Comparative Cytotoxic Effects of Nanocomposites Prepared Using Nano- Hydroxyapatite(Ha)/Chitosan/Vitamin K2

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**Abstract*:*** Due to their synergistic qualities, the combination of nHA, chitosan, and vitamin K2 in nanocomposites has attracted considerable attention among the wide range of nanomaterials. Due to their synergistic qualities, the combination of nHA, chitosan, and vitamin K2 in nanocomposites has attracted considerable attention among the wide range ofnanomaterials. Nano hydroxyapatite, a mineral form of calcium phosphate, is a biocompatible material notable for its likeness to the mineral component of genuine bone. Chitosan is anaturally occurring polysaccharide that is produced from chitin and has outstandingbiocompatibility, biodegradability, and antimicrobial properties. The fat-soluble vitamin K2 is crucial for bone metabolism and blood coagulation regulation. The combination of these materials in nanocomposites may have advantageous synergistic effects for applications like drug delivery and bone tissue regeneration.The research aims to assess the comparative cytotoxic effect ofnanocomposites prepared using nano hydroxyapatite (nHA), chitosan, and vitamin K2 using the brine shrimp lethality test. The brine shrimp lethality test is a widely used bioassay forpreliminary toxicity screening of various substances due to its simplicity, cost-effectiveness, and rapid results.The results of the brine shrimp lethality test will provide valuable information on the comparative cytotoxic effect of nanocomposites based on nHA, chitosan, and vitamin K2. The obtained LC50 values will indicate the toxicological safety profile of the nanocomposites andtheir potential implications for biomedical applications. In the present study it is seen that with increase in concentration the number of naupalli survived after 24 hours reduced except in vitamin k. Vitamin K2 had the highest survival rate(9.6 out of 10 survived on an average in all concentrations) and best results and concentration 40g/ml is considered to be the safest.The findings may help guide further in vitro and in vivo studies to explore the safety and efficacy of these nanocomposites for various biomedical purposes.In conclusion, nanocomposites prepared using nano hydroxyapatite, chitosan, and vitamin K2 hold significant promise for use in implants, particularly in bone tissue engineering and dental implantology. These nanocomposites offer a multifunctional approach to improve implant biocompatibility, osseointegration, and bone regeneration. Further research and clinical studies are necessary to validate the efficacy and safety of these nanocomposites for various implant applications.

