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# Cytotoxic Effect and Antimicrobial Activity of Chitosan Nanoparticles and Hafnium Metal Based Composite: Two Sides of the Same Coin- An *In vitro* Study

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Author VR designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SR and DN managed the analyses of the study. Author DG managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

Chitosan (CTS) is a biocompatible polymer that has been widely researched for tissue engineering purposes. It has demonstrated a significant role in bone tissue engineering in the last two decades. Being a natural polymer obtained from chitin, a major component of crustacean exoskeleton, it has varied uses. Lately, attention has been given to chitosan composite materials due to its minimal foreign body reactions, antibacterial nature, biocompatibility, biodegradability, and the ability to be molded into various shapes and forms. It can be used as porous structures, suitable for cell

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ingrowth and osteoconduction. The aim of this research was to assess the biocompatibility of a chitosan nanoparticle and hafnium metal-based composite and project its use for bone tissue engineering. In the present study, we have prepared chitosan nanoparticles and their based hafnium composite and it was analyzed for its cytotoxic effect using brine shrimp lethality assay and antimicrobial activity using the disc diffusion method. There was a significant difference between the concentrations used ($p<0.01$), when One way ANOVA statistical analysis was performed. The current study substantiates the antimicrobial activity and highlights the possible cytotoxicity of the CTS and hafnium composite.

*Keywords: Chitosan; hafnium; composite; cytotoxic effect; antimicrobial activity; brine shrimp lethality assay.*

## 1. INTRODUCTION

Research on biomaterials for dental implants and bone substitutes has expanded considerably over the last few decades [1–3]. The establishment of a load-bearing biomaterial must be integrated with natural bone. Biocompatibility, osteoconductivity, high porosity and biomechanical compatibility, are essential criteria the implanted biomaterial should possess [3–5]. The best bioactive biomaterials in bone tissue engineering, renowned for their excellent biocompatibility with the human body environment include Chitosan (CTS) and hydroxyapatite (HAp) [6–8].

Chitosan is a biocompatible polymer that has been researched upon for tissue engineering objectives [9–12]. The first discovery of chitosan dates back to the middle of the eighteenth century, but the compound did not reach its fame until the 1930s, when its crystalline structure was discovered [13–15]. Chitosan an aminoglucopyran, is composed of randomly distributed *N*-acetylglucosamine and β-(1,4)-linked glucosamine residues. The transfection efficiency of chitosan/DNA nanoparticles depends on several factors such as the degree of deacetylation and molecular weight of the chitosan, pH, protein interactions, charge ratio of chitosan to DNA (N/P ratio), cell type, nanoparticle size and interactions with cells [16]. Though the compound was originally used only in limited applications, chitosan and chitosan-based composites are currently used more diversely in different fields including water treatment, cosmetics, food, paper, and textile industries, agriculture, photography products, fuel cells and batteries, detergents, gene therapy, cancer therapy, drug and vaccine delivery and biotechnology [11,17–21].

Hafnium is a passive metal with a number of interesting properties, such as high ductility and strength, as well as resistance to corrosion and mechanical damage [22–24]. However, the behavior of hafnium in the biological environment has not been studied in great depth. Thus, further studies of hafnium coating under biological conditions are needed in order to determine the suitability of this material, for biomedical applications. On the other hand, composites demonstrate tailored physical, biological, and mechanical properties as well as expectable degradation behavior [25–27]. The apt selection of a particular composite for a given application demands a thorough understanding of pertinent cells and/or biocompatible response.

Previously our department has published extensive research on various aspects of prosthetic dentistry [28–38], this vast research experience has inspired us for the present investigation. We have prepared chitosan nanoparticles and their based hafnium composite to evaluate the benefits of the new combination. This novel composite was prepared and analyzed for its cytotoxic effect using brine shrimp lethality assay and antimicrobial activity using the disc diffusion method. Similar attempt is not reported in the literature and hence this study was initiated with aim to assess the biocompatibility of a chitosan nanoparticle and hafnium metal-based composite and project its use for bone tissue engineering.

## 2. MATERIALS

### 2.1 Synthesis of Chitosan Nanoparticles

Raw materials for the chitosan preparation were chitosan nanoparticle, distilled water, glacial acetic acid and hafnium metal particles (Fig. 1). 500 mg of chitosan dissolved in 49.5 mL of double distilled water. 0.5 mL of glacial acetic acid was added to this solution (Fig. 2a). A 500 mg of hafnium metal was added to this preparation and kept in a magnetic stirrer for 24

hours for the preparation of composite (Fig. 2a, b, Fig. 3).

