Research Article

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Development of mucoadhesive thiomeric chitosan nanoparticles for the targeted ocular delivery of vancomycin against *Staphylococcus aureus* resistant strains

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Abstract: This study aims to formulate mucoadhesive vancomycin loaded thiolated chitosan (TCS) nanoparticles. These nanoparticles are mucoadhesive and enhance the retention of the drug at the ocular site. For this purpose, TCS loaded vancomycin nanoparticles were prepared by the ion-gelation method and were characterized for their size, shape, polydispersity index, mucoadhesion, cellular uptake and anti-inflammatory activity. The average size of the synthesized nanoparticles was found to be 288 nm with positive zeta potential. Moreover, 85% vancomycin was successfully encapsulated in TCS nanoparticles by using this method. A 2-fold increase in mucoadhesion was found as compared to non-thiolated vancomycin formulation (p < 0.05). Zone of inhibition of vancomycin loaded TCS was also significantly improved compared to non-thiolated chitosan nanoparticles and vancomycin alone. In-vivo anti-inflammatory evaluation via histopathology resulted in ocular healing. Based on the results, it is inferred that TCS nanoparticles are a promising drug delivery carrier system for ocular delivery of vancomycin.

Keywords: Chitosan; Thiolated chitosan; Vancomycin; Ocular drug delivery; Mucoadhesion; Anti-inflammatory activity

1 Introduction

In the last decade pharmaceutical scientists garnered their attention towards challenging delivery of drugs through the ocular route [1]. Eye is a sensitive organ and acts as a barrier to drug delivery owing to its complex anatomy [2, 3]. Most common barriers are nasolacrimal drainage, tear turnover and reflex blinking. These barriers lead to reduced topical bioavailability and only 5% of the drug reaches the ocular tissues [4]. Therefore, some alternative routes are followed like intravitreal injections, periocular injections, and systemic administration but as the small volume of eye and blood retinal barriers makes systemic administration unworkable; to achieve the desired amount the repeated administration may lead to endophthalmitis, hemorrhage, and retinal detachment. The new drug delivery systems prolong drug efficacy in the specific area, encourages targeted therapy, bio recognition, and convenient administration fundamentally shows improvement in the drug related parameters [5, 6].

Nanotechnology is an emerging field in the arena of medicine, which basically contains the overall project to formulate a new drug, initiates with formulation of the new drug entity up to its evaluation and finally its applications in medicine [7, 8]. Nanoparticles retain distinctive physical and chemical features due to their great surface area and nanoscale size [9]. Nanoparticles (NPs) are wide class of
materials that include particulate substances, which have one dimension less than 100 nm at least. The modification of nanocarriers can be beneficial for many small and large biomolecules to be encapsulated [10]. This modification reduces irritation, and makes them more compatible with ocular tissues; therefore, these can easily be introduced in the eyes without any harm, and studies proved them the best candidates for intracellular delivery [11, 12]. In comparison with conventional therapy, nanomedicines can be used in advanced techniques like targeted drug delivery, sustained drug delivery moreover improves the physicochemical properties like solubility of both small and large bio molecules hence reduces side effects and improves quality of life [13–15]. Among nanoparticles, polymeric nanoparticles are widely exploited for drug delivery designing owing to their biocompatible and non-toxic nature [16, 17]. Chitosan (CS) is a cationic polysaccharide derived from crusta ceous skeleton and utilized as a nanocarrier for a variety of drugs [18, 19]. Thiolation of CS imparts mucoadhesive properties that enhance residence time of formulation at delivery site. CS contains an amino group in its structure that can form a linkage with thiol groups to form thiolated chitosan (TCS). Mucoadhesion of TCS is allied with non-covalent interaction between thiol group and glycoproteins of the mucous. Ocular drug delivery system based on thiolated chitosan showed greater mucoadhesion as well as good in vivo compatibility [9, 20]. Vancomycin is an anti bacterial agent that is utilized in the treatment of many ocular infections and it shows good activity against Gram positive bacteria particularly resistant strains methicillin resistant staphylococcus aureus (MRSA). Vancomycin is a promising antibacterial agent belonging to BCS class III drugs [21]. Their clinical applications in ocular delivery are reduced owing to its natural properties that is highly hydrophilic and large molecular weight. These properties of vancomycin result in reduced bioavailability and limited corneal penetration. These challenges address the need of developing a formulation for vancomycin that improves its ocular retention and penetration [22, 23].

