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Mucoadhesive liposomes as ocular delivery system: physical, microbiological, and in vivo assessment

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Abstract

Background: Mucoadhesive drug delivery is a promising strategy to overcome ocular biopharmaceutical constraints. *Objective and methods*: Ciprofloxacin HCl-loaded reverse phase evaporation liposomes were coated with different concentrations and molecular weights of mucoadhesive biocompatible chitosan polymer to form chitosomes. This colloidal mucoadhesive system was evaluated in vitro and in vivo with respect to deliver the antibiotic to ocular surface. *Results and conclusion*: The results obtained pointed out that liposome coating process resulted in entrapment efficiency reduction and higher chitosan concentration, and molecular weight showed a more pronounced effect. No morphological differences between coated and uncoated liposomes were observed. Diffusion was the drug release mechanism from chitosomes. Concerning rheological behavior, pseudoplastic flow was characteristic to the prepared chitosomal dispersions. In addition, chitosan coating improved the ocular permeation of ciprofloxacin HCl. Microbiologically; this formulated system enhanced antimicrobial activity of ciprofloxacin HCl against both Gram-positive and Gram-negative bacteria. Moreover, this mucoadhesive system was able to inhibit the growth of *Pseudomonas aeruginosa* in rabbits' eyes for 24 hours when compared to the marketed preparation. In vivo bacterial conjunctivitis model elucidated that symptoms were controlled by the prolonged release formulation such as that done by the marketed product.

Key words: *Chitosan coat; chitosomes; ciprofloxacin HCl; corneal permeation; in vivo model; liposomes; ocular delivery*

Introduction

There is a considerable logic in attempting to retain delivery systems in the front of the eye, given the enormous loss of an instilled drug solution that topically occurs. A starting point in a retentive system is to consider erodible and nonerodible inserts, with long duration, and they substantially modify drug bioavailability. However, therapeutically, they are not well tolerated by patients and hence, they are not perceived as a desirable next-generation topical ocular delivery system. A more advisable dosage is in a drop form, which creates little to no refractive index problem for vision and needs be dosed no more frequently than once or twice daily[1].

The most frequent approach to achieve improvement in drug efficacy to the eye is the use of viscosified solutions. Nevertheless, viscosity alone cannot significantly prolong the residence time. This can be considered, in part, as the premise of using bioadhesive polymers to enhance drug absorption. The capacity of some polymers to adhere to the mucin coat, covering the conjunctiva and the corneal surfaces of the eye, forms the basis for ocular mucoadhesion. These systems markedly prolong the residence time of a drug in the conjunctival sac. Bioadhesive polymers are macromolecular hydrocolloids with numerous hydrophilic functional groups and posses the correct charge density. These bioadhesive polymers may be natural, synthetic, or semi-synthetic in origin[2].

Considering the fact that the cornea and conjunctiva have a negative charge, it was proposed that the use of mucoadhesive polymers, which may interact intimately with these extraocular structures, would increase the concentration and residence time of the associated drug[3].

Among the wide variety of mucoadhesive polymers, the cationic polymer, chitosan has been a good candidate.

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It is a bioadhesive vehicle suitable for ophthalmic formulations, as it exhibits several favorable biological properties such as biodegradability, nontoxicity, and biocompatibility[4]. As well as its ability to increase membrane permeability both in vitro and in vivo and be degraded by lysozymes[5]. In fact, because of its positive charges at neutral pH, an ionic interaction with the negative charges of sialic acid residues of mucus has been proposed as its mechanism of mucoadhesion[6].

Coating of liposomes (lipid-based system) with chitosan solution (polymer based system) has been used to increase the stability toward drug release[7] and for targeting purposes[8].

Different authors had reported that liposomes encapsulation enhanced the activity of certain antibiotics against bacteria[9,10]. Liposomes, because of its ability to accumulate in the mononuclear phagocyte system, increased the bactericidal activity of drugs against such intracellular pathogens as *Salmonella* spp., *Francisella* spp., and *Listeria monocytogenes*. Proteoliposomes and cationic liposomes were investigated for their potential targeting ability to the bacterial biofilms produced by *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus epidermidis*, and oral bacteria. Fluid liposomal drug formulations were developed to increase the bactericidal efficacy of antibiotics by promoting effective interaction between the bacteria and the liposomes. Compared with free drug, various liposomal forms of fluoroquinolones and aminoglycosides yielded reductions in minimal inhibitory concentration (MIC) for Gram-positive and Gram-negative bacteria[9].

The aim of this study was to design a system consisting of ciprofloxacin HCl-loaded liposomes with bioadhesive chitosan polymer to form chitosomes. Our hypothesis was that, this system, which presumably could combine the properties of liposomes and chitosan, would be easily applied as eye drops, well tolerated by ocular tissues and prolonged the residence time of ciprofloxacin HCl in the eye which can be applied once or twice daily. In addition, the in vitro antimicrobial activity of the prepared liposomal and chitosomal formulations against Gram-positive and Gram-negative bacteria was evaluated. Moreover, efficacy of the prepared formulations against induced bacterial conjunctivitis using an in vivo rabbit model was investigated.

