

Original Article

Synergistic Effects of Zinc-Doped Chitosan and Whitlockite Nanoparticles in Bone Regeneration: A Comprehensive *in vitro* Evaluation

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Bone tissue plays a crucial role in supporting body structure, protecting organs, and regulating metabolic processes. Conditions like osteoporosis, infections, and fractures require innovative solutions for effective bone healing. Recent advancements in biomaterials and nanotechnology, particularly with hydroxyapatite (HAP) and magnesium-substituted whitlockite (WH), present promising avenues for bone regeneration. Zinc, essential for bone health and possessing antibacterial properties, has been shown to enhance osteoblast differentiation through BMP-2 signaling and influence the RANKL/RANK/OPG pathway. In this study, we synthesized zinc-doped chitosan (Zn-Ch) and a calcium-magnesium phosphate (WH) composite using co-precipitation with subsequent neutralization to achieve uniform zinc distribution. The WH-ZnCh scaffold was fabricated by varying ZnCh concentrations (1%, 2%, and 3%) and assessing their impact on crystallinity, chemical interactions, and microstructure. Characterization techniques included X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and energy-dispersive X-ray spectroscopy (EDS) to analyze structural, chemical, and microstructural properties. X-ray diffraction (XRD) revealed a reduction in WH crystallinity with increasing ZnCh concentrations, indicating a transition towards an amorphous structure. Fourier transform infrared spectroscopy (FTIR) confirmed chemical interactions between ZnCh and WH, with significant peak shifts in the 3% composite. Scanning electron microscopy (SEM) displayed an interconnected porous scaffold, ideal for tissue engineering, while energy-dispersive X-ray spectroscopy (EDS) confirmed the homogeneous distribution of calcium, zinc, and other key elements in the composite. MTT assays indicated that lower concentrations of Zn-Ch enhanced cell viability, while higher concentrations exhibited cytotoxic effects. This study successfully synthesized zinc-doped chitosan/whitlockite nanoparticles, revealing decreased crystallinity and enhanced bioactivity through XRD, FTIR, SEM, and EDS analyses. The scaffolds demonstrated a favorable porous structure and mechanical integrity, promoting cell proliferation. The MTT assay confirmed optimal cell viability at 50% concentration, highlighting the nanoparticles' antioxidant properties in reducing oxidative stress. Biocompatibility assessments indicated no significant cytotoxic effects, affirming their safety for bone regeneration. Overall, these multifunctional nanoparticles offer a promising strategy for overcoming challenges in bone tissue engineering, warranting further investigation into their *in vivo* applications and clinical integration.

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Introduction

Bone tissue plays a vital role in the human body, not only providing structural support for muscles and enabling mobility, but also protecting critical organs, housing bone marrow, and regulating

several physiological processes such as mineral homeostasis and endocrine functions. It contributes to the regulation of insulin sensitivity, glucose tolerance, and even cognitive functions. [1] Thus, maintaining the integrity of bone tissue is crucial for overall health and well-being. However, bone tissue can be compromised by a variety of pathological conditions, including infections like osteomyelitis and periodontitis, traumatic injuries such as fractures, cancer, and systemic disorders like osteoporosis. These conditions often lead to impaired bone healing or regeneration, highlighting the need for effective therapeutic interventions. [2]

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Advances in biomaterials and nanomedicine have opened new frontiers in bone regeneration and tissue engineering. The development of bioactive materials that can support bone healing, restore function, and promote osteogenesis is critical in addressing skeletal anomalies. In particular, two key minerals dominate the inorganic composition of bone: hydroxyapatite (HAP) and whitlockite (WH). While HAP has been widely studied for its role in bone physiology, WH, which accounts for approximately 20–35% of the total bone mineral content, is garnering increasing attention for its unique properties. The differences in crystal structure and physicochemical characteristics between HAP and WH suggest that they play distinct roles in bone metabolism. [3]