**Fig. 1. Materials used for Chitosan preparation**

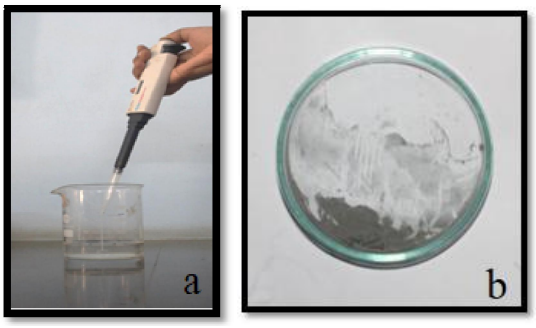


**Fig. 2. CTS- hafnium composite preparation;**
**2a. Preparation of chitosan**
**2b. Hafnium metal particles**

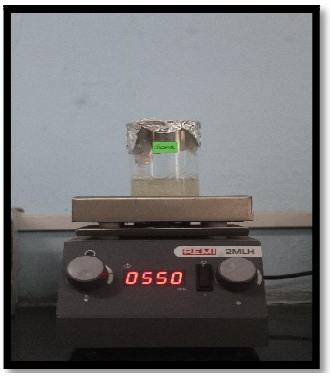


**Fig. 3. Preparation of Chitosan-Hafnium composite**

## 2.2 Preparation of Test Organism

Brine shrimp eggs have been obtained from Sla ®, and India has been used as a test species 38–42. Synthetic seawater was formulated by sorting and dispersing 36 g of sea salt in 1 liter of distilled water for the spawning of shrimp eggs. The seawater was placed in a special plastic bin (hatching chamber) with such a separation for dark and light areas. Shrimp eggs were placed to just the dark side of the platform, while the bulb above other side (light) tends to attract the harvested shrimp. The shrimp was encouraged to spawn for 2 days and mature as nauplii (larva). After two days, whenever the shrimp larvae are available, 5 ml of the artificial seawater and diverse concentrations of composite viz. 5, 10, 20, 30, and 50 µg/mL were prepared.

## 2.3 Brine Shrimp Lethality Assay (BSLA) for the Studied CTS-hafnium Composite

Ten nauplii were used in each test. Three replications were used for each concentration and the blank control was always included. Control groups were used in cytotoxicity study to validate the test method and ensure that the results obtained were only due to the activity of the test agent and the effects of the other possible factors were nullified. After 24 hours, using a dissection microscope, the number of surviving shrimps was counted and recorded [38–42].

## 2.4 Antimicrobial Activity for the Studied CTS-hafnium Composite

The antimicrobial activity was tested using the agar disc diffusion method [43–47]. If the test sample possesses antimicrobial activity, the bacteria is killed or growth is hampered and there will be a clear area around the wafer where the bacteria have not grown enough to be visible This is referred to as zone of inhibition and in this study 3 concentrations of the CTS-hafnium composite (50 ,100 and 150 µg/mL)were used in a single disc for 2 most common oral micro biota viz, Streptococcus mutans and lactobacillus. The distance from the center of each concentration of CTS-hafnium composite is measured to determine its antimicrobial potential. Inhibition produced by the test sample is compared with that produced by known concentration of a reference antibiotic compound.

# 3. RESULTS AND DISCUSSION

## 3.1 Cytotoxicity

Cytotoxic Effect from the brine shrimp lethality test done it is noted that on the 1st day five of nauplius survived, while on day 2 it got

decreased to three nauplius, and on day 3 only one nauplii remained to survive. The mean of three replications used for each concentration viz, 5 µg/mL (3.67 ± 0.58), 10 µg/mL (1.33 ± 0.58), 20 µg/mL (1 ± 0.00), 30 µg/mL (1 ± 0.00), 50 µg/mL (0 ± 0.00) and the blank control (10 ± 0.00) was tabulated (Table 1).

As the concentration of the nanoparticles increased, the toxicity got decreased and nauplius survived. When the concentration of nanoparticles decreased, the toxicity increased, and nauplius died. Hence, from the current study, it is noted that, as we used less concentration it caused only half the amount of toxicity (Fig. 4). There was a significant difference between and within the concentrations used ($p<0.01$), when One way ANOVA statistical analysis (IBM SPSS Statistics 20®) was performed (Table 2). Hence, if the concentration is above 50%, it can be used for biomedical applications.

