multifunctional scaffolds that not only support bone regeneration but also provide antimicrobial protection [19, 20]. Recent advancements in the synthesis of WH nanoparticles have enabled direct comparisons between WH, hydroxyapatite (HAp), and β-tricalcium phosphate (β-TCP), two commonly used biomaterials in bone tissue engineering [21, 22]. Studies have shown that WH-reinforced scaffolds demonstrate superior bone-specific differentiation and regenerative capabilities compared to HAp-reinforced scaffolds. Furthermore, WH implants, with their intermediate resorption rate, promote bone regeneration that is comparable to or superior to that of both HAp and β-TCP [23]. This makes WH a highly promising material for use in bone regeneration, particularly in clinical situations where infection control and mechanical strength are critical [13, 24]. The present study aims to explore the properties of alginate-incorporated whitlockite particles, particularly when combined with antibiotics, to assess their potential in addressing both bone regeneration and bacterial contamination challenges. The study will focus on characterizing the structural and compositional features of these materials and evaluating their potential applications in the biomedical and tissue engineering fields.

Materials and Methods

Synthesis of Whitlockite (WH)

The synthesis of Whitlockite (WH; Ca18Mg2(HPO4)2(PO4)12) began with the preparation of calcium and magnesium nitrate solutions. A total of 2.96 grams of calcium nitrate ($Ca(NO_3)_2$) was dissolved in 25 mL of distilled water under constant stirring to ensure complete dissolution. Simultaneously, 0.449 grams of magnesium nitrate ($Mg(NO_3)_2$) was dissolved in 3.5 mL of distilled water in a separate beaker and stirred until fully dissolved. In another container, 2.5641 grams of diammonium hydrogen phosphate ($(NH_4)_2HPO_4$) were dissolved in 20 mL of distilled water. The calcium and magnesium nitrate solutions were then slowly added to the diammonium hydrogen phosphate solution under continuous stirring to ensure homogeneity. This step was performed gradually to prevent premature precipitation and to maintain uniformity in the solution.

Once the solutions were combined, the pH of the mixture was adjusted to 6 using ammonia solution (NH_4OH), carefully adding the ammonia dropwise while monitoring the pH with a pH meter. After reaching the desired pH, the solution was stirred for an additional hour to ensure complete reaction and homogenization. The mixture was then autoclaved at 200°C for 15 minutes to promote crystallization and the formation of the Whitlockite composite. After autoclaving, the mixture was allowed to cool to room temperature naturally. The resulting precipitate was collected by filtration and washed thoroughly with ethanol several times to remove any unreacted materials or impurities. The washed precipitate was then dried overnight at 60°C to obtain the final calcium-magnesium-phosphate composite in powder form.

Preparation of Alginate-Whitlockite (Al-WH) Scaffold

To prepare the alginate-Whitlockite composite scaffold, 2 grams of sodium alginate were dissolved in 100 mL of distilled water. The solution was stirred at room temperature for one hour to ensure the complete dissolution of the sodium alginate, forming a viscous alginate solution. Following this, the synthesized Whitlockite (WH) powder was added to the alginate solution in three different concentrations: 0.1%, 0.2%, and 0.3% by weight. The mixture was stirred for an additional two hours to ensure uniform dispersion of the WH particles throughout the

alginate matrix. The well-mixed alginate-Whitlockite solution was then transferred into molds and frozen at -20°C overnight to maintain the structural integrity of the scaffold during the subsequent freeze-drying process.

The frozen scaffolds were lyophilized to remove all moisture, resulting in a porous, dry scaffold with interconnected pores, ideal for tissue engineering applications. This porous structure is essential for enhancing cell attachment and nutrient exchange within the scaffold.

Crosslinking of the Alginate-Whitlockite Scaffold

The lyophilized alginate-Whitlockite scaffold was subjected to crosslinking using a 0.5 M calcium chloride ($CaCl_2$) solution. The scaffold was immersed in the calcium chloride solution for 3 minutes, during which ionic cross-linking occurred between the calcium ions and the alginate, stabilizing the scaffold's structure. After crosslinking, the scaffold was washed thoroughly with distilled water to remove any residual calcium chloride, which could otherwise interfere with subsequent biological applications. The scaffold was then lyophilized once more to obtain the final, stable alginate-Whitlockite composite scaffold.