Whitlockite ($\text{Ca}_{18}\text{Mg}_2(\text{HPO}_4)_2(\text{PO}_4)_{12}$) is a magnesium-substituted calcium phosphate mineral that is naturally present in human bone tissue. This mineral, which is second only to hydroxyapatite in prevalence, exhibits a unique rhombohedral crystal structure and has been shown to play a critical role in bone formation and mineralization. Synthetic forms of whitlockite [WH] are highly bioactive and have demonstrated great potential in bone regeneration and tissue engineering applications. [4] Recent studies suggest that WH is capable of enhancing osteoblast activity and promoting bone healing by facilitating calcium and phosphate ion exchange at the bone-implant interface, thereby improving osseointegration. [5]

In addition to calcium and phosphate, magnesium and zinc are essential trace elements that contribute to bone health. Zinc, in particular, is crucial for numerous cellular functions, including enzyme activity, protein synthesis, and immune response. The recommended daily intake of zinc for adults is around 15 mg, with dietary sources typically providing sufficient levels. [6] Zinc plays a key role in bone tissue production by acting as a co-factor for alkaline phosphatase, an enzyme that is critical for bone mineralization. Moreover, zinc has been found to regulate osteoblast differentiation through the activation of the BMP-2 signaling pathway, which in turn induces the expression of the Runx2/Cbfa1 transcription factor, a key regulator of bone formation. [7,8]

Apart from its physiological function in the human body, zinc has strong antibacterial properties when it is present in ionic and nanoparticle forms, especially zinc oxide and zinc sulfide. Its selective toxicity against both Gram-positive and Gram-negative bacteria has been demonstrated, but its effects on human cells are insignificant. [7]. Zinc ions have a variety of inhibitory effects on the activities of intact bacterial cells, including the generation of glucosyltransferase, polysaccharide synthesis, transmembrane proton translocation, and acid tolerance. It can increase the permeability of bacterial cell membranes to protons and decrease the generation of Adenosine Triphosphate (ATP) in glycolyzing cells. Furthermore, zinc has well-documented antibacterial properties, particularly in its ionic and nanoparticle forms, such as zinc oxide and zinc sulfide. Its selective toxicity against both Gram-positive and Gram-negative bacteria, coupled with minimal cytotoxic effects on human cells, makes zinc an attractive candidate for use in biomedical applications. Zinc ions disrupt bacterial cell functions by inhibiting glucosyltransferase activity, polysaccharide synthesis, and acid tolerance mechanisms, leading to reduced ATP production and increased membrane permeability. These antibacterial properties, along with zinc's role in promoting bone regeneration, underscore its potential in the development of advanced biomaterials. [9]

Recent studies have explored the incorporation of zinc into various biocompatible scaffolds to enhance bone regeneration. For instance, Wang et al. developed a novel composite framework comprising

zinc-doped whitlockite (ZnWH), gellan gum, and human-like collagen. This composite demonstrated significant potential in promoting osteogenic differentiation through the controlled release of ZnWH nanoparticles, which stimulated human bone marrow mesenchymal stem cells (hBMSCs) to increase the expression of alkaline phosphatase (ALP), osteocalcin (OCN), and osteopontin (OPN). These findings highlight the promising role of zinc-doped biomaterials in enhancing bone tissue regeneration. [10] This study aims to further investigate the potential of zinc-doped chitosan/whitlockite nanoparticles in bone regeneration. Specifically, we focus on evaluating the antibacterial, antioxidant, and cytotoxic properties of these zinc-doped biomaterials, with the ultimate goal of developing an effective and biocompatible solution for bone tissue engineering.

Materials and Methods

Preparation of Zinc-Doped Chitosan

To synthesize zinc-doped chitosan (Zn-Ch), a total of 2 grams of chitosan was first dissolved in 100 mL of 0.1 M acetic acid. The dissolution process was carried out under constant stirring at room temperature for a period of 2 hours, ensuring that the chitosan was fully dissolved and the solution was homogenous. This step is crucial to achieve a consistent base for the incorporation of zinc ions.

Subsequently, an equal volume of 0.01 M zinc nitrate solution was slowly introduced into the chitosan solution. The zinc nitrate solution acts as the source of zinc ions, which are known to enhance the biological properties of chitosan by promoting antibacterial activity and improving biocompatibility. The resulting mixture was then carefully titrated with 1 M NaOH solution to adjust the pH to neutral (pH = 7). The slow addition of NaOH, under continuous stirring, helps to prevent the formation of undesirable precipitates, ensuring that zinc ions are uniformly distributed within the chitosan matrix.