The aim of present research work was to prepare and characterize TCS-based nanoparticles for the ocular delivery of vancomycin. TCS improves the mucoadhesion and ocular bioavailability of the drug [24].

2 Materials and Methods

Vancomycin was obtained from Global Pharmaceuticals (Manufactured by Livzon Group Fuzhou Fuxing Pharmaceutical Co. Ltd). Chitosan (160 kDa), N-hydroxy succin imide (NHS), thioglycolic acid (TGA) and artificial mucin were obtained from Sigma Aldrich (Germany). 1-ethyl-3-(3- dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was obtained from Penicon Inc. (Pakistan). Cystamine dihydrochloride was purchased from Daesthesia (China). Potassium dihydrogen phosphate (KH2PO4), rhodamine, sodium dihydrogen phosphate (H2Na2P), dipotassium hydrogen phosphate (K2HPO4) and sodium tripolyphosphate (TPP) were obtained from Scharlau (Germany). Sodium hydroxide (NaOH), sodium bicarbonate (NaHCO3) and calcium chloride (CaCl2) were supplied by Merck (Germany). Distilled water was provided by Pharmaceutics Lab (Department of Pharmacy, QAU, Islamabad, Pakistan). Deionized water was supplied by Wilshire Laboratories (Lahore, Pakistan).

2.1 Preparation of thiolated chitosan

Thiolated chitosan (TCS) was prepared by amide bond formation between thio glycolic acid and amino group of chitosan [25]. Briefly, 1% w/v CS solution was prepared in 1% (w/w) acetic acid solution. Then, 500 mg of TGA was added to the chitosan solution. To activate the carboxylic acid moieties, 100 mM of EDAC was added as a coupling agent. 10 M NaOH solution was used to adjust the pH to 5.5. Then, the solution was incubated for 3.5 h under continuous stirring. After incubation, the solution was dialyzed through membrane assembly for 3 days with a light protection environment. Crude thiolated polymer was then dialyzed again with 5 mM HCl. To break ionic interactions between the positively charged polymer and the negatively charged sulfhydryl groups, 1% NaCl was added to the dialyzing medium. Finally, the sample was dialyzed again with 1 mM HCl to adjust the pH of the solution. The final product was lyophilized and stored at 4°C for further use [25].

2.2 Preparation of vancomycin loaded thiolated chitosan nanoparticles

Vancomycin loaded thiolated nanoparticles were prepared by ion-gelation method. For this purpose, 0.1% w/v solution of TCS was prepared in distilled water. Then, 1 mg of vancomycin was added to the TCS solution, followed by stirring on a magnetic hot plate at 750 rpm. Furthermore, TPP was added as a cross-linker through a syringe, again followed by stirring for 30 min at 750 rpm. Formulation was optimized via Design Expert software by changing TPP concentrations while keeping all other parameters constant [26].
2.3 Characterization

2.3.1 Particle size, zeta potential and polydispersity index

The size, charge and PDI of the prepared nanoparticles were determined by using Malvern zeta sizer version 7.12. Sample was diluted in a ratio of 1:10 with deionized water and results were recorded.

2.3.2 Morphology of nanoparticles

Surface morphology was determined by scanning electron microscopy (SEM). Formulation was lyophilized for the SEM analysis.

