Letters in Applied NanoBioScience
Open-Access Journal (ISSN: 2284-6808)

Article
Volume 15, Issue 1, 2026, 18
https://doi.org/10.33263/LIANBS151.018

Nanomicellar Delivery of Pilocarpine Prodrug for Improved Glaucoma Therapy in Rabbits

Ajay Kumar Gupta [1,2,*], Mona Gupta [1]

[1] Department of Chemistry, University of Delhi, Delhi-110007, India; akgupta25@hotmail.com;
[2] Present Address: Centre for Advanced Research Studies, Ganpat University, Kherva, Dist. Mehsana - 384012, Gujarat, India
* Correspondence: director.research@ganpatuniversity.ac.in;

Received: 26.10.2024; Accepted: 25.02.2026; Published: 30.03.2026

Abstract: Copolymeric micelles composed of a copolymer of N-isopropyl acrylamide (NIPAAM), N-vinyl pyrollidone (VP), and acrylic acid (AA) cross-linked with N, N'-methylene bis acrylamide (MBA) have been used as carriers for an anti-glaucoma drug, pilocarpine prodrug (bispilocarpic acid 1,2-ethylene monoester). Up to 40% w/w of the pilocarpine prodrug has been entrapped in these copolymeric micelles, with an entrapment efficiency of around 70%. The size of the pilocarpine prodrug-encapsulated copolymeric micelles was measured by dynamic light scattering and found to be temperature-dependent. At 25°C, these micelles have an average size of around 35 nm in diameter with a narrow size distribution. The transmission electron microscope image shows that these micelles are spherical. About 45% of the prodrug was released from the copolymeric micelles in phosphate-buffered saline (PBS, pH 7.2) in 6 hours, compared to only about 18% in 2 hours. *In vitro*, corneal permeation and corneal hydration studies have indicated (i) enhanced corneal permeability compared to an aqueous suspension of an equal amount of prodrug and (ii) no corneal damage, respectively. Pilocarpine prodrug-loaded copolymeric micelles achieved more efficient ocular delivery by reducing the drug's irritation potential and improving its intraocular pressure (IOP)- lowering response. The nanoparticle solution achieved a maximum reduction in IOP from 33.0-35.0 mmHg to 22.0 mmHg after 6 hours and lasted for more than 9.0 hours, whereas with the control formulation, the maximum reduction in IOP was to 24.0–26.0 mmHg after 2.0 hours, which lasted only 4.5 hours. From the results, it may be concluded that this micellar formulation can be effectively used for anti-glaucoma therapy with higher therapeutic efficacy at a much lower concentration of the drug.

Keywords: copolymeric micelles; ocular delivery; pilocarpine prodrug; corneal permeation; intraocular pressure.

1. Introduction

Pilocarpine is a direct-acting cholinergic agonist used for the control of elevated intraocular pressure (IOP) associated with glaucoma [1,2], which increases the outflow facility of aqueous humor. Unfortunately, being highly water-soluble (log $P_{app} \sim 0.01$) [3], pilocarpine from its aqueous formulation, when applied topically, does not penetrate into the eye and is lost by physiological drainage [4]. Ocular absorption of pilocarpine can be enhanced substantially either by increasing its lipophilic character [5] or incorporating it into some drug carrier [6–8] or both. By converting a drug into its prodrug derivative, its water solubility, hydrophobicity, and pKa values can be changed [9]. The disadvantages of these pilocarpine ester derivatives are significant ocular irritation and low aqueous solubility at physiological pH values.

To overcome these drawbacks, literature has reported utilizing many new strategies such as hydrogels [10–12], mucoadhesive polymers [13,14], liposomes and nanoparticles [15,16], which improve the ocular bioavailability of the drugs. Numerous ophthalmic formulations have been reported in the literature using alkylcyanoacrylate nanoparticles [17,18]. In all these studies, the ocular bioavailability was not found to be satisfactory [16,18], probably because these nanoparticles do not have combined properties of ultra-low size (preferably below 50 nm diameter), high entrapment efficiency and sustained release of the drug under physiological conditions (i.e. pH and temperature of the eye) after topical administration in the eye.

