Chitosan Nanoparticles as Vehicle for Prolonged Ocular Delivery of Ofloxacin

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Abstract: Ofloxacin is a synthetic broad-spectrum fluoroquinolone antibiotic, frequently used for the treatment of ocular infections. In the present study, ionic gelation technique was employed as the method of preparation of ofloxacin loaded chitosan nanoparticles using different concentrations of chitosan and sodium tripolyphosphate (TPP) and the potential of these nanoparticles in sustained ocular drug delivery was investigated. Different drug-polymer ratios were used to prepare five batches of nanoparticles at two concentrations of chitosan solutions (0.2% w/v and 0.4% w/v). The formulated nanoparticles were evaluated for morphology by Scanning Electron Microscopy, and subjected to particle size analysis and zeta potential measurement by Zetasizer. Drug content, encapsulation efficiency and in vitro drug release studies were also done. LCS-OF3 nanoparticles prepared with 0.2% chitosan solution and drug-chitosan ratio 3:1 were checked for pH and osmolality for their suitability in in vivo study after that subjected to in vivo drug release study in rabbits, the results of which clearly indicate the controlled and prolonged drug release from LCS-OF3 nanoparticles when compared to that from commercially available ofloxacin eye drops.

Keywords: Chitosan, ionic gelation, nanoparticles, ocular delivery, ofloxacin, trisodium polyphosphate.

INTRODUCTION

Despite the preference given to topical administration in eye diseases due to the simplicity of application and patient adherence, the poor ocular bioavailability on local administration to the ocular surface posed by many anatomical and physiological constraints is a well-known fact [1]. The therapeutic efficacy of ocularly administered drugs can be improved by loading them in colloidal carriers as these drug reservoirs solve the poor retention issue of the instilled drug solution through their small particle size and bioadhesive properties thus increasing the contact time and controlling the drug release [2]. Many different colloidal delivery vehicles like liposomes, micelles, nanoemulsions and polymeric nanoparticles, have been exposed as drug carriers in ophthalmic use [3-7]. Out of all the colloidal carriers, polymeric nanoparticles are frequently studied as an ocular delivery carrier because of their potential advantages such as biocompatibility, biodegradability, safety, mucoadhesion, greater stability of both the loaded drug and drug product, relative ease of preparation, and low cost of production [1, 2].

Chitosan is a useful polymer in ocular drug delivery due to its permeability enhancing properties and its acceptable biocompatibility and biodegradability by lysozymes [8]. The other promising characteristics of chitosan related to ophthalmic delivery include its hydrophilicity, spreadability over the corneal surface, bioadhesive, antimicrobial and wound healing properties and better acceptance on topical administration on ocular surfaces [9]. The unique structural advantageous characteristics make chitosan an acceptable carrier material for different types of formulations such as in situ gelling systems, micro & nanoparticles and inserts in ocular therapeutics [10]. Chitosan’s cationic polyelectrolytic nature provides a strong electrostatic interaction with mucus and negatively charged mucosal surfaces [11]. At neutral pH, chitosan can converted into a hydrogel so that there is more possible formation of hydrogen bonds or ionic linkages between chitosan’s cationic amino groups and mucin’s anionic sialic acid residues [9]. Moreover, as cornea and conjunctiva have a negative charge, chitosan can increase the retention of the drug loaded particles on the eye surface through mucoadhesion [12]. In addition to mucoadhesion, chitosan also serves as a permeability enhancer by improving the transcorneal penetration through either facilitated intra/interepithelial cellular drug transport across cornea or the structural changes in the proteins associated with the tight junctions between the corneal epithelial cells, resulted out of the cationic chitosan [9].

Chitosan and modified chitosan have been shown to be a potential choice of polymer to prepare mucoadhesive nanoparticles for sustained and local delivery to ocular sites [13]. Chitosan nanoparticles have a higher precorneal retention than chitosan solutions, and depending on the size, the nanoparticles may enter the corneal epithelium to a certain depth by a paracellular or transcellular pathway [8]. Chitosan nanocarriers have been reported to be prepared for indomethacin and brimonidine tartrate to prolong the precorneal delivery of these therapeutics.
residence time and improve the ocular bioavailability through slow gradual release of the drug increasing the delivery to both external and internal ocular tissues [14, 15].

