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# SYNTHESIS, CHARACTERIZATION, ANTIBACTERIAL PROPERTIES, AND CYTOTOXICITY OF TERBIUM-DOPED HYDROXYAPATITE CRYSTALS FOR BIOMEDICAL APPLICATIONS

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## ABSTRACT

**Background:** Hydroxyapatite (HAp) is widely recognized for its biocompatibility, osteoconductivity, and similarity to natural bone mineral. However, its limited antimicrobial properties and mechanical stability restrict its broader biomedical applications. Recent advances in rare earth element doping, such as with terbium (Tb), have shown potential to enhance these properties by introducing antimicrobial and bioactive functionalities. **Aim:**This study aims to synthesize, characterize, and evaluate the biocompatibility and antimicrobial properties of terbium-doped hydroxyapatite (Tb-HAp) nanoparticles for potential biomedical applications. **Materials and Methods:**Tb-HAp nanoparticles were synthesized using a co-precipitation method, maintaining a Tb/(Tb + Ca) ratio of up to 10%. The nanoparticles were characterized using FTIR spectroscopy, SEM imaging, and EDS analysis. Biocompatibility was assessed using the MTT cytotoxicity assay, while antimicrobial activity was evaluated against S. aureus, S. mutans, and P.gingivalis using the agar well diffusion method. **Results:**FTIR confirmed successful incorporation of Tb into the HAp matrix without altering its structural integrity. SEM analysis revealed a rough, porous morphology favorable for cell attachment and osseointegration. The MTT assay indicated higher cell viability for Tb-HAp than undoped HAp, demonstrating improved biocompatibility. Antimicrobial testing showed larger inhibition zones for Tb-HAp against all tested microorganisms compared to undoped HAp and standard antibiotics, highlighting its enhanced antibacterial efficacy. **Conclusion:**The incorporation of terbium into hydroxyapatite significantly improves its antimicrobial and biocompatible properties, making it a promising candidate for applications in dental and orthopedic implants, tissue engineering, and infection-resistant coatings. Further studies are warranted to evaluate long-term stability, systemic effects, and scalability for clinical translation.

***Keywords*****:** nanoparticle, terbium-hydroxyapatite crystals, hydroxyapatite,terbium

## INTRODUCTION

Hydroxyapatite(HAP) [Ca10(PO4)6(OH)2, HAP] is chemically and structurally similar to inorganic substances found in natural bone and teeth.[1] This resemblance, in combination with additional features such as biodegradability, biocompatibility, osteoinductivity, and osteoconductivity,[2-6] has validated HAP nanoparticles (HAP NPs) to be a versatile biomaterial. They have been used in a variety of applications, comprising drug delivery systems,[7,8] bone repair,[9.10] bone tissue engineering, and dental applications.[11,12]

The fundamental constraint of HAP is its low stability, which limits its potential for use in broader biomedical settings.[13] Therefore,several studies have been carried out in order to enhance characteristics of HAP crystals by doping them with other nanoparticles. Chemically synthesized HAP, due to its hexagonal crystal structure,

enables the substitution of foreign ions of identical size and charge, making it a versatile host material which can be customized to various substrates and dopants.[14] Silver, cobalt, magnesium, zinc, etc are some of the metal nanoparticles used as dopants. According to available evidence, doping metal nanoparticles with hydroxyapatite can improve biocompatibility, osteoinductivity, mechanical stability, bone tissue integrity, antibacterial properties, and other characteristics, thereby increasing their potential applications.[13,15]

In recent advances, rare earth (RE) metals doped with HAP have garnered attention due to their unusual and unique physicochemical properties.[16] Terbium is one of the rare earth elements (lanthanide series) with high luminescence at low energy levels and signal transduction capabilities. Studies have previously investigated the application of this property in biological imaging.[16,17] In addition to this property, recent research has shown that terbium nanoparticles exhibit antimicrobial capabilities due to reactive oxygen species (ROS) that cause oxidative damage to cell membranes and nuclei, as well as ion release and surface charge interaction, resulting in enhanced antioxidant qualities. Terbium nanoparticles(Tb NP) have been used in tissue engineering, wound healing, and anticancer therapy due to their potential for angiogenesis, cell proliferation, growth factor promotion, biocompatibility, cytotoxicity mitigation, and anti-inflammatory properties. These nanoparticles represent a promising future for nanomaterials.[18]

The current study's rationale is to combine the exceptional characteristics of hydroxyapatite crystals and terbium ions to generate a novel material with superior properties such as enhanced antibacterial properties while preserving its biocompatibility.