Copolymeric micelles have been increasingly investigated as delivery carriers for hydrophobic drugs over the last decade [1]. These copolymeric micelles are formed as a result of self-assembly of amphiphilic copolymers in spherical shape having a core–shell-type structure. The core is hydrophobic and can be used to load hydrophobic drugs, thereby enabling the delivery of poorly soluble drugs. The outer shell is hydrophilic, with a brush-like corona, making these micelles water-soluble [3]. This paper describes the preparation and characterization of cross-linked copolymeric micelles (size less than 50 nm diameter) made of N-isopropyl acrylamide (NIPAAM), N-vinylpyrrolidone (VP), and acrylic acid (AA) containing a prodrug of pilocarpine encapsulated into the polymeric network. In this study, we synthesised pilocarpic acid diesters to overcome the stability concerns of pilocarpic acid monoesters. These diesters were highly stable and had shelf-lives of more than 5 hours (pH 5-6, 25°C). The prolonged action is due to the fact that diesters are first converted to the corresponding monoesters by enzymatic hydrolysis under physiological conditions [3]. Also, the *in vitro* release behaviour, transcorneal permeation, ocular irritation, and in vivo intra-ocular pressure (IOP) lowering effects of the drug-loaded copolymeric micelles have been reported in this paper. NIPAAM has been used to form stable micellar aggregates with hydrophobic cores mainly composed of the isopropyl moiety [19]. VP renders the hydrogel property of the copolymer [20], and AA has been added to make the copolymer pH-sensitive [21]. The incorporation of acrylic acid also gives mucoadhesiveness to the copolymer [22], which may impart the increased retention time of the polymer on the corneal surface.

2. Materials and Methods

2.1. Materials.

N-isopropylacrylamide (NIPAAM) was purchased from Ranbaxy Acros (India). The compound was stabilized with 500 ppm p-methoxyphenol. It was crystallized from a saturated solution of n-hexane before use. N, N'-methylene *bis*acrylamide (MBA) was the product of Sigma (Dorset, U.K.), and was used as received. Acrylic acid (AA), n-hexane, sodium monohydrogen phosphate, dihydrogen phosphate, and ferrous ammonium sulphate (FAS) were purchased from Sigma (Dorset, U.K.); N-vinylpyrrolidone (VP) was purchased from Fluka (Dorset, U.K.). 99.8% pure ethanol was purchased from Merck, Germany. Before use, acrylic acid and vinyl pyrrolidone were distilled under low pressure. Pilocarpine hydrochloride was purchased from Sigma (Dorset, U.K.) and was used as supplied.

2.2. Synthesis of pilocarpine prodrug.

The bispilocarpic acid ester was prepared by esterifying the sodium salt of pilocarpic acid (sodium pilocarpate) with alkyl dihalogenide as described elsewhere [23]. Sodium pilocarpate was synthesized as described by Bundgaard *et al*., with some modifications [24].

2.2.1. Synthesis of sodium pilocarpate.

To a solution of pilocarpine hydrochloride (0.196 g, 0.80 mmol) in 2.0 mL of water, kept in an ice-water bath, 0.9 mL of ice-cold 2.0 M sodium hydroxide (NaOH) was added gradually in four steps. The solution was kept undisturbed at 0-4°C for one hour. Excess NaOH was neutralized by adding 1.0 M hydrochloric acid (HCl) (to pH ~ 9.0). The solution was then lyophilized immediately to prevent isomerization of sodium pilocarpate to sodium isopilocarpate. The lyophilized solid powder was slurried in 3.0 mL of ethanol and stirred at 60°C for about 10 minutes. The resulting suspension was cooled to 4°C, and the precipitated sodium chloride was separated by filtration. The ethanol in the filtrate was vacuum-evaporated, and the resulting compound was dried in a vacuum desiccator to obtain sodium pilocarpate as a white, semicrystalline, hygroscopic material.