Following pH adjustment, the solution was subjected to elevated temperature conditions (60°C) and stirred for 2 hours to promote the interaction between chitosan and zinc ions. This thermal treatment helps facilitate the formation of zinc-chitosan complexes. After the reaction was completed, the mixture was allowed to cool down to room temperature.

To isolate the zinc-doped chitosan, the solution was centrifuged at 4000 rpm for 10 minutes, separating the solid precipitate from the liquid. The precipitate was collected and washed multiple times with distilled water to eliminate any unreacted chemicals. A final ethanol wash was applied to further purify the product, ensuring that no residual impurities were present. The purified precipitate was then dried to obtain a solid form of zinc-doped chitosan (Zn-Ch), which was stored for further use.

Wh preparation

The calcium-magnesium-phosphate (WH) composite was synthesized through a co-precipitation method. Initially, 2.96 grams of calcium nitrate ($\text{Ca}(\text{NO}_3)_2$) was dissolved in 25 mL of distilled water, and 0.449 grams of magnesium nitrate ($\text{Mg}(\text{NO}_3)_2$) was dissolved in 3.5 mL of distilled water. Both solutions were stirred separately until complete dissolution of the salts was achieved, which is essential to ensure homogeneity during the later stages of composite formation.

In a separate container, 2.5641 grams of diammonium hydrogen phosphate ($(\text{NH}_4)_2\text{HPO}_4$) was dissolved in 20 mL of distilled water. This phosphate source is key to the formation of calcium phosphate,

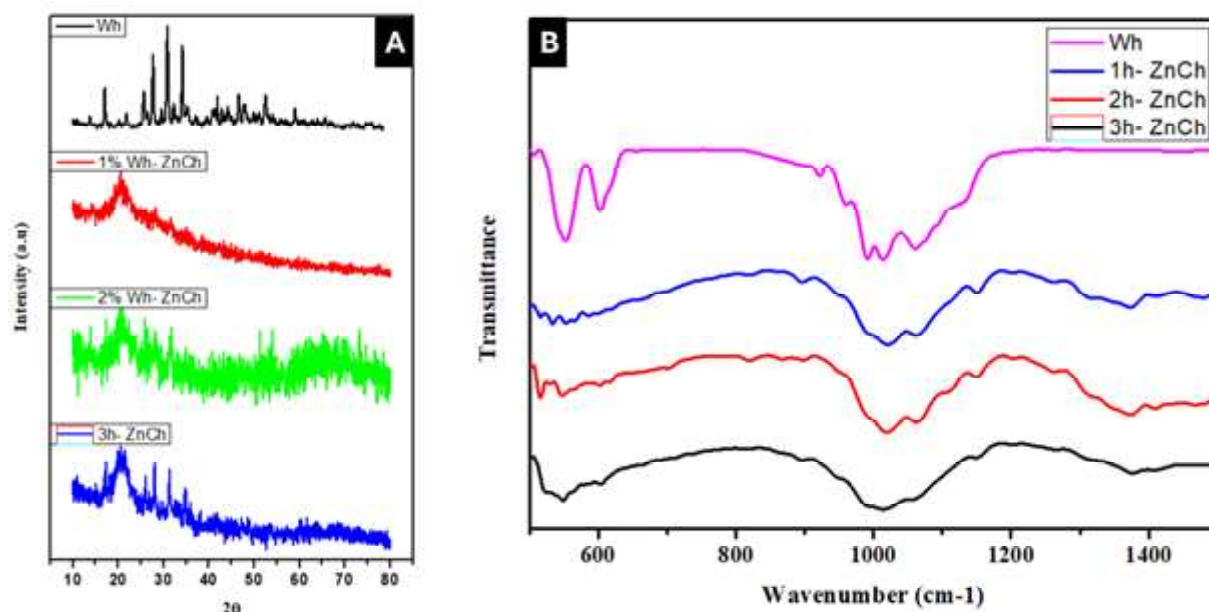


Figure 1: (A) X-ray diffractogram and (B) FTIR Spectra of the representative scaffolds and Whitlockite

a bioactive material commonly used for bone regeneration due to its osteoconductive properties. The solutions of calcium nitrate and magnesium nitrate were then slowly added to the diammonium hydrogen phosphate solution under constant stirring to promote a uniform distribution of calcium and magnesium ions throughout the mixture.