Materials and methods

Materials

Chitosan of high, medium, and low molecular weights was purchased from Sigma-Aldrich (Steinheim, Switzerland). The phospholipid, Lipoid E 80, was a gift from Lipoid Company (Ludwigshafen, Germany), and Ciprofloxacin HCl, CPF, was purchased from Bayer Co. (Leverkusen, Germany). Cholesterol 99% was purchased from Winlab (Leicestershire, UK), dicetyl phosphate from Sigma-Aldrich, uranyl acetate-2-hydrate from Allied Signal (Riedel-Dehaen, Germany), and Spectrapore® 2 dialysis membrane from Spectrum laboratories Inc. (Houston, TX, USA). All other solvents and materials used were of analytical grade.

Microorganisms

The standard strains used in this study were *Staphylococcus aureus ATCC 6538P* and *Pseudomonas aeruginosa ATCC 9027*. The clinical isolates were isolated from different clinical specimens obtained through the courtesy of the Department of Microbiology, Faculty of Medicine, Alexandria University. The culture media used were Nutrient agar, Nutrient broth, and Cetrimide agar (Oxoid Ltd., Basingstoke, UK)

The used animals were Albino rabbits (weight between 1.5 and 2 kg, Medical Research Centre, Alexandria University, Alexandria, Egypt).

Methods

Preparation of ciprofloxacin HCl-loaded liposomes

Liposomal dispersions were prepared by reverse phase evaporation (REV)[11]. Previously optimized components of liposomes were utilized in this study[12]. Concerning neutral liposomes (F1), phospholipid (lipoid E 80) and cholesterol (2:1) were dissolved in chloroform : diethyl ether (1:1). Aqueous phase (buffer solution contains ciprofloxacin HCl) was added such that the organic-to-aqueous phase ratio was 6:1. The mixture was sonicated at 40°C either for 5 or 10 minutes in an ultrasonic bath (Julabo sonicator, model USR-3; Julabo Labortechnik, Ceelbach, Germany). A stable emulsion was produced, from which the organic solvent was slowly removed at reduced pressure at 45°C by a rotary evaporator (Rotavapor, type R110; Buchi Company, Postfacn, Switzerland). Liposomal dispersions formed were maintained for 1 hour at a temperature exceeding the phospholipid transition temperature (40°C). The composition of the negatively charged liposomes (F2) was phospholipid, cholesterol, and dicetylphosphate (2:1:0.5 molar ratio) and the same procedure was followed.

Preparation of chitosan-coated liposomes (chitosomes)

An appropriate amount of chitosan was dissolved in 0.5% (v/v) of glacial acetic acid. Chitosan solution was added dropwise into liposomes dispersion under controlled stirring rate (100 rpm) at room temperature (25°C), followed by incubation at 10°C for 1 hour. The final concentrations of the lipid and chitosan were half of the original solutions[13].

To study the factors, which may influence chitosomes, the following variables were studied:

1. Chitosan concentration: 0.1% and 1% solutions.
2. Chitosan molecular weight: high, medium, and low.
3. Surface liposomes charge either neutral or negative.

A total of nine chitosomal dispersions were prepared (Table 1).

Determination of entrapment efficiency percent

The entrapment efficiency (EE%) was determined by indirect method, by using dialysis bags of cellulose acetate[14,15]. Each formulation of 3 mL was placed into the dialysis bag, which was then transferred into 300 mL of a cold phosphate buffer solution adjusted to pH 4.5. The reservoir medium was stirred at 150 rpm for 3 hours with a magnetic stirrer. Samples of 10 mL were withdrawn and analyzed at λ_{max} of 277 nm using UV spectrophotometer (Perkin Elmer Lambda 3B, Perkin Elmer, New York, NY, USA).

The entrapped ciprofloxacin HCl was then calculated and expressed as EE% as follows:

$$\%\,\text{Entrapment Efficiency(EE\%)} = \frac{Q_t - Q_d}{Q_t} \times 100, \quad (1)$$

where Q_t is the theoretical amount of ciprofloxacin HCl and Q_d is the amount of ciprofloxacin HCl dialyzed.

Morphological evaluation and particle size analysis of chitosomes

Ciprofloxacin HCl-loaded chitosomes were morphologically studied using optical microscope (Euromed, Hague, Holland) and transmission electron microscope (TEM) (model JEM-100S; Joel, Tokyo, Japan). The mean size and size distribution of the selected chitosomes were studied using laser diffractometer (CilasL100, model 1064 liquid; Quantachrom, UK France).

Table 1. Composition of different chitosomal formulations.

Formula code (C)	Chitosan concentration (%, w/v)	Chitosan molecular weight	Type of surface charge of liposomes
C1	0.1	High	Negative
C2	1	High	Negative
C3	0.1	Medium	Negative
C4	1	Medium	Negative
C5	0.1	Low	Negative
C6	1	Low	Negative
C7	1	High	Neutral
C8	1	Medium	Neutral
C9	1	Low	Neutral

Determination of sedimentation volume

Chitosomes were transferred to a 5-mL measuring cylinder incubated at 20°C. The sedimentation was recorded 1 day after preparation. The value of sedimentation volume (F) was then computed from the following equation[16]:

$$F = \frac{V_u}{V_0}, \quad (2)$$

where V_u is the volume of sediment solid at fixed time and V_0 is the total volume of the dispersion.