Chitosan micro-or nanoparticles can be formed by means of ionic gelation brought out by electrostatic interactions between the positively charged chitosan chain and polyanions employed as cross-linkers. The polyanion triply phosphate (TPP) is extensively used for mostly pharmaceutical applications due to the proven toxicity of the other covalent cross-linking stabilizers like glutaraldehyde and other organic molecules [16].

Chitosan nanoparticles are the proven promising drug delivery vehicle in ocular applications for different drugs [17]. In the present study, the chitosan nanoparticle as a new vehicle for the improvement of the delivery of the antibiotic ofloxacin to the ocular mucosa was investigated.

Ofloxacin is a synthetic broad-spectrum fluoroquinolone antibiotic which is frequently used for the treatment of ocular infections including acute and subacute conjunctivitis and bacterial keratitis [18]. Ofloxacin is available as eye drops (0.3% w/v), the dose being two drops of in the affected eyes for every four hours in mild to moderate infections or hourly in severe infections [19]. Besides loss of drug from tear flow, lacrimal and nasal drainage leading to lesser absorption, this conventional eye drops requires higher frequency of instillation causing discomfort to patience resulting in patient non-adherence to the administration schedule. On administration chitosan nanoparticles loaded with ofloxacin prolong the ocular stay of the drug through controlled release of the drug and mucoadhesion of the nanoparticles both caused by the polymer chitosan which will ultimately result in reduced frequency of administration and improved ocular bioavailability of ofloxacin.

MATERIALS AND METHODS

Materials

Ofloxacin was gifted by Pharma Fabricon, Madurai, India. Chitosan (low molecular weight, deacetylation-85.16%) was purchased from Otto chemicals, Mumbai, India. Sodium triply phosphate (TPP) was procured from Finar Chemicals, India. All other reagents and chemicals used were of pharmaceutical grade.

Preparation of Nanoparticles

The method of preparation of nanoparticles in the present study was ionic gelation, which involves the reaction between a cationic amino group of chitosan and anionic counter ion of sodium triply phosphate (TPP) [20]. Chitosan was dissolved in 1% acetic acid at two different concentrations (0.2% w/v and 0.4% w/v) and different drug: polymer ratios were used to prepare five batches of nanoparticles (Table 1). Batches LCS-OF1, LCS-OF2, and LCS-OF3 were the formulations prepared with 0.2% w/v of chitosan in drug: polymer 1:1, 2:1, and 3:1 respectively. The other two batches HCS-OF1 and HCS-OF2 were prepared with 0.4% w/v chitosan in drug: polymer ratio 1:1 and 1.5:1 respectively. Aqueous solution of TPP (0.84mg/ml) was used after adjusting pH to 3 with 0.1 N HCl. To the Drug-chitosan solution, aqueous TPP solution was added and stirred using magnetic stirrer (Remi Equipments Pvt. Ltd., India) until an opalescent suspension was formed [21].

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Scanning Electron Microscopy

The surface and shape of the nanoparticles were examined by Scanning Electron Microscopy (SEM) (Hitachi-s-3000 N, Hitachi High Technologies America, Inc, USA) at different magnifications. Samples were mounted on aluminum stubs and coated with gold in Hitachi HUS 5 GB vacuum evaporator (Hitachi High Technologies America, Inc, USA) and observed in Hitachi-s-3000N SEM at an acceleration voltage of 10KV.

Particle Size and Polydispersity Index

The particle sizes of the nanoparticles of the five formulations were determined by photon correlation spectroscopy (PCS) with a Zetasizer ZS 90 (Malvern Instruments, Malvern, UK). The mean particle size and the width of the size distribution in terms of the polydispersity index (PDI) can be obtained by PCS [22]. In order to get a sufficient scattering intensity, the samples were suitably diluted with demineralized particle-free water prior to the measurement.