2.3.3 Encapsulation Efficiency

Encapsulation efficiency was determined by centrifugation of the vancomycin loaded chitosan nanoparticle formulation at 13500 rpm for 1 h. Supernatant was then collected and free drug concentration in the supernatant was determined using a UV-Visible spectrophotometer (Agilent 8543) at λ\text{max} of 290 nm. The same procedure was repeated for vancomycin loaded thiolated chitosan nanoparticles. Upon analysis of free drug concentration in the supernatant, encapsulated drugs can be determined indirectly by using the following formula:

\[
\%\text{Encapsulation Efficiency} = \frac{W_1 - W_2}{W_1} \times 100
\]

\(W_1\): Total vancomycin added
\(W_2\): free vancomycin in the supernatant

2.3.4 FTIR

Fourier transform infrared (FTIR) analysis was performed by using a Bruker alpha-P spectrophotometer (USA). Scanning range of FTIR was 4000-500 cm\(^{-1}\). FTIR spectrum was obtained for unmodified chitosan and thiolated chitosan to assess the presence of required functional groups [27].

2.3.5 Differential Scanning Calorimeter (DSC)

Stability of modified and unmodified chitosan was assessed by differential scanning calorimeter (DSC) (SD Q600, TA Instruments, Delaware, USA). Temperature range of 50-300°C was set with a heating rate of 10°C/min [28].

2.3.6 In-vitro dissolution

Release of vancomycin from CS and TCS nanoparticles was evaluated by using phosphate buffer (pH = 7.4) as a medium with the help of non-shaking water bath apparatus. Drug loaded nanoparticles were placed in a dialysis bag that was then positioned in the buffered medium. For comparison purposes, release of marketed formulation was also evaluated by using the same method. Release studies were conducted for 48 h with frequent sampling at regular intervals of 1, 2, 3, 4, 5, 6, 8, 12, 24 and 48 h. Collected samples were analyzed with a UV-Visible spectrophotometer at 290 nm.

2.3.7 Mucoadhesive rheological behavior

Mucoadhesion study was performed by comparing the viscosity of the prepared formulation with that of a reference mucin solution with the help of a viscometer. The cone plate viscometer was utilized to assess the rheological behavior of the formulations. For this study, 5 ml of freshly prepared mucin solution was added to 1 ml formulation of thiolated and non-thiolated chitosan. These mixtures were incubated for 6 h at 37 ± 0.5°C. Reference was pure mucin dispersion [29]. 400 µL of each sample was analyzed by cone plate viscometer at a shear rate of 50 s\(^{-1}\) after regular time intervals of 1, 2, 4 and 6 h. Rheological characteristics were measured by following formula:

\[\eta = \eta_{\text{mix}} - \eta_{\text{ref}}\]

\(\eta_{\text{mix}}\) is the apparent viscosity of formulation of TCS or non-thiolated chitosan.
\(\eta_{\text{ref}}\) is the apparent viscosity of reference mucin dispersion.

2.3.8 Evaluation of zone of inhibition

Zone of inhibition of prepared formulations was measured by a well diffusion method. *Staphylococcus aureus* (*S. aureus*) strain was utilized to assess zones of inhibition. Helmuten agar was used as a growth medium for colonies of *S. aureus* strain. Plates with formulation were incubated for 24 h [30].

2.3.9 Cellular uptake test

Ocular uptake through fluorescence microscope observation along with quantitative assessment was done for the rhodamine-tagged thiolated chitosan nanoparticles.
2.3.10 Ocular irritation evaluation

Modified draize test for ocular irritation was investigated in albino rabbit eyes with the approval of Riphah International University ethical considerations and monitored respectively.

2.4 In-vivo anti-inflammatory activity via Histopathological evaluation

\textit{In-vivo} anti-inflammatory assay was conducted on female rabbits (6–8 weeks) divided in 3 groups (n = 5) under the approval of Bioethical Committee Riphah International University, Islamabad, Pakistan, following NIH guidelines. The anti-inflammatory action of thiolated nanoparticles solution for eye was evaluated in an animal model after inducing inflammation. Moreover, negative control was phosphate buffer (PBS) and positive control included diclofenac sodium eye drops (5 mg:5 ml). Likewise, the anti-inflammatory action of free vancomycin solution and thiolated nanoparticles solution for eye were assessed via histopathological evaluation.

2.5 Stability studies

Stability studies were performed to investigate the leakage of drug from the non-thiolated and thiolated nanocarriers for 3 months in 10 ml glass vials at 25 ± 2°C followed by protection from light. Samples were drawn and the encapsulated amount of drug was determined along with the physical appearance and the pH.