In vitro release of ciprofloxacin HCl from chitosomes

Membrane diffusion technique was used for the in vitro release study[17]. Dialysis membrane was clamped between donor and receiving compartments. The upper compartment (donor) was exactly fitted on the surface of the lower compartment (receiving); the receptor phase was phosphate buffer saline, pH 7.4. The procedure was carried out in a thermostatically controlled water bath (M.B.H. & Co., Staufen, Germany) at a temperature of 34 ± 0.5°C and shaken at 25 rpm; 1 mL of each preparation or solution control was placed into the cap on the membrane; 6 mL of freshly prepared PBS equilibrated at 34 ± 0.5 °C was used as release medium. Samples were withdrawn at predetermined time intervals and analyzed spectrophotometrically at λ_{max} 272 nm using phosphate buffer (pH 7.4) as a blank. After each sample, equal volume of fresh receptor fluid was replaced into the receptor chamber to maintain a constant volume.

Kinetics of ciprofloxacin HCl release from the prepared liposomal dispersions were examined based on the magnitude of correlation coefficients obtained after application of zero-order, first-order, and Higuchi diffusion models.

Rheological evaluation of chitosomes

The rheological studies were carried out using Brookfield digital viscometer (model DV-II; Brookfield Engineering Laboratories Inc., Middleboro, MA, USA). Sample was placed in a suitable beaker and the instrument continuously sheared the material at various rates using suitable spindles. Measurements were done over a range of speed of 3–30 rpm each for 60 seconds.

Ex vivo permeation studies

Membrane diffusion technique was used for ex vivo permeation studies using diffusion cell assembly, which was composed of two parts: the upper part was exactly fitted on the surface of the lower part and the cornea from adult albino rabbit (1–2 kg) with small part of

scleral rim was isolated from the rabbit eye and fixed between them. The cell assembly was placed in a thermostatically controlled water bath, at a temperature of 34 ± 0.5°C and shaken at 25 rpm.

Chitosomal dispersion was placed in the upper compartment of the cell on the separated cornea; the diffusion medium used was 6 mL freshly prepared isotonic phosphate buffer pH 7.4 preequilibrated at 34 ± 0.5°C.

Samples (3 mL) were withdrawn at different time intervals. After each sample, equal volume of fresh receptor fluid was added to maintain a constant volume. The sampling time was fixed for 6 hours, after which corneal damage was expected to occur[18,19]. Samples were analyzed spectrophotometrically at λ_{max} 272 nm, using isotonic phosphate buffer pH 7.4 as a blank.

The apparent permeability coefficient (P_{app}) was determined according to the following equation[18,19]:

$$P_{app} = \Delta Q / \Delta t (3600 \times A \times C_0), \quad (3)$$

where, $\Delta Q/\Delta t$ is the permeation rate of drug across the cornea obtained from the slope of the straight line of corneal permeability (ΔQ) versus time (t); A the exposed corneal surface area (cm^2); and C_0 the initial concentration of the drug in the donor compartment.

The experiments were carried out in triplicate.

Determination of the minimum inhibitory concentration of liposomes and chitosomes against the tested microorganisms using agar dilution technique

The prepared formulation was twofold serially diluted with sterile distilled water to the final volume of 2 mL. One milliliter of each dilution of the formula was aseptically well mixed with 9 mL sterile molten agar and poured in sterile petri dishes. A control plate was carried out side by side with the experiment by replacing the formula with sterile distilled water. The plates were allowed to set for 5 minutes for complete solidification.

The microbial culture was 10^2 diluted using sterile saline, and aliquots of 10 µL of the dilution were then dropped onto the surface of the dried plates using a micropipette fitted with sterile disposable plastic tips and allowed to stand at room temperature (25°C) until the drops were absorbed and incubated in an inverted position at 37°C for 24 hours.

The plates were then visually inspected for microbial growth and the MIC was taken as the lowest concentration of the formula resulting in a complete inhibition of the growth or not more than two discrete colonies per spot[20].

In vivo evaluation

Single-dose study. Albino rabbits of either sex were used for single-dose study. The animals were placed in restraining boxes during the experiment, which allowed their heads to move freely and their eye movements were not restricted.

Suspension of *P. aeruginosa* was prepared to give 0.5 McFarland standard[21]. Liposomes coated with 1% (w/v) medium-molecular-weight chitosan and marketed preparation (Ciloxan® eye drops; Bayer Health care, Leverkusen, Germany) were tested. Bacterial suspension of 40 µL was instilled in the right eye of each rabbit and the left eye was served as control. After allowing the growth to proceed to about 18 hours (the log phase), one drop (equivalent to 50 µL) of each formulation was then instilled. Cotton swabs from eyes were aseptically taken at 2, 4, 8, and 24 hours and then was streaked on sterile cetrimide agar plates. Incubation was carried out at 37 ± 0.5°C for 24 hours and checked for growth.