Characterization Techniques

The structural and compositional characteristics of the prepared Whitlockite and alginate-Whitlockite composite scaffolds were analyzed using various characterization techniques. X-ray diffraction (XRD) was used to determine the crystalline structure and phase composition of the synthesized Whitlockite. Fourier-transform infrared spectroscopy (FTIR) was employed to identify the functional groups and confirm the chemical composition of the materials. Scanning electron microscopy (SEM) provided detailed information on the surface morphology and pore structure of the scaffolds, while energy-dispersive X-ray spectroscopy (EDS) was used to analyze the elemental composition and distribution of calcium, magnesium, and phosphate within the composite.

MTT Assay for Cytotoxicity Assessment

To evaluate the biocompatibility of the alginate-Whitlockite scaffolds, an MTT assay was performed following the ISO 10993-6:2016 standard. Human osteoblastic MG-63 cells were seeded into a 96-well plate at a concentration of 10,000 cells per well and incubated with extracts from the scaffold for 24 hours. After incubation, the MTT solution was added to each well and incubated for an additional 4 hours at 37°C. The resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO), and the absorbance was measured at 570 nm using a microplate reader.

Results

The X-ray diffraction (XRD) analysis (Figure 1A) of the alginate-whitlockite (Wh-Al) composites reveals distinct structural characteristics across different concentrations of Whitlockite. The XRD pattern for pure alginate exhibits a broad, amorphous peak, which is typical of its non-crystalline nature. In contrast, the pattern for pure Whitlockite shows sharp, well-defined crystalline peaks, confirming its crystalline structure. When Whitlockite is incorporated into the alginate matrix at concentrations of 1%, 2%, and 3%, the XRD patterns reflect the successful integration of Whitlockite within the alginate matrix. The broad amorphous peak from alginate remains evident across all composite samples. However, crystalline peaks corresponding to Whitlockite are also observed, with their intensity varying according to the Whitlockite concentration. As the concentration of Whitlockite increases, the intensity of these crystalline peaks becomes more pronounced, indicating a stronger crystalline phase in the composite. This

suggests a consistent interaction between the alginate and Whitlockite, with Whitlockite contributing to the crystalline structure within the otherwise amorphous alginate matrix.

