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Anti Inflammatory Properties of Terbium Doped Hydroxyapatite Crystals for Biomedical Applications - In Vitro Study

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KEYWORDS

Nanoparticle, Terbium-Hydroxyapatite Crystals, Hydroxyapatite, Terbium, Anti Inflammatory

ABSTRACT:

Objective: This study aimed to evaluate the anti-inflammatory properties of terbium-doped hydroxyapatite (Tb-HAp) in vitro, with the goal of determining its ability to attenuate inflammation and improve the biocompatibility of HA-based biomaterials for bone and dental applications.

Materials and Methods: Tb-HAp was synthesized using the coprecipitation method with varying concentrations of terbium (0%–10%). Anti-inflammatory activity was assessed through three assays: protein denaturation inhibition, heat-induced hemolysis (membrane stabilization), and trypsin inhibition, with aspirin used as the positive control.

Results: In the protein denaturation inhibition assay, Tb-HAp exhibited a dose-dependent increase in inhibition. At 100 µg/mL, inhibition was 36.15%, which rose to 85.95% at 500 µg/mL. The IC50 value was calculated to be 259.67±1.47 µg/mL, indicating that higher concentrations were required to inhibit 50% of protein denaturation. In comparison, aspirin at 100 µg/mL showed a higher inhibition of 95.69%. The heat-induced hemolysis assay showed a similar dose-dependent response. At 100 µg/mL, inhibition was 39.24%, which increased to 81.74% at 500 µg/mL, with an IC50 of 194.41±1.83 µg/mL. Aspirin at 100 µg/mL achieved 94.43% inhibition, indicating superior membrane stabilization. Lastly, the trypsin inhibition assay revealed a dose-dependent increase in inhibition, starting at 16.46% at 100 µg/mL and reaching 70.05% at 500 µg/mL. The IC50 for this assay was 350.14±1.56 µg/mL, while aspirin showed 94.01% inhibition at 100 µg/mL, outperforming Tb-HAp at the same concentration.

Conclusion: Tb-HAp demonstrated significant anti-inflammatory activity in all three assays, showing a dose-dependent response. While it was less potent than aspirin, it displayed potential for improving the biocompatibility of hydroxyapatite-based biomaterials. These findings suggest that Tb-HAp could be an effective material for reducing inflammation and enhancing tissue integration in biomedical and dental applications

1. Background

Hydroxyapatite (HAP) [Ca10(PO4)6(OH)2] has a similar chemical structure and composition to the inorganic components in natural teeth and bone.[1,2] This makes HA a good option for application in bone tissue engineering, where it is said to have excellent osteoconductive, osteoinductive and osteogenic properties. In addition to this, HA's biodegradability and biocompatibility makes it ideal for a wide range of biomedical applications, like bone regeneration, drug delivery systems, and dental applications.[3-5]

Although synthetic hydroxyapatite (HA) has reported to have excellent potential in bone tissue engineering, it is limited by its low fracture toughness, poor mechanical strength, and brittleness, which make it not appropriate for load-bearing applications. In order to overcome these flaws, various studies have been conducted to improve its biological, mechanical and physicochemical

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properties. The addition of another material will modify the crystallinity, lattice structure, and dispersal kinetics of HA, which enhances its mechanical properties and biological activity, such as promoting better bone and tissue regeneration.[6-9]

Along with these mechanical drawbacks, another major flaw with HA's utilization in bone tissue engineering is the high risk of infection due to pathogenic microorganisms. Infections and inflammation at the implant site can hinder the healing process and lead to complications.[10] As a result, modifying HA structure to improve not only its structural properties and qualities but also to impart antibacterial and anti-inflammatory qualities is necessary. Dopants added to HA can help modulate the immunological response, reduce inflammation, and reduce bacterial colonization, resulting in better healing results and fewer adverse effects from infection and inflammation.[6,11,12,13,14]

Numerous research studies have suggested that nanoparticles of calcium phosphate can be doped with lanthanide by substitution of the Ca2+ ions, a method that also enhances the biological properties of HAp.[15]

Terbium (Tb, atomic number 65) is a rare earth element with promising biomedical and dental applications. A small amount of Tb^{+} ions may significantly enhance the characteristics of materials, such as hydroxyapatite (HA), while preserving their fundamental physicochemical and biological properties. Tb-HAp has excellent in vitro biocompatibility for various cell lines. Tb-HAp show potential in biomedical and dental applications due to their improved material characteristics, biocompatibility, and tissue integration.[16]

This study aims to evaluate the anti-inflammatory properties of terbium-doped hydroxyapatite (Tb-HA) crystals in vitro, with the objective of determining whether Tb-doped HA can reduce inflammation and improve the biocompatibility of HA-based biomaterials.

