Synthesis and characterization of vancomycin-loaded chitosan nanoparticles for drug delivery

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Abstract
Chitosan is a linear polysaccharide with prominent physicochemical and biological properties such as biocompatibility, biodegradability, nontoxicity, nonimmunogenicity, bioadhesion, and antibacterial, antifungal, and hemostatic activity. Due to these properties, it has found many applications in cosmetic, textile, and food industries; agriculture; biotechnology; and pharmaceutical industry and medicine, especially in biomedical applications. The special chemical structure of chitosan allows some specific modifications, and by reducing the size of chitosan particles to nano-size, it becomes an excellent drug nanocarrier. Vancomycin is a typical antibiotic used for bacterial infections caused by gram-positive bacteria. In this work, chitosan nanoparticles (CSNPs) were prepared via ionotropic gelation using tripolyphosphate (TPP) as a cross-linker. The effect of chitosan and TPP concentration on the size of chitosan nanoparticles was studied, and CS/TPP ratio of 1:1 with an average size of nanoparticle about 100 nm was selected. The prepared samples were characterized using DLS, FTIR, TGA, DSC, and SEM techniques. The results confirmed that vancomycin has been loaded successfully on chitosan nanoparticles. Also, it is observed that 40% of vancomycin is released burstly in the first 9 h and after that the drug release is continued gradually to receive 90% at 100 h.

Keywords Drug release · Vancomycin · Chitosan nanoparticles · Cross-linker
Introduction

Chitosan is a linear polysaccharide which is obtained by partial deacetylation of chitin in an alkali environment [1–4]. Chitosan with its physicochemical and biological properties such as biocompatibility, biodegradability, nontoxicity, nonimmunogenicity, bioadhesion, and antibacterial, antifungal, and hemostatic activity has attracted the attention of many researchers [2, 5–7]. Due to these properties, chitosan has found many applications in cosmetic, textile, and food industries; agriculture; biotechnology; and pharmaceutical industry and medicine, especially in biomedical applications [2–4, 8–11].

Chitosan has a special chemical structure which makes it unique over other polysaccharides. The structure of chitosan allows some specific modifications via amine or hydroxyl functional groups, which can be loaded with drug directly or through linker [6].

It is found that nanostructures have real potential as effective drug delivery systems, especially in the treatment of microbial infections, due to their advantages over conventional antibiotic formulations [12–14]. Nanoparticles (NPs) due to their small size (1–100 nm) can carry high drug loads as nanovehicles. Also, NPs are used to deliver proteins, peptides, enzymes, genes as well as vaccines [5]. Chitosan nanoparticles (CSNPs) are widely studied in the field of drug, protein, and gene delivery [4, 6]. Small particle size, compactness, and high surface area are the unique physical and chemical properties of chitosan nanoparticles which are associated with many biomedical applications such as drug release and mucosal remodeling [15]. CSNPs are prepared by different methods such as ionotropic gelation, polyelectrolyte complexation, reverse micellar, emulsion solvent diffusion, and electrospaying techniques [1, 6].

One of the most studied methods for preparing chitosan nanoparticles is ionotropic gelation, because it is simple and easy and the aqueous medium used in this method eliminates the hazards of using an organic solvent. Ionotropic gelation is based on cross-linking of cationic chitosan amino groups to a polyanionic cross-linker. Tripolyphosphate (TPP) is mostly used as a negatively charged polyanion to cross-link with chitosan. The obtained complex is used as a drug nanocarrier [1, 4, 5].

Vancomycin is a glycopeptide antibiotic (Scheme 1) that blocked the synthesis of peptidoglycan in the bacterial cell wall. It has a strong bactericidal activity against gram-positive resistant pathogen. Vancomycin is used only parenterally in clinical therapies, because the continuous oral dosing increases vancomycin concentration and leads to toxicity and development of resistance [16–19].

One of the diseases in which vancomycin is used is osteomyelitis. Osteomyelitis is an inflammatory bone disease caused by pathogenic microorganisms, mostly Staphylococcus aureus, and leads to progressive bone destruction and loss. The disease is diagnosed by the formation of bacterial plaque around the infected area. Depending on the duration of the infection, osteomyelitis is classified into acute or chronic. The pathogenic microorganisms can adhere to and even invade mammalian cells. Methicillin-resistant S. aureus and multidrug-resistant mycobacteria
are drug-resistant pathogens, which can develop persistence in the intracellular locations after drug treatment. To the best of our knowledge, many studies have been done on the vancomycin-loaded chitosan-based nanoparticles, chitosan film, and nanofibers [16–22], but less research has been done on vancomycin-loaded chitosan nanoparticles.

