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ORIGINAL ARTICLE

Chitosan/carbomer nanoparticles- laden in situ gel for improved ocular delivery of timolol: in vitro, in vivo, and ex vivo study

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Abstract

Due to the small capacity of the eye cavity and the rapid drainage of liquid into the nasolacrimal duct, patients must frequently administer the drops. Nanoparticles (NPs) and in situ gel systems have each proven their ability to achieve eye retention independently. In this study, timolol-loaded chitosan-carbomer NPs were prepared using the polyelectrolyte complexation method, and incorporated into a pH-responsive in situ gel system made of carbomer. The rheological behavior of NPs-laden in *situ gel* was examined at room and physiological conditions. Characteristics such as zeta potential, surface tension, refractive index, mucoadhesive properties, drug release, transcorneal permeability, and intra-ocular pressure (IOP) lowering activity were investigated on NPS and NPs-laden in situ gel formulations. The optimum gained NPs system had an encapsulation efficiency of about 69% with a particle size of 196 nm. The zeta potential of the NP and NPs-laden in situ gel were −16 and +11 mV respectively. NPs-laden in situ gel presented enhanced viscosity at physiological pH. All physicochemical properties were acceptable for both formulations. NPs and NPs-laden in situ gel systems proved to sustain drug release. They showed mucoadhesive properties which were greater for NPs-laden in situ gel. IOP reduction by NPs-laden in situ gel was significantly higher and more long-lasting than the timolol solution and NPs. In conclusion, the developed NPs-laden in situ gel is a promising carrier for ocular drug delivery due to the slow release of drug from nanoparticles, its mucoadhesive properties, and high viscosity acquisition in contact with precorneal film, which lead to improved therapeutic efficacy.

Keywords Glaucoma · Chitosan · Carbomer · Nanoparticles · In situ gel · Ocular drug delivery

Introduction

Timolol maleate (TM), a non-selective beta-adrenergic blocker, has been considered first-line treatment for open-angle glaucoma and hypertension for many years due to its role in lowering intraocular pressure (IOP). Although currently, due to the lower risk of systemic side effects of prostaglandins, it is recommended to start the treatment of these diseases with a topical prostaglandin, but timolol is still used in patients who do not respond adequately to this drug class or for whom they are contraindicated. Also, in many patients, with the aim of increasing the effect, timolol is prescribed in combination with prostaglandins [1, 2]. Eye drop solutions are the most common ophthalmic preparations available for TM. Eye drops do not have adequate ocular bioavailability due to shedding tears or drainage into the nasolacrimal duct, which loses approximately 80% of the instilled dose [3]. So that ocular bioavailability of timolol after topical administration on albino rabbit eyes has been reported to be 1.22–1.51% [4].

On the other hand, the drainage of TM into the nasolacrimal duct results in systemic side effects limiting its use, especially in patients suffering from heart diseases or bronchial asthma [5]. Many attempts have been made to improve

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the therapeutic effectiveness of ophthalmic preparations, including their incorporation into colloidal systems such as nanoparticles (NPs) [6–12]. Due to high drug loadings, targeted delivery, slow and controlled drug release, longer eye retention time, and better permeability, nanoparticles in ophthalmic products provide higher ocular bioavailability than conventional eye drops [11].

Chitosan (CS) is a cationic polysaccharide with desirable properties such as biocompatibility, biodegradability, mucoadhesive feature, and ability to enhance the paracellular transport and absorption through the mucosal membranes such as eyes, nose, buccal, and gastro-enteric system [13–17]. Its mucoadhesive property prolongs the contact between chitosan-based nanoparticles and the mucosal surfaces and increases drug retention time in situ. Therefore, CS-based systems are acknowledged as suitable delivery systems for ocular administration [15]. One of the widely developed methods for the formation of CS NPs is polyelectrolyte complexation (PEC) which offers the advantage of a simple and mild preparation without the use of organic solvents or high shear forces [14, 18].

Carbomer (carbopol) is a polyanion agent that can interact with positively charged amine groups of CS and form CS/carbomer NPs. Carbomer is a generic name for synthetic high molecular weight polymers of acrylic acid. It provides several advantages such as high viscosity in low concentrations, bioadhesive properties, and patient compliance, and has been widely used in designing controlled drug delivery systems [19, 20].

Rapid dissolution and short retention time for drug penetration caused by high glass transition temperature and water solubility of carbomer are its main disadvantages. In CS/carbomer NPs, the partial formation of an electrostatic complex between CS and carbomer results in a delayed carbomer dissolution rate overcoming the above disadvantages [19, 21]. Carbomer is also used as a polymer to prepare pH-induced in situ gelling systems due to its ability to undergo sol-to-gel phase transition in response to an increase in pH [20].

The present work aimed to obtain an ophthalmic delivery system for TM with improved retention time and sustained drug release using a chitosan/carbomer NPs-laden in situ gel system.