2.6 Statistical data analysis

Results were statistically evaluated by one-way analysis of variance (ANOVA) and student t-test with significant p value less than 0.05. All results were presented as the mean of three samples with standard deviation (x± SD).

3 Results and Discussion

Vancomycin loaded thiolated nanoparticles were prepared by ion-gelation method. TCS was synthesized via addition of TGA and EDC for 3 h as shown in Figure 1.

![Figure 1: Formulation of vancomycin-loaded TCS nanoparticles via ionic-gelation method. Initially, chitosan was converted into thiolated chitosan via using carboddimide chemistry and then TPP was added for conjugating it with vancomycin to form vancomycin-loaded TCS nanoparticles.]

3.1 Nanoparticles characterization

3.1.1 Particle size, zeta potential and polydispersity index

The average particle size of the optimized formulation was 288 nm with a PDI of 0.385 as shown in Figure 2. Zeta potential of this formulation was +10.4 mV, indicating an overall positive charge on the nanoparticles. Positive charge on zeta potential depicted the cationic nature of the nanoparticulate system and this system is responsible for killing of resistant bacterial strains [22]. Table 1 shows the results from zeta potential characterization of vancomycin loaded TCS nanoparticles.

![Table 1: Zeta potential of vancomycin loaded TCS nanoparticles.]

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Zeta Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>8.1±0.6</td>
</tr>
<tr>
<td>TCS</td>
<td>10.4±0.9</td>
</tr>
</tbody>
</table>

3.1.2 Morphology of nanoparticles via SEM

SEM observations revealed that the nanoparticles prepared by using TCS have a smooth surface and are spherical in shape, as depicted in Figure 3. Thiolation enables the nanoparticles to appear in a stabilized uniform morphol-
Figure 2: Particle size of vancomycin loaded TCS nanoparticles.

Figure 3: SEM image of vancomycin loaded loaded thiolated chitosan nanoparticles.

3.1.3 FTIR and DSC

FTIR spectra of CS and TCS are compared in Figure 4A. Given that the amino groups in CS reacted with the carboxyl groups of TGA, resulting in an amide bond formation, additional peaks corresponding to this amide bond and peaks of thiol groups (from TGA) were observed in the spectrum of TCS: S-S disulfide bond formation was confirmed by the presence of a peak at 1000 cm\(^{-1}\). C-NH amide bond formation was confirmed by the C=O stretching at 1645 cm\(^{-1}\) and the deformation of the N-H stretching signal at 3225 cm\(^{-1}\) compared to neat CS. Further, the presence of thiol groups was indicated by the -SH stretching peak at 2496 cm\(^{-1}\) [32].

Figure 4: [A] FTIR spectra of CS (a) and TCS (b), [B] DSC of CS (a) and TCS (b).

3.1.4 Encapsulation efficiency

Results from encapsulation studies depicted in Figure 5A indicate that 78% vancomycin was encapsulated in CS nanoparticles and 85% in TCS. Higher encapsulation efficiency leads to the increased stabilization of the drug in the polymeric matrix. All this stabilization and increased encapsulation points toward a thiolation mechanism.

3.1.5 In-vitro drug release

In-vitro drug release of vancomycin loaded CS and TCS nanoparticles are shown in Figure 5B. For comparison, release of vancomycin alone is also plotted. In the case of TCS, 75% of the drug was released within 24 h and almost 90% of the drug was released after 48 h, while for CS the percentages of drug release were smaller (about 60% and 70%, respectively). Table 1 shows mathematical models proposed to predict the kinetics for drug release, including zero order, first order, Higuchi, Korsmeyer-Peppas and Hixon Crowell [25]. Commercial vancomycin release followed a first order kinetics while vancomycin loaded CS and DSC results of unmodified CS and modified CS are compared in Figure 4B. DSC thermograms of CS showed an endothermic peak between 80 and 120°C, named as dehydration temperature (TD), which is assigned to the loss of water associated with the hydrophilic groups of CS. The exothermic peak that begins at about 280°C indicates the degradation of the polymer. The DSC thermograms revealed that modification of CS with thiol groups enhances its thermal stability, which ultimately enhances the stability of the formulation prepared by using this polymer [8].
TCS nanoparticles release fitted better to Korsmeyer-Peppas model ($R^2 = 0.942$). This model describes drug release from polymer matrix by a power law. However, initially, water enters the polymer by diffusion, followed by polymer swelling which ultimately causes drug dissolution and release into the medium as shown in Figure 5B. It is hence concluded that, the release of Vancomycin from pure drug solution showed Korsmeyer-Peppas model as shown in Table 2.