Pharmacodynamic activity. Bacterial conjunctivitis was induced in rabbits' eyes by exposing them to bacteria strain of *P. aeruginosa*. Treatment was initialized 24 hours later. Three rabbits were used for the evaluated formulation. The right eye of each rabbit received one drop of the tested preparation every 24 hours for 4 days. Considering the control group, two to three drops of the marketed product (Ciloxan® eye drops) was applied three times a day for the duration of study. Eyes of each animal were visually observed and graded every day for the duration of the study for the following parameters, that is, redness of the mucous membrane, lacrimal secretion, mucoidal discharge (whitish to yellowish-white semisolid discharge), response to ocular stimulus (assessed by shining torch light on the eye), and swelling of eyelids (Table 2)[22].

Results and discussion

Morphological evaluation and particle size analysis

The prepared liposomes were characterized and optimized in terms of EE%, particle size and release characteristics by our group in a previously published research[12].

Liposomes were spherical particles which had single-walled bilayer enclosing an aqueous compartment

Table 2. Assessment scale of experimentally induced bacterial conjunctivitis in rabbits' eye.

	Grade		
Parameter	0	1	2
Redness of the mucous membrane	Absent	Mild	Severe
Lacrimal secretion	Normal	More than normal	Abnormal
Mucoidal discharge	Absent	Little	More
Response to ocular stimulus	Normal	Fast	Very fast
Swelling of the eyelid	Absent	Slight	Edema

while the existence of smooth polymer layer surrounding the surface of chitosomes was well visualized with both optical and transmission microscopes (Figure 1).

As stated in Table 3, the particle size of chitosomes in each formulation was increased with respect to the original liposomes size, suggesting the formation of coating layer on the liposomes surfaces. The mean particle size diameters (μm) of chitosomes increased inversely with increasing chitosan molecular weight (C2, C4, C6) and increased with increasing chitosan concentration (C3, C4).

When the chitosan solution was added to the colloidal dispersion of liposomes, the chitosan molecules adhered to the liposomes surface. Polymer-coated vesicles were produced and it was probably formed by electrostatic interaction between positively charged chitosan and the opposite charge on the liposome surface. The chitosan covered the surface of liposomes by forming ion-complex in the liposome formulation[23], which increased with increasing chitosan concentration[24].

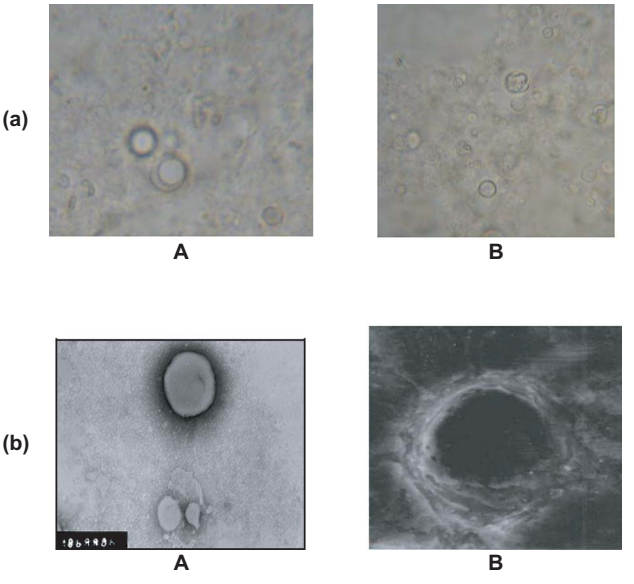

Figure 1. Representative images of liposomes (A) and chitosomes (B). Images were obtained by optical (a) and transmission electron microscopes (b).

Table 3. Entrapment efficiency, particle size, and sedimentation volume of different chitosomal formulations.

Formula code	Entrapment efficiency (%)	Reduction in entrapment efficiency (%)	Particle size (μm)	Relative sedimentation volume
F1	53.220 ± 0.538	—	6.230	0
F2	71.400 ± 0.247	—	7.230	0
C1	61.350 ± 1.242	14.076 ± 1.739	—	0.200
C2	60.280 ± 0.642	15.574 ± 0.898	21.720	0
C3	66.710 ± 0.459	6.569 ± 0.643	7.480	0.100
C4	64.050 ± 0.764	10.294 ± 1.070	7.970	0
C5	69.820 ± 0.568	2.212 ± 1.070	—	0
C6	67.500 ± 0.851	5.462 ± 1.192	8.150	0
C7	49.930 ± 0.388	6.181 ± 0.636	—	0.400
C8	50.190 ± 1.525	5.693 ± 2.865	6.670	0.150
C9	52.460 ± 0.524	1.428 ± 0.985	—	0.150

The vesicle size of chitosomes with low-molecular-weight chitosan (C6) was slightly larger (8.15 μm) than others. This may be explained on the basis that the smaller molecules of chitosan did not cover the surfaces of liposomes, resulting in insufficient physical barriers to prevent coalescence. The insufficient coverage that might result in uneven surface charges of liposomes facilitates the aggregation and coalescence of liposomes[25]. Chitosomes made up with high-molecular-weight chitosan (C2) showed the exceptional mean particle size (21.72 μm) which could be explained based on the fact that at a higher molecular weight, bridging effects predominated[26].