Materials and methods

Materials

Timolol maleate was commercially purchased from Sina Darou Laboratories Company, Iran. Low molecular weight chitosan (deacetylation degree 95% and viscosity < 25 cps) which was obtained from fresh North Atlantic shrimp (Pandalus borealis shells) was purchased from Primex, Iceland. Carbomer 940 was purchased from Fluka, Switzerland. Hydroxypropyl methylcellulose FM4 (HPMC) was obtained from COLORCON Co., Germany. Mucin from the porcine stomach (Type II) was commercially purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were of analytical grade.

Preparation of CS/carbomer nanoparticles

CS was dissolved in a 1% (w/v) acetic acid solution to make CS concentrations of 0.02 and 0.1% (w/v) [19, 22]. TM was then added to CS solutions. The carbomer was dissolved in sodium phosphate buffer (pH 5.8), containing 0.01% benzalkonium chloride as a preservative [23, 24], to obtain carbomer solutions of 0.02 and 0.04% (w/v). Each of the prepared CS solutions, containing TM, was added drop-wise to carbomer solutions with a volume ratio of 1:5 (CS: carbomer) under magnetic stirring at room temperature [19]. The four obtained formulations were then homogenized at 12000 rpm using a homogenizer (IKA T25 ULTRA-TURAX, Laboratory equipment, Germany). The final TM concentration in all formulations was 0.5% (w/v) based on 'timolol' in regard to the TM concentration in the conventional marketing drop. CS: carbomer weight ratios of (1:1), (1:2), (1:5) and (1:10) were used in this study. The resulted NPs were lyophilized to protect against leakage of the drug during long-term storage.

Characterization of nanoparticles

The particle size and zeta potential of all CS/carbomer nanoparticles were measured using a dynamic light scattering system (DLS, Zetasizer 3000 HS; Malvern Instruments Ltd., UK).

Encapsulation efficiency (EE)

EE of the prepared NPs formulations was determined by indirect method. Briefly, Amicon® ultra centrifugal filters (3k) containing formulations were centrifuged (MPW 350R, Poland) at 15,000 g for 30 min, and free TM in the supernatant was measured by UV-Vis spectrophotometer (Biochorm, England) at 295 nm. The EE of the NPs was calculated with the following equation [11].

$$\mathrm{EE}\,(\%) = \frac{\text{Total amount of TM} - \text{Free amount of TM}}{\text{Total amount of TM}} \times 100$$

Table 1 HPMC and carbomer content of the designed in situ gel formulations

Formulation (%w/v)	IG1	IG2	IG3	IG4	IG5	IG6	IG7	IG8	IG9
HPMC	0.1	0.2	0.3	0.1	0.2	0.3	0.1	0.2	0.3
Carbomer	0.1	0.1	0.1	0.2	0.2	0.2	0.3	0.3	0.3

Table 2 Content of the NPs laden in situ gel formulations

Formulation	CS: carbomer ratio in NPs	HPMC (%w/v)	Carbomer (%w/v)
CC2-IG1	1:10	0.1	0.1
CC2-IG2	1:10	0.2	0.1
CC2-IG3	1:10	0.3	0.1

The spectrophotometric method was validated for TM measurement and its analytical characteristics are presented in Fig. S1 and Tables S1-S2.

Atomic force microscopy (AFM)

Topographic images of CS/carbomer nanoparticles were obtained at room temperature using a commercial Nanowizard II Multi-Mode AFM (JPK, Germany) which was operated in tapping mode. A silicon cantilever/tip (APPNANO, USA) with a tip height of 14 to 16 µm, radius of curvature of 6 nm, and typical resonance frequency between 200 and 400 kHz was used in these experiments. Nanoparticle suspension was diluted with ethanol and homogenized for 20 min at 150 w using a probe sonicator. One drop of the sample was poured on a piece of mica plate and allowed to dry in air. The surface images were obtained at fixed resolution (512 × 512 data points) with a scan rate of 1 Hz.

Preparation of the blank in situ gel samples

Carbomer and HPMC were dissolved in distilled water to obtain different concentrations as indicated in Table 1 [23, 25]. The solutions were allowed to be hydrated overnight under a stirrer to ensure the complete dissolution of the polymers.

By adding NaOH (1 M), the pH of all formulas was brought to 7.4 (physiological pH condition of eye), and the consistency of the solution was visually checked and scaled based on the time the gel started to form and the time the gel remained [26, 27]. Formulations with measurable initial and secondary viscosities were selected for further evaluation.

Preparation of the NPs-laden in situ gel formulation

The mix solutions of HPMC (0.1, 0.2, or 0.3% w/v) and carbomer 0.1% w/v were prepared as in situ gel bases. The lyophilized form of the selected NPs based on particle size, EE, and zeta potential was added to the selected in situ gel systems (Table 2).