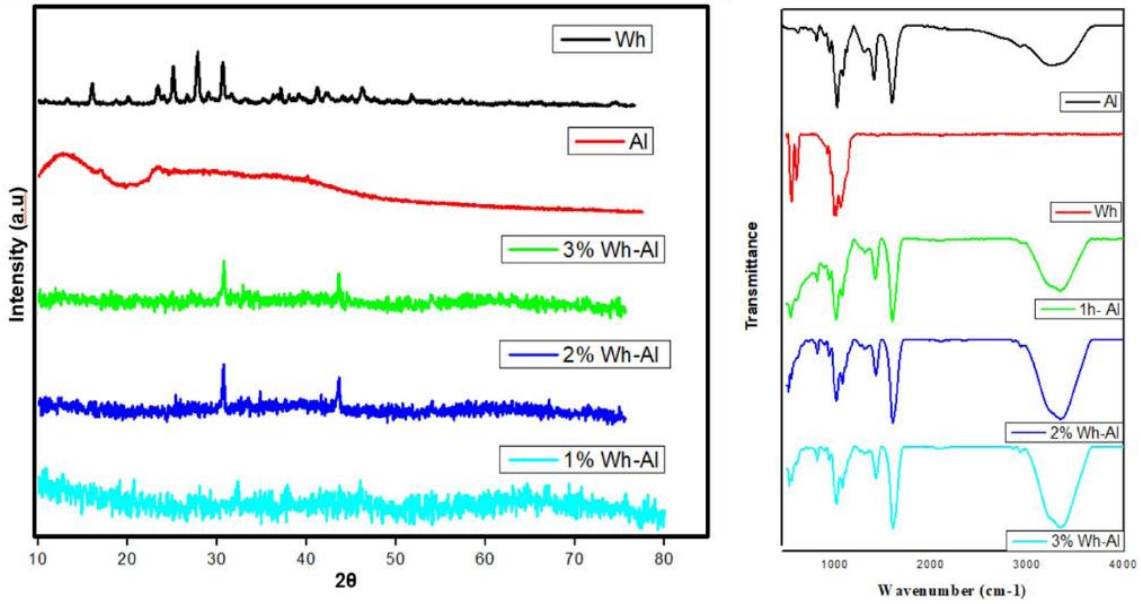

Figure 1. (A) The X-ray Diffractogram and (B) shows the FTIR Spectra of the Representative Scaffolds.

FTIR analysis further corroborates the findings of XRD. The FTIR spectrum (Figure1 B) for pure alginate exhibits characteristic broad absorption peaks, particularly in the region around 3300 cm^{-1}, which corresponds to O-H stretching, and around 1600 cm^{-1}, corresponding to the C=O stretching of carboxylate groups. The spectrum for pure Whitlockite, on the other hand, shows distinct absorption bands in the 1000–1100 cm^{-1} range, which are characteristic of phosphate (PO_4^{3-}) groups present in calcium phosphate materials. The FTIR spectra of the 1%, 2%, and 3% Wh-Al composites reveal absorption peaks from both alginate and Whitlockite, confirming the successful incorporation of Whitlockite into the alginate matrix. Minor shifts in peak positions are observed, particularly in the phosphate and hydroxyl regions, suggesting some level of interaction between the two components at the molecular level. Despite these interactions, the fundamental chemical structures of both alginate and Whitlockite remain largely unchanged, indicating stable incorporation without significant alteration to the individual molecular identities of the materials.

The morphology and elemental composition of the alginate-Whitlockite composites were examined using scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) spectroscopy. The SEM images (Figure 2) revealed a fibrous and layered microstructure, typical of biocompatible materials such as hydroxyapatite and calcium phosphate compounds, which are widely used in biomedical applications. These structures provide evidence of a porous network that could support cell attachment and proliferation, making the composite suitable for tissue engineering applications. The EDX spectra confirmed the elemental composition of the composites, with dominant peaks for oxygen, calcium, and phosphorus, which are key components of calcium phosphate compounds like Whitlockite. Additionally, the presence of magnesium and zinc in certain samples suggests some level of material complexity, potentially enhancing the bioactivity of the composites.

The cytotoxicity of the alginate-Whitlockite composites was assessed using the MTT assay (Figure 3), with concentrations of 1%, 2%, and 3% of Wh-Alg tested. The results, as depicted

in the bar graph, indicate similar cytotoxicity levels for all composite concentrations, with values ranging between 0.23 and 0.25 absorbance units. The control sample exhibited a lower cytotoxicity level of approximately 0.18, while the positive control showed the lowest cytotoxicity at around 0.05. Despite some variability within the measurements, as indicated by the error bars, the data suggest that the Wh-Alg composites maintain moderate levels of cytotoxicity. The cellular viability of 1%Wh-Al at concentrations of 25%,50%, and 75% is gradually increased compared to control meanwhile the cellular viability of 2%Wh-Al and 3%Wh-Al at the same concentrations is decreased compared to 1%Wh-Al. These levels, although higher than those of the control and positive control groups, remain within acceptable ranges for biocompatible materials, making the Wh-Alg composites promising candidates for further investigation in tissue engineering and regenerative medicine applications.