**Keywords:** nano hydroxyapatite, chitosan, vitamin K2, brine shrimp lethality test

# Introduction

The ability of bone to combine mechanical properties with the ability to regenerate makes it a marvel of nature. Bone is a highly vascular, organized, and dynamic tissue that creates complex structures with linked porosity networks at sizes ranging from the nano to the macro. Bonerepair is a common clinical issue, as evidenced by the nearly 2.2 million bone transplantations performed annually worldwide in fields like dentistry, orthopedics, and neurosurgery[(Shakir et al., 2016)](https://paperpile.com/c/VtC5q7/2SqQa)Thedesign of structures and technologies for bone regeneration, which can encourage the growth of new bone tissue and repair bone abnormalities, has received a lot of interest recently. Due to its great biocompatibility and role as the primary component of bone tissue, hydroxyapatite (HA), or Ca10(PO4)6(OH)2, is the biomaterial for medical applications that has been the subject of the most research in the recent years[(Basirun et al., 2018)](https://paperpile.com/c/VtC5q7/4UVM0)It has a crystalline bio-apatite structure that resembles the mineral found in human bone. To enhance their mechanical and biological qualities,hydroxyapatite precursors are frequently mixed with different natural and synthetic polymers in the domain of biomedical applications.[(Szcześ et al., 2017)](https://paperpile.com/c/VtC5q7/CEi22)According to reports, chitosan (CS), because of itsbiocompatibility, bioresorbable, and antibacterial activity, is a prime choice for bone tissue engineering[(Sultankulov et al., 2019)](https://paperpile.com/c/VtC5q7/lKhgI)The main effects of vitamin K on bone health are preservation and enhancement of bone quality. [(Evaluation Composite Restoration Posterior Teeth Proanthocyanidin Pretreatment Liner Using Fédération Dentaire Internationale Criteria: Split-Mouth Randomized Controlled Trial, n.d.; Pranati et al., 2021; Sakthi, 2021)](https://paperpile.com/c/VtC5q7/ycgLE+oAYUK+CCeBr)) The mechanisms might involve increasing the collagen matrix, activatingosteocalcin, and affecting osteoblast and osteoclast differentiation. Among the vitamin Khomologs, MK-7 has the highest activity and bioavailability in people. MK-7 is therefore believed to improve bone health when taken orally[([No Title], n.d.)](https://paperpile.com/c/VtC5q7/Cp7zp) The use of composite material design in hard tissue engineering offers a viable method of achieving biological and structural resemblance to native bone tissue. Combining two or more components with dissimilar physicochemicalcharacteristics can expand their practical uses beyond what can be achieved by a single component[(Ramakrishna et al., 2001)](https://paperpile.com/c/VtC5q7/uzA73) This study marks the first of its kind to explore the synergistic effects of combining Vitamin K2 with nHAP and Chitosan to evaluate cytotoxicity. These nanocomposites could find applications in wound dressings, biomedical coatings, and implant materials, to effectively combat infections and reduce the incidence of healthcare-associated infections.Despite the fact that nanoparticles have many uses, there is growing discussion and controversy in the scientific and regulatory sectors about what happens to them in biological systems andthe potential harm that these substances may do to living things. [(Hsieh et al., 2012)](https://paperpile.com/c/VtC5q7/EcgD2) There is a growing commercial usage of these materials, which exposes people both directly and indirectly.[(Nel et al., 2006)](https://paperpile.com/c/VtC5q7/PXY66) To assure the production and application of nanomaterials, a comprehensive understanding of particle kinetics and toxicity is necessary for any usage of nanoparticles in living creatures.[(Keerthana & Ramesh, 2021; Murugesan, 2021; Tiwari & Jain, 2021)](https://paperpile.com/c/VtC5q7/cbgfT+rwfkx+OCdhw)[(Keerthana & Ramesh, 2021; Murugesan, 2021; Subramanian et al., 2021; Tiwari & Jain, 2021)](https://paperpile.com/c/VtC5q7/cbgfT+rwfkx+OCdhw+qkCux)Testing protocols and established standards must also be followed safety as well as thoroughtraining for employees on the possible hazards of nanotechnology. Lethal brine shrimp testing is still a common tool in toxicology applications and research today[(Costa-Lotufo et al., 2005)](https://paperpile.com/c/VtC5q7/VJoqR) In Artemia toxicity assays,nanoparticles are inexpensive, easily accessible, straightforward, and durable. To clarify the impacts on brine shrimp toxicity and safety, researchers employed a variety of nanoparticle types. The deadly effects of chemically produced nanoparticles have been evaluated using brine shrimp toxicity. It also provides a brief overview of brine shrimp biology and the assessment of NP toxicity by in vitro and in vivo evaluation. [(G. & Ganapathy, 2022; Kumar & Ramesh, 2021)](https://paperpile.com/c/VtC5q7/kBbDh+GuV3j))The primary objective of this study is to examine the comparative cytotoxic effect ofnanocomposites prepared using nano hydroxyapatite, chitosan, and vitamin K2. Through in vitro cytotoxicity evaluations, we aim to assess the potential biocompatibility and safety of the nanocomposites for use in biomedical applications. Specifically, we will examine the impact of varying concentrations of the nanocomposites on the viability, proliferation, and apoptosis of different human cell lines, including osteoblasts, chondrocytes, and endothelial cells.