Rheological assessment

Rheological behaviors of the formulations were evaluated at 25˚C before and after pH adjustment at 7.4 using a Brookfield viscometer (Model LVDV-II + PRO, USA). The spindles No. 61 and 34 were used for low- and high-consistency samples, respectively. Viscosity was measured at varying speeds (0.1 to 200 rpm) [28]. The rheological behavior of the samples was determined by fitting the viscosity data on the following Newtonian and non-Newtonian equation:

$$\mathrm{Log}\,\delta = N \log \mathrm{r} - \log\eta$$

Where δ, τ, η, and N represent shear rate, shear stress, viscosity coefficient, and flow index, respectively [29]. $N = 1$ indicates Newtonian behavior while N less than 1 corresponds to shear thickening flow, and for pseudoplastic material, N is more than 1 [29–31].

Characterization of NPs-laden in situ gel systems

Surface tension, pH value, and refractive index were evaluated at room temperature. The surface tension of the formulations was determined by the De Nouy ring method (CSC Scientific Company, USA) [32]. The refractive indices were determined using a PrismaTech benchtop refractometer (Model BPTR-50, Iran). The measurements were made in triplicate.

Assessment of mucoadhesive property

The mucoadhesive evaluation was carried out using a turbidimetry-based method. A solution of type-II porcine mucin (0.1% w/w) was freshly prepared and mixed with an equal volume of the selected formulations using vortex. The turbidity of the porcine mucin solution, the formulations, and the mucin-formulation mixtures was measured using a UV-Vis spectrophotometer at 650 nm within 8 h [10, 33–36].

In vitro drug release study

A Franz diffusion cell was used for this study. The receptor was filled with 10 ml simulated tear fluid (STF, composition: sodium chloride 0.0670 g, sodium bicarbonate 0.200 g, calcium chloride, $2H_2O$ 0.008 g, and purified water q.s. 100 g) as the release medium [37, 38]. An acetate cellulose membrane (cut off = 12 kDa) was placed between the

donor and receptor. 0.3 ml of each formulation was placed in the donor along with an equal volume of STF to simulate the tear fluid pH and its dilution effect and the test was done at 32˚C. It is notable that the lyophilized form of NPs was used in these experiments. TM solution (0.5%) in sodium phosphate buffer at pH 5.8 (TMS) was chosen as a control. 0.5 ml of the release medium was drawn at various time intervals (0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7, 8, and 24 h) and replaced with 0.5 ml STF. The content of TM was determined by UV-Vis spectrophotometer (Biochorm, England) at 295 nm. The drug release experiments were performed in triplicate [28, 32].

In vivo intra-ocular pressure lowering activity

The study was performed on 12 adult male rabbits (weighing 2 to 2.3 kg). The animal experiments were conducted in full compliance with the regulatory principles of the ethics committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1401.126). The rabbits were housed with access to food and water and were maintained on a 12 h light-12 h dark cycle in a temperature-controlled room, at 22–26 °C [32]. Through experiments, the rabbits were divided into three groups, each consisting of 4 rabbits: Group I received CC2; Group II received CC2-IG3, and Group III received 0.5% TMS. The formulations were sterilized by UV irradiation for 30 min before instillation [39].

Each group received 50 µL of the formulation in the left eye, while, the right eye remained untreated. An IOPen® tonometer (Medicel AG, Swiss Technology for Surgery, Luchten, Switzerland) was used to measure intraocular pressure. IOP of both eyes was measured right before instillation and at regular intervals after instillation up to 8 h. The change in IOP (ΔIOP) was expressed as follows:

ΔIOP = IOP untreated eye-IOP treated eye [27, 32, 40–42].

Ocular irritancy test

The cornea, iris, and conjunctiva of all 12 rabbits (3 previously treated groups) were macroscopically monitored for up to 72 h for ocular irritancy. The total irritation score was the average of the sum scores of three parts (cornea, iris, and conjunctival) [43]. During the experiments, the rabbits were housed in separate standard cages in a light-controlled room (12 h light-12 h dark cycle) at 22–26 °C and 50 ± 5% relative humidity with no restriction of food or water.

Ex vivo transcorneal permeation

The study was performed using rabbit cornea. Whole eyeballs were immediately excised after the rabbits were slaughtered. Then the corneas were carefully removed along with 2–3 mm of surrounding scleral tissue, and washed with cold saline. In the transcorneal permeation study, the same model and diffusion method used in the in vitro release study were utilized, with the difference that the cellulose acetate membrane was replaced with a cornea with its epithelial surface facing the donor [28, 44].

The apparent permeability coefficient (P_{app}) and steady-state flux (J_{ss}) of the drug through cornea were calculated from the following equation.

$$J_{ss} = \frac{dQ}{dt} = P_{app}.C_d$$

Where Q is the cumulative amount of drug passed through the corneal surface area (S) unit at time t and C_d is the drug concentration in the donor phase under sink conditions. If C_d remains constant, a linear relationship between Q and t is established and Jss can be calculated from the slope of the linear equation. Failure to establish a linear relationship indicates that the C_d is variable, in which case Papp is calculated based on the following equation:

$$lnC_d = lnC_0 - \frac{SP_{app}t}{V}$$

Where C_0 and V are initial drug concentration and volume of the donor phase, respectively [45].