2.2.2. Synthesis of bispilocarpic acid ester.

Sodium pilocarpate (0.20 g, 0.8 mmol) was dissolved in 20.0 mL dimethylsulphoxide, DMSO. Dichloroethane (0.3 mmol) was added in DMSO over a period of 1.0 hour. The above solution was stirred at room temperature for 48-72 hours and poured into 10.0 mL of distilled water. The mixture was extracted with two 10.0 mL portions of chloroform. The combined chloroform extracts were washed with 10.0 mL of distilled water, 10.0 mL of 2% sodium bicarbonate solution, and 10.0 mL of distilled water, respectively. Chloroform was evaporated under reduced pressure, and the resulting residue was dried on calcium chloride in a vacuum desiccator to give bispilocarpic acid 1,2-ethylene monoester (Figure 1).

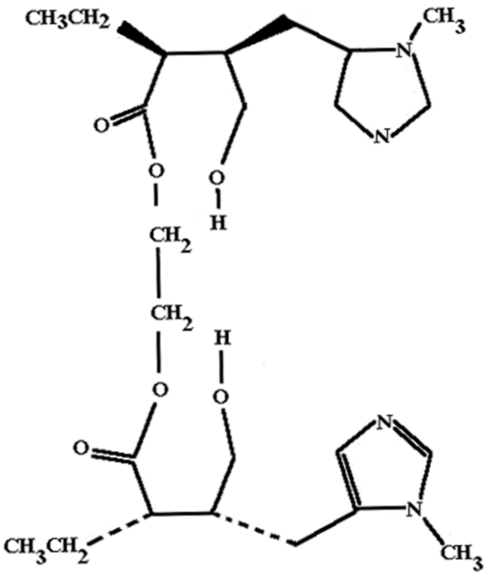


Figure 1. Structure of pilocarpine prodrug (bispilocarpic acid 1,2-ethylene monoester) used in the present investigations. These diesters are hydrophobic and stable, which can be encapsulated inside the micellar formulation for effective anti-glaucoma therapy.

The monoester was purified from a chloroform/ether mixture to give the pure prodrug of pilocarpine (pKa ~ 6.9). The formation of bispilocarpic acid ester (Figure 1) was characterized through Fourier-transformed infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy [24]. It was found that the prodrug derivative (log P = 2.765) was much more lipophilic than pilocarpine (log P = 0.01) as determined from partitioning between 1-octanol and phosphate buffer saline (PBS, pH 7.2) at room temperature.

2.3. Synthesis of copolymer.

A copolymer of NIPAAM, VP, and AA was synthesized through a free radical polymerisation mechanism as described previously [24]. Briefly, water-soluble monomers, i.e., NIPAAM, VP, and AA were used in an 85.7:9.5:4.8 molar ratio, and the polymer was cross-linked with MBA. In a typical experimental protocol, we used 900 mg NIPAAM, 100 μL freshly distilled VP, and 50 μL AA in 100 mL water. Polymer chains were cross-linked by adding 150 μL of MBA (0.049 g/mL). 100 μL of ferrous ammonium sulphate (FAS) (0.05% w/v) and 150 μL of ammonium persulphate (APS) (20% w/v) solutions were added to begin the polymerization process. The polymerisation process was carried out at 30°C for 24 hours in a nitrogen gas atmosphere. After the polymerisation reaction was complete, the solution was then dialyzed using a 12 kDa cut-off dialysis membrane. The sample was then frozen in liquid nitrogen and freeze-dried to obtain dry powder for further use. The above method produced void copoly(NIPAAM-VP-AA) micelles cross-linked with MBA.