The aim of the present work was to prepare and evaluate vancomycin-loaded chitosan nanoparticles. In this respect, chitosan nanoparticles were prepared through an ionotropic method and then loaded with vancomycin. The prepared samples were characterized using Fourier transform infrared spectrometry (FTIR), dynamic light scattering (DLS), scanning electron microscopy (SEM), X-ray diffraction (XRD), thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), and UV–Vis spectrophotometry.

**Experimental**

**Materials**

Low molecular weight chitosan (80–85% deacetylation), sodium tripolyphosphate (TPP), and acetic acid were obtained from Sigma–Aldrich Chemicals Co., Germany. Vancomycin was purchased from Razavi Pharmaceutical Co., Iran.
Preparation of chitosan nanoparticles

In order to prepare chitosan nanoparticles, low molecular weight chitosan was dissolved at 25 °C by adding acetic acid (2 M) gradually and stirring at 9000 rpm (Heidolph, Germany). By using NaOH (2 M) solution, the solution pH was adjusted to 5.5. To remove any undissolved chitosan, the solution was filtered using a 0.45-µm cellulose acetate filter.

TPP solution was prepared by dissolving 0.25 wt% TPP in double-distilled water and passed through a 0.25-µm cellulose acetate filter.

Three different concentrations of chitosan (0.1, 0.2, and 0.3 wt%) were prepared to evaluate the effect of the chitosan-to-TPP ratio on nanoparticle size. Then, 2 ml of TPP at a concentration of 0.2 wt% was added dropwise by burette to 4 ml of chitosan solution at a rate of 0.2 ml/min. To prepare the chitosan nanoparticles, TPP solution was added drop by drop in CS/TPP volumetric ratios (1:2, 1:3, 1:4) to the chitosan solution and stirred at room temperature at 9000 rpm using a high-speed stirrer.

Some of the prepared samples were used to determine the nanoparticle size, and the remaining was centrifuged (Sigma, Germany) for 20 min. Then, the samples were washed with water and centrifuged again to remove unreacted gradients. Then, the nanoparticles were air-dried at ambient temperature and analyzed. Each of the reported results is an average of three repetitions of the test.

Loading of vancomycin

To prepare vancomycin-loaded CSNPs, vancomycin was added at a concentration of 0.5 μg/ml to chitosan solution at a concentration of 0.2 mg/ml. After that, TPP solution with a concentration of 0.45 mg/ml was added to the chitosan solution containing vancomycin.

Characterization

To evaluate the linking of functional groups in chitosan, chitosan nanoparticles, and drug to the nanoparticles, a Fourier transform infrared spectrometer (Perkin Elmer, Germany) was used.

The particle size and the nanoparticle size distribution were determined using dynamic light scattering (Cordovan Tech, France). For this purpose, the solution was sonicated for 3 h after the formation of nanoparticles and immediately subjected to DLS test.

The morphology investigation of the prepared sample was performed using a scanning electron microscope (SEM) (Seron Technology, South Korea). The
prepared nanoparticles’ crystallinity was determined using an X-ray diffractometer (XRD) (Inel Inc., France).

Melting point, glass transition temperature, and the weight loss of the prepared samples were determined by a differential scanning calorimeter (DSC) and thermal gravimetric analyzer (TGA) (Sanaf, Iran). The test was done at a heating rate of 10 °C/min under a nitrogen flow of 40 ml/min, and the specimen was heated from room temperature to 300 °C.

**Drug release study**

UV–Vis spectroscopy (280-4 Alph, Germany) at 280 nm was used to measure vancomycin dispersion in the nanocrystalline membrane. For this purpose, a membrane containing nanoparticles (15 mg weight) was placed in 30 ml phosphate solution and incubated at 37 °C with shaking at 60 rpm. One milliliter of the nanoparticles was recovered at predetermined intervals, and the volume of fresh PBS equivalent was added to the suspension. The drug solution concentrations used were 20, 40, 80, 120, 160, and 200 ppm. The standard curve in Fig. 1 was used to calculate vancomycin concentration in each sample. The released drug from the sample (1 ml) was measured at predicted times of 20, 40, 60, 100, 150, and 210 min as well as 5, 7, 10, 22, 29, 36, 48, 54, 75, and 100 h. All experiments were repeated three times.