## MATERIALS AND METHODS

The present study was approved by the institutional Ethics Committee, Chennai, Tamil nadu, India.

### SYNTHESIS OF HYDROXYAPATITE NANOPARTICLE

Terbium-substituted hydroxyapatite nanoparticles were generated using a coprecipitation method. The nanoparticles were prepared using the following coprecipitation procedure: To get a transparent solution, dissolve Ca(NO3)2·4H2O with terbium-(III) nitrate pentahydrate in distilled water and stir continuously at room temperature. The above-mentioned solution was then combined dropwise with an ammonium phosphate dibasic solution obtained under the same conditions as before. During this method, the Ca/P and (Ca + Tb)/P ratios were naturally maintained at 1.67. The atomic ratio Tb/(Tb + Ca) ranged from 0% to 10%. The reaction mixture was stirred continuously for 6 h. Meanwhile, the resultant solution was adjusted to a pH of approximately 10.5 by adding the needed amount of NH4OH. The same process was used to prepare undoped HAp without adding a terbium precursor. The resultant solutions were stored for 24 h. After maturation, the suspensions were filtered, washed with distilled water to a pH of 7.0 and dried in a hot air oven at 90°C for 15 h.[17]

### CHARACTERISATION OF TbHapNp

The synthesised nanoparticle in powder form was sent for characterisation. The surface properties of TbHapNp is evaluated using scanning electron microscope (JSM-IT800 Schottky Field Emission Scanning Electron Microscope - HR-SEM; Model: JEOL, JSM IT-800. USA). The chemical composition of TbHapNp was evaluated using Energy Dispersive X-ray (EDS) analysis. Fourier transform infrared spectroscopy (FTIR) was used to evaluate the functional groups present in TbHapNp. The Bruker Alpha II FTIR (Bruker Optics) spectrometer along with platinum attenuated total reflectance (ATR) was utilized to study the TbHapNp.

### CYTOTOXICITY TESTING

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide" assay (MTT assay) was used to evaluate the cytotoxicity of different concentrations of TbHapNp. It is basically a colorimetric test that is done to evaluate the metabolic activity of cells as an indicator of cell viability,proliferation and cytotoxicity. The MTT gets converted to formazan if NAD(P)H-dependent oxidoreductase enzymes are present in the viable cells. A solubilization solution is used to break down the insoluble formazan crystals that are formed. Multiwell spectrophotometer is utilized to determine the absorbance of the coloured solution at 500-600 nm. The solution will be darker if the majority of the cells are viable and metabolically active.

### ANTIMICROBIAL TESTING

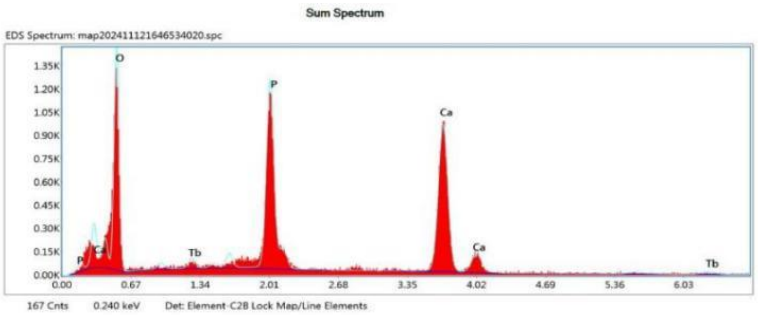
The antimicrobial effects against S.mutans, S.aureus and P.gingivalis were tested utilising the agar well diffusion method. All the three bacterias were stored in separate nutrient broth flasks which contain all the necessary nutrients for preventing microbial development. About 140 microliters of each bacteria was uniformly distributed on separate nutrient agar plates and was labeled with the respective bacteria name. The synthesised TbHapNp powder was utilized in two different concentrations - Low concentration(LC50uL) and High concentration(HC- 100uL). For comparing the antimicrobial property commercially available, common antibiotics like amoxicillin were tested against gram positive bacteria. The three agar plate containing the

bacteria was incubated for about 24 hours at 37 degree celsius for the bacteria growth to occur. Antimicrobial property was confirmed by the presence of a zone of inhibition around the well.