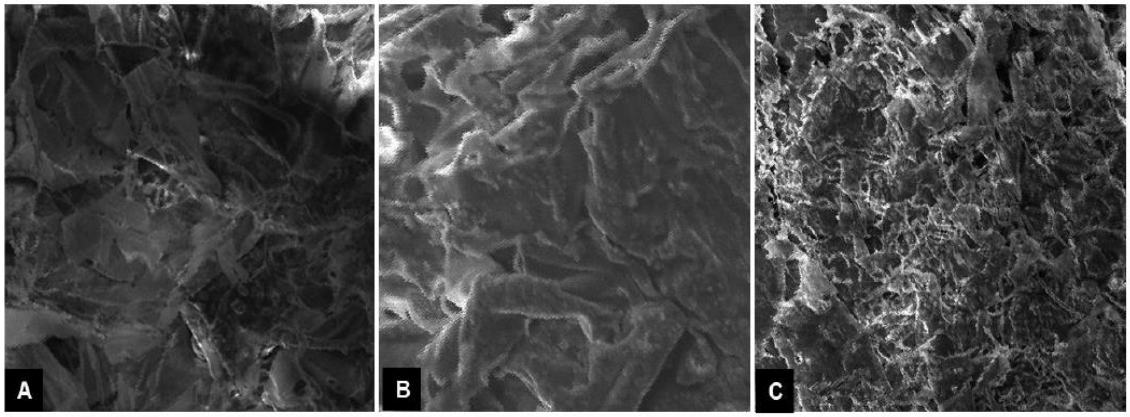

Figure 2. Scanning Electron Microscopic Images of the Whitlockite Incorporated Scaffolds A) 1% Wh - Al, B) 2% Wh- Al, C) 3% Wh- Al.

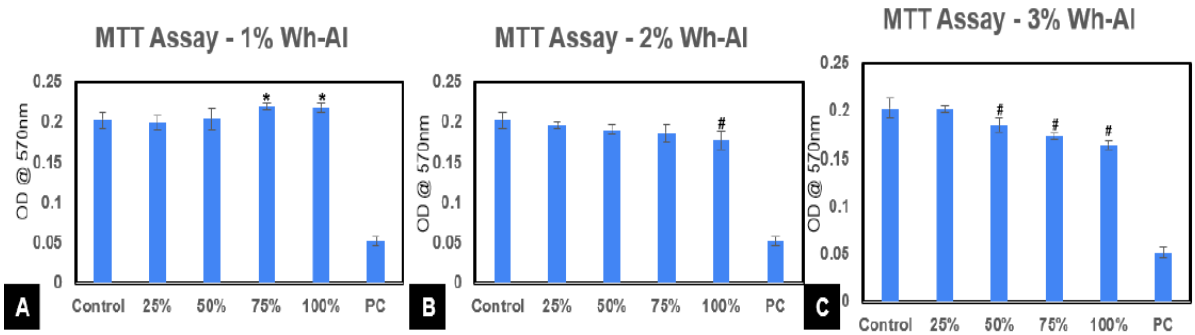

Figure 3 -Biocompatibility Assessment of the Fabricated Scaffolds by Indirect MTT Assay. A) 1% Whitlockite-Alginate Concentration B) 2% Whitlockite- Alginate Concentration C) 3% Whitlockite - Alginate Concentration. MTT Assay Results Compared the Cytotoxicity of Scaffolds with the Control Group, Indicating the Viability of Cells Treated with the Whitlockite-Alginate. Where the * Indicates the Significantly Increased Cellular Viability of Cells Compared to the Control and the # Means the Cellular Viability is Reduced Significantly Compared to the Control

Discussion

The findings of this study demonstrate that the alginate-Whitlockite (WH) composite scaffold exhibits promising characteristics for potential applications in bone tissue engineering. The successful incorporation of

Whitlockite into the alginate matrix, confirmed by XRD, FTIR, SEM, and EDS analyses, suggests that the composite not only retains the structural integrity of both components but also enhances the scaffold's mechanical and biological properties, making it a suitable candidate for promoting bone regeneration.