**Results and discussion**

**Preparation of CSNPs**

In this work, chitosan nanoparticles were prepared by ionotropic gelation. This method involves ionic interactions between the positive charge of CS and the negative charge of TPP as a cross-linker. In fact, as shown in Fig. 2, the positively charged chitosan amino groups were cross-linked with the negatively charged tripolyphosphate anions and vancomycin drug particles were entrapped in the nanoparticle. The chitosan molecules were gelled when they cross-linked ionically [1, 6, 22]. Ionic cross-linking of chitosan is a noncovalent interaction which can occur in

![Graph](image-url)
the presence of negatively charged multivalent ions (polyanions) like TPP. Noncovalent or physical cross-linking is more promising for pharmaceutical applications than covalent cross-linking, because it is reversible and may avoid toxicity of the reagents [23].

Previous studies showed that the particle size and surface loadings have been affected by the chitosan concentration, the ratio of CS/TPP, and the pH of the solution. The primary aim was to determine the conditions that the nanoparticles can be produced with optimal characteristics, such as proper nanoparticle size and minimum polydispersity index. The particle size affected the ability of the drug delivery system to penetrate the tissue and effective drug release [23, 24].

It was found that by increasing the CS concentration, the size of the formed particles increases. This also occurs with increasing TPP concentration. In the present study, the suspension of CS–TPP nanoparticles with concentrations of 0.1, 0.2, and 0.3 mg/ml CS as well as TPP with a concentration of 0.45 mg/l was prepared. The effect of different concentrations of these two solutions on particle size and morphological properties was investigated [25].

**Dynamic light scattering (DLS)**

As was mentioned before, many factors affected the size and distribution of chitosan nanoparticle including molecular weight of chitosan, CS/TPP volumetric ratio, the additional conditions of TPP to chitosan, chitosan and TPP concentration, the pH of chitosan, ambient temperature, and agitation rate. DLS technique was used to measure the hydrodynamic diameter in the nanometer range. Table 1 shows the effect of CS/TPP ratio on the size of chitosan nanoparticle. According to the results, the CSNPs sample with a size of 99.73 nm and a CS/TPP ratio of 1:1 was selected for further analysis.

Dynamic light scattering test was also performed after preparing vancomycin-loaded chitosan nanoparticles. It is observed in Fig. 3 that the nanoparticles size increased due to drug loading and reached about 100 nm.
The nanoparticles size depends greatly on the volumetric ratio of CS/TPP, and with increasing this ratio from 1:1 to 2:1, the nanoparticle size has changed from 325.84 nm to 99.73 nm. The DLS test was also performed after loading vancomycin on the nanoparticles. It was observed that the size of the nanoparticles increased slightly due to the drug loading and the average nanoparticle size reached 100.88 nm (Fig. 3).

| Chitosan conc. (mg/ml) | TPP conc. (mg/ml) | CS/TPP (v/v) | Stirrer velocity (rpm) | Temperature (°C) | Nanoparticle diameter (nm) |
|-----------------------|------------------|--------------|------------------------|------------------|----------------------------|
| 0.1                   | 0.45             | 1:1          | 6000                   | 25               | 325.84                     |
| 0.2                   | 0.45             | 1:1          | 9000                   | 25               | 680.93                     |
| 0.3                   | 0.45             | 1:2          | 9000                   | 25               | 478.97                     |
| 0.2                   | 0.45             | 1:1          | 9000                   | 25               | 99.73                      |

Fig. 3 Dynamic light scattering of (a) chitosan nanoparticles and (b) vancomycin-loaded chitosan nanoparticles.
Scanning electron microscopy (SEM)

The SEM images of chitosan nanoparticles with and without vancomycin are shown in Fig. 4. As shown in Fig. 4a, the image of unloaded CSNPs revealed a smooth and homogenous surface. Surface nanoparticles represent a very uniform and spherical shape morphology with an average size of 86 nm. SEM image of vancomycin-loaded chitosan nanoparticles is shown in Fig. 4b. The image also reveals a smooth and homogenous surface without any crystallized vancomycin. On the other hand, the drug is distributed homogenously at a molecular level, which corresponds to the results obtained by some researchers [16, 27].