Concerning the effect of surface charge of liposomes on mean particle size, formulae C4 and C8 were studied for comparison. Smaller particle size was observed for neutral liposomes than for negative ones, which could be seen from the view of chitosan bilayer interaction. Negative liposomes adsorbed more chitosan (thicker adsorptive layer) than neutral liposomes, as electrostatic attraction was the predominant interaction mechanism, whereas in neutral liposomes hydrophobic forces dominated. Similar results were obtained for leuprolide[16].

Entrapment efficiency

Effect of chitosan coating process on liposomes EE% is summarized in Table 3. The entrapment efficiencies were ranged from 49.93% to 69.82%, which were lower than initial EE% of uncoated liposomes (71.4% and 53.22% for negative and neutral liposomes, respectively).

To study this effect percentage reduction in EE% were calculated as follows:

$$\%\,\text{Reduction in EE} = \frac{\text{initial EE\%} - \text{EE\% of chitosomes}}{\text{initial EE\%}} \times 100. \quad (4)$$

The lower chitosan concentration, formulae C1, C3, and C5 (0.1%, w/v), showed a higher EE% and lower reduction in EE% in comparison to higher chitosan concentration, formulae C2, C4, and C6 (1% w/v), for all chitosan molecular weights under investigation.

These results could be explained by the interaction of positively charged substances with lipid membranes depending on electrostatic attraction at the head group level[27]. Chitosan had strong affinity for the phospholipids of vesicle bilayer. Ciprofloxacin HCl is a water-soluble drug which was added to the aqueous compartment of liposomes. High percentage of the drug was ionized at pH 4.5. Because of these conditions, both ciprofloxacin

HCl and chitosan had positive charge and they competed with each other to interact with the negatively charged phospholipids of the bilayer. This type of competition had already been reported by other authors with leuprolide[16].

The mutual repulsion between the cationic drug molecules and the positively charged chitosan matrix prevented any kind of interaction between them. In addition, the presence of protons resulted from the dissociation of ciprofloxacin HCl contributed to this repulsive force[28]. At 1% (w/v) chitosan concentration, there was a higher number of positively charged molecules competing with drug molecules than that at lower concentration (0.1%, w/v), so the former showed a lower EE% with higher reduction in EE% compared to the later.

Chitosomes made up with chitosan with low molecular weight showed a higher EE% and lower reduction in EE% in comparison to those made up with higher chitosan molecular weight. This could be attributed to the fact that, low molecular weight chitosan could not cover the surfaces of liposomes sufficiently as done with the higher molecular weight chitosans[26,29]. As, there was not enough interaction between negatively charged phospholipids bilayers and cationic chitosan molecules, subsequently, less cationic ciprofloxacin HCl replaced from its binding sites, lower chitosan molecular weight showed higher EE%.

To study the influence of charge type of liposomes on EE% of chitosan, neutral liposomal formula was coated with 1% (w/v) chitosan of different molecular weights. Reduction in EE% was calculated and compared with negative liposomes.

Concerning negatively charged liposomes, the interaction between chitosan and bilayers possessed stronger ionic interaction; thus stronger competition occurred between cationic drug and chitosan. This led to a greater reduction in EE% value in comparison to neutral liposomes in which chitosan/liposomes interaction was depended on hydrophobic interaction rather than ionic one. Similar results were observed by Guo et al.[16]

Relative sedimentation volume

The sedimentation volumes of all samples were determined as a probe of formulation stability. The sedimentation volumes of all chitosomes made up with negatively charged liposomes were around 0 (Table 3). Such liposomes possessed a chitosan coat having a positive charge, which played a role in the resistance to flocculation. While at 0.1% (w/v) chitosan concentration (C1), polymer bridge resulted in flocculation. Furthermore, the thickness of the adsorption layer was important; as chitosomes made up with negatively charged liposomes had thicker adsorptive layer than those made up with neutral ones, the thick adsorptive layer on the liposomes kept the particles apart and led to stabilization[16]. At higher concentrations, surface coverage by the adsorbed chitosan was sufficient to prevent polymer bridge flocculation. The adsorbed polymer film now serves to stabilize the particles against particle–particle interaction presumably by the mechanism of steric stabilization[24].

Rheological studies

Liposomal dispersion showed a low apparent viscosity (15 cP), which did not allow sufficient retention time of the dosage form[30]. While all chitosomal dispersions were shear thinning systems (with higher apparent viscosity values), an increase in shear stress was observed with increase in angular velocity (exhibited a pseudoplastic rheology) as shown in Figure 2. It has been assumed that the instillation of a solution influences the normal behavior of tears as little as possible, and as tears show a pseudoplastic behavior, pseudoplastic properties are recommended. This property offered the advantage of low viscosity during blinking and stability of tear film during interblinking periods[31].

In vitro release studies

A system involving polymer coating of liposomes offered a versatile drug delivery system in which the release profile could be controlled by various parameters, depending on the properties of both liposomes and the coating polymer. This coated liposomal system could serve as a ‘depot’ for a sustained release of bioactive material over an extended period of time. On the top of that, it is a strategy to modulate drug release and eliminate the burst effect[32].