2.4. Loading of pilocarpine prodrug.

Before loading the prodrug in the micelles, the critical micelle concentration of the copolymer was determined as 500 ng/mL [21] using pyrene as a fluorescent marker molecule. A poorly water-soluble diester of pilocarpine was loaded into copoly(NIPAAM-VP-AA) micelles as follows: 100 mg of the freeze-dried copolymeric dry powder was dispersed in 10 mL of distilled water and stirred continuously to obtain an optically clear micellar solution. Pilocarpine prodrug was dissolved in chloroform (100 mg/mL) and added to the copolymeric micelle solution slowly with constant stirring. The solution was then warmed gently to 40-50°C to evaporate the chloroform completely, then cooled to room temperature. Because of the hydrophobicity of the inner core of the micelles, the hydrophobic prodrug got entrapped into the hydrophobic core of the micelles. The prodrug-loaded copolymeric micelles were dialyzed in 5 litres of double-distilled water for 4 hours, with the water changed every 1 hour to remove the unentrapped drug. The dialysed solution was lyophilized immediately to get dry powder for subsequent use.

2.5. Calculation of entrapment efficiency (E%).

The E% of copoly(NIPAAM-VP-AA) micelles loaded with pilocarpine prodrug was determined as follows: Unentrapped free prodrug was separated fro the pilocarpine prodrug loaded Copoly(NIPAAM-VP-AA) micelles by using Millipore UFP2THK24 (100 kD cut off) membrane filter, and the amount of free prodrug in the filtrate was determined by using a UVIKON 930 spectrophotometer at λ_{max}= 215 nm. The entrapment efficiency (E%) was calculated as:

$$E\% = ([PP]_{total} - [PP]_{free}) \times 100 \; / \; [PP]_{total} \qquad (1)$$

Where, [PP]= Pilocarpine prodrug concentration.

2.6. Measurement of size and morphology of the copolymeric micelles.

2.6.1. Dynamic light scattering (DLS) measurements.

The average size and size distribution of the prodrug-loaded copoly(NIPAAM-VP-AA) micelles were determined by DLS measurements. A Brookhaven (USA) dynamic light scattering instrument with a BI 200 SM Goniometer and BI8000 autocorrelator was used for the DLS studies. An air-cooled argon ion laser that was operated at 488 nm was used as a laser light source. The lyophilized powder of prodrug-loaded copolymeric micelles was dispersed in a buffer solution of pH 7.2 and filtered through a Millipore, USA, filtration unit having an average pore size of 0.2 μm. The scattered light intensity was measured at 90° to the incident beam by the DLS instrument and analyzed using a 128-channel digital correlator. The self-diffusion of particles was determined from the time-dependent autocorrelation function of the scattered intensity, and the Stokes-Einstein equation was used to calculate particle size. The instrument was checked and calibrated with a suspension of standard latex particles of known size, supplied by Brookhaven Ltd.

2.6.2. Transmission electron microscopy (TEM) studies.

A TEM image of the drug-loaded copoly(NIPAAM-VP-AA) micelles was obtained using a Philips (USA) model EM300 transmission electron microscope at 85,000× magnification and 80kV. Lyophilized powder of prodrug-loaded copolymeric micelles was dispersed in distilled water (~5 mg/mL), and a drop of this solution was kept on a membrane-coated grid surface by using a filter paper (Whatman No.1). A drop of 1% w/v phosphotungstic acid, as a staining reagent, was thereafter added to the surface of the grid. Excess fluid from the grid surface was removed after one minute, and it was air-dried at room temperature before putting it into the electron microscope.

2.7. In vitro release behavior in aqueous buffer.

10 mg of pilocarpine prodrug-loaded copolymeric micelles, lyophilised powder, was dispersed in 1.0 mL of 0.1 M phosphate-buffered saline (pH 7.2) and transferred to dialysis tubing (3.5 kDa cut-off membrane). The dialysis bag was then placed in a bottle containing 50 mL of buffer solution. The bottle was placed in the shaking water bath and stirred at 25°C and 50 rpm. After every 1 hour, 1.0 mL solution was removed, and pilocarpine prodrug concentration (after partitioning in 1-octanol) was determined spectrophotometrically at λ_{max}= 215 nm. The release medium was replaced with fresh buffer every hour for 6 hours to maintain sink conditions during the release experiments.