Zeta Potential

Zeta potential measurements were performed in Malvern Zetasizer 3000HS (Malvern Instruments, Malvern, UK). The nanoparticles were diluted with double-distilled water and taken in disposable cuvettes for measuring electrophoretic mobility at dispersant dielectric constant of 79.7. Zeta potential is calculated from the obtained electrophoretic mobility values applying Henry equation,

\[ U_z = 2\varepsilon f(Ka) / 3\eta \]

where \( Z \) is zeta potential, \( U_z \) is electrophoretic mobility, \( \varepsilon \) is the dielectric constant, \( \eta \) is the viscosity of the medium and \( f(Ka) \) is the Henry’s function. In this case, \( f(Ka) \) is 1.5 and is referred to as Smoluchowski approximation [23].

Drug Entrapment Efficiency

The drug loaded nanoparticles were initially isolated from un-entrapped free ofloxacin by using a membrane filter unit (Minstar single use filter unit) for the determination of drug content (i.e., the amount of drug entrapped in the nanoparticles) and the entrapment efficiency. The quantity of ofloxacin in filtrate was measured spectrophotometrically at 292nm [24]. The amount of ofloxacin entrapped in the nanoparticles was calculated as the difference between the total quantity of the drug used to prepare the nanoparticles and the amount present in the supernatant [12].

The entrapment efficiency (EE %) was calculated as

\[ EE\% = \left\{ \frac{(\text{Ofloxacin total} - \text{Ofloxacin free})}{\text{Ofloxacin total}} \right\} \times 100 \]

In vitro Drug Release Study

Diffusion is one of the commonly used techniques to study the in vitro drug release from controlled ocular drug delivery systems [25]. The dialysis membrane used for diffusion
study (pore size -2.4nm, molecular weight 12000-14000Da, Himedia, India) was sealed on both ends, to contain the nanoparticle formulation 1ml and was immersed in the receptor medium of simulated tear fluid (STF-10ml), the composition of which was NaCl 0.67g, NaHCO3 0.2g, receptor medium of simulated tear fluid (STF-10ml), the composition of which was NaCl 0.67g, NaHCO3 0.2g, CaCl2.2H2O 0.008g and water up to 100g [18]. The receptor fluid was maintained at 37 °C and was stirred in a magnetic stirrer. At regular intervals the whole 10ml of the reservoir fluid was withdrawn and replaced with fresh 10ml of STF. The samples were analyzed for drug content at 292nm. In vitro drug release studies were done in triplicate on all the five batches of nanoparticles prepared and also for the commercial formulation.

Sterilization of Nanoparticles and Test for Sterility

Optimized ofloxacin chitosan nanoparticles formulation LCS-OF3 was centrifuged at 12000 rpm (RM 12C/Remi Instruments Ltd, India) and lyophilized in CHRIST Lyophilisier (Alpha 1-2 model, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) for 24 hours to obtain the powder form. The exposure of the nanoparticles to UV radiation (UV –B lamp, O Panel 00, UV-B 313, Q-Lab corporation, USA) was done for 30 minutes at a distance of 10cm after which the nanoparticles (equivalent to 30mg of ofloxacin) were suspended in 10ml of double distilled water aseptically by sonication (Bath sonicator, Sonica, Soltec – 2200 MH, Soltec Soluzioni Tecnologiche, Italy). The pH (Univercel Biochemicals pH meter, M606, India) and osmolality (Advanced osmometer model 3250, Advanced Instruments Inc, USA) of the nanoformulation were noted.

The test for sterility is intended for detecting the presence of viable forms of bacteria, fungi and yeast in sterilized preparations. Moreover, in the present study sterility testing was required to check the efficiency of sterilization technique followed. The Test for Sterility was carried out as per Indian Pharmacopoeia, 1996 [26]. Fluid thioglycollate medium was used to detect aerobic bacteria & fungi and soya bean-casein digest medium was used to inspect the presence of the anaerobic bacteria. The sterility and growth promotion ability of the culture media had been confirmed previous to sterility testing. The sterilized nanoparticle preparation was directly inoculated into the culture media aseptically and incubated for 14 days and observed for microbial growth during that period.