FTIR spectroscopy

The characteristic peaks of FTIR spectra of CS, CSNPs, vancomycin, and vancomycin-loaded chitosan nanoparticles are given in Table 2. As given in Table 2, a peak at 3360 cm\(^{-1}\) is observed for O–H group overlapped with N–H group stretching vibrations. Usually, O–H group and N–H group bands are separated, but due to the presence of hydrogen bonds in chitosan, the two peaks are overlapped. The peak at 2963 cm\(^{-1}\) is assigned to the C–H stretching vibration mode. The absorption peaks at 1633 and 1594 cm\(^{-1}\) are attributed to C=O bending vibration and N–H bending vibration of protonated amino (–NH\(_2\)) group, respectively. The C–N stretching vibration of the amine group was observed at 1230 cm\(^{-1}\), and the asymmetric C–O–C stretch was observed at about 1158 cm\(^{-1}\). A weak peak observed at 1420 cm\(^{-1}\) is attributed to the bending vibration of O–H, and the peak at 1370 cm\(^{-1}\) is assigned to the bending vibration of C–H deformation [26, 28].

For CSNPs, the peak at 3427 cm\(^{-1}\) is attributed to O–H group overlapped with N–H group stretching vibrations. The peak becomes wider and shifts to a higher wavenumber, which indicates that hydrogen bonding is increased. The peak at 1594 cm\(^{-1}\) for bending vibration of N–H in CS shifts to 1635 cm\(^{-1}\), and the new peak at 1553 cm\(^{-1}\) is attributed to N–O–P stretching vibration, which implies the cross-linking of TPP anions with ammonium groups of chitosan to form chitosan nanoparticles [28].

Fig. 4  SEM images of (a) chitosan nanoparticles and (b) vancomycin-loaded nanoparticles
In Table 2, vancomycin shows characteristic broader peaks attributed to OH stretching vibration of R–CH₂–CH₃, COOH, and R–NH–R at 3290 cm⁻¹, carboxyl stretching vibration of amide group at 1655 cm⁻¹, long alkyl chain at 1494 cm⁻¹–1126 cm⁻¹ band, R–O–R stretching vibration at 1010 cm⁻¹, and aromatic peak at 1552 cm⁻¹.

For drug-loaded CSNPs in Table 2, there is a merged N–H stretching vibration peak at 3431 cm⁻¹ and carbonyl stretching peak shifts to 1563 cm⁻¹–1341 cm⁻¹. The similar R–O–R stretching peaks are observed at 1056 cm⁻¹ and aromatic peak at 1637 cm⁻¹. It seems due to the inter- and intramolecular interactions, the shifting and overlapping of absorption peaks assigned to the successful loading of vancomycin on CSNPs.

### X-ray diffraction (XRD) analysis

The X-ray patterns of chitosan, chitosan nanoparticles, vancomycin, and vancomycin-loaded CSNPs are shown in Fig. 5. As can be seen, CS has weak diffraction peaks at 2θ = 12° and sharp diffraction peaks at 2θ = 22°, which indicate the high degree of crystallinity of chitosan (Fig. 5a). The broad peak in CSNPs is due to the addition of TPP to chitosan that was resulted in the formation of amorphous CSNPs. Thus, it is evident that the ionic gelation and interactions of CS and TPP reduced the
folding and crystallization ability of CS chains (Fig. 5b) [29]. This pattern change can be assigned to the modification of molecules arrangement in the crystal lattice. This intensity reduction is more obvious in the vancomycin-loaded CSNPs pattern. The vancomycin pattern is shown in Fig. 6d. As shown in Fig. 6c, the pattern of

![X-ray diffraction pattern of (a) chitosan, (b) chitosan nanoparticles, (c) vancomycin-loaded chitosan nanoparticles, and (d) vancomycin](image)

**Fig. 5** X-ray diffraction pattern of (a) chitosan, (b) chitosan nanoparticles, (c) vancomycin-loaded chitosan nanoparticles, and (d) vancomycin

![TGA thermogram of chitosan, chitosan nanoparticles, vancomycin, and vancomycin-loaded CSNPs](image)

**Fig. 6** TGA thermogram of chitosan, chitosan nanoparticles, vancomycin, and vancomycin-loaded CSNPs
vancomycin-loaded CSNPs shows a stronger peak than that of CSPNs peak at $2\theta = 25^\circ$, due to the presence of vancomycin.

**Thermogravimetric analysis (TGA)**

The TGA thermogram of chitosan, chitosan nanoparticles, vancomycin, and vancomycin-loaded chitosan nanoparticles is shown in Fig. 6. It is clear that for chitosan, the first weight loss about 3.5% is occurred at 50 °C to 100 °C which is attributed to the loss of absorbed or bound water severe weight loss and related to the hydrophilic nature of chitosan [30]. The decomposition is initiated at 200 °C, and an obvious weight loss occurred from 200 °C to 450 °C which probably corresponds to the dehydration of the anhydro-glucosidic ring. The residue remaining at the end of the experiment (at 800 °C) is about 22%. As can be seen for chitosan nanoparticle, the thermal behavior is changed. The water elimination is occurred within 50 °C to 100 °C with 7.5% weight loss. It seems more weight loss at the dehydration stage of CSNPs than CS due to its higher hydrophilicity that resulted in more bound water. CSNPs is decomposed from 150 °C to 250 °C with 60% remaining residue.

