Encapsulation efficiency of Chitosan Tripolyphosphate nanoparticles containing Saw scaled viper snake venom

RUNNING TITLE

Nanoparticles and its encapsulation efficiency containing snake venom

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ABSTRACT

Chitosan Tripolyphosphate (CS/TPP) nanoparticle is a biodegradable and nontoxic polysaccharide, used as a carrier for drug delivery. The morphology and particle size measurements of the nanoparticles were studied by field emission scanning electron microscopy (FE-SEM) and FTIR (Fourier Transform Infrared Spectra). This study aims to evaluate the impact of Saw Scaled Viper venom encapsulation on various factors and loading capacity, in addition to explore the physicochemical structure of nanoparticles. FTIR confirmed that tripolyphosphoric groups of TPP linked with ammonium groups of CS in the nanoparticles. Our results showed that CS can react with TPP to form stable cationic nanoparticles. The

results showed that encapsulation efficiency of venom at a different concentration 20, 40, 60, 500 and 1000 µg/ ml were achieved for CS/TPP nanoparticles at a different concentration of 1.5, 2 and 3 mg/ml. The cytotoxicity of CS/TPP nanoparticles was evaluated by MTT (-3 (4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) assay.

KEYWORDS: Chitosan Tripolyphosphate, Nanoparticle, Saw Scaled viper, Fourier Transform Infrared Spectra.

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INTRODUCTION

Chitosan was a natural biodegradable, biocompatible and non-toxic biopolymer extracted from the shells of crustaceans. Recently, Chitosan (CS) hydrophilic nanoparticles (CS-NPs) had received much attention for delivery of therapeutic peptides, proteins, antigens, oligonucleotides, and genes by intravenous, oral, and mucosal administration[1] following the work of Mumper[2] , chitosan and its derivatives had been examined extensively for medical and pharmaceutical applications in artificial organs, targeted drug delivery, drug transport, protein delivery gene transfer and so on [3-6]. CS is a positive charged and is mucoadhesive in nature[7-8]. Hence, it was used extensively in drug delivery applications [9-13]. In acidic pH amino groups can undergo protonation, thus makes them soluble in water. Solubility of CS depends upon the distribution of free amino and N-acetyl groups. It has good adhesion, coagulation ability, and immunostimulating activity [14].

Chitosan/tripolyphosphate nanoparticles (CS/TPP) have been used as an alternative to chitosan to encapsulate peptides, proteins, pDNA, insulin, and siRNA [15-23]. Chitosan-TPP nanoparticles entrapped siRNA have been found to be better vector siRNA delivery vehicles compared to chitosan siRNA complexes[21]. Csaba et al. [22] has adapted ionic gelation technique for the encapsulation of different nucleic acids (plasmid DNA and short oligonucleotides) into chitosan-TPP nanoparticles and evaluated their potential as gene delivery nanocarriers. These CS/TPP nanoparticles have shown promising results as nasal delivery vehicles for insulin[23], antigens such as tetanus toxoid [24] and diphtheria toxoid [25], where found a prompt and facilitated administration of therapeutic proteins. Additionally, recent data have shown that Chitosan nanoparticles can be used as new ocular drug delivery systems [2]6.

Chitosan nanoparticles have a long shelf-life [27], can be obtained by a very mild ionic gelation method, and has been reported to possess an excellent capacity for the association of proteins [28-29]. Chitosan has been extensively examined for its potential in the development of controlled-release drug delivery systems [30].

In the present study is to produce biodegradable nanoparticles for loading of Saw scaled viper venom and to evaluate their potential as antigen delivery systems. This study is to analyse the different concentration of CS/TPP nanoparticles loading with various concentration of venom to evaluate the encapsulation efficiency of nanoparticles.

MATERIALS AND METHODS

Chitosan from shrimp shell were purchased from Sigma Aldrich (Bangalore) (Low molecular weight [LMW]). Deacetyl degree was a minimum of 85% and Sodium tripolyphosphate (TPP), acetic acid were purchased from (Merck, Bangalore). The lyophilized snake venom of Russell's viper was obtained from Irula's Snake Catcher's Society, Chennai, Tamil Nadu, India with proper permission (No.WL1/7/2006 dt 13.11.2006) and preserved in desiccator at 4°C for further use. Triple distilled water was used for all the experiments.