### 3.1.6 Mucoadhesion study with the help of rheological behavior

Mucoadhesion behavior can be assessed in-vitro by rheology of the formulation. Rheology is an in-vitro parameter that is utilized to analyze mucoadhesive properties to mucus membranes [33]. In the present study, rheological properties were determined by cone plate viscometer and the results are depicted in Figure 5C. As can be observed, TCS nanoparticles showed significantly higher mucoadhesion as compared to non-thiolated CS and reference mucin.

### 3.1.7 Zone of inhibition

Zone of inhibition for vancomycin, vancomycin loaded CS and TCS nanoparticles was measured by well diffusion method using *S. aureus*. Zone of inhibition for TCS nanoparticles was found to be 10 mm, which is significantly higher than that of CS nanoparticles and vancomycin alone ($p<0.05$), as shown in Figure 5D.

### 3.1.8 Cellular uptake test

Within the context of in-vitro corneal permeation evaluation, the cellular uptake features of rhodamine-linked thiolated nanomicelles were assessed. By increasing incubation period from 5 to 90 min, the red fluorescence of rhodamine was increased as shown in Figure 7B proving that the cellular uptake by rhodamine-linked thiolated nanocarriers were incubation period dependent. The fluorescence strength of cells incubated with rhodamine-linked thiolated nanocarriers were much stronger in comparison with the free rhodamine-vancomycin solution.

### 3.1.9 Ocular irritation studies

During the entire test significant ocular endurance was noticed and no irritation or tear shedding was observed following 13 ophthalmic administrations in the rabbit eyes by the thiolated nanoparticles. In the cornea, conjunctiva or iris no injury was noticed as shown in Figure 6A. Regarding the Draize test the average total score remained less than 02 all over the whole test. There were no corneal lesions through the whole experiment as explored by the fluorescence microscope examination.

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**Table 2: Mathematical models proposed to predict the kinetics for drug release.**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Zero order</th>
<th>First order</th>
<th>Higuchi</th>
<th>Korsmeyer-Peppas</th>
<th>Hixon Crowell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R^2$</td>
<td>$k_1$</td>
<td>$R^2$</td>
<td>$k_2$</td>
<td>$R^2$</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.643</td>
<td>11.01</td>
<td>0.982</td>
<td>0.275</td>
<td>0.942</td>
</tr>
<tr>
<td>CS nanoparticles</td>
<td>0.554</td>
<td>3.047</td>
<td>0.901</td>
<td>0.061</td>
<td>0.939</td>
</tr>
<tr>
<td>TCS nanoparticles</td>
<td>0.306</td>
<td>1.965</td>
<td>0.904</td>
<td>0.082</td>
<td>0.921</td>
</tr>
</tbody>
</table>

**Figure 5:** [A] Encapsulation efficiency of vancomycin loaded CS and TCS nanoparticles, [B] In-vitro release of vancomycin, vancomycin loaded CS and TCS nanoparticles. [C] Rheological behavior of CS and TCS nanoparticles and reference [D]. Zone of inhibition for vancomycin, vancomycin loaded CS and TCS nanoparticles.
3.1.10 In-vivo anti-inflammatory assay via Histopathological evaluation

In-vivo anti-inflammatory assay was conducted on female rabbits (6–8 weeks) divided in 3 groups (n = 5) under the approval of Bioethical Committee Riphah International University, Islamabad, Pakistan, following NIH guidelines. The anti-inflammatory effectiveness of the free vancomycin solution and the thiomeric chitosan ocular solution against phosphate buffer solution as negative control and diclofenac sodium ophthalmic drops as positive control is shown in Figure 6C in histopathological evaluation. Additionally, the histopathology examination showed that the cornea treated with thiomeric NPs displayed a protected epithelial layer having no stromal bulging (corneal edema).