Physical stability test

The physical stability of the optimized NPs and lyophilized NPs were investigated from the point of view of EE and particle size changes in a period of three months at room temperature and in the refrigerator.

Statistical analysis

All studies were carried out in triplicate and data were reported as a mean ± SD. One-way ANOVA and multiple comparisons (Tukey's test) were used to assess the significance of the differences between the various groups, and $p < 0.05$ was considered statistically significant.

Table 3 Particle size, encapsulation efficiency, and zeta potential of TM NPs (mean ± SD, $n = 3$)

Formulation	CS: carbomer	EE (%)	Particle Size (nm)	Zeta potential (mv)
CC1	1:1	39.62 ± 8.68	278.6 ± 14.3	+ 22.2
CC2	1:2	69.98 ± 16.23	196.3 ± 11.0	+ 15.7
CC3	1:5	44.32 ± 20.42	154.0 ± 22.6	-23.8
CC4	1:10	46.57 ± 25.21	1340.5 ± 323.6	-19.0

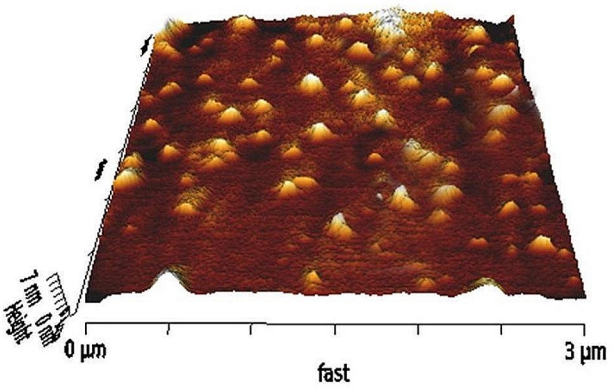

Fig. 1 AFM image of CS/carbomer nanoparticles (CC2)

Results

Particle size, drug encapsulation efficiency, and Zeta potential of nanoparticles

As can be seen in Table 3, all tested formulations, except CC4, resulted in nanometer-sized particles. The average encapsulation efficiency (EE) of the designed formulations was between 39.62 and 69.98%. The maximum average EE was for CC2 formulation. Zeta potential values were positive for CC1 and CC2, while the other two formulations had negative zeta potentials. Since all formulations (except CC4) had acceptable particle size, CC2 with positive zeta potential and maximum average EE was used as the selected TM-loaded nanoparticles formulation.

Table 4 The consistency of in situ gel samples (IG1-IG9) at pH 7.4

Formulation	Carbomer (% w/v)	HPMC (% w/v)	Consistency
IG1	0.1	0.1	+ +
IG2	0.1	0.2	+ +
IG3	0.1	0.3	+ + +
IG4	0.2	0.1	+ + + +
IG5	0.2	0.2	+ + + +
IG6	0.2	0.3	+ + + +
IG7	0.3	0.1	+ + + +
IG8	0.3	0.2	+ + + +
IG9	0.3	0.3	+ + + +

++ Gelation after few seconds, remains for few hours; +++ Gelation immediately, remains for few hours; ++++ Production very stiff gel

Morphology of the nanoparticles

Figure 1 displays the tapping mode AFM height image of CS/carbomer NPs (CC2) in a dry state on the mica plate. The topography of CC2 shows the size of nanoparticles is around 200 nm, which confirms the result obtained by the DLS method (Table 3).

Rheological and viscosity assessment

All formulations, IG1-IG9, had sufficient fluidity, but at pH 7.4 a sudden increase in consistency occurred. The observed changes in consistency are shown in Table 4. Formulations IG4 to IG9 with higher levels of carbomer concentration were observed to provide highly consistent gels at pH 7.4 without fluidity when the container was inverted. These formulations under physiological condition create stiff gels that can be annoying to the eyes. However, formulations IG1 to IG3 with minimum secondary consistency were chosen for further viscosity evaluation.

Rheograms of IG1, IG2, and IG3 at initial and physiological pH are shown in Fig. 2 (a-c). Based on visual