Synthesis of CS/TPP nanoparticles

CS/TPP nanoparticles were prepared by ionic gelation process following the protocol

described by Mohammadpourduunighi et al. [32]. Chitosan was dissolved at a concentration of 3 mg/ml in 0.5% acetic acid solution. A solution of TPP at the concentration of 1.0 mg/ml was prepared with deionized water. Then, 10 ml of TPP solution was added dropwise under constant stirring to 25 ml of the chitosan solution. An opalescent suspension was formed spontaneously under the aforementioned conditions. Nanoparticles were separated by centrifugation at 16,000 rpm for a period of 30 min at 14°C. The supernatant was discarded and the wet pellet of CS/TPP nanoparticles was collected. The pellet was washed serially with 20%, 75% and 100% , followed by freeze drying and stored at 4°C for further studies.

Characterizations of CS/TPP Nanoparticles

The morphology of the CS/TPP nanoparticles was observed by field emission scanning electron microscopy (FE-SEM) (AMRAY 1910) equipped with a backscattered electron detector at 15-30 kV. For SEM images, the samples were sputter-coated with about 15nm Au using a Polaron coater system. The Fourier Transform Infrared Spectra (FTIR) of CS and CS/TPP was recorded on a Perkin- Elmer FTIR spectrometer (SPECTRUM 1000) using KBr pellets at a resolution of 4 cm^{-1} to evaluate the cross-linking of CS with TPP. The CS and CS/TPP nanoparticles were mixed with KBr in the ratio of 1:150 and ground in a mortar by hand with a pestle. The powder was pressed into pellets under a pressure of 4 tonnes. The IR absorbency scans were analysed between 500 to 4000 cm^{-1} for changes in the intensity of the sample peaks.

Cytotoxicity Assay

The cytotoxicity of CS/TPP nanoparticles were evaluated by heart cell line of catla (SICH). SICH cells were seeded at a density of 5.0×10^4 cells/well in 24-well flat-bottomed micro assay plates (Falcon Co., Becton Dickinson, Franklin Lakes, NJ) and incubated for 24 hr. CS/TPP nanoparticles were added and incubated for 4 h at 28°C. After incubation, the mixture was replaced with 500 µl of fresh Leibovitz's L-15 (GIBCO) medium containing 2% serum. After 24 hr incubation, 120 µl of 2 mg/ml of MTT solution in 1 × PBS was added. Plates were then incubated for an additional 4 h at 28°C. MTT-containing medium was removed and 200 µl of acidified alcohol (100 µl of concentrated HCl in 100 ml of isopropanol) was added to dissolve the formazan crystal formed by live cells. Absorbance was measured at 570 nm in a microplate reader (Thermo Lab Systems). The cell viability (%) was calculated according to the following equation:

$$\text{Cell viability } (\%) = \frac{\text{OD570 (sample)}}{\text{OD570 (control)}} x100$$

where the OD 570 (sample) represents the measurement from the wells treated with CS/TPP nanoparticles and the OD 570 (control) represents the measurement from the wells treated with PBS buffer only.

Preparation of Venom - loaded CS/TPP nanoparticles

Venom loading in CS/TPP nanoparticles system was done by the methods of incubation. Hence, CS/TPP nanoparticles were formed first via TPP coacervation, and the nanoparticle containing solution at different concentration (1.5, 2 and 3 mg/mL) was then mixed with solutions containing venom at different concentrations (20, 40, 60, 500, and 1000 µg/mL). The mixed solutions were gently stirred for 60 min to allow venom adsorption on the nanoparticles. In this method, venom loading was solely via adsorption on the surface of nanoparticles. Venom encapsulation in this work was all conducted at pH 5.5 unless where otherwise stated.

Evaluation of Encapsulation Efficiency

In this study encapsulation efficiency was studied. The venom loaded CS/TPP nanoparticles were carefully transferred to centrifuge tubes and nanoparticles were separated by centrifugation at 20,000 rpm for 30 min at 10°C. The supernatant was collected and the unbound venom content in the supernatant was quantified by UV spectrophotometer at 595 nm. The

encapsulation efficiency (EE) was calculated using the following equation as described by Gan and Wang (2007).

$$EE = \frac{\text{Total amount of Venom} - \text{Free amount of Venom in supernatant}}{\text{Total amount of Venom}} \times 100$$

Venom release from the nanoparticles *in vitro*

The *in vitro* venom release profiles of CS/TPP nanoparticles were determined as follows: the various concentration of venom (20, 40, 60, 500, 1000 µg/ mL) was loaded in various concentration of CS/TPP nanoparticles (1.5, 2 and 3 mg/mL) were treated with chitosanase digestion (3µg) .Then the sample were incubated at 37°C for 60 minutes under continous stirring. The amount of venom released from the nanoparticles was evaluated by UV spectrophotometry.