3.1.11 Stability Studies

The stability of newly developed thiolated and non-thiolated drug loaded nanoparticles was evaluated. The media used for this study was simulated tear fluid (pH = 7.4). Storage condition of 25°C under light protection showed time dependent release of vancomycin from thiolated nanocarriers. After 3 months of storage, 87.00 ± 2.43% of drug remained encapsulated in thiolated polymeric nanomicelles while only 67.36 ± 4.78% drug remained encapsulated in nanocarriers indicating the stability under these conditions as shown in Figure 7 and Table 3. However, comparative Table 4 is now provided for comparing results of the with that of previously reported one.

**Table 3:** Stability Studies of non-thiolated and thiolated nanocarriers. SD n = 3.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Physical appearance</th>
<th>pH</th>
<th>% Entrapment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Thiolated</td>
<td>Clear</td>
<td>7.24±4.78</td>
<td>87±1.78</td>
</tr>
<tr>
<td>Thiolated</td>
<td>Clear</td>
<td>7.4±0.05</td>
<td>67±4.18</td>
</tr>
</tbody>
</table>

**Table 4:** Comparative table comparing results of the with that of previously reported one.

<table>
<thead>
<tr>
<th>Nanoparticulate System</th>
<th>Performance</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucoadhesive Thiomeric Chitosan Nanoparticles</td>
<td>Targeted delivery for Staphylococcus blepharitis</td>
<td>No disadvantages.</td>
</tr>
<tr>
<td>Thiolated Pluronic based nanomicelles</td>
<td>Targeted delivery for Staphylococcus blepharitis</td>
<td>Costly</td>
</tr>
<tr>
<td>Cyclosporine A (CsA) nanoparticles (NPs) for the treatment of inflammation of the eye surface</td>
<td>Increased bioavailability and bioavailability</td>
<td>Non-biodegradable</td>
</tr>
<tr>
<td>Drug-laden contact lenses</td>
<td>Commercialization</td>
<td>Drug leaching</td>
</tr>
<tr>
<td>Resveratrol-loaded mucoadhesive lecithin/chitosan nanoparticles (RMLCNs)</td>
<td>Prolonged ocular drug delivery</td>
<td>Difficult method to follow</td>
</tr>
<tr>
<td>A temperature-triggered, cross-linked nano hydrogel formulation</td>
<td>Increased Bioavailability</td>
<td>Instability</td>
</tr>
</tbody>
</table>
4 Conclusions

Vancomycin loaded thiolated nanoparticles were successfully manufactured by ionic gelation method. In-vitro characterization of vancomycin loaded thiolated pluronic PF68 nanomicelles was confirmed by FTIR, SEM, DSC and XRD. Thiol moieties in the nanoparticles were synthesized using thiol modified polymers form disulfide linking with cysteine in the ocular glycoproteins of mucus. The synthesized formulation was mucoadhesive with sustained delivery, prolonged retention and increased drug stability. Moreover, vancomycin loaded TCS nanoparticles improve zone of inhibition against *S. aureus* with enhanced cellular uptake as well as anti-inflammatory action. Thus, these nanoparticles have great potential to be employed as a gateway to deliver vancomycin through the ocular route with improved retention for the treatment of ocular diseases.

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**Author Contributions:** All the authors had played important role in preparing this article. FJ has contributed in experimentation and writing; while FJ, SZ, SA, RA, SP and IMI review this paper. FJ, SZ, SA, RA, IMI, A.R S.P and AMD-P, had participation in writing and revision of the manuscript; and GS had participation of ideology developing along with FJ.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**


**Ethical approval:** Animal handling was conducted according to the guidelines of Bio-Ethical Committee (BEC) of Riphah International University, Pakistan (Ethical code: Ref. No.REC/RIPS/2019/30).

**Data availability statement:** The data are available on reasonable request from the correspondence Author.