Table 1. Composition, size, zeta potential, polydispersity index, encapsulation efficiency of all batches of chitosan nanoparticles.

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Drug: Polymer Ratio</th>
<th>Particle Size Z-average value (nm) (Mean ± SD) (n=3)</th>
<th>Polydispersity Index</th>
<th>Zeta Potential (mV)</th>
<th>Entrapment Efficiency (EE %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCS-OF1</td>
<td>1:1</td>
<td>163.2±66.2</td>
<td>0.165</td>
<td>+33.8±9.09</td>
<td>50.0±5.9</td>
</tr>
<tr>
<td>LCS-OF2</td>
<td>2:1</td>
<td>191.6±69.3</td>
<td>0.131</td>
<td>+32.3±5.91</td>
<td>61.5±4.3</td>
</tr>
<tr>
<td>LCS-OF3</td>
<td>3:1</td>
<td>253.6±57.2</td>
<td>0.051</td>
<td>+29.5±4.67</td>
<td>72.3±5.8</td>
</tr>
<tr>
<td>HCS-OF1</td>
<td>1:1</td>
<td>298.5±97.5</td>
<td>0.107</td>
<td>+38.9±14.1</td>
<td>65.0±7.4</td>
</tr>
<tr>
<td>HCS-OF2</td>
<td>1.5:1</td>
<td>319.9±113.4</td>
<td>0.126</td>
<td>+34.6±12.8</td>
<td>66.7±6.2</td>
</tr>
</tbody>
</table>

LCS – Low concentration of chitosan – 0.2%w/v, HCS - High concentration of chitosan 0.4% w/v.

In vivo Studies

Selection of Animals

Male albino New Zealand rabbits weighing approximately 1-2kg were selected for the study. They were free from any form of ocular damage prior and throughout the whole study. The animals were taken care of and maintained according to the guidelines of “Committee for the purpose of Control and Supervision of Experiments on Animals” (CPCSEA), New Delhi. The study protocol was approved by the Institutional Animal Ethics Committees, Ultra College of Pharmacy, Madurai registered with CPCSEA (IAEC approval number: UCP/IAEC/2007/007) for animal experimentation. The animals were placed in the laboratory thirty minutes prior to the experiments for acclimatization to the light (light cycle was 12 hours light and 12 hours dark), temperature (28 °C±2 °C) and relative humidity (60±15%).

Animal Study

Six healthy rabbits (3 per group) were positioned without disturbing normal eye movement. Commercial eye drop solution and the selected nanoparticle formulation was instilled onto the cornea of the unanaesthetized animals of Group 1 and Group 2 respectively with an amount of 20μl using an adjustable micropipette. The tear fluid was collected using sterile Whatmann no. 51 paper discs placed in the inferior lid margin of the rabbits. Tear samples were collected at 5,10,15,20,25,30,45 and 60 minutes after instillation. The collected discs were inoculated in petri plates with media containing microbial strain Staphylococcus aureus and observed for the zone of inhibition [27-29]. The means of the zones of inhibition of Group I & II were compared by student’s t test.

RESULTS AND DISCUSSION

Ionic gelation with TPP is a rapid and scalable method of preparation of chitosan nanoparticles [30]. The formation of nanoparticles by ionic gelation of chitosan with TPP depends on the pH and concentration of chitosan and TPP solutions [20, 21]. At acidic pH the presence of polyphosphate anions is high and also ionization of amine group of chitosan would be greater thus increasing the ionic interaction between chitosan and TPP. The formation and yield of nanoparticles are good with moderate concentrations of chitosan and TPP.
Ofloxacin loaded chitosan nanoparticles were prepared by using either 0.2% or 0.4% w/v Chitosan. When the concentration of chitosan and TPP as gelating agent are appropriate, an opalescent suspension representing a colloidal dispersion was obtained [31]. Therefore in our study sufficient amount of TPP at 0.84mg/ml concentration was used to
obtain nanoparticles. Lyophilization was carried out with the prepared LCS-OF3 nanoparticles and the redispersability was satisfactory.