2. Materials And Methods

Synthesis of Tb-HAp

Tb-HAp was produced employing the coprecipitation technique. To make a transparent solution, dissolve Ca(NO3)2·4H2O and terbium-(III) nitrate pentahydrate in distilled water and stir. Stir continuously at room temperature. The previously mentioned solution was then added dropwise to an ammonium phosphate dibasic solution prepared under exactly the same settings as before. Throughout this process, the Ca/P and (Ca + Tb)/P ratios were kept at 1.67. The atomic ratio Tb/(Tb + Ca) varied between 0% and 10%. The resultant mixture was agitated continuously for 6 hours. Meanwhile, the resultant solution was modified to a pH of roughly 10.5 by adding the appropriate amount of NH4OH. The same technique was utilized to make undoped HAp without using a terbium precursor.The resulting solutions were kept for 24 hours. Following maturation, the suspensions were filtered, rinsed with distilled water to pH 7.0, and dried using a hot air oven at 90°C for 15 hours. [14]

3. Anti-Inflammatory Testing

1) Protein denaturation inhibition assay

The protein denaturation inhibition assay was carried out using the approach of Sakat et al. 2010 [17] with a few modifications. Aspirin was utilized as the positive control. Approximately 500 µL of 1% bovine serum albumin was introduced to the sample at various concentrations (100-500 µg). The reaction mixture was maintained at room temperature for 10 minutes before being heated to 50 degrees Celsius for 20 minutes. After the heat reaction, the solution was brought down to room temperature, and the absorbance was measured at 660 nm.

$$\% \text{ of inhibition} = [(\text{OD of control} - \text{OD of test sample})/(\text{OD of control})] \times 100$$

2) Heat-induced hemolysis (membrane stabilization)

0.5 mL of 10% RBC was introduced to samples that ranged between 100 to 500 µg. the resultant solution was stored in a water bath at 55 degrees Celsius for about 30 minutes. After centrifugation at 3000 rpm for about 10 minutes at room temperature, the supernatant underwent spectrophotometric assessment at 560 nm. Aspirin was chosen as the positive control.

$$\% \text{ of inhibition} = [(\text{OD of control} - \text{OD of test sample})/(\text{OD of control})] \times 100$$

3) Trypsin inhibition assay

To a serial concentration ranging from 100-500 µg of sample, add 0.5 mL of 1% bovine serum albumin and incubate at room temperature for about 5 minutes. The

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reaction was halted by adding 250 µL of trypsin (0.5 mg/mL) subsequently centrifuged at 2500 rpm for about 10 minutes at room temperature. The final solution was collected, and absorbance was measured at 210 nm. In this study, aspirin was used as a positive control.

% of inhibition = [(OD of control - OD of test sample)/ (OD of control)] × 100

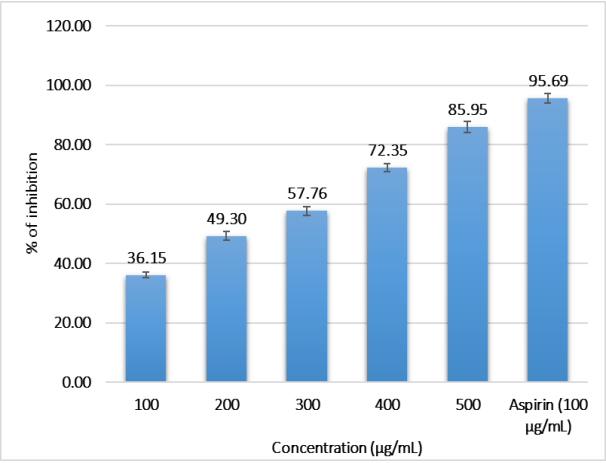
4. Results

Protein Denaturation Inhibition Assay

The Protein Denaturation Inhibition Assay reveals a dose-dependent gradual rise in inhibition, with 36.15% inhibition at 100 µg/mL and 85.95% at 500 µg/mL. The IC50 value of 259.67±1.47 µg/mL indicates that the Tb-HAp requires greater concentrations to produce 50% inhibition of protein denaturation which is comparable to Aspirin. At 100 µg/mL, Aspirin(control) inhibits protein denaturation by 95.69%, making it more effective than Tb-HAp material.(Table 1)(Graph 1)