**Table 1. Surviving shrimps for each concentration (5, 10, 20, 30, 50 µg/mL)**

| Groups | Concentrations of CTS-hafnium and neutral | Surviving shrimps |
|---|---|---|
| CONTROL | Neutral | 10 ± 0.00 |
| | 5 µg/ml | 3.67 ± 0.58 |
| TEST | 10 µg/ml | 1.33 ± 0.58 |
| | 20 µg/ml | 1 ± 0.00 |
| | 30 µg/ml | 1 ± 0.00 |
| | 50 µg/ml | 0 ± 0.00 |

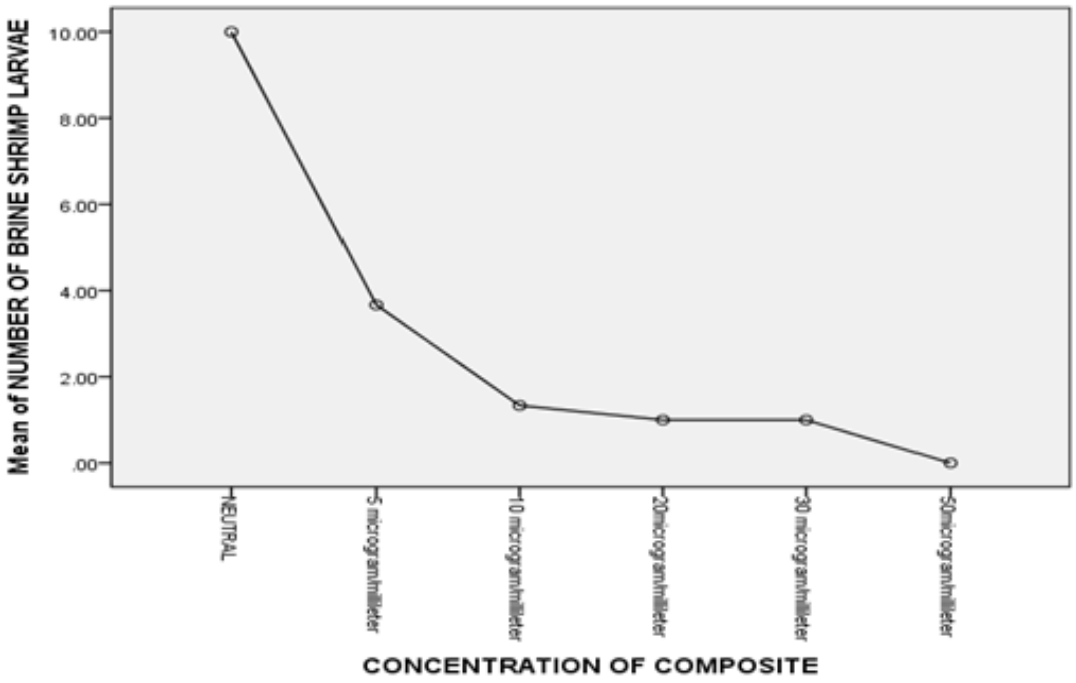


**Fig. 4. Graph showing number of brine shrimp larvae present at different concentrations of the composite solution viz. 5, 10, 20, 30, and 50 µg/mL**

**Table 2. ANOVA showing differences in means of various concentrations used. There was a significant difference between and within the concentrations used (One way ANOVA, $p<0.01$)**

| Number of brine shrimp larvae | | | | | |
|---|---|---|---|---|---|
| | Sum of Squares | Df | Mean Square | F | Sig. |
| Between Groups | 207.167 | 5 | 41.433 | 372.900 | .000 |
| Within Groups | 1.333 | 12 | .111 | | |
| Total | 208.500 | 17 | | | |

### 3.2 Antimicrobial Activity

The mean measured distance from the center for each concentration of CTS-hanium composite for *S. mutans* bacteria (Fig. 5) and lactobacillus bacteria were noted (Fig. 6). The zone of inhibition values measured for *S. mutans* for each concentration were 31 mm, 13 mm, 14 mm, 16 mm and for Lactobacillus the values were 28 mm, 13 mm, 16 mm, 17mm for reference antibiotics, 50,100 and 150 µg/mL respectively.