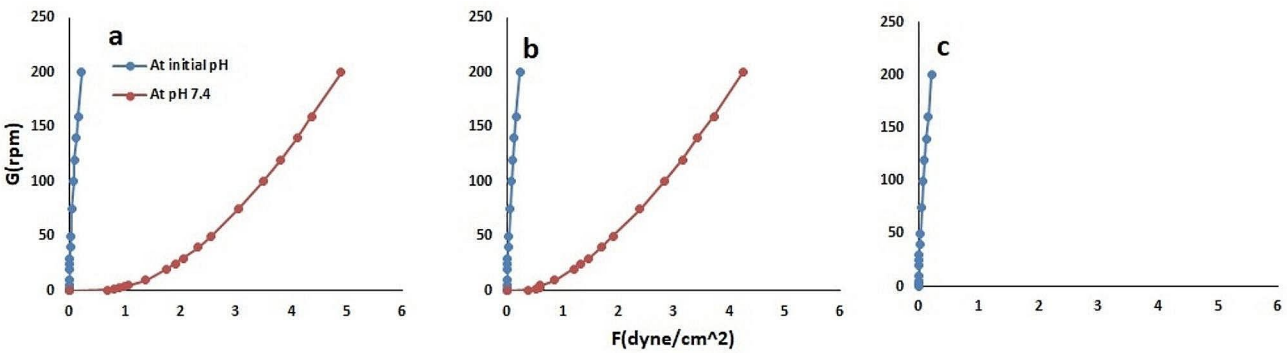

Fig. 2 Rheograms of in situ gels at initial and physiological pH: (**a**) IG1, (**b**) IG2, and (**c**) IG3

Table 5 Rheometric parameters of the selected in situ gel and nanoparticles laden in situ gel formulations at initial and secondary pH (7.4)

pH	Formulation	*N*	Ŋ (Cp)
Initial	IG1	0.861	7.1
	IG2	0.820	8.1
	IG3	0.739	10.5
	CC2-IG1	0.708	6.4
	CC2-IG2	0.808	7.6
	CC2-IG3	0.751	13.5
Secondary	IG1	1.888	432.5
	IG2	2.533	1426.9
	IG3	-	-
	CC2-IG1	0.990	6.8
	CC2-IG2	1.015	9.4
	CC2-IG3	1.042	19

Table 6 Physicochemical characteristics (zeta potential, pH, refractive index, and surface tension) of the optimized formulations

Formulation	Zeta potential	pH	Refractive Index (nD)	Surface tension (*N*/m)
CC2	+15.7	5.16 ± 0.060	1.3350 ± 0.0000	0.064 ± 0.000
CC2-IG3	-11.1	5.04 ± 0.025	1.3353 ± 0.0000	0.068 ± 0.000

Data are presented as mean ± SD ($n = 3$)

Table 7 Turbidometric measurement of the interaction of mucin and the formulations (mean ± SD, $n = 3$)

Sample	Abs (nm)	A_{pre}	ΔA
Mucin solution	0.220 ± 0.001	-	-
CC2	0.040 ± 0.005	-	-
CC2-IG3	0.123 ± 0.010	-	-
Mucin/CC2	0.416 ± 0.013	0.260	0.156
Mucin/CC2-IG3	0.566 ± 0.047	0.343	0.223

evaluation, IG3 had a higher secondary viscosity than IG1 and IG2 at pH 7.4, as well as compared to its primary viscosity. It should be noted that due to the high consistency of IG3 at this pH, this viscosity cannot be measured by the same method. Table 5 represents N and η values for selected in situ gel (IG) and NPs-laden in situ gel formulations at initial and physiological pH. As can be seen, all formulations had higher viscosity coefficients after pH adjustment. The results showed a direct relation between HPMC concentration and viscosity, although, NPs-laden IG formulations showed less viscosity increment after pH adjustment.

Characterization of NPs and NPs-laden in situ gel formulations

The pH, refractive index, and surface tension of two selected formulations are reported in Table 6.

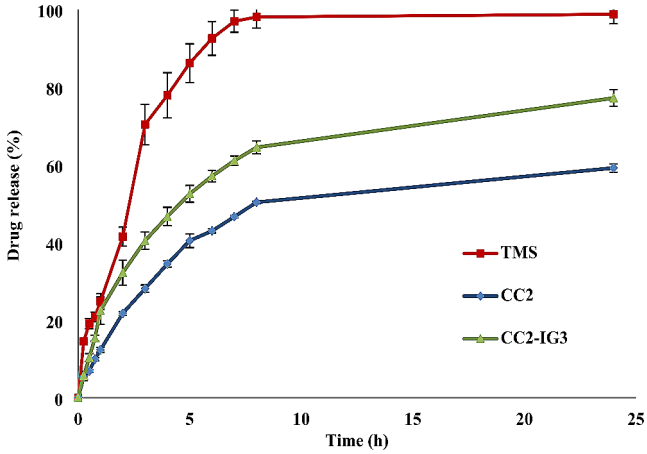

Fig. 3 In vitro release of TM from CC2, CC2-IG3, and TMS (mean ± SD, $n = 3$)

Mucoadhesive capacity

As depicted in Table 7, the absorbance (A) of both CC2-mucin and CC2-IG3-mucin dispersions was greater than their predicted absorbance (A_{pre}), which was calculated from the sum of the separate absorbances of the mucin solution and the formulations. The positivity of the difference between the measured and predicted absorbance values (ΔA) is representative of the action between the mucin and the formulation components and confirms the mucoadhesive ability of the formulation [34, 35]. However, the larger ΔA value obtained for CC2-IG3 indicates the higher bioadhesive strength of this formulation compared to CC2.