Table 1 - Protein Denaturation Inhibition Assay

S. No.	Concentration (µg/mL)	% of inhibition	IC50 (µg/mL)
1	100	36.15	259.67±1.47
2	200	49.30	
3	300	57.76	
4	400	72.35	
5	500	85.95	
Aspirin	Aspirin (100 µg/mL)	95.69	



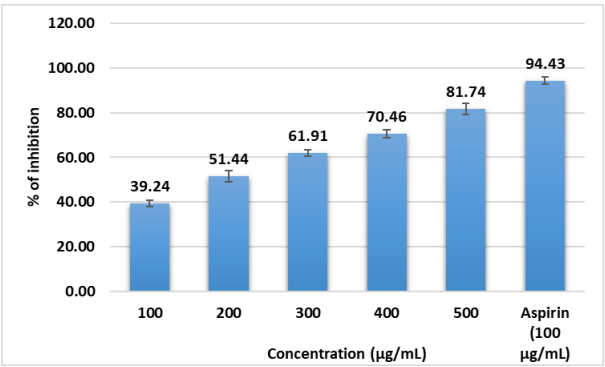
Graph 1 - Protein Denaturation Inhibition Assay

Heat-induced hemolysis (membrane stabilization)

The Heat-induced Hemolysis (Membrane Stabilization) Assay shows a dose-dependent gradual increase in the percentage of inhibition as the concentration of Tb-HAp rises. At 100 µg/mL, the inhibition is 39.24%, which increases progressively to 81.74% at 500 µg/mL, suggesting effectiveness of Tb-HAp in stabilizing the membrane and preventing hemolysis as the concentration rises. The IC50 value of 194.41±1.83 µg/mL implies that the concentration needed to achieve 50% inhibition of hemolysis. In comparison, Aspirin at 100 µg/mL achieves 94.43% inhibition, implying that while Tb-HAp shows a significant dose-dependent effect, Aspirin exhibits a higher potency in stabilizing the membrane and preventing hemolysis at this concentration.(Table 2)(Graph 2)

Table 2 - Heat-induced hemolysis

S. No.	Concentration (µg/mL)	% of inhibition	IC50 (µg/mL)
1	100	39.24	194.41±1.83
2	200	51.44	
3	300	61.91	
4	400	70.46	
5	500	81.74	
Aspirin	Aspirin (100 µg/mL)	94.43	



Graph 2 - Heat-induced hemolysis

Trypsin inhibition assay

The Trypsin Inhibition Assay reveals a dose-dependent rise in inhibition as the concentration of Tb-HAp increases. At 100 µg/mL, the inhibition is 16.46%, and it gradually increases to 70.05% at 500 µg/mL, indicating Tb-HAp's rising ability to inhibit trypsin activity at higher concentrations. The IC50 value of 350.14±1.56

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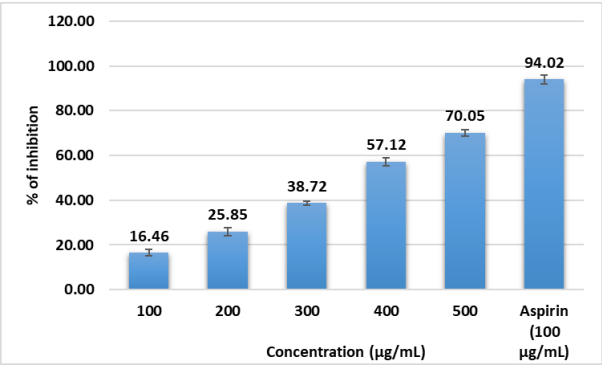
µg/mL reveals that Tb-HAp has good potency because this concentration is required to produce 50% inhibition of trypsin. Meanwhile, at 100 µg/mL, Aspirin inhibits trypsin with a potency of 94.01%, thereby outperforming Tb-HAp at the same concentration. (Table 3)(Graph 3)

Table 3 - Trypsin inhibition assay

S. No.	Concentration (µg/mL)	% of inhibition	IC50 (µg/mL)
1	100	16.46	350.14±1.56
2	200	25.85	
3	300	38.72	
4	400	57.12	
5	500	70.05	
Aspirin	Aspirin (100 µg/mL)	94.01	



Graph 3 - Trypsin inhibition assay

5. Discussion

Recent advances in biomaterials research have focused on modifying hydroxyapatite (HA) crystals through doping with other metals. Hydroxyapatite, a calcium phosphate compound resembling the mineral component of bone, is widely known for its excellent osteoconductive property and biocompatibility. [18] However, it has many limitations—such as brittleness, limited antibacterial activity, and suboptimal anti-inflammatory properties—have prompted researchers to explore the incorporation of additional elements to enhance its performance, particularly in the field of bone tissue engineering.