2.8. In vitro transcorneal permeation studies.

Excised goat corneas were used to determine the *in vitro* transcorneal permeation studies [25]. The goat eyeballs were brought in cold normal saline from the local butcher shop within 1 h of the animal's death. Forceps were used to delicately remove the lens and then the iris, leaving the cornea as a transparent film. The goat corneas were isolated, and 2-4 mm of surrounding scleral tissue was retained with the membranes. The isolated corneas were thoroughly cleaned with refrigerated normal saline. A Franz all-glass diffusion cell was used to study the corneal permeation experiment. It has two chambers: the upper one is the donor, while the bottom one is the receptor chamber. The cornea was then mounted between the donor and receptor chambers of the diffusion cell in such a way that the epithelial layer of the cornea faces towards the donor chamber of the diffusion cell. Corneal area available for permeation was 0.64 cm^2. One millilitre of prodrug-loaded copolymeric micellar solution (10.0 mg/mL, PBS buffer, pH 7.4) was placed in the donor chamber, i.e., on the top of the cornea. The opening of the donor chamber was sealed with parafilm to prevent evaporation of the liquid formulation of the drug. The receptor was filled with 50 mL of PBS (pH=7.4), and all air bubbles were removed. The pH of the buffer solutions was similar in both chambers. Mixing was achieved by bubbling a mixture of 95% O_2 and 5% CO_2, and the chambers were thermostated at 37°C. Water was constantly passed through the outer jacket of the receptor cell, while receptor cell solution was continuously mixed to maintain uniformity. At 30-minute intervals over the 6h period, a 2.0 mL sample was withdrawn from the receptor chamber through the sampling port, and an equal volume of buffer was refilled to maintain sink conditions. The drug content of the sample withdrawn was determined spectrophotometrically at λ_{max}=215 nm. Pilocarpine prodrug dispersed in aqueous buffer solution was used as the control. Each experiment was repeated three times, and the results were presented as mean ± S.D.

2.9. Corneal hydration (H%) studies.

At the end of the corneal permeation experiment, the cornea was washed several times with cold normal saline, freed of the scleral ring, and its weight was measured. The corneal membranes were then dried in an oven at 80°C until constant weight was achieved to determine corneal hydration. H% was calculated as follows:

$$H\% = (W_w - W_d) \times 100 / W_w \qquad (2)$$

Where, W_w = weight of wet cornea and W_d = weight of dry cornea.

2.10. Ocular irritation studies.

All Animal studies were conducted after obtaining approval from the Institutional Animal Ethics Committee (IAEC) of Ganpat University, Gujarat, India, in compliance with CPCSEA guidelines. Adult New Zealand rabbits of both sexes weighing 2.0-2.5 kg were used in this study (N=5 per group). The rabbits were kept singly in the cages and fed standard feed. All experiments were conducted in accordance with the ARVO guidelines on the use of animals in ophthalmic research. Pilocarpine prodrug-loaded copoly(NIPAAM-VP-AA) micelles were dispersed in water. Sodium chloride was used to make the isotonic solution with a pH of 7.4. The prodrug-loaded micellar solution was equivalent to 1% w/v pilocarpine. Isotonic solutions of pilocarpine hydrochloride and pilocarpine prodrugs were also prepared and used as control formulations. The right eye was used as a control, while the contralateral eye received a drop of 50 µl of the test solutions. After instillation of eye drops, the discomfort level was measured on the basis of whether the eyes were closed or half-open. These studies were termed as eyelid closure studies [5]. Five rabbits were used for each formulation in these studies.