## RESULTS

### FTIR SPECTRA

The FTIR spectrum of HAp without Tb reveals a wide variation in the 3000-3400 cm-1 area, which is similar to the -OH groups. The peak positions at 1085, 1020, and 958 cm-1 match to the stretching mode of P-O. The frequencies 602 cm-1, 562 cm-1, and 474 cm-1 correspond to the bending mode of O-P-O. The band at 873 cm-1 is caused by the [HPO4]2- ions . The FTIR spectra of Tb-doped HAp is identical to that of pure HAp. In the FTIR spectra of Tb-HAp samples, CO32− has frequencies at 872 and 1426 cm−1. This may be attributed to the CO32− compounds substituting the PO43−compositions, indicating a partnership between HAp and carbon dioxide from the air. The creation mechanism of Tb-HAp, which is connected to the substitution of Ca ions with Tb ions, has not been fully described based on the current work.




Smart Quant Results

| Element | Weight % | Atomic % | Net Int. | Error % | Kratio | Z | A | F |
|---|---|---|---|---|---|---|---|---|
| O K | 26.76 | 46.20 | 111.53 | 10.33 | 0.0840 | 1.1396 | 0.2756 | 1.0000 |
| TbM | 1.59 | 0.28 | 2.67 | 14.38 | 0.0119 | 0.6451 | 1.0991 | 1.0561 |
| P K | 20.50 | 18.28 | 137.23 | 3.90 | 0.1898 | 0.9739 | 0.9466 | 1.0045 |
| CaK | 51.15 | 35.24 | 134.98 | 4.33 | 0.4780 | 0.9450 | 0.9878 | 1.0012 |

**Figure 1.** Depicts the FTIR spectra of the HAp and Tb-HAp samples.

### SEM ANALYSIS

Both nanoparticles had an agglomerated and defect-like morphology, with a rough surface and inconsistent particle dispersion across the entire morphology. HAp nanoparticles had a rod-like morphology, while Tb-HAp had a distorted rod-like morphology. The chemical composition, as well as the percentages of Ca, P, Tb, and O, are combined with the base. The uniform distribution Ca and P components were spread across the entire surface of the Tb-HAp. The rough surface of the Tb-HAp could promote biocompatibility. The porosity size and shape of the Tb-HAp nanoparticles may aid in osseointegration and cell proliferation. (Figure 2 and 3)