# Material and methods

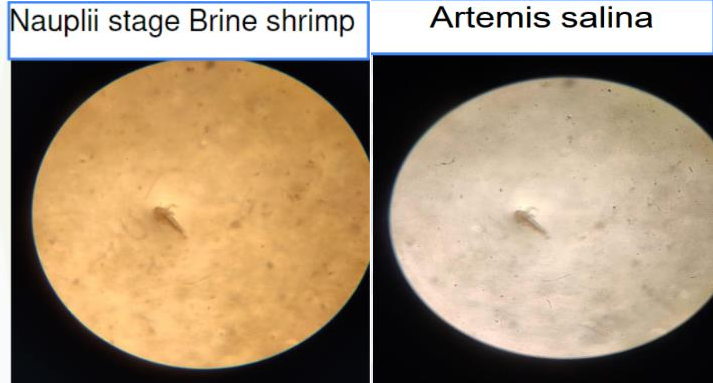
## Sample Preparation

Nano Hydroxyapatite (nHA): Nano hydroxyapatite will be synthesized using a well- established method, such as the precipitation or sol-gel technique.[(Li et al., 2023)](https://paperpile.com/c/VtC5q7/d2WYd)The particle size of nHA will be confirmed through scanning electron microscopy (SEM) or transmission electron microscopy (TEM) analysis(Saadh et al., 2024).

Chitosan: Chitosan will be extracted from a reliable and sustainable source, such as shrimp shells. The degree of deacetylation will be determined using Fourier-transform infrared spectroscopy (FTIR).[(Rinaudo, 2006)](https://paperpile.com/c/VtC5q7/KKWy9)

Vitamin K2: Pure vitamin K2 will be obtained from a reputable supplier and confirmed through high-performance liquid chromatography (HPLC) analysis(Almatrafi et al., 2024).

Nanocomposite Preparation: The nanocomposites will be fabricated by mixing appropriate ratios of nHA, chitosan, and vitamin K2. The materials will be homogenized using a suitable technique, such as sonication or mechanical stirring, to ensure uniform dispersion.



**Figure1:** Nauplii stage brine shrimp and artemis salina

## Brine Shrimp Lethality Test

Brine Shrimp Culture: Brine shrimp eggs (Artemia salina) will be hatched in artificial seawater to obtain nauplii. The culture will be maintained at an optimal temperature (around 25-30°C) and illuminated with a light source to ensure proper growth and development.

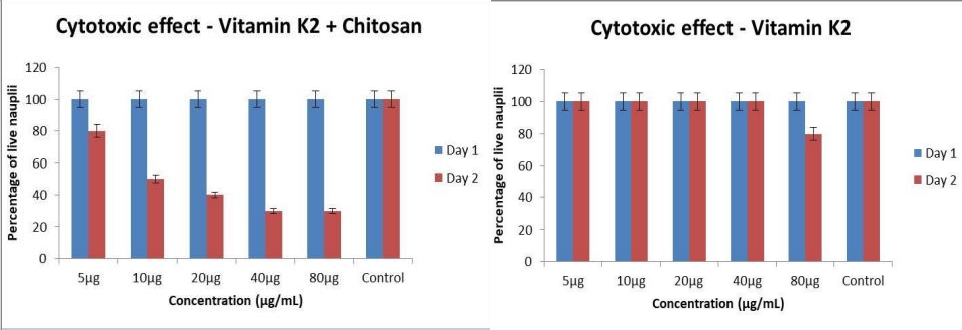
Test Solutions Preparation: Different concentrations of the nanocomposites will be

prepared in artificial seawater to obtain a range of doses. A negative control (artificial seawater) and a positive control (e.g., potassium dichromate) will also be included in the experiment.

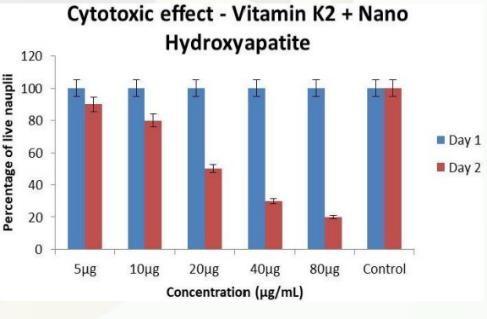
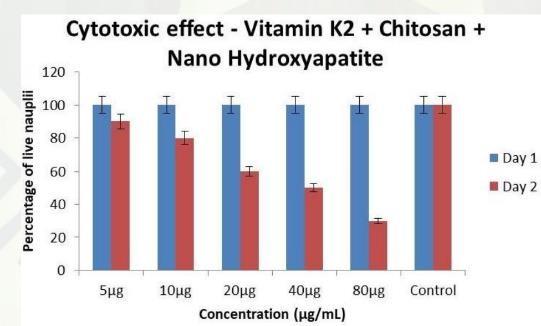
Exposure of Brine Shrimp Nauplii: A predetermined number of brine shrimp nauplii will be exposed to each concentration of the nanocomposites and controls for a specific period (e.g., 24 hours).