2.11. In vivo measurements of intraocular pressure (IOP) reduction: betamethasone model.

The intraocular pressure reduction experiments were carried out with New Zealand albino rabbits weighing 2.0-2.5 kg [N=5 per group]. The potency of pilocarpine prodrug-loaded micellar formulations was assessed using animals with elevated IOP values. In this study, we used a method similar to that of Jackson *et al.* [2]. Hydrodynamic properties in this model are quite similar to those in open-angle glaucoma in humans. Ocular hypertension in the animals was induced by subconjunctival injection (syringes 1.0 mL GLASS VAN INSULIN, canules 27G X 3 /4, Hindustan Syringes & Medical Devices Ltd., India) of 0.5mL betamethasone sodium phosphate injection IP (Betnesol®, Glaxo India Ltd., Nasik, India) into the left eye. Subconjunctival injection minimizes systemic cortisone effects and reduces the animal death rate. Weekly injections were given over 3 weeks. An increase in ocular pressure was observed after the third week of glucocorticoid treatment, which remained stable for 2 weeks. Only rabbits who achieved an increase of 30 mmHg or above in IOP values were chosen for the IOP reduction experiments. A tonometer (RIESTER, Germany) was used at different intervals to take the baseline tonometric measurements over a period of 9 hours. Efforts were made to ensure that a variance of not more than 2 mmHg is recorded. Animals with higher variations in IOP values were excluded from the experiments. Ten microlitres of Xylocaine® Topical 4% (ASTRA-IDL, Sweden) was administered before each measurement in order to anaesthetize the corneal surface. A commercial eye drop (Pilocar, 4.0% w/v pilocarpine eye drop, FDC Ltd., India) was used as the control formulation. A standard curve of parent pilocarpine drug was plotted using a spectrophotometer at λmax = 215 nm. The concentration of pilocarpine prodrug in copolymeric micelles was determined from the standard curve of pilocarpine. The concentration of pilocarpine in the commercial preparation was obtained from the manufacturer's information.

Each formulation was tested by instilling 50 μL into the lower conjunctival sac, and intraocular pressure (IOP) was measured at predetermined time intervals. To maintain the constant circadian variations, all studies were performed at the same time of day. After administration, IOP was measured and compared with the baseline IOP recorded at the beginning of the experiments. IOP values were measured at 0.5, 1.0, 2.0, 3.0, 4.5, 6.0, 7.5, and 9 hours.The results were plotted as the IOP values versus time, and every time point depicts the mean value of IOP recorded for 5animals.

2.12. Statistical analysis.

Each experiment was repeated three times. The statistical analysis of experimental data utilized the Student’s t-test, and the results were presented as mean ± S.D. Statistical significance was accepted at a level of $p<0.05$.

3. Results and Discussion

3.1. Synthesis and characterization of NIPAAM-VP-AA copolymeric micelles.

Copolymerization of NIPAAM, VP, and AA was performed by free-radical polymerization in water. The copolymer was characterised as described previously [24]. The critical micelle concentration (CMC) of the copolymer was determined to be 500 ng/mL [24] using pyrene as a fluorescent marker. CMC is the minimum concentration at which copolymeric chains spontaneously self-assemble and form micellar structures in solution.

As a result of micelle formation, pyrene is loaded into the hydrophobic core of the micelle. The copolymer has amphiphilic properties with both water-soluble and water-insoluble groups. Hydrophobicity in the copolymer arises from the isopropyl groups of NIPAAM and $–CH_2$-CH_2- backbone of the copolymer, whereas the amide bond of NIPAAM, pyrrolidone groups of VP, and carboxyl group of acrylic acid contribute towards the hydrophilicity of the copolymer. When dissolved in water at concentrations above the copolymer's CMC, it self-associates into micelle-like structures. This is due to the fact that the water-soluble segments get dissolved in water whereas water insoluble isopropyl groups come together by hydrophobic interactions resulting in the formation of copolymeric micelles with inner hydrophobic core that could be used to dissolve poorly water soluble molecule like pilocarpine prodrug into it. Upto 40% w/w pilocarpine prodrug could be entrapped into these copolymeric micelles with entrapment efficiency around 70%. The prodrug loaded copolymeric micelles were lyophilised immediately for further use.