The pH of the mixture was adjusted to 6 by adding ammonia solution (NH₄OH). A controlled pH environment is vital for the precipitation of the desired calcium-magnesium-phosphate phase, avoiding the formation of undesirable side products. After stabilizing the pH, the solution was stirred for an additional hour to ensure complete reaction and homogeneous mixing of the constituents.

To enhance crystallization and ensure the formation of a well-defined composite structure, the mixture was autoclaved at 200°C for 15 minutes. Autoclaving at high temperatures promotes the nucleation and growth of crystals, thus improving the composite's mechanical properties and bioactivity. Once autoclaving was complete, the mixture was allowed to cool to room temperature. The precipitate formed during the process was thoroughly washed with ethanol to remove any unreacted chemicals or impurities. Finally, the washed precipitate was dried overnight to yield the calcium-magnesium-phosphate composite, which would later be combined with Zn-Ch to form the scaffold.

Preparation of WH+Zn-CH Composite Scaffold

To prepare the WH+Zn-CH composite scaffold, 400 mg of zinc-doped chitosan (Zn-Ch) was dissolved in 40 mL of 1% acetic acid. The mixture was stirred for 1 hour at room temperature to ensure complete dissolution and uniform dispersion of Zn-Ch. The addition of acetic acid helps to solubilize the chitosan and zinc complexes, providing a workable solution for scaffold fabrication.

After ensuring complete dispersion of Zn-Ch, WH powder was gradually introduced into the solution. The WH powder was added at three different concentrations (0.1%, 0.2%, and 0.3%) to create

varying composites for testing. The mixture was stirred continuously for 2 hours to ensure homogeneous distribution of the WH particles within the Zn-Ch matrix.

To create a porous scaffold structure, the mixture was frozen overnight at -20°C. Freezing induces the formation of ice crystals, which later sublimate during the lyophilization process, leaving behind a porous network within the scaffold. The frozen mixture was subsequently lyophilized, resulting in a dry, porous composite scaffold with Zn-Ch and WH integrated.

Neutralizing the Scaffold

The scaffold was neutralized to remove residual acetic acid and cross-link the chitosan matrix. This was achieved by immersing the scaffold in 0.5 M NaOH prepared in 70% ethanol. The use of ethanol helps to control the swelling of the scaffold, ensuring that the structure remains intact during neutralization. The immersion allowed for neutralization of any remaining acetic acid and further cross-linking of the chitosan, enhancing the mechanical strength and stability of the scaffold.

Once neutralized, the scaffold was removed from the NaOH solution and placed on filter paper to drain excess liquid. It was then rinsed thoroughly with distilled water to wash away any remaining NaOH and ethanol. The pH of the scaffold was carefully monitored, and additional rinsing was performed if necessary to achieve a pH in the range of 7 to 8, which is suitable for biological applications.

The neutralized scaffold was then frozen again at -20°C overnight and lyophilized to remove any residual moisture, yielding the final WH+Zn-CH composite scaffold ready for subsequent biological testing and evaluation.

Results

XRD Analysis

The X-ray Diffraction (XRD) analysis (Figure 1A) was conducted to

assess the crystallinity and structural changes in the WH-ZnCh composites as a function of zinc content. The XRD patterns revealed a notable decrease in crystallinity and an increase in amorphous characteristics as the zinc concentration increased across the samples. The pure WH sample, represented by the black curve, exhibited sharp, well-defined peaks, indicative of a highly crystalline and ordered structure typical of calcium-magnesium-phosphate (WH) materials. These peaks correspond to specific crystal planes, reflecting the well-ordered arrangement of the atomic lattice.