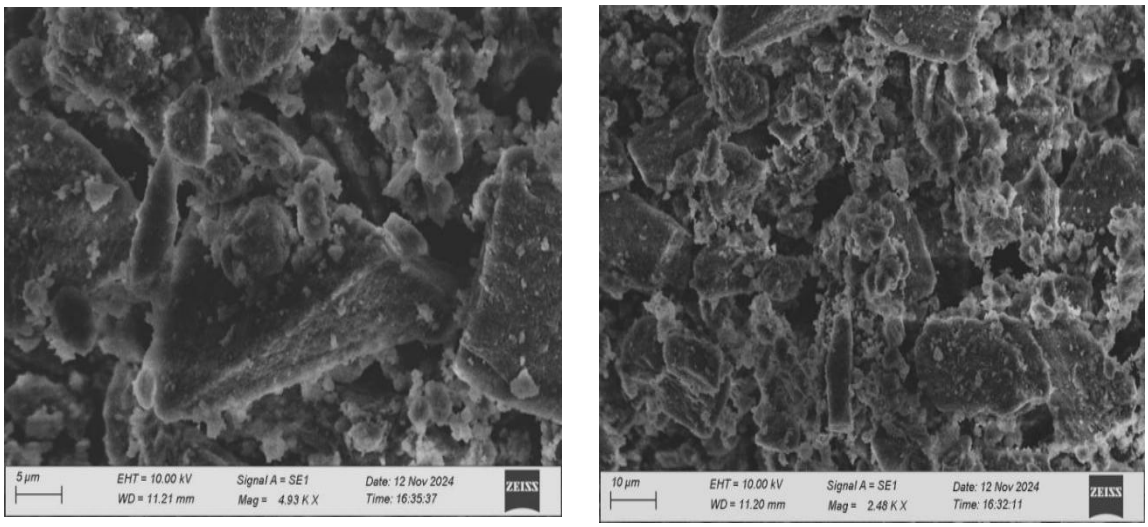



**Figure 2.** Depicts SEM images of hydroxyapatite and Tb-doped hydroxyapatite, as well as their chemical composition

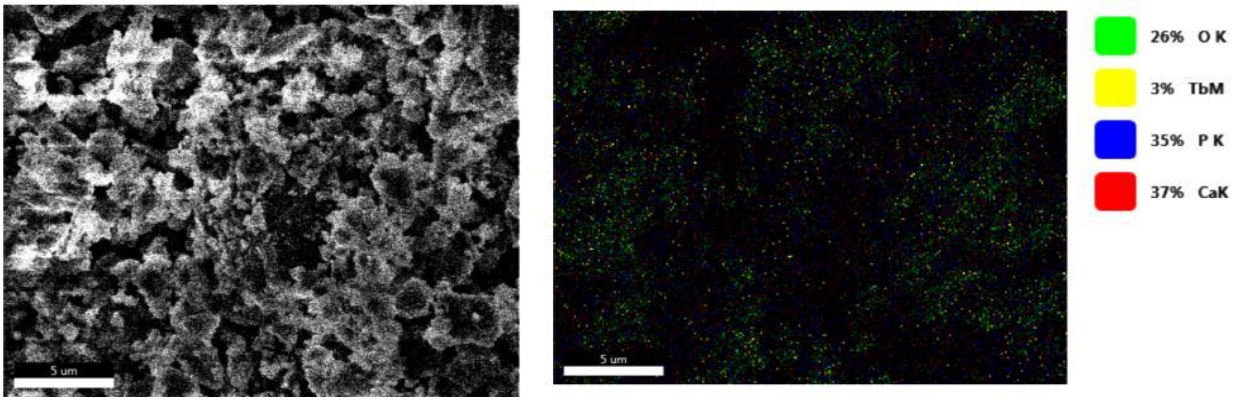
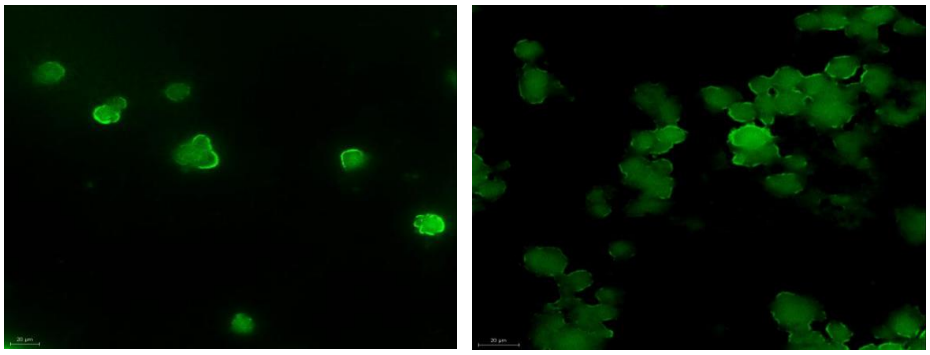



**Figure 3. SEM EDAX**

## CYTOTOXICITY

MTT assay and Confocal Laser Scanning Microscopy Analysis were utilized to assess cell viability, morphology, and interactions with nanoparticles. The MTT and microscopy results are presented in the figure (Figure 4). The Tb-HAp and HAp nanoparticles increase biocompatibility and achieve the required cell viability, as evidenced by the MTT results. The Tb nanoparticles had a higher viability than the HAP nanoparticles, although the HAp had a viability rate of roughly 75% when compared to the control(Figure 5). The increased number of cells in the Tb-HAp sample is conclusive proof that Tb-HAp enhances cell adhesion, proliferation, and attachment. The rough surface of the Tb-HAp prevented cell adherence on the HAp surface, allowing the live tissues and nanoparticle surfaces to interact strongly.