The prodrug-loaded copolymeric micelles in phosphate buffer saline (pH 7.2) were found to be stable for up to 1 month at 5±3°C. However, the lyophilized powder can be stored for a longer duration under refrigeration. The lyophilized powder can be easily reconstituted in water or buffer before usage.

3.2. Size and morphology of drug-loaded copolymeric micelles.

Figure 2 shows the typical size distribution of copoly(NIPAAM-VP-AA) micelles as measured by the dynamic light scattering method. As measured by DLS, the average hydrodynamic size of the micellar formulation was found to be around 35 (4.3 nm diameter at 25°C), with a uniform size distribution exhibiting a unimodal profile.

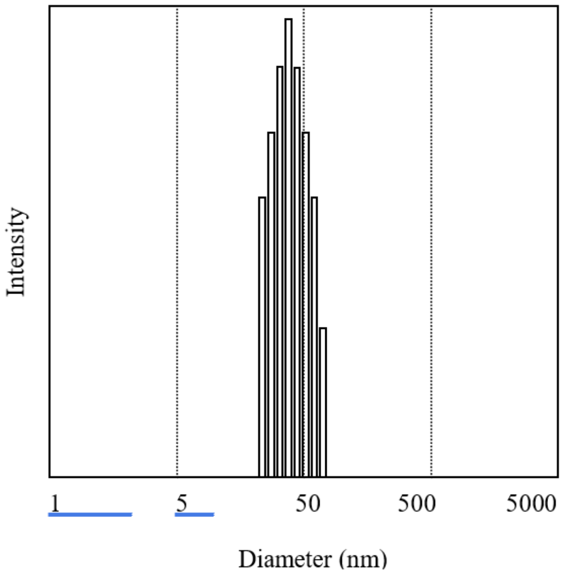
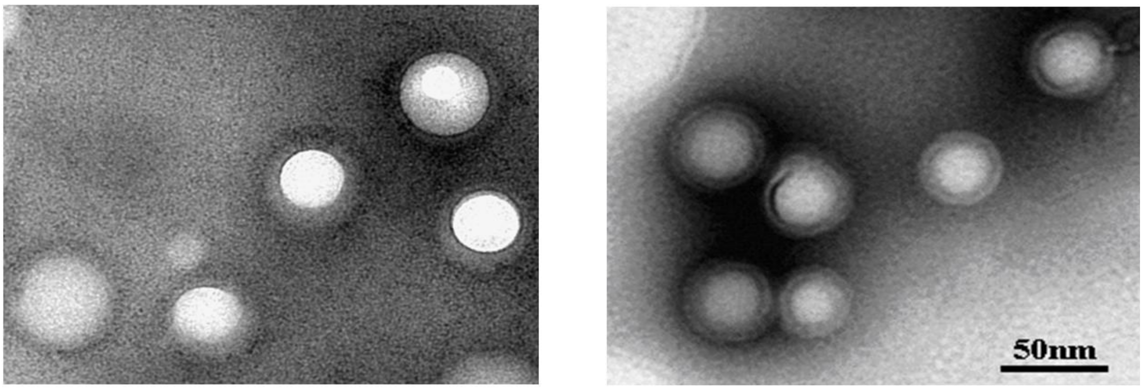


Figure 2. Typical size distribution of pilocarpine prodrug-loaded copoly(NIPAAM-VP-AA) micelles as determined by dynamic light scattering measurements. DLS measurements show that the particles have a size of about 35 nm at 25°C with a narrow size distribution.

TEM picture of the stained samples of void and prodrug-loaded copolymeric micelles is shown in Figure 3(a) and 3(b). As shown in the picture, the copolymeric micelles are predominantly monodisperse and spherical, with an approximate diameter of 40 ± 3.4 nm, consistent with the size obtained from DLS measurements. However, the size of the copolymeric micelles remains unchanged after drug loading (Figure 3b). From DLS and TEM studies, it was established that the size and morphology of the copolymeric micelles remained unchanged before and after lyophilization, and that aggregation of the copolymeric micelles did not occur during lyophilization.