In contrast, the 1% WH-ZnCh composite, represented by the red curve, showed broader peaks with reduced intensity compared to the pure WH sample. This broadening suggests the presence of amorphous phases in the material, likely caused by the incorporation of zinc-doped chitosan (ZnCh), which disrupts the regular crystalline arrangement of WH. The lower intensity peaks imply that ZnCh interferes with the crystal growth of WH, introducing disorder into the composite.

As the zinc concentration increased to 2% (green curve), the XRD pattern showed even broader and less intense peaks, indicating a further reduction in crystallinity and an increase in the amorphous phase. The reduced sharpness of the peaks suggests a higher degree of structural disorder in the composite, signifying that ZnCh content significantly influences the loss of the WH crystalline structure.

The 3% WH-ZnCh composite (blue curve) demonstrated the most substantial loss of crystallinity. The peaks were the broadest and least defined, reflecting a highly amorphous structure with minimal remaining crystalline phases. This result suggests that the introduction of 3% ZnCh leads to significant structural disruption within the WH matrix, potentially forming a new material phase dominated by amorphous characteristics. The progressive loss of crystallinity with increasing ZnCh concentration is consistent with the hypothesis that ZnCh interferes with the WH crystal lattice, promoting the formation of a more disordered composite structure.

FTIR analysis

FTIR spectroscopy (Figure 1B) was employed to identify functional groups and assess the chemical interactions between WH and ZnCh

in the composite scaffolds. The pure WH sample, shown by the pink curves, exhibited distinct and well-defined peaks corresponding to functional groups characteristic of the calcium-magnesium-phosphate structure. Key absorption bands include those for Si-O and C-O stretching vibrations, which are typical of WH materials and play a crucial role in their bioactivity and mechanical properties.

The incorporation of ZnCh into the WH matrix resulted in notable changes in the FTIR spectra. The 1% WH-ZnCh composite, represented by the blue curve, showed a decrease in transmittance and slight peak shifts. These changes indicate chemical modifications within the material, likely due to the interaction between the zinc-doped chitosan and the WH matrix. The reduced transmittance suggests a denser material, while the peak shifts may reflect the formation of new bonds or the alteration of existing functional groups due to the introduction of ZnCh.

In the 2% WH-ZnCh composite (red curve), more pronounced changes were observed. The peaks became broader, and additional shifts were detected, indicating stronger interactions between WH and ZnCh. The alterations in the spectra suggest that the increased ZnCh content enhances the formation of new functional groups, potentially leading to the development of a composite with improved chemical and mechanical properties. The interaction between WH and ZnCh at this concentration appears to be more extensive, promoting the formation of a more integrated composite structure.

The most significant changes were observed in the 3% WH-ZnCh composite (black curve). In this sample, the FTIR spectrum exhibited a high degree of peak shifting, broader absorption bands, and reduced permeability, reflecting significant chemical modifications. The higher concentration of ZnCh likely leads to the formation of a new composite structure with altered interconnectivity between the functional groups of WH and ZnCh. This result suggests that at higher zinc concentrations, the chemical environment of the composite is drastically changed, leading to a new material with potentially different properties compared to the lower ZnCh-containing composites.