The cumulative amounts (%) of ciprofloxacin HCl as a function of time from different chitosomal dispersions are illustrated in Figure 3. The optimized formulation (F2) showed an initial burst release of about 20.4% of ciprofloxacin HCl after 1 hour, followed by a more gradual release for the following 24 hours. After coating with chitosan, all chitosomes showed a slower release profiles than the uncoated liposomes, which provided an evidence for coat formation. In addition, burst effect was eliminated, which could be explained by the competition that occurred between the cationic drug and the chitosan, which reflected on the EE% of chitosomes. As mentioned before, the initial burst release was because of desorption of drug molecules from liposomal surfaces; hence, the surface molecules were eliminated via competition, and the initial burst was eliminated[24].

This effect of coating was more pronounced in negatively charged liposomes than neutral liposomes, as strong electrostatic interaction occurred between cationic

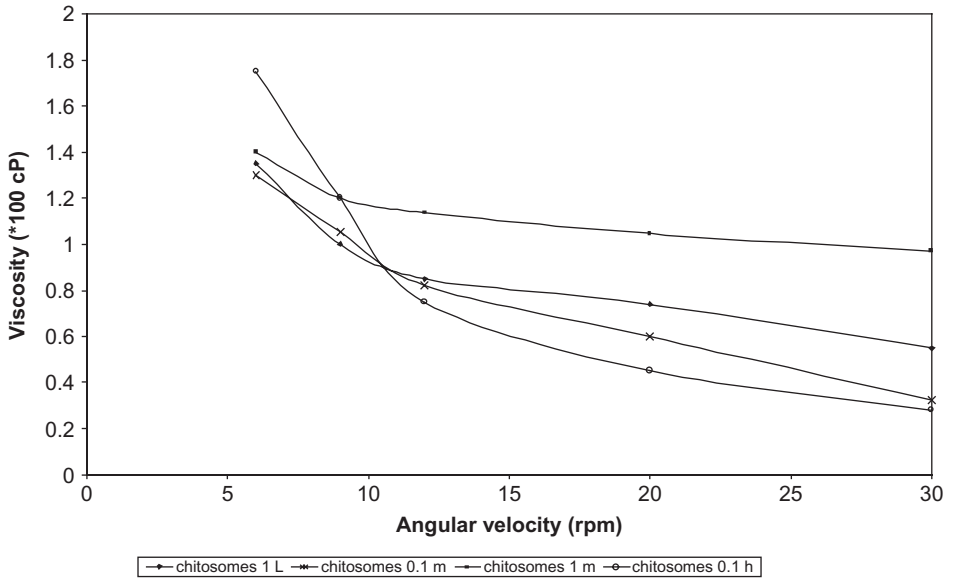

Figure 2. Effect of coating with different chitosan concentrations and molecular weights on the rheological properties of chitosomes at 25°C.

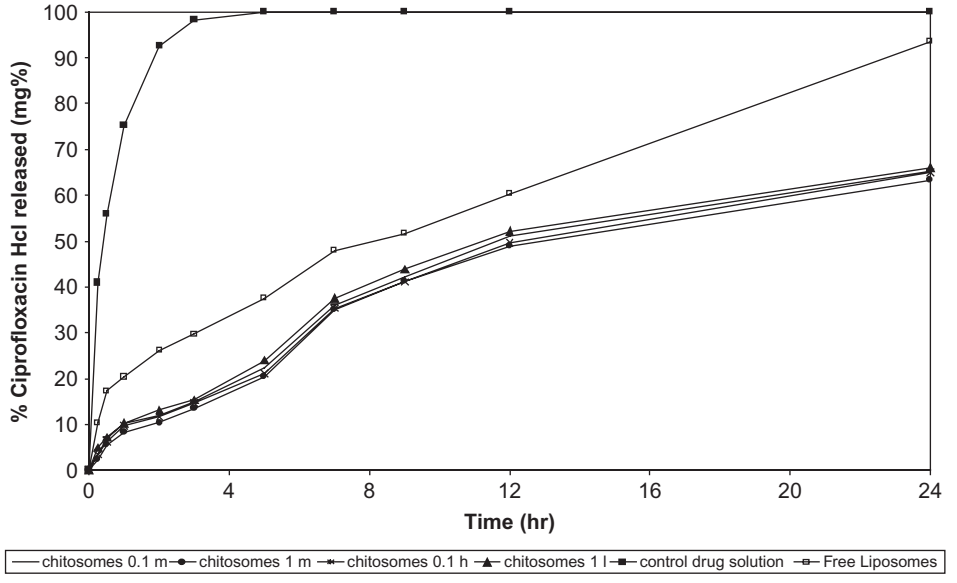

Figure 3. In vitro release profiles of different chitosomal formulations (C1, C3, C4, C6) into isotonic phosphate buffer pH 7.4.

drug and negatively charged liposomes, whereas in neutral liposomes, hydrophobic interaction between chitosan and neutral membranes was the predominate mechanism of coat formation[16].