RESULTS

The results revealed that CS had the strongest absorbance at 1738 cm^{-1}, indicating the presence of NH_2 group. To compare the IR spectra of CS and CS/TPP nanoparticles, the signal intensity of this NH_2 band decreased upon introducing TPP, while a new band was observed at about 1643 cm^{-1} in the IR spectrum of CS/TPP nanoparticles. According to literature, the CS film modified by phosphate also displayed a similar IR spectrum owing to the interaction between the phosphate and chitosan. At the same time, the band at 1383 cm^{-1} almost disappeared in CS/TPP nanoparticles due to the loss of free hydroxyl of CS, indicating that strong hydrogen bonds within and between the CS/TPP nanoparticles formed. Moreover, the band at 1240 cm^{-1} resulting from the –COOH groups of TPP was not observed. All the above observations indicated that CS was cross-linked with TPP.

The FE-SEM of CS/TPP nanoparticles revealed a very homogeneous morphology with quite uniform and spherical shape. The size of particles ranged from 30 to 60 nm. The FTIR and FE-SEM result has been published in our previous literature vimal et al. (31).

Cytotoxicity of CS/TPP nanoparticles was evaluated by MTT assay and the results are shown in Fig. 1. The viability of catla heart cells incubated with the CS/TPP nanoparticles was found to be more than 95% in the concentrations ranging from 1.5, 2 and 3 mg/ml. No significant morphological changes were observed in catla heart cells treated with CS/TPP nanoparticles and the results are shown in Fig. 2.

The efficacy of different concentrations of CS/TPP nanoparticles (1.5, 2, and 3 mg/mL) loaded with different concentration of snake venom (20, 40, 60, 500, and 1000 µg/mL). We observed that the CS/TPP nanoparticles at a concentration of 1.5 mg/mL yielded low encapsulation efficiency and formed aggregates of large diameters and with the highest concentration of CS/TPP nanoparticles (2, 3 mg/mL) the encapsulation efficiency was high. Therefore, the formation of nanoparticles is possible only with specific concentrations of CS and TPP. The influence of different concentration of CS/TPP nanoparticles (1.5, 2 and 3 mg/mL) with a loading capacity of venom at low (20, 40 and 60 µg/mL) and high concentration (500, 1000 µg/mL) were evaluated to study the encapsulation efficiency and the results are shown in Fig. 3.

The percentage of unbound encapsulation efficiency of venom at a concentration of (20, 40, 60, 500, and 1000 µg/mL) loaded with CS/TPP (1.5, 2, and 3 mg/mL) nanoparticles and the results are shown in Fig. 4. The results of our present study confirmed that encapsulation efficiency was (89 %) while increasing the concentration of CS/TPP nanoparticles (3 mg/mL) and loading capacity of the venom at a concentration of (500, and 1000 µg/mL). Our study indicates that, the loading capacity of a venom increased when the MW of CS/TPP is increased (2, 3 mg/mL) in a constant venom concentration (500 and 1000 µg/ml) Fig. 5.

The *in vitro* release profiles of encapsulated various concentration of venom (20, 40, 60, 500, and 1000 µg/mL) was loaded in various concentration of CS/TPP nanoparticles (1.5, 2,

and 3 mg/mL) was studied. *In vitro* release system of venom loaded nanoparticles was studied using chitosanase treatment. The percentage of venom released from CS/TPP nanoparticles and the results are shown in Table.1.

DISCUSSION

The ionic gelation method was followed to prepare CS/TPP nanoparticles using low molecular weight chitosan. The size of the nanoparticles ranged from 30 to 60 nm. Csaba et al. [22], used ionic gelation method and obtained smaller CS/TPP nanoparticles (93 nm) using low molecular weight chitosan. Our results can be compared with previous studies on ionic gelation process for the preparation of CS/TPP nanoparticles [1, 21, 22, 34].

The results of FTIR analysis of CS/TPP nanoparticles showed a high peak of 1738 cm^{-1} in CS, indicating the presence of NH_2 group. The signal intensity for the NH_2 decreased to 1643 cm^{-1} after addition TPP as observed by Lam et al.[35], and Mohammadpourdounighi et al.[32]. Lam et al. [35] was observed the peaks of 1650 cm^{-1} and 1636 cm^{-1} for amino group in CS and CS/TPP, respectively [35]. Mohammadpourdounighi et al. [32] was found that the 1595 cm^{-1} peak of N-H bending vibration shifts to 1540 cm^{-1} in CS/TPP nanoparticles after addition of TPP.