SEM analysis

SEM analysis was performed to evaluate the microstructure and

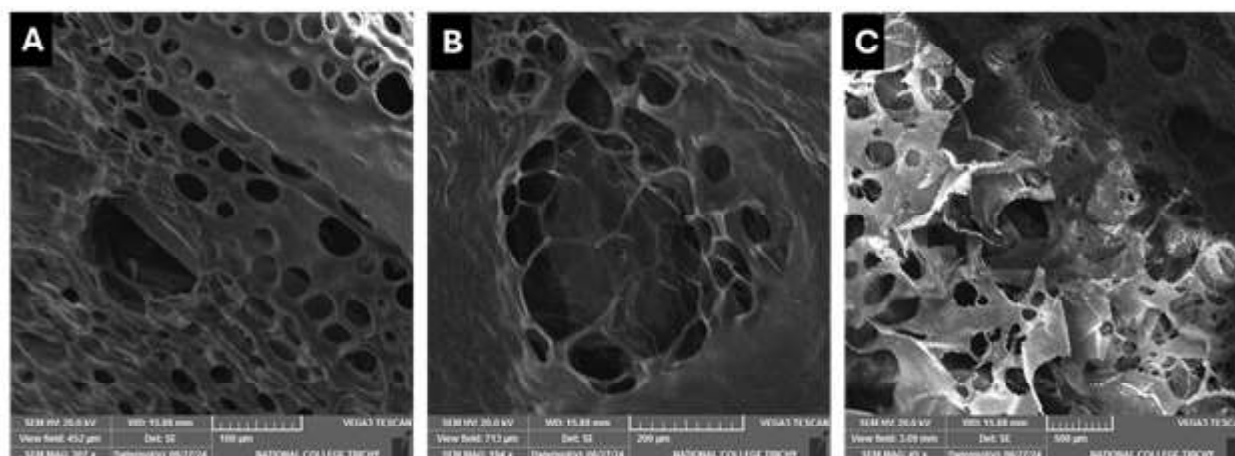


Figure 2: Scanning Electron Microscopic images of the Zinc-doped chitosan/whitlockite incorporated scaffolds A) 1% WH-ZnCH, B) 2% WH-ZnCH and C) 3% WH-ZnCH

surface morphology of the WH-ZnCh composite scaffolds. The SEM images revealed that the scaffolds possess a controlled pore configuration, which is a critical attribute for tissue engineering applications. A well-defined and interconnected porous network is essential for promoting cell growth, tissue ingrowth, and nutrient exchange. The pores within the scaffolds are uniformly distributed, which facilitates the transport of nutrients, oxygen, and waste, crucial for successful tissue regeneration.

The pore size and distribution in the scaffolds also play a role in their mechanical properties. The thick walls of the scaffold contribute to its mechanical strength, ensuring that the scaffold can support cell proliferation and withstand physiological loads. The orientation and arrangement of the pores suggest that the scaffold can bear mechanical stress while maintaining permeability, a dual requirement for effective tissue engineering scaffolds.

In some formulations, a more variable pore structure was observed, likely due to processing conditions or shrinkage during the scaffold fabrication process. This variability can potentially compromise the mechanical reliability of the scaffold. However, the overall porous architecture remained favorable for tissue regeneration, and the processing parameters can be optimized to achieve a more consistent pore structure in future scaffold designs.

EDS analysis

Energy Dispersive Spectroscopy (EDS) analysis was performed to evaluate the elemental composition of the WH-ZnCh composite scaffolds and to investigate the distribution of critical elements across different samples. At 1% concentration, the predominant elements detected included nitrogen (N), carbon (C), oxygen (O), calcium (Ca), zinc (Zn), magnesium (Mg), and phosphorus (P), with nitrogen being the most concentrated element, indicating the presence of ZnCh, while calcium and oxygen highlighted the calcium phosphate structure characteristic of WH. The detection of zinc peaks at varying energy levels suggested multiple oxidation states, confirming the integration of ZnCh into the composite. At 2% concentration, it displayed a similar elemental profile, emphasizing peaks for calcium, oxygen, and nitrogen, with zinc again indicating its incorporation into the scaffold matrix, potentially enhancing the composite's biological properties. At 3% concentration, however, the elemental composition varied slightly, with increased prominence of nitrogen and carbon while showing reduced peaks for calcium and oxygen. Notably, zinc exhibited stronger peaks at higher energies, suggesting a higher concentration of ZnCh or a shift in the primary phase of zinc within the scaffold. Magnesium and phosphorus remained at low levels in all concentrations, indicating their minor incorporation compared to the other elements. The integration of zinc into the WH matrix appears to

play a significant role in influencing elemental distribution, enhancing both the mechanical and biological properties of the composite scaffold.

The assessment of cell viability using the MTT assay for zinc-doped chitosan/whitlockite (Wh-ZnCh) nanoparticles at concentrations of 1%, 2%, and 3% revealed distinct effects on cellular activity. For the 1% Wh-ZnCh formulation, the baseline cell viability was measured at approximately 0.19 optical density (OD). At a 25% concentration, a slight increase in OD indicated a potential stimulation of cell growth, reflecting an initial positive response. Notably, the 50% concentration resulted in a significant enhancement in OD, suggesting effective cellular proliferation or increased metabolic activity. However, at 75% concentration, the OD reverted to control levels, implying that no further stimulation of cell activity occurred. The 100% concentration exhibited only a marginal elevation in viability compared to the control, indicating a lack of enhanced activity. The positive control, which represented a cytotoxic condition, demonstrated substantial cytotoxicity, with an OD of approximately 0.03.