24 HOURS



48 HOURS

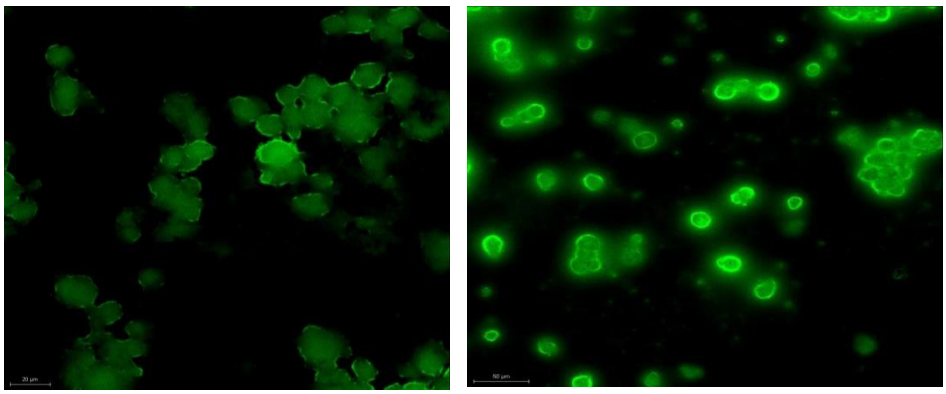


**Figure 4** **C**ell line analysis - MTT ASSAY and confocal laser scanning microscopy images

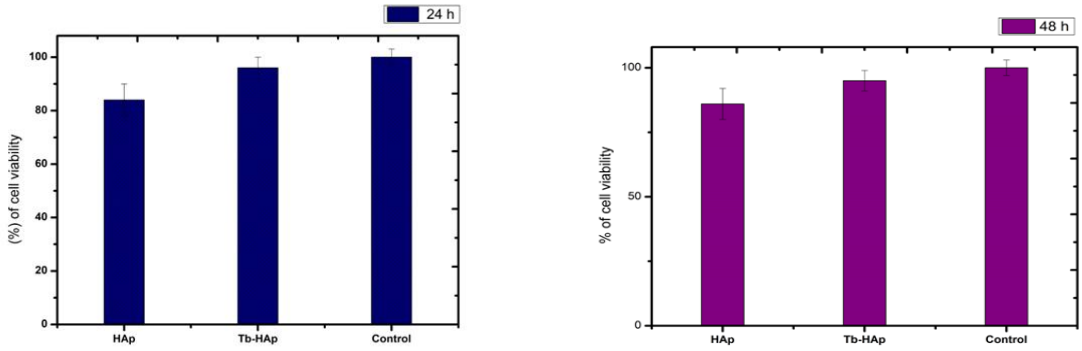



**Figure 5.** Graphical representation of cell viability after 24 hrs and 48 hrs

**ANTIBACTERIAL PROPERTY**

Tb-doped hydroxyapatite nanoparticles may be a suitable material for the development of dental tissue engineering because they provide an intricate array of therapeutic ions in the composite, resulting in targeted antibacterial actions against oral microbes. Hydroxyapatite was already known to possess antimicrobial properties. Aside from HAp-based nanomaterials, extra Tb rare earth metals in the produced nanomaterial showed outstanding activity. It was expected that the synthesized Tb/HAp nanoparticles would exhibit synergistic antibacterial properties. The antibacterial activity was tested using a culture of three Gram-positive bacteria (*S. aureus, S. mutans and P.gingivalis* ) and on an agar medium at 37 ℃ for 24 hours. The antibacterial negative control agent diffused from the disc into the agar, and there was no significant difference in the inhibitory zones of plates after 24 hours of incubation. However, in the positive control, large inhibitory zones were seen between the generated nanomaterial composites, which were significantly smaller than the discs of HAp alone and Tb-HAp, which were completely visible to the naked eye. Furthermore, reasonable antibacterial activity and considerable inhibition zones were observed with all microorganisms, as shown in Figure 6. These findings suggest that Tb has a synergistic impact and can enhance antimicrobial activity more effectively than both HAp and positive control (Table 1).

**Figure 6.** Antibacterial testing - zone of inhibition visible

**Table 1. Antibacterial testing**

| **Zone of Inhibition measurement (mm)** | | | | |
|---|---|---|---|---|
| **Microorganisms** | **Positive control Antibiotics (Erythromycin/ Penicillin)** | **Negative control (DMSO)** | **HAp** | **Tb-HAp** |
| ***S. aureus*** | 12 mm | Nil | 12 mm | 14 mm |
| ***S. mutans*** | 11 mm | Nil | 10 mm | 13 mm |
| ***P.gingivalis*** | 11 mm | Nil | 12 mm | 14 mm |

## DISCUSSION

This study investigated terbium-doped hydroxyapatite (Tb-HAp) nanoparticles synthesized through co-precipitation and evaluated their physicochemical, antimicrobial, and biocompatible properties. Key findings revealed that Tb-HAp nanoparticles outperformed undoped hydroxyapatite (HAp) in antimicrobial activity and cell viability, affirming their potential for biomedical applications. FTIR spectroscopy verified the substitution of Ca ions with Tb ions without altering the crystalline structure. SEM analysis revealed that Tb-HAp nanoparticles exhibited a rough, porous morphology conducive to cell adhesion and osseointegration. Cytotoxicity assays using the MTT method showed superior cell viability for Tb-HAp, indicating enhanced biocompatibility. Antimicrobial testing demonstrated significant inhibition of *S. aureus*, *S. mutans*, and *P.gingivalis*, with Tb-HAp outperforming both undoped HAp and commercial antibiotics in some cases.