Coating of liposomes with chitosan provided two control points for modifying the rate of release of the drug; the first being the lipid bilayer and the second being the coating layer. From release profile, it could be emphasized that the cumulative amount of drug released from all formulae (release pattern) was similar with slight differences, which may be because of the influence of chitosan concentration along with its molecular weight. For instance, formula C4 showed the slowest pattern of release among the other formulae. This is because of the formation of complete coat as chitosomes formed from higher chitosan concentration (1%, w/v) with medium molecular weight, which resulted in formation of a thicker coating layer. Formula C3 showed a faster release than formula C1 despite the use of the same chitosan concentration (0.1%, w/v). This demonstrated the viscosity influence, as C1 had a higher viscosity as compared to C3, so viscosity of the preparation acted as a physical barrier to drug molecules diffusion throughout the formulae[33].

It could be concluded that medium-molecular-weight chitosan was stabilizing the original liposomal structure. Similar results were observed by Filipovic-Grcic et al.[23]

The overall curve fitting showed that the drug release from all the studied chitosomal formulations followed *Higuchi* model. This indicated that drug was released by diffusion from chitosomes (with $r^2 > 0.97$).

Ex vivo permeation studies

The amount of drug permeated would be the total rate at which the vesicles permeate the cornea and the rate and extent of drug release from the vesicles to the surface of the cornea (forming the effective concentration gradient) or after passing through the cornea into aqueous humor[18].

The apparent permeability coefficient (P_{app}) could be used as a parameter for the corneal permeability. There was a direct proportionality between the percentage total cumulative amounts permeated at the end of the permeation experiment and the apparent permeability coefficient values. Relative P_{app} values were also calculated by dividing the values of the apparent permeability coefficient by the P_{app} of the drug solution (Table 4).

The liposomal formulation showed a permeation rate lower than that of the drug solution. For negatively charged liposomes, ciprofloxacin HCl permeability was about 1.5-fold lower than that for the free drug. This could be explained by the fact that, corneal surface was negatively charged, this led to expelling of negatively charged liposomes from the corneal surface and deposited loosely on the corneal surface. This was in agreement with results observed for acyclovir[34].

Concerning the chitosomal formulation, the amount permeated along with the apparent permeability coefficient was higher than that for the free drug (relative P_{app} = 1.737). These results were attributed to the presence of chitosan coat. Chitosan is a transcorneal absorption promoter, which increased the diffusion of hydrophilic drugs, probably through disruption of tight junctions. Chitosan, unlike other enhancers, appears to be well tolerated by the corneal cells. Additionally, its action is reversible[35].

In vitro antimicrobial activity of chitosomes containing ciprofloxacin HCl

The in vitro susceptibility of liposomal formulation of ciprofloxacin HCl was studied. The bactericidal activities of the prepared liposomes against two reference strains and six clinical isolates of *P. aeruginosa* and one *S. aureus isolate* (fluoroquinolones resistant) are presented in Table 5. Empty liposomes showed no antibacterial activity in all tested organisms. Concerning negatively charged liposomes (F2), the MICs of liposomes, for the reference and isolated strains, were higher than those for free ciprofloxacin HCl.

It was reported that unilamellar liposomes containing antibiotics exhibited bactericidal activity against extracellular pathogens in the treatment of various infections[21]. It was shown that ciprofloxacin HCl encapsulated in neutral liposomes exhibited much higher antibacterial efficacy against both Gram-positive and Gram-negative bacteria than anionic vesicles. For Gram-negative bacteria, approximately twofold or more increase in MIC for *P. aeruginosa* strains was observed for anionic liposomes (F2). Negatively charged liposomal ciprofloxacin HCl antagonized the antimicrobial activity of the drug, which may be because of electrostatic repulsion between the anionic surface of the liposomal bilayers and the negatively charged outer membrane of Gram-negative bacterial cells or, in case of Gram-positive

Table 5. In vitro activity of chitosome formulations containing ciprofloxacin HCl against reference strains and clinical isolates in nutrient agar.

	MIC values (μg/mL)		
Bacterial strain	Free drug	Liposomes (F2)	Chitosomes (C4)
Staphylococcus aureus ATCC 6538P	0.125	0.500	0.063
S. aureus isolate	8.000	16.000	2.000
Pseudomonas aeruginosa ATCC 9027	0.0625	0.500	0.063
P. aeruginosa P1	0.781	3.125	0.390
P. aeruginosa P2	25.000	50.000	3.125
P. aeruginosa P3	6.250	12.500	0.390
P. aeruginosa P4	25.000	50.000	3.125
P. aeruginosa P5	1.563	0.781	1.563
P. aeruginosa P6	1.563	0.781	0.781

Table 4. Corneal permeability and apparent permeability coefficient of selected chitosomal preparation.

Formulation	Corneal permeability (% total cumulative amount permeated)	Apparent permeability coefficient (P_{app}). 10^6 cm/s	Relative P_{app} values
Drug solution	17.160 ± 0.489	5.188 ± 0.228	—
Liposomes (F2)	12.691 ± 0.331	4.412 ± 0.113	0.836 ± 0.034
C4	26.136 ± 0.716	8.632 ± 0.354	1.737 ± 0.096

bacteria, electrostatic repulsion occurred with peptidoglycan[9].