In the 2% Wh-ZnCh formulation, the baseline viability remained consistent at 0.19 OD. At 25% concentration, there were no observable cytotoxic effects as viability remained similar to baseline levels. A peak in OD was recorded at the 50% concentration, suggesting significant stimulation of cellular activity. The 75% concentration maintained stable viability, but a noticeable decline was observed at 100% concentration, indicating mild cytotoxic effects. The positive control again confirmed significant cytotoxicity.

For the 3% Wh-ZnCh formulation, baseline viability remained steady at 0.19 OD. Consistent with previous findings, the 50% concentration induced the highest metabolic activity. A slight decrease in viability was recorded at the 75% concentration, while the 100% concentration indicated some cytotoxic effects, although it did not reach the cytotoxicity levels observed in the positive control, which consistently demonstrated significant cytotoxicity.

Overall, these results suggest that while lower concentrations of Wh-ZnCh nanoparticles are generally non-cytotoxic and may promote cell viability, higher concentrations can induce cytotoxic effects, particularly at the 100% level.

Discussion

Bone tissue plays a vital role in various physiological processes, including support for musculature, locomotion, protection of vital organs, and maintenance of mineral homeostasis. Pathological conditions such as osteomyelitis, fractures, and osteoporosis can severely compromise bone integrity, leading to significant morbidity.

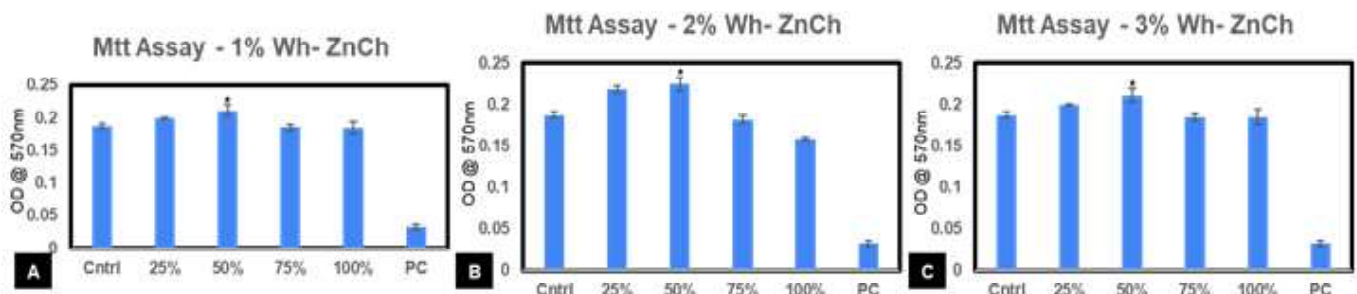


Figure 3: Biocompatibility of the fabricated scaffolds assessed by indirect MTT Assay. A) 1% WH-ZnCH, B) 2% WH-ZnCH, C) 3% WH-ZnCH

Recent advancements in biomaterials and nanomedicine offer promising therapeutic avenues for addressing these skeletal anomalies. This study focuses on the synthesis and characterization of zinc-doped chitosan/whitlockite (Zn-Ch/WH) composite nanoparticles, which were developed to enhance bone regeneration through their antibacterial, antioxidant, and cytotoxic properties.

The successful synthesis of Zn-Ch and WH composites was confirmed through various characterization techniques, including X-ray diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), and Energy Dispersive Spectroscopy (EDS). The XRD analysis indicated a decrease in crystallinity with increasing zinc content, which suggests that the incorporation of zinc-doped chitosan disrupts the crystalline structure of WH. This observation aligns with previous studies that report similar trends in composite materials, where the introduction of organic components leads to amorphization of the inorganic matrix [11]. The resultant amorphous structure may enhance the material's bioactivity, as amorphous calcium phosphate has been shown to exhibit improved solubility and reactivity compared to its crystalline counterparts.