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The prepared nanoparticles are perfectly smooth and spherical in shape and in different size ranges as revealed by SEM photographs (Fig. 1).

**Particle Size and Polydispersity Index**

The mean size of the prepared nanoparticles along with the polydispersity index is presented in Table 1. Z-Average value is the mean value of the hydrodynamic diameter of the nanoparticles. The concentration of chitosan solution used was found to affect the particle size, greater the concentration, larger the nanoparticles size, the reason being the break-up of concentrated chitosan solution of increased viscosity to larger globules [32]. Ofloxacin content also influences the size of the nanoparticles proportionately. The polydispersity index of the nanoparticle formulations are low suggesting a narrow size distribution and LCS-OF3 has monodisperse size distribution with PDI less than 0.1.

**Zeta Potential**

The zeta potential of a colloidal sample which is the potential within hydrodynamic shear or slipping plane of the electrical double layer of a charged particle determines whether the particles within a liquid will tend to flocculate (stick together) or not. The zeta potentials of all the five formulations are presented in Table 1. A positive potential value of Z is due to the cationic chitosan and the difference in values among the batches owes to the amount of chitosan present in the final nanoparticles i.e. the drug: polymer ratio. The higher positive zeta potentials indicate the stable nature of the formulations, a high positive potential would inhibit the aggregation of the nanoparticles thus maintaining the colloidal dispersion [33]. Ocular tolerability of nanoparticles of positive zeta potential of values around 30 has already been proved with moxifloxacin loaded chitosan-dextran sulphate nanoparticles in Hen’s egg test-chorioallantoic membrane (HET-CAM) test [34].

**Drug Entrapment Efficiency**

Ofloxacin entrapment efficiency of the nanoparticles ranged from 50-72.3% which are presented in Table 1. LCS-OF3 nanoparticles had the highest drug content among the five formulations.

**In vitro Drug Release Study**

The drug release from the commercial eye drops was complete within 90 minutes, whereas ofloxacin entrapped in chitosan nanoparticles was slow and extended up to 6 hrs. The graphical representation of drug release from nanoparticles is illustrated in (Fig. 2).

Results of drug release study reveal that about 54-87% was released from LCS-OF1 to HCS-OF2 within 1 hour. A pattern of bimodal release as shown by an initial burst release followed by a slow sustained delivery was observed with all formulations. This would be helpful in accentuating the antimicrobial activity of ofloxacin through facilitating the attainment of a drug level above minimum inhibitory concentration and then maintaining that level for continuous action which observation has already been made in another study also [35]. Out of the five formulations, a complete and controlled delivery of ofloxacin along with an initial release comparable to that of commercial eye drops was noticed with LCS-OF3. Based on relatively greater drug entrapment efficiency and preferable drug release pattern LCS-F3 was selected for *in vivo* study for which purpose they were sterilized.

**Sterilization of Nanoparticles and Test for Sterility**

Sterilisation by ionizing radiations, heat, steam and chemical methods can be suitably applied for chitosan in clinical applications [36, 37]. In the present study, just previous to animal studies, UV sterilization of the freeze-dried

**Fig. (2).** *In vitro* ofloxacin release from nanoparticles. LCS-Low concentration of Chitosan (0.2%w/v); HCS-High concentration of Chitosan (0.4%w/v).
nanoparticles spread like a thin film at a closer distance to UV source for half an hour was done. Also Sterility test was carried out for the UV radiation treated nanoparticles to ensure the sterility of the nanoparticles. The results show the absence of aerobic & anaerobic bacteria and fungi proving the sterility imparted by UV sterilization (Fig. 3).