Mortality Assessment: After the exposure period, the number of dead and surviving nauplii in each group will be recorded. The lethality or mortality rate will be calculated for each concentration.

# Statistical Analysis



1. *(b)*



1. *(d)*

**Figure 2:** Cytotoxicity activity of all the groups (A: VitK2+chitosan, B:Vit K2, C: Vitamin K2+ chitosan+nHAP D: Vitamin K2 + nHAP) at varying concentrations. X axis represents the concentrations of the respective test compounds at 25 µL, 50 µL, and 100 µL, while the Y axis represent percentage of live nauplii

## Ethical Considerations

The brine shrimp lethality test is a widely accepted and ethical method for preliminary toxicity screening. No vertebrate animals will be used, ensuring compliance with ethical

guidelines for animal experiment. The obtained data will be subjected to statistical analysis using appropriate software (e.g., SPSS 23). The lethal concentration (LC50) values will be determined through regression analysis to estimate the concentration of nanocomposites required to cause 50% mortality in brine shrimp nauplii. The results have been presented

graphically using dose-response curves to visualize the toxicity profile of the nanocomposites.

## Quality Control and Replicates

The experiment will be carried out in triplicate or more, and the mean values will be computed, to guarantee the accuracy of the data. Each set of studies will contain both positive and negative controls to ensure that the brine shrimp lethality test is accurate.

# Results

The data on the death rate of brine shrimp larvae at various observational intervals and concentration levels are shown in the table. To determine the LC value, the total deaths after 24 hours were subjected to a probit analysis using the SPSS application 23. It can be seen that there were 10 nauplii on day 1 and that fewer nauplii persisted after 24 hours. Additionally, it was observed that as concentration increased, nauplii numbers reduced. Additionally, vitamin K2 had the highest survival rate and best results and concentration 40g/ml is considered to be the safest.

# Discussion

The comparative cytotoxic effects of nanocomposites prepared using nano-hydroxyapatite (HA), chitosan, and vitamin K2 are an essential aspect of understanding their potentialbiocompatibility for various biomedical applications. In this discussion, we will analyse the implications of the cytotoxicity findings and their significance. HA is considered biocompatible, with minimal cytotoxicity. However, the particulate size and surface characteristics can influence its biocompatibility. Chitosan is a biopolymer derived from chitin and has gained attention invarious biomedical applications due to its biocompatibility. In addition to perhaps improving the nanocomposites' mechanical qualities, chitosan may also increase their biocompatibility. When chitosan is coated bio actively on implant surface, it can create stronger titanium surfaces thatare more capable of osseointegration than uncoated titanium alloys. [(Kasabwala et al., 2021; Rajeshkumar & Lakshmi, 2021; Varghese et al., 2023)](https://paperpile.com/c/VtC5q7/v8vCK+RiQsC+6fz17) Using chitosan in conjunction with other polymer compounds of mineralization can significantly boost the amount and quality of bone formation surrounding the implant surfaces by increasing thenumber of bi-layers[(Duraisamy, 2021a)](https://paperpile.com/c/VtC5q7/Yyd9N) In the initial stages of bone healing, the hydroxyapatite coating applied to implant surface enhances osseointegration. The strong bone-bonding capacity of metal implants is facilitated by the HA. At a later stage of recovery, the titanium implant will likewise have the same amount of bone contact. The impact of bone formation and bone-bonding strength via HA crystallinity is not significantly covered. Because it offers superior strength, durability, andosteoconductive qualities, the HA coating is the most attractive of the lot due to its increased crystallinity[(Duraisamy, 2021b)](https://paperpile.com/c/VtC5q7/FJTqR) Vitamin K2 plays a role in bone metabolism and may enhance bone regeneration in nanocomposites. The choice of vitamin K2 as an additive could positively influence bone cell behaviour and, at the same time, introduce concerns about its cytotoxic potential at higherconcentrations. [(Ajay et al., 2023; Chokkattu et al., 2023; Padarthi et al., 2023)](https://paperpile.com/c/VtC5q7/FbkhQ+8FUvT+7k9Ou) In the future, it is reasonable to assume that hydroxyapatite and vitamin K2, vitamin D, chitosan coated titanium dental implants may have better biocompatibility andosseointegration properties and hence, it is of interest to prepare titanium dental implants coated with nanohydroxyapatite and Vitamin K2 and to study their biocompatibility andosseointegration.[(Devi & Duraisamy, 2020)](https://paperpile.com/c/VtC5q7/OIITh)The current study demonstrates that the newly hatched naupalii larvae are distinctive for cell cytotoxicity evaluations. This methodology is usually favoured in studies. The brine shrimp lethality test for marine natural products is similar to another method of egg hatch inhibition13, however it's shown to be significantly less sensitive in detecting the toxicity of macroalgae in the extract. Before undertaking this test, a number of criteria need to be taken into account.