The XRD results clearly show that the alginate-Whitlockite composite exhibits distinct crystalline peaks corresponding to Whitlockite, while the broad amorphous peak from alginate remains present. As the concentration of Whitlockite increases, the intensity of the crystalline peaks becomes more pronounced, indicating a stronger crystalline phase within the otherwise amorphous alginate matrix. This integration of Whitlockite into the composite is crucial, as Whitlockite's crystalline nature is known to contribute to the mechanical stability of bone tissue scaffolds. The XRD patterns also align with previous studies that have highlighted the crystalline structure of Whitlockite and its ability to enhance the mechanical properties of biomaterials used in bone regeneration [25].

FTIR analysis further confirms the successful incorporation of Whitlockite into the alginate matrix, with characteristic absorption bands from both alginate and Whitlockite visible in the spectra. The minor shifts observed in the phosphate and hydroxyl regions suggest some degree of molecular interaction between the two components, which may contribute to the improved mechanical properties of the scaffold. However, the fundamental chemical structures of both materials remain intact, indicating that the scaffold maintains its stability during the synthesis process. These findings are consistent with other studies that have demonstrated the stable incorporation of calcium phosphate materials into biopolymer matrices without compromising their chemical integrity [26].

The SEM analysis revealed a porous and fibrous microstructure in the alginate-Whitlockite composite, which is essential for promoting cell attachment, proliferation, and nutrient exchange. The presence of a porous network in the scaffold is a key factor in tissue engineering, as it allows for the infiltration of cells and the delivery of nutrients and growth factors necessary for bone regeneration. [27, 28]. The layered structure observed in the SEM images is typical of biocompatible materials such as hydroxyapatite and calcium phosphate compounds, which are widely used in biomedical applications. The elemental composition analysis, conducted via EDS, confirmed the presence of calcium, magnesium, and phosphate, further supporting the successful incorporation of Whitlockite into the scaffold.

The porous nature of the scaffold is particularly advantageous for bone tissue engineering, as it mimics the natural architecture of bone, which is characterized by a hierarchical, porous structure that facilitates cellular activities such as osteogenesis and angiogenesis. Previous studies have emphasized the importance of scaffold porosity in enhancing bone regeneration, and the results of this study are in line with those findings [29–31].

The MTT assay performed on human MG-63 osteoblastic cells demonstrated that the alginate-Whitlockite scaffold exhibits excellent biocompatibility, with no significant cytotoxic effects observed after 24 hours of incubation. This is a crucial finding, as biocompatibility is a fundamental requirement for any material intended for use in tissue engineering. The non-cytotoxic nature of the scaffold, combined with its porous structure and ability to promote cell attachment, suggests that it could provide a suitable environment for bone regeneration [32, 33].

The inclusion of magnesium in the Whitlockite structure likely contributes to the enhanced biocompatibility of the scaffold, as

magnesium is known to play a critical role in bone metabolism and the regulation of osteoblast and osteoclast activity. Several studies have reported that magnesium-enriched biomaterials promote osteogenic differentiation and enhance bone regeneration by stimulating the proliferation and differentiation of osteoblastic cells while simultaneously inhibiting osteoclastic activity [34, 35]. The results of this study are consistent with these findings, suggesting that the alginate-Whitlockite composite has significant potential for use in bone tissue engineering.

The scaffold's alginate component, known for its ability to encapsulate and release bioactive molecules, could potentially be used to deliver antibiotics in a controlled manner, thereby providing both regenerative and antimicrobial benefits. Future studies should explore the scaffold's antimicrobial efficacy, particularly in preventing infections associated with bone implants.

Conclusion

The alginate-Whitlockite composite scaffold developed in this study demonstrates significant potential for bone tissue engineering applications. The successful incorporation of Whitlockite into the alginate matrix enhances the scaffold's mechanical properties, while its porous structure and biocompatibility make it suitable for promoting cell attachment and proliferation. The scaffold's potential for use in antimicrobial applications further increases its appeal in clinical settings where infection control is critical. Future research should focus on in vivo studies to evaluate the scaffold's long-term performance in bone regeneration and its ability to prevent bacterial infections.

Conflict of Interest

Nil.

Acknowledgement

Nil.

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