**Fig. 5. Zone of inhibition of the different concentrations (50,100 and 150µg/mL) of CTS-hafnium composite and the reference antibiotic for *S. mutans* bacteria**

**Fig. 6. Zone of inhibition of the different concentrations (50,100 and 150µg/mL) of CTS-hafnium composite and the reference antibiotic for lactobacillus bacteria**

The current study we have prepared chitosan nanoparticles and its based hafnium composite and it was analyzed for its cytotoxic effect using brine shrimp lethality assay. The studied composite had antimicrobial activity but was cytotoxic at higher concentrations. CTS with various composites can be potential bone implant materials with good osteoconductivity, osteoinductive and osteogenic properties. The structural, mechanical, chemical interaction and in vitro study of CTS, with various composite preparations have been carried out for other industrial purposes [48–51]. Although many CTS composite materials have been developed, questions persist with their biocompatible properties. Hence, much research is in progress to address the gap in the development of these properties.

The brine shrimp lethality assay (BLSA) is proven as a useful tool for preliminary assessment of toxicity [52–55]. It is a comprehensive bioassay for the bioactive compounds of natural and synthetic origin [56–58]. It has advantages of being rapid (24 hours), inexpensive, and simple (e.g., no aseptic techniques are required). It easily employs a large number of organisms for statistical validation and requires no special equipment and a relatively small amount of sample (2-20 mg or less). Moreover, it does not require animal serum as is needed for other cytotoxicity tests.

For bone engineering purposes, Leena et al prepared Silibinin-loaded chitosan nanoparticles (SCN) were using the ionic gelation technique, and the scaffolds (Alg/Gel-SCN) were synthesized by the conventional method of freeze drying. The scaffolds were subjected to physicochemical and material characterization studies. The addition of SCN did not affect the porosity of the scaffolds, yet increased the protein adsorption, degradation rates, and bio-mineralization. These scaffolds were biocompatible with mouse mesenchymal stem cells. The scaffolds loaded with 50 µM Silibinin promoted osteoblast differentiation, which was determined at cellular and molecular levels [59].

Hong ZQ investigated the efficiency of the use of chitosan nanoparticles containing plasmid-bone morphogenetic protein 2 (pBMP2) sequences (CNPBs) to induce the differentiation of bone marrow stem cells (BMSCs) into osteoblast-like cells that may be able to promote ectopic bone formation. pBMP2s were constructed, and chitosan nanoparticles were incubated with 50, 100 or 200 µg/ml pBMP2.Ectopic bone formation was observed following the integration of polyglycolic acid (PGA) scaffolds with CNPBs and BMSCs, which were implanted into the dorsal muscles of Sprague-Dawley rats. Exposure to CNPBs led to the transfection of BMSCs with BMP2. The transfected BMSCs possessed the characteristic phenotypes of osteoblasts. Therefore, CNPBs may be a promising method of promoting the formation of novel bone tissue [60].

**Table 3. Mean of distance of zone of inhibition) of CTS-hafnium composite and the reference antibiotic control**

| | | Distance of zone of inhibition ( in mm ) | |
|---|---|---|---|
| **Groups** | **Concentrations of CTS-hafnium and reference antibiotic** | ***Streptococcus mutans*** | ***Lactobacillus* sp** |
| CONTROL | ANTIBIOTIC | 13 | 13 |
| TEST | 50 µg/mL | 14 | 16 |
| | 100 µg/mL | 16 | 17 |
| | 150 µg/mL | 31 | 28 |

Moradikhah F et al used a cross-junction microfluidic device for preparation of alendronate-loaded chitosan nanoparticles with desired characteristics to introduce a suitable element for bone tissue engineering scaffolds. The osteogenic effects of prepared selected nanoparticles on human adipose stem cells (hA-MSCs) were evaluated by assessment of alkaline phosphatase (ALP) activity, calcium deposition, ALP and osteopontin gene expression. They concluded that the prepared nanoparticles significantly enhanced osteogenic differentiation of hA-MSCs and can be a suitable compartment of bone tissue engineering scaffolds [61].

Composite Chitosan-based materials have been found to have a predominant role in bone tissue engineering in recent years. Limited evidence exists with substantial research work to address the cytotoxicity of CTS-metal composites.

## 4. CONCLUSION

The present study demonstrated the antimicrobial activity and highlighted the possible cytotoxicity of the CTS and hafnium composite. Though challenges still exist, the addition of hafnium metal to improve the properties of CTS would surely support and stimulate the function of natural bone. The development of research on the efficacy of CTS-hafnium composite may open great possibilities for future bone tissue engineering and hence should be explored for further osteoblastic activity in all bone bioengineering experimentation.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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