![Fig. (3). Sterility testing: Absence of growth of aerobic bacteria and fungi.](image)

The pH of the redispersed nanoparticles in double distilled water was 5.5. This pH can well be tolerated when instilled into eyes as the normal tears having a pH between 6.9 and 7.5 would restore the physiological pH [38]. The osmolality of the nanosuspension was 177 mOsm. Lacrimal secretions have osmolality in the range of 280-293 mOsm and solutions of osmolality less than 100 mOsm and more than 640 mOsm will be eye-irritants [39]. However hypotonic solutions with lower osmolality would improve bioavailability of water-soluble drugs by solvent-drag effect [40]. Consequently sterile LCS-OF3 nanoparticles formulation was used in in vivo studies.

**In vivo Studies**

In the present study in vivo release of ofloxacin was examined in rabbits’ eye and the level of drug released was determined in terms of zones of inhibition of the growth of *Staphylococcus aureus* in agar-disc method. The results are given in Table 2 and (Fig. 3). Though the zone of inhibition with commercial eye drops was slightly greater than that with LCS-OF3 nanoparticles *i.e.*, it did not show any sustainable antibacterial effect at 15 min sampling. But the LCS-OF3 formulation showed prolonged and almost same inhibition till 30 minutes. Lower but Clear inhibition zones were also observed up to 1 hour. In vivo study clearly indicates that the LCS-OF3 nanoparticles released the drug in a sustained and constant manner when compared with that of commercial formulation which would require frequent instillation. The reasons for this timed antibacterial action may be the adherence of the nanoparticles on the conjunctiva, which leads to the increase in the contact time. As the drug is in the matrix of chitosan it will also stay adhered to the conjunctiva thus showing more therapeutic efficacy and decrease in the loss of drug through lachrymal drainage system. This will be useful in reducing the frequency of administration and in improving patient compliance.

![Table 2. In vivo drug release study -Antimicrobial activity of ofloxacin released in rabbits eye – Agar Diffusion study.](image)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Diameter of zone of inhibition (mm)</th>
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<tbody>
<tr>
<td></td>
<td>Group 1 (Commercial eye drops)</td>
</tr>
<tr>
<td>5</td>
<td>26.0±1.15</td>
</tr>
<tr>
<td>10</td>
<td>13.25±1.26</td>
</tr>
<tr>
<td>15</td>
<td>8.0±0</td>
</tr>
<tr>
<td>20</td>
<td>8.0±0</td>
</tr>
<tr>
<td>30</td>
<td>6.0±0</td>
</tr>
<tr>
<td>45</td>
<td>6.0±0</td>
</tr>
<tr>
<td>60</td>
<td>6.0±0</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.D (n=4). * Values are significantly different when compared to Group 1 at p < 0.001 (Student’s t test).

Previous studies demonstrated the presence of chitosan in an ophthalmic solution or as nanoparticle resulted in a significant increase in the precorneal residence time due to mucoadhesion enabling therapeutic drug levels to be maintained over longer period of time [27, 41]. Our study also reinforces this finding thus proving that chitosan nanoparticles is a promising delivery system for controlled release of ofloxacin to the eye. Moreover the slow controlled release of ofloxacin will alleviate the major problem of white crystalline deposit on cornea on instilling ofloxacin ophthalmic solution caused by a reduction of solubility due to pH change on ocular surface [42].

**CONCLUSION**

The ofloxacin loaded chitosan nanoparticles can be prepared by ionic gelation method using TPP as gelating agent. Nanoparticles prepared are of perfect sphere shape in the acceptable size range with respect to ocular use. The size and zeta potential of the nanoparticles were found to depend on the concentration of chitosan. The drug release from nanoparticles is prolonged when compared to conventional eye drop, following a mixed pattern of initial burst release followed by slow release. The residence time of the prepared
nanoparticles on the ocular surface revealed by in vivo studies on rabbit is more than that of conventional eye drops. Our study has provided evidence that chitosan nanoparticles can be potentially used as a vehicle to deliver ofloxacin to ocular tissues in a controlled fashion.

**CONFLICT OF INTEREST**

The authors confirm that this article content has no conflicts of interest.

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