Morphologically the CS/TPP nanoparticles prepared in the present work were found to be spherical in shape as observed [33, 36]. Lam et al. [35] prepared the CS/TPP nanoparticles with the size of 50 – 70 nm. Mohammadpourdounighi et al. (2010) found that the loading capacity of venom increased with CS in a constant venom concentration (500 µg/mL). Chitosan effectiveness in coagulating solids and proteins were inversely proportional to its MW[37]. In the study of CS/TPP nanoparticles revealed that the highly viscous nature of the gelation medium hinders encapsulation of venom. Relatively lower adhesiveness of CS/TPP nanoparticles at a concentration promotes encapsulation of snake venom and gelation between CS and TPP. The results showed that encapsulation efficiency of venom at a concentration 20, 40, 60, µg/ mL were achieved for low binding efficiency with CS/TPP nanoparticles at a different concentration of 1.5, 2, 3 mg/mL [32].

Cytotoxicity of CS/TPP nanoparticles was evaluated by the 3- (4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) assay using catla heart cell line. The results showed that CS/TPP nanoparticles were poorly cytotoxic and caused less than 10% cell death. It has been reported that CS/TPP nanoparticles were non toxic than other cationic polymers such as poly-L-lysine and polyethyleneimine [21, 22]. These studies including our present study suggest that CS/TPP nanoparticle is non toxic and hence is a suitable biocompatible reagent for sustained release. Based on the published literature [22] cytotoxicity and biological effect of CS/TPP, we conclude that CS/TPP is useful as a carrier system.

CONCLUSION

In conclusion, ionic gelation method was used to prepare CS/TPP nanoparticles. The FTIR showed that CS was cross-linked with TPP, an uniformly size distribution and good dispersion were observed. CS/TPP nanoparticles are suitable for the simultaneous encapsulation efficiency of snake venom.The results of our present study suggested that the CS/TPP nanoparticles were synthesed in our lab can able to use as an alternative for traditional adjuvant systems for future medicines.

CONFLICT OF INTEREST

All authors have completed the Unified Competing Interest and declare: No support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

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Figure Captions

Figure.1**.**Percentage of cell survival of catla heart cells exposed to different concentrations of CS/TPP nanoparticles (mg/ml) by MTT assay.

Figure.2**.** Morphology of fish heart cell line exposed to different concentrations of CS/TPP nanoparticles (mg/ml)

Figure.3. Influence on encapsulation efficiency of venom loaded CS/TPP nanoparticles (1.5, 2 and mg/mL).

Figure.4. Percentage of unbound venom in the CS/TPP nanoparticles (1.5, 2 and mg/mL).

Figure.5. Influence of CS/TPP nanoparticles concentration on encapsulation efficiency (venom concentrations 500 µg/mL and 1000 µg/mL.

Figure.1.

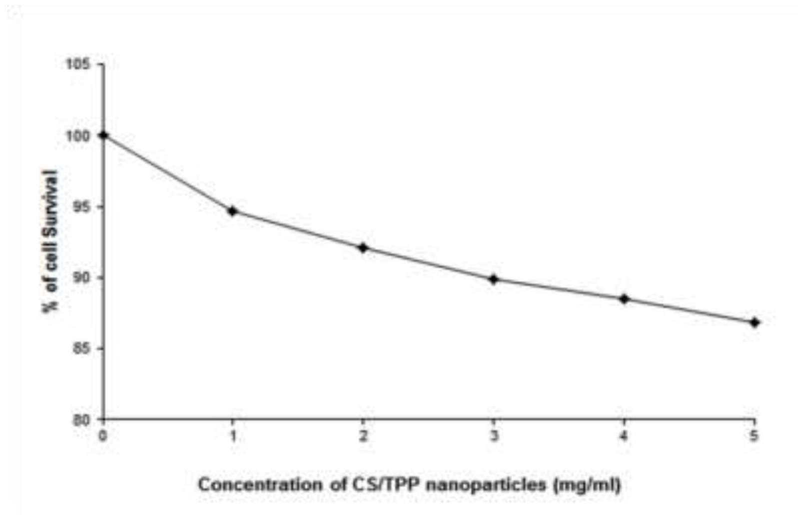

Figure.2.