Vancomycin is decomposed at 155 °C to 560 °C with 20% final remaining residue. The thermal behavior pattern of drug-loaded CSNPs is similar to CSNPs the decomposition of which is occurred from 152 °C to 280 °C. In general, the results confirm that thermal stability of CSNPs is higher than that of CS, which can be corresponded to the interactions due to the cross-linking of CS molecules with TPP. Also, the cross-linker reduces the decomposition temperature. These results are consistent with the results of other researchers [28, 30].

**Differential scanning calorimetry (DSC)**

The DSC results of chitosan, chitosan nanoparticles, vancomycin, and vancomycin-loaded chitosan nanoparticles are shown in Fig. 7. It is obvious that vancomycin has a melting point ($T_m$) of 240 °C and a glass transition temperature ($T_g$) of 72 °C. The melting point and glass transition temperatures for chitosan are 230 °C and 67.5 °C, for chitosan nanoparticles 240 °C and 75.7 °C, and for vancomycin-loaded chitosan nanoparticles 248 °C and 70 °C, respectively. We had wider characteristics at a melting temperature of 67.5 and glass temperature of 230 °C. Thermography of chitosan nanoparticles. The melting temperature of glass and its nanoparticles reached 75.7°C and 240 °C, and the chitosan nanoparticles with drug showed marked signs of vancomycin at 70 °C and 248 °C.

As can be seen, vancomycin thermogram shows a melting point ($T_m$) of 75.3 °C and a glass transition temperature ($T_g$) of 250.6 °C, while for chitosan these temperatures are 67.5 °C and 230 °C, respectively. Due to the nanoscale of chitosan nanoparticles, their $T_m$ and $T_g$ have risen to 75.5 °C and 240 °C, respectively. The thermogram of vancomycin-loaded chitosan nanoparticles exhibited all characteristic peaks of vancomycin at 72 °C and 250 °C; therefore, there is no interaction between vancomycin and chitosan.
Drug release

Diffusion studies on vancomycin-loaded CSNPs were performed in three stages. The study lasted for 100 h. The concentration was calculated by measuring ultraviolet light at regular intervals, converted to milligrams, and Fig. 8 is plotted to determine the percentage of drug release over time. NPs containing 5 mg of vancomycin appeared to release all of the vancomycin containing at the end of the release study. It took CSNPs 100 h to release 100% of the drug. About 40% of the active substance was released in the first 9 h and then slowly released. After 100 h, the work continued uninterrupted and remained constant. It is observed that the release of all vancomycin in NP is not possible. It was observed that CSNP, which released 40% vancomycin loaded in the first 9 h, slowed the process to 90% vancomycin. This difference in NP produced is thought to be due to a change in the amount of vancomycin in it. The vancomycin in the solution provides a stronger bond to the NP, thus slowing its release.
The vancomycin release from chitosan nanoparticles was evaluated for 100 h. A burst release of vancomycin from chitosan nanoparticles was observed at the initial stage. Forty percentage of vancomycin was released in the first 9 h. Then, the release is continued gradually, and finally, 90% of vancomycin was released in 100 h. It seems that chitosan nanoparticles reduce the drug release rate and vancomycin-loaded CSPNs are suitable for sustained drug release.

Conclusion

Chitosan nanoparticles are widely used in biomedical applications, especially in drug delivery systems. Chitosan nanoparticles were prepared through ionotropic gelation using TPP as a cross-linker, and vancomycin, which is a typical antibiotic used for bacterial infections caused by gram-positive bacteria, was loaded. The effect of chitosan concentration and TPP concentration on the size of chitosan nanoparticles was studied, and the CS/TPP volumetric ratio of 1:1 with an average size of nanoparticle about 100 nm was selected. The prepared samples were characterized using DLS, FTIR, TGA, DSC, and SEM techniques. The results confirmed that vancomycin has been loaded on chitosan nanoparticles and there was not any interaction between vancomycin and chitosan. Also, it is observed that 40% of vancomycin is released burstly in the first 9 h and after that the drug release is continued gradually to receive 90% at 100 h. Thus, it seems that chitosan nanoparticles reduce the drug release rate and are potentially viable for sustained drug release and more research in this field can continue.

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