Doping hydroxyapatite with metallic ions such as zinc, magnesium, silver, copper, and rare earth elements has been shown to significantly improve its mechanical strength, bioactivity, antibacterial efficacy, and anti-inflammatory capabilities. These modifications not only enhance faster and more effective bone regeneration but also decrease the likelihood of implant-associated infections and inflammation, which are major causes of implant failure. As a result, doped HA materials are being actively investigated for a broad range of biomedical and dental applications, including bone grafts, dental implants, coatings for orthopedic devices, and tissue engineering scaffolds.[19,20]

To the best of our knowledge, the anti-inflammatory effects of terbium-doped hydroxyapatite (Tb-HAp) nanoparticles have not yet been thoroughly investigated. Terbium (Tb), is a rare earth element that has attracted attention in biomedical research due to its luminescent, angiogenic, and antioxidant properties. Although there is limited evidence on the anti-inflammatory properties of terbium doped hydroxyapatite, similar studies involving rare metals doped with HA have reported that rare earth ions can contribute to immunomodulatory effects.

The present study tested the anti-inflammatory properties of terbium-doped hydroxyapatite (Tb-HAp) using various in vitro assays. Tb-HAp showed dose-dependent anti-inflammatory effects in all three tests, but was less effective than aspirin (control group). Tb-HAp reduced protein denaturation but required greater quantities than aspirin to achieve similar effects. In the heat-induced hemolysis testing, Tb-HAp significantly inhibited hemolysis, although aspirin demonstrated a higher level of inhibition at similar dose. Tb-HAp also inhibited trypsin activity, but aspirin was superior to Tb-HAp in this assay as well. Tb-HAp possesses anti-inflammatory potential, although it requires higher concentrations to have effects similar to aspirin. This implies that Tb-HAp could be a biocompatible material with moderate anti-inflammatory properties suitable for biomedical and dental applications.

The findings of the present study was supported by a review by Mohanto et al who had reported various biomedical benefits of terbium based nanoparticles. They had highlighted its uses in wound healing, tissue engineering and cancer treatment. They also reported that these nanoparticles were biocompatible, exhibited anti-inflammatory properties, enhanced cell proliferation, altered growth factor activity and promoted angiogenesis. [20] A study by Pandi et al evaluated the antioxidant,anti cancer, antidiabetic and anti inflammatory properties of terbium nanoparticles. They

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reported that terbium nanoparticles showed similar activity as some of the common medications such as ascorbic acid (antioxidant), acarbose (antidiabetic), and diclofenac sodium (anti-inflammatory). [21]

Comparable insights were provided by Mondal et al. and Bordea et al., whose work reported that nano-hydroxyapatite facilitated tissue integration by regulating the activity of monocytes and macrophages and could act as a carrier for anti-inflammatory drugs, making it suitable for immune-interactive roles in bone regeneration and healing.[22,23] Shang et al. [24] emphasized that the immunoinflammatory response is a pivotal step in bone regeneration, and HA-based materials can be used to interact with immune cells—especially macrophages—to foster an osteoimmune microenvironment favorable for healing. A study by Sanmiguel et al [25] concluded that hydroxyapatite/silver (HA/Ag) nanocomposites exhibit bioactivity, antimicrobial efficiency, and anti-inflammatory properties while maintaining low cytotoxicity. They significantly reduced inflammatory markers (IL-1, TNF-α) and nitric oxide release.

More studies, particularly in-vivo testing and comparisons with other dopants, is needed to validate Tb-HAp's therapeutic viability in reducing inflammation and enhancing regeneration outcomes in hard tissue applications.

6. Limitations

This study was limited to in vitro assays, which may not fully represent in vivo conditions. The anti-inflammatory mechanism of Tb-HAp was compared with only aspirin. Therefore further in vitro and in vivo testing needs to be done to validate the current study findings

7. Conclusion

The current study reports the dose dependent anti inflammatory effects of Terbium doped hydroxyapatite crystals. Although less effective than aspirin, Tb-HaP appears to be a promising alternative with moderate anti-inflammatory properties suitable for future biomedical and dental applications.

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