**Table1:**Extract concentrations of different combinations and the death rate of brine shrimp on day 1 and after 24 hrs The test results demonstrate that several extract concentrations, including 80 g/mL, 40 g/mL, 20 g/mL, 10 g/mL, and 5 g/mL, will kill brine shrimp larvae.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **S.No.** | **Combination** | **Concentration** | **Number survived DAY1** | **Number survived DAY 2 (after 24Hrs)** |
|  |  |  |  |  |
| 1. | K2 + nHAP | 5μL | 10 | 8 |
|  |  | 10μL | 10 | 9 |
|  |  | 20μL | 10 | 5 |
|  |  | 40μL | 10 | 3 |
|  |  | 80μL | 10 | 2 |
|  |  |  | CONTROL | 10 |
| 2. | K2 + Chito + nHAP | 5μL | 10 | 8 |
|  |  | 10μL | 10 | 9 |
|  |  | 20μL | 10 | 6 |
|  |  | 40μL | 10 | 5 |
|  |  | 80μL | 10 | 3 |
|  |  |  | CONTROL | 10 |
| 3. | VIT K2 | 5μL | 10 | 10 |
|  |  | 10μL | 10 | 10 |
|  |  | 20μL | 10 | 10 |
|  |  | 40μL | 10 | 8 |
|  |  | 80μL | 10 | 10 |
|  |  |  | CONTROL | 10 |
| 4. | K2 + Chito | 5μL | 10 | 8 |
|  |  | 10μL | 10 | 5 |
|  |  | 20μL | 10 | 4 |
|  |  | 40μL | 10 | 3 |
|  |  | 80μL | 10 | 3 |
|  |  |  | CONTROL | 10 |