In vitro TM release profile

The cumulative percentage of TM released as a function of time from both selected formulations and TMS is shown in Fig. 3. In the case of TMS as control, about 98% of TM was released and reached a plateau within 7–8 h, while in CC2 and CC2-IG3 formulations the total drug release was 59% and 77% at 24 h, respectively.

Various models were used to analyze the kinetics of TM release from the formulations, which are presented in Table 8. The goodness of fit of each model was assessed by examining the coefficient of determination (R-squared) [44].

Kinetics modeling of the release profiles (Table 8) showed a first-order model as the best fit for drug release from TMS, suggesting that the release of TM is influenced by the concentration gradient. However, drug release from CC2 and CC2-IG3 showed highest correlation with Higuchi model. For better investigation, the obtained results were checked with Korsmeyer-Peppas kinetics equation, and the "n" values were obtained between 0.43 and 0.85 for both

Table 8 Various kinetics models for TM release from different formulations

Formulation	Kinetics model	Fitting equation	R^2	n
TMS	Zero-order	Q = 0.119 t + 0.178	0.9246	-
	First order	ln(1-Q) = -0.488 t + 0.198	0.9779	-
	Higuchi	Q = 0.411 $t^{1/2}$ - 0.103	0.9720	-
	Peppas	ln Q = 0.627 ln t − 1.224	0.9720	0.627
CC2	Zero-order	Q = 0.060 t + 0.069	0.9680	-
	First order	ln(1-Q)= -0.085 t + 0.053	0.9890	-
	Higuchi	Q = 0.204 $t^{1/2}$ -0.069	0.9962	-
	Peppas	ln Q = 0.690 ln t– 2.069	0.9932	0.690
CC2-IG3	Zero-order	Q = 0.074 t + 0.119	0.9398	-
	First order	ln(1-Q) = -0.124 t -0.094	0.9845	-
	Higuchi	Q = 0.256 $t^{1/2}$ + 0.057	0.9936	-
	Peppas	ln Q = 0.682 ln t − 1.734	0.9767	0.682

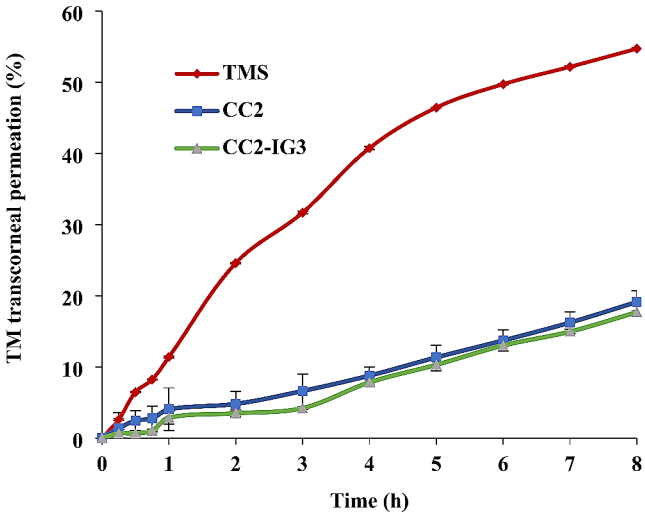

Fig. 4 Ex vivo transcorneal TM permeation from CC_2, CC_2-IG_3, and TMS (mean ± SD, $n = 3$)

Table 9 The permeability parameters of the formulations (mean ± SD, $n = 3$)

Formulation	J_{ss} (mg/cm^2.h)	P_{app} (cm/h)
CC2	0.158 ± 0.003	0.047 ± 0.001
CC2-IG3	0.157 ± 0.003	0.046 ± 0.001
TMS	*	0.225 ± 0.021

*Due to the non-establishment of a linear relationship between Q and t, it was not possible to calculate J_{ss} for TMS and its P_{app} was calculated from the logarithmic equation

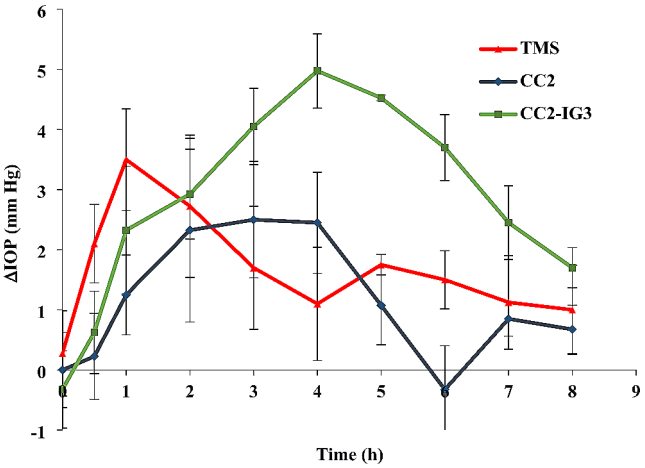

Fig. 5 IOP reduction following ocular application of CC2, CC2-IG3, and TMS in rabbit's eye (mean ± SD, $n = 4$)

developed formulations. The results indicated an anomalous release process i.e. combination of both Fickian and case II transport mechanisms. Therefore, TM release was controlled by diffusion of the solid drug dispersed in the nanoparticle matrix and erosion of polymeric matrix [46, 47].