FTIR analysis further elucidated the interaction between WH and Zn-Ch. The spectral changes, such as peak shifts and changes in transmittance, imply the formation of new chemical bonds and interactions, which could contribute to the mechanical strength and bioactivity of the composite. Previous research has similarly demonstrated that the interactions between organic and inorganic phases in composite materials can significantly influence their properties [12]. The observed broadening of absorption bands in the FTIR spectra of higher Zn-Ch concentrations underscores the complexity and potential synergistic effects between the components.

The SEM analysis of the scaffolds revealed a well-defined porous structure, which is critical for promoting cellular activities, including nutrient transport and tissue ingrowth. Pore size and distribution have been shown to directly influence cell behavior and proliferation in tissue engineering applications. [13] The scaffolds' mechanical integrity, supported by their thick walls, ensures that they can withstand physiological loads while maintaining permeability. While some variability in pore structure was noted, the overall architecture remained favorable for tissue engineering applications. This finding is consistent with the notion that optimizing fabrication parameters can help achieve a more uniform scaffold structure, enhancing the reproducibility and reliability of the material. The EDS analysis confirmed the successful incorporation of zinc into the composite scaffolds, with varying elemental profiles at different concentrations. The predominant presence of nitrogen and carbon at the higher Zn-Ch concentrations suggests a substantial integration of chitosan into the scaffold, which could enhance its biological properties.

The MTT assay results demonstrated that lower concentrations of Zn-Ch/WH nanoparticles promote cell viability and metabolic activity, while higher concentrations could induce cytotoxic effects. This finding aligns with the principle of dose-dependent responses observed in biomaterials, where lower doses facilitate cellular functions and higher doses may be detrimental. [9] The observed peak in viability at the 50% concentration across formulations indicates an optimal range for enhancing osteogenic differentiation and supporting cell proliferation.

Oxidative stress, driven by reactive oxygen species (ROS), is a well-known impediment to bone regeneration and tissue repair. [14] ROS-induced damage to osteoblasts can hinder bone formation

and prolong healing. In this study, the nanoparticles' antioxidant properties were attributed to both zinc and chitosan, which are capable of neutralizing ROS, thereby preserving cell viability and enhancing osteoblast differentiation. By protecting bone-forming cells from oxidative damage, these nanoparticles help maintain a healthy cellular environment essential for bone regeneration. [15–17]

The cytotoxicity assessment of the zinc-doped nanoparticles provides additional confidence in their biocompatibility, which is critical for clinical translation. The nanoparticles did not exhibit any significant cytotoxic effects on osteoblasts, further supporting their safety in bone regeneration applications. The chitosan matrix in the formulation offers a biodegradable scaffold that supports cell adhesion and proliferation. [18–21] Meanwhile, whitlockite, a calcium phosphate mineral that closely mimics the inorganic composition of natural bone, enhances the bioactivity and osteoconductivity of the nanoparticles, facilitating their integration into the host bone tissue [22]. The presence of zinc further enhances osteoblast activity through the activation of pathways such as BMP-2 and Runx2, which are critical in bone formation. [10]

Moreover, the combination of chitosan, whitlockite, and zinc creates a multifunctional platform that leverages the individual strengths of each material. Chitosan provides a biodegradable and biocompatible structure [23,24], and whitlockite delivers osteoconductive and bioactive properties. [25] and zinc plays a critical role in both antimicrobial defense and promoting osteogenic differentiation. The synergistic effects of these components result in a composite material that not only supports bone regeneration but also actively mitigates potential complications, such as infections and oxidative stress, that could otherwise hinder the healing process.

Conclusion

In conclusion, zinc-doped chitosan/whitlockite nanoparticles represent a promising material for bone regeneration applications. Their multifunctionality—spanning antibacterial, antioxidant, and biocompatible properties—addresses the critical challenges of bone tissue engineering. The findings of this study lay a solid foundation for further research, particularly regarding in vivo performance and scalability to clinical applications. Future studies may explore the nanoparticles' efficacy in animal models and their potential for integration into clinical protocols, potentially revolutionizing approaches in regenerative medicine.

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