Every egg resembles a tiny particle of fine sand. If maintained in a cold, dry environment, eggs can remain viable for several years. Whenever there is a salt solution present, eggs will hatch intolarvae. [(Dharman et al., 2023; S. Sindhu et al., 2023; Sreenivasagan et al., 2023)](https://paperpile.com/c/VtC5q7/zi4uF+TWDWJ+wEUZk) The amount of brine fluctuates between 2 to 4%. Instead of iodized salt or reagent grade salt, use of sea salt yields the best results. It is advised to use seawater. Distilled water could be used as an alternative if regular water is unavailable. If the tap water has chlorine in it, don't use it. To dechlorinate the water in this situation, tap water has to be left to stand for approximately overnight. For eggs to hatch, the pH level of the water must be adjusted. The ideal pH range is8.0 ± 0.5. To prevent larvae from dying owing to pH drops during incubation, the pH should be corrected using sodium hydroxide or sodium carbonate. The eggs will surface on their own. The development of the larvae commences when a salt solution penetrates the eggs. We require oxygen. The floating eggs get oxygen directly from the air for their development. Larvae hatch at 24 to 30 hours of high aeration and continuous light during 36 to 48 hours of incubation at room temperature (28 to 30°C). In liquid columns, larvae can freely travel around. In most cases, it islocated at the upper air-liquid contact. [(Ramakrishnan et al., 2023; Shenoy & Maiti, 2023; J. S. Sindhu et al., 2023)](https://paperpile.com/c/VtC5q7/LvEaw+ex2B4+7WLEa) The plastic tube that extends to the bottom of the pot is filled with air, which helps the eggs move continuously. Larvae were given no food during this study's period of time. The adverse effects of the plant extract or malnutrition may beaccountable for the larvae's mortality. Control samples using only larvae were used to verify the plant extract's lethal effect. As long as the newly hatched larvae are still receiving nutrition from the yolk sac, they can all survive lasting up to 48 hours sans nourishment. Adding regular yeast every 2 days will provide larvae with nourishment. Low sodium chloride concentrations were tested by Michael et al. (1956) in the larval culture fluid. Sodium hydroxide was used to bringthe solution's pH to 10.0. The culture solution's composition was changed after that. 30°C is the ideal temperature for cultivation.!4

## Potential applications of nanocomposites in implants

Dental Implants: The use of nanocomposites in dental implants can improve the integration of the implant with the surrounding bone, reducing the risk of implant failure and promoting long-term implant success. Additionally, the controlled release of vitamin K2 from thenanocomposites may further enhance bone formation and remodelling around the implant[(Venugopalan, 2021)](https://paperpile.com/c/VtC5q7/Y46zI)Drug Delivery Systems: Nanocomposites containing chitosan can be engineered as drugdelivery carriers, allowing the controlled release of therapeutic agents, growth factors, or antibiotics directly to the implant site. This localized drug delivery approach can improve the efficacy of treatments while minimizing systemic side effects.Bone Scaffolds: Nanocomposites can serve as the basis for 3D-printed bone scaffolds,providing a biomimetic environment that supports cell attachment, proliferation, and tissue regeneration. The combination of nHA and chitosan within the nanocomposite can promote cell adhesion and differentiation, leading to enhanced bone formation within the scaffold.

## Advantages of Brine Shrimp Assay

Rajabi et al. (2015) state that artemia has several advantages, such as its ability to utilize various nutrition sources and wide geographic dispersion, which make it perfect for toxicity approaches.It can readily accommodate a large number of nauplii for statistical demonstration; the eggs are viable at a reasonable price very fast and remain viable in dry storage for years.This assay does not use animals for testing since it does not require animal agglutinin.Its compact size allowed for easy laboratory operations, including its sophisticated adaptability to a range of experimental conditions. • A strong understanding of biology and ecology[(Hnamte et al., 2020)](https://paperpile.com/c/VtC5q7/e6BqF)

## Disadvantages of Brine Shrimp Assay

Regulated settings for pH, light, aeration, temperature, and salinity during studies.The comparable geographic position of the cysts.The Artemia nauplii were the same age at the beginning of each test.Both positive and negative controls are crucial to the assay's operation and are needed to maintain standard compliance and assess the larvae's sensitivity[(Hnamte et al., 2020)](https://paperpile.com/c/VtC5q7/e6BqF)

# Conclusion

In conclusion, nanocomposites prepared using nano hydroxyapatite, chitosan, and vitamin K2hold significant promise for use in wound dressings, biomedical coatings, and implant materials, to effectively combat infections and reduce the incidence of healthcare-associated infections.Vitamin K2 hold significant promise for use in implants, particularly in bone tissue engineering and dental implantology. These nanocomposites offer a multifunctional approach to improve implant biocompatibility, osseointegration, and bone regeneration. To confirm the effectiveness and safety of these nanocomposites for use in a variety of implant applications, furtherinvestigation and clinical testing are required.

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