Antimicrobial Properties The enhanced antimicrobial properties of Tb-HAp align with earlier research on doped hydroxyapatite materials. Wu et al. (2023) reported that hydroxyapatite co-doped with terbium and zinc showed synergistic antibacterial effects due to ROS-mediated bacterial membrane disruption.[15] Similarly, the findings by Gu et al. (2022) indicated that rare earth-doped HAp nanoparticles enhance antibacterial activity, particularly against Gram-positive bacteria, through ion release and surface charge interactions. [16] Other studies, such as those by Tosan et al. (2021), have highlighted that metal-doped hydroxyapatite enhances mechanical stability and antimicrobial efficacy, with terbium contributing to the generation of reactive oxygen species (ROS), disrupting bacterial DNA, and damaging cell membranes. These mechanisms correlate with the significant inhibition zones observed for Tb-HAp nanoparticles in this study.[17-19]

Biocompatibility and Cytotoxicity Biocompatibility is a hallmark of hydroxyapatite-based materials, but doping with terbium significantly improves cell viability and proliferation. Paduraru et al. (2021) demonstrated that terbium-doped HAp promotes cell adhesion and proliferation due to its rough surface and ion release.[18] This study's findings also align with Mohanto et al. (2024), who noted that terbium nanoparticles enhance angiogenesis and cell proliferation, making them promising for bone regeneration and wound healing applications.[17] Further, studies by Liu et al. (2021) on the controlled delivery of doxorubicin using hydroxyapatite scaffolds showed that doping hydroxyapatite improves its interaction with biological tissues, which is consistent with the increased cell viability observed in Tb-HAp nanoparticles.[21]

Material Morphology and Stability SEM analysis in this study showed that Tb-HAp nanoparticles had a rough, porous morphology, facilitating better cell attachment. This is in agreement with Zhong et al. (2022), who found that porous, rough surfaces in hydroxyapatite scaffolds enhance osseointegration and osteo-immunomodulation .[20] Other studies, such as Fihri et al. (2017), highlighted that metal doping in HAp improves not only the morphology but also the mechanical stability, crucial for load-bearing biomedical implants.[24,25-29]

Comparison of Rare Earth Doping Rare earth elements, including terbium, exhibit unique luminescent and antibacterial properties. Studies by Ibrahim et al. (2020) and Marion et al. (2020) emphasized the versatility of rare earth-doped HAp for applications like imaging, therapy, and antimicrobial coatings.[22,23] Gu et al. (2022) further reported that terbium ions enhance fluorescence and antibacterial activity without compromising biocompatibility.[16] This study confirms previous findings by Nevarez Rascon et al. (2021), who demonstrated that nanostructured HAp-based materials improve the performance of dental adhesives and implants.[21,30-34] The current findings expand this knowledge by showing that terbium-doped variants could further enhance antibacterial protection, critical in preventing peri-implant infections.

While this study provides valuable insights, it has several limitations. The antimicrobial assays were conducted in vitro, which may not fully capture the material's efficacy in complex biological systems. Cytotoxicity tests employed a single cell line, limiting the generalizability of the results. Furthermore, the study examined a narrow range of terbium doping concentrations (0–10%), leaving higher doping levels unexplored. Long-term stability and potential toxicity of Tb-HAp nanoparticles in vivo were also not evaluated, which is critical for clinical translation.

Future research should address these limitations by conducting long-term in vivo studies to evaluate the

biocompatibility and systemic effects of Tb-HAp. Expanding the doping concentration range could optimize material properties for specific applications. Co-doping terbium with other elements, such as zinc or strontium, may yield synergistic effects and further enhance antimicrobial and osteogenic capabilities. Additionally, incorporating Tb-HAp into advanced scaffold designs or coatings for orthopedic and dental implants could unlock new therapeutic applications. Lastly, scalable synthesis methods should be developed to ensure cost-effective production for clinical use.

## DECLARATION

**Conflict of Interest**

There are no conflicts of interest.

**Financial support**

None

**Competing Interests**

The authors have no competing interests to declare.

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