Ex vivo transcorneal TM permeation profile

The profiles of transcorneal permeation of the drug from TMS, CC2, and CC2-IG3 are depicted in Fig. 4 and the permeability parameters of the formulations are shown in Table 9.

TMS presented higher drug permeation than the selected formulations and there was no significant difference between CC2 and CC2-IG3 in terms of corneal permeability parameters ($P > 0.05$).

In vivo IOP lowering activity

The pharmacodynamics evaluation is presented as the change in IOP (ΔIOP) versus time (Fig. 5). The application of TMS resulted in a sudden decrease in IOP of about 3.5 mm Hg in 1 h. After that, an increase in IOP was observed, which may be due to the rapid elimination of the drug from the site of action. CC2 formulation lowered the IOP at a slower rate to about 2 mmHg at the end of 2 h, and the effect persisted for about 2 h. Thereafter, a gradual increase in the IOP was observed. In the case of CC2-IG3, the peak effect was obtained at 4 h with the highest IOP reduction value of about 5 mmHg. However, the IOP reduction was greater and more long-lasting by the CC2-IG3 formulation compared to TMS and CC2.

Ocular irritation

The observations did not show significant inflammation, redness, or macroscopic irritation in the rabbits' eyes. The obtained eye cumulative irritation scores were 0.4, 0.25, and 0.3 for TMS, CC2, and CC2-IG3, respectively [43]. Given that their cumulative irritation score is less than 3, they are considered non-irritant. Due to the non-toxic nature of the used polymers (chitosan, HPMC and carbomer) as well as the safe pH and RI range of the final formulation, no irritation was observed in the eyes of rabbits for CC2-IG3 as presented in Figs S6.

Stability test

The study of physical stability of CC2 and lyophilized CC2 was conducted at room temperature and in the refrigerator for 3 months. The results are shown in Figs S2-S5. As can be seen, for non-lyophilized NPs, the EE has significantly decreased over time in both storage conditions, which indicates the gradual leakage of TM from the NPs in the vehicle. The particle size of NPs also increased significantly with time. However, the lyophilized NPs showed very high stability to changes in EE and particle size. In none of the cases, the storage conditions in the refrigerator did not show an advantage over the room, and the results and particle size did not differ significantly in both environments.

Discussion

The Polyelectrolyte complexation method was used to form NPs. It is a safe and green process without the use of organic solvents, surfactants, or cross-linkers. The PECs were formed in water based on electrostatic (ionic) interaction between positively charged ammonium groups of chitosan and negatively charged carboxyl groups of carbomer [19]. Particle size analysis confirmed the formation of nanometer-sized particles (except for CC4).

Zeta potential is the electrical potential difference between the charge on a particle at the shear plane and the liquid that surrounds it [48]. Among the NPs formulations, both CC1 and CC2 which were prepared using higher CS concentration (0.1%) showed positive zeta potentials. In the case of CC2-IG3 although it contained CC2, a negative zeta potential was recorded (Table 6). This may be due to the excessive amount of polymers, especially carbomer, added to prepare the in situ gel.

CC2 NPs showed bio-adhesive properties, which could result from the interaction of the positively charged NPs with negatively charged sialic acid on the surface of the eye [49]. This feature leads to their long-term residence and drug delivery in the eye. Despite the negative zeta potential, mucoadhesive evaluation still confirmed bioadhesive properties for CC2-IG3. It can be assumed that the mucoadhesive characteristic of CC2-IG3 is related to carbomer and is caused by the electrostatic and hydrophobic interactions, and hydrogen bonds between polyacrylic acid and mucin, which increase the mucoadhesive capacity of carbomer. Moreover, based on diffusion theory, bioadhesive polymer chains interpenetrate into mucin glycoprotein chains, and form semi-permanent bonds with the mucous surface [50–52].