The antibacterial efficacy of chitosomes containing ciprofloxacin HCl prepared using 1% (w/v) chitosan with medium molecular weight was evaluated in vitro. The bactericidal activities of the prepared chitosomes against two reference strains and six clinical isolates of *P. aeruginosa* and one *S. aureus* (fluoroquinolones resistant) are illustrated in Table 5. MIC values of chitosomes were higher by two- to fourfold compared to MICs for free drug in all tested organisms especially for *P. aeruginosa*-resistant strains, that is, sub-MIC activity. These results reflected that presence of chitosan enhanced the antibacterial action of negatively charged liposomes containing ciprofloxacin HCl, as chitosan by itself had antimicrobial activity[36]. Also this sub-MIC antimicrobial efficacy was not correlated with rapid drug release from the preparation but rather with electrostatic interaction between the cationic chitosomes and the bacterial cells[9].

In vivo evaluation

Concerning single-dose study

The development of chitosomes formulation was aimed to increase the dosing interval of the drug administration into the eye (once daily), so they were evaluated using single-dose protocol. The tested formulation showed a markedly improved effect when compared to the marketed eye drops (Table 6). They were able to prevent the growth of *P. aeruginosa* in rabbit eye for 24 hours. Growth was examined in all animals after 2 hours of post-instillation of the formulation. Marketed product (Ciloxan®) did not inhibit the growth after 4 hours, this necessitates frequent administration of the dosage form while the prepared formulation was able to inhibit *P. aeruginosa* growth for the entire duration of the study (24 hours). Prolonged release observed previously for the prepared formulations might be the reason for the 24-hour bacterial growth inhibition.

Using animal model, bacterial conjunctivitis was induced by instillation of *P. aeruginosa* in rabbits' eyes (Figure 4). Treatments were carried out by chitosomes along with the marketed product. The eyes were observed for different parameters on score basis up to 4 days. Improvement in most symptoms was occurred after first day in case of prepared prolonged release formulation compared to the second day of treatment in the case of marketed eye drops. These can be expected from the enhanced antibacterial activity of the prepared formulations as discussed before. Although many changes in the measured parameters were statistically nonsignificant, the treatment with the prolonged systems resulted in earlier curing of symptoms than marketed preparations[21,37].

Thus, symptoms mainly associated with conjunctivitis were controlled by the prolonged release formulations such as that done by the marketed product.

Table 6. In vivo single-dose study of the prepared formulations ($n = 3$ animals).

	Time interval (hours)			
Formulation	2	4	8	24
Marketed product	−3[a]	−3	+3[b]	+3
Chitosomes (C4)	−3	−3	−3	−3

[a](− sign) indicates absence of *Pseudomonas aeruginosa* growth in the tested animal.
[b](+ sign) indicated the growth of *P. aeruginosa* in the tested animal.

Conclusion

Through this research, it could be concluded that prolonged release mucoadhesive liquid ocular dosage form based on ciprofloxacin HCl liposomes was successfully formulated and evaluated. Chitosomes were prepared by coating liposomes with chitosan. Chitosomes prepared with 1% (w/v) medium-molecular-weight chitosan showed prolonged release for 24 hours by diffusion as the mechanism of release. Chitosan coating improved the ocular permeation of ciprofloxacin HCl. This formulated system enhanced antimicrobial activity of ciprofloxacin HCl

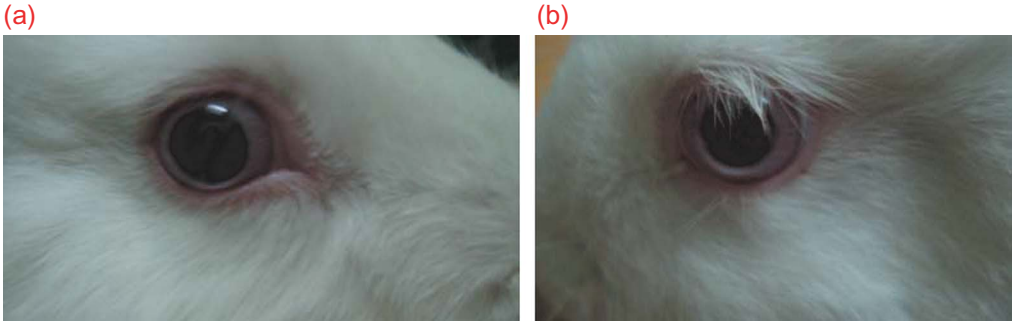

Figure 4. Normal rabbit eye showing no mucoidal exudates (a); bacterial conjunctivitis in rabbit eye showing mucoidal exudates (b).

against the tested microorganisms. Moreover, the formulated system was able to inhibit the growth of *P. aeruginosa* in rabbits' eyes for 24 hours when compared to the marketed preparation. Thus, chitosomal formulation can be viewed as a valuable alternative to conventional eye drops by virtue of its ability to enhance precorneal residence time and, consequently, ocular bioavailability. The ease of administration coupled with the capability of providing sustained release could result in a decrease in administration frequency, which improved patient compliance.

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Declaration of interest

The authors report no conflicts of interest.

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