The administration of ophthalmic preparations should have the least interference with the pseudo-plastic character of the precorneal film. Ocular shear rate is about 0.03 s^{-1} during inter-blinking periods and can reach very high shear rates of about 4250–28,500 s^{-1} during blinking, thus viscoelastic fluids with high viscosity under low shear rate conditions and low viscosity under high shear rate conditions are often preferred [53]. Consistency evaluation confirmed the sol-to-gel transition of all IG1 to IG9 samples after pH adjustment to about 7.4. The results further show that the concentration of carbomer has a more decisive effect on the secondary viscosity than HPMC, which is consistent with the characteristic of carbomer as a polymer sensitive to increasing pH. When the carbomer is neutralized with an alkali, the polyacrylate branched chains interconnected by cross-links start to hydrate and partially open due to electrostatic repulsion to form a fine gel mass that absorbs and retains water [54]. Rheological assessment for IG1, IG2, and IG3 samples indicated dilatant and pseudoplastic behavior at initial and secondary pH, respectively. Formulations CC2-IG1, CC2-IG2, and CC2-IG3 also showed dilatant behavior at initial pH and unexpected Newtonian behavior after pH adjustment. In physiological pH, TM-loaded NPs-laden in situ gel formulations obtained lower viscosities compared to other in situ gel systems. It seems that the positive zeta potential of CC2, which is caused by chitosan strands and timolol ions, interacts with the negative charge of the carboxyl groups of carbomer in the continuous phase. This interaction interferes with the formation of carbomer-water hydrogen bonds and reduces the final viscosity of the gel.

In the release study, both CC2 and CC2-IG3 formulations showed slower drug release than TMS. This means that the designed nanoparticles can play a role in controlling the release of TM because the encapsulated TM cannot be rapidly released from the NPs. The NPs-laden in situ gel formulation was expected to have a more sustained release than the NPs formulation due to the retention of the nanoparticle system in its polymer network, but the results were different. The higher amount of drug release from CC2-IG3 compared to CC2 may be due to the presence of TM released in the in situ gel during storage time.

The transcorneal permeation test showed no significant difference between the permeability of CC2 and CC2-IG3 formulations ($P > 0.05$). It seems in situ gelation did not affect this process. However, drug penetration was faster for TMS than them. This is contrary to the common expectation that chitosan nanoparticles can increase corneal permeability to drugs [12]. Probably, corneal permeation is a function of drug release from the NPs, and the nanoparticles either do not pass through the cornea or release the drug slowly after passing.

In clinical studies, TMS caused a rapid decrease in IOP, and the effect was faster than CC2 and CC2-IG3. These results are consistent with the in vitro data, so that the release rate of TM from TMS was much faster than that from other formulations. Despite rapidly lowering IOP, TMS showed a short duration of effect, indicating rapid removal of the drug from the eye. Hence, it was not able to sustain the activity for a long period of time requiring repeated administration of the formulation.

Compared with TMS, the lower rate of IOP reduction by CC2 is probably because the drug is entrapped inside the NPs and requires more time for release. On the other hand, its longer effect could be caused by the interaction of the positive charges of CS nanoparticles with the negative charges of the mucosal sialic acid residues, leading to long term ocular retention of CC2 (35). However, CC2-IG3 formulation showed a much higher effect intensity and durability compared to TMS and CC3. This may be due to having a greater mucoadhesive property and achieving high viscosity at eye pH.

pH is a concern as a parameter that affects tolerability and efficacy. The pH value of tear fluid is about 7.4 and due to the buffering capacity of tears, pH values in the range of 4 to 8 can be tolerated by the eye [55]. The developed formulations had pH values of 5.16 (CC2) and 5.04 (CC2-IG3) which corresponded to the acceptable range and probably did not cause eye discomfort due to irritating pH.

Ideal eye drops should have refractive index values matching the range especially not higher than 1.47 [56]. As reported in Table 5, the refractive index values of both developed formulations are not expected to cause visual impairment.

Surface tension was measured as a very important parameter in the effectiveness of eye formulations. Less surface tension causes better distribution of the product on the cornea and better contact between them [32]. The surface tension of the lacrimal fluid ranges from 0.04 to 0.05 N/m and for water it is from 0.068 to 0.072 N/m [57]. The two developed formulations had a surface tension in the range of water's surface tension.

Conclusion

In this study, the PEC method, which is a simple and safe method without using cross-linkers, organic solvents and surfactants, was used to prepare the formulations. Both CC2 and CC2-IG3 formulations proved to have the property of mucoadhesive, and sustaining drug release. These features can be effective in prolonging their ocular therapeutic effect. However, the reduction in IOP with CC2-IG3 formula was greater and the duration of effect was longer compared to CC2. CC2-IG3 benefits from the advantages of both nanoparticles and in situ gel systems, including controlled release, mucoadhesive properties, and high viscosity at physiological conditions.

Considering characteristics accepted in ophthalmic products such as pH, refractive index value, non-irritancy, and safety, CC2-IG3 shows potential as a promising ophthalmic drug delivery system to prolong therapeutic activity, although further investigation through pharmacokinetic studies is required to determine its impact on ocular bioavailability.

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Data availability The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval The present study was designed and conducted with the permission of the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (2 July 2022/IR.AJUMS. REC.1401.126).

Consent for publication All authors are satisfied with this publication.

Competing Interests The authors declare no conflict of financial or non-financial interests.

Conflict of interest All authors declare that they have no conflict of interest.

Ethical standards The experiments comply with the current laws of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

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