(a) **(b)**

Figure 3. Transmission electron microscope picture of **(a)** void; **(b)** pilocarpine prodrug-loaded copoly(NIPAAM-VP-AA) micelles. The image indicates that the void copolymeric micelles are spherical and nearly monodisperse, with an average diameter of 40 ± 3.4 nm, consistent with the size obtained from DLS measurements.

3.3. In vitro release behavior in aqueous buffer.

The *in vitro* release profile of the pilocarpine prodrug from copoly(NIPAAM-VP-AA) micelles at pH 7.2 was determined at 25°C over different time intervals, as shown in Figure 4. The figure shows that the drug release rate from the copolymeric micelles increases over time. The percentage release at pH 7.2 at 2, 3, 4, and 6 hours was found to be 18, 25, 33, and 45%, respectively. The enhanced release of the drug was assumed to result from two mechanisms: (i) enhanced water solubility of the prodrug and (ii) the swelling of the copolymer in PBS due to ionization of AA groups [1]. Since the copolymer used to form these micelles is crosslinked with MBA, cleavage of the bonds between the copolymer and the crosslinking agent in PBS releases the entrapped prodrug [24]. The prodrug is insoluble in water, but in PBS, the ester groups of the released prodrug are hydrolyzed, and pilocarpine is formed upon closure of the lactone ring. Prodrug solubility in aqueous buffer is increased [5]. Also, highly ionized acrylic acid groups at the micellar surface are responsible for the increased swelling of the polymeric matrix.

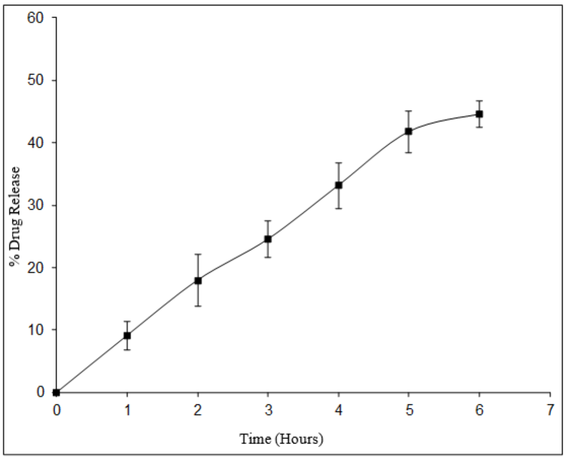


Figure 4. Release profile of pilocarpine prodrug from crosslinked copolymeric micelles in PBS (pH 7.2) at 25°C (N=5, $p<0.05$). The free prodrug released from the nanoparticles was filtered through a Millipore filter, and its concentration was determined spectrophotometrically.

This observation was further substantiated by zeta potential measurement. In acidic pH (i.e., pH 4.0), the zeta potential of the copolymeric micelles was found to be 0.6 mV because carboxylic groups are highly protonated in this pH range. However, in PBS at pH 7.2, the zeta potential changed to -11.2 mV due to the ionization of the carboxyl groups on the copolymeric micelle surfaces. These negative charges increase the porosity of the copolymeric matrix, which favors the faster diffusion of the entrapped drug.

3.4. In vitro transcorneal permeation studies.

The efficacy of the copolymeric micelles to increase the corneal penetration of pilocarpine prodrug was assayed by measuring the transcorneal flux of the drug through isolated goat cornea. Figure 5 depicts the amount of prodrug permeated from an aqueous suspension of the prodrug and from the same concentration of prodrug loaded into micelles. Results shown in Figure 5 indicate that the inclusion of the drug in the polymeric micelles significantly increases its penetration rate across the cornea. From the figure, it can be seen that about 100 µg of prodrug is permeated within 5.0 hours from an aqueous suspension of the drug. On the other hand, the prodrug entrapped in copolymeric micelles permeates to an extent of 80 (g in the initial 1 hour) and reaches a maximum value of 250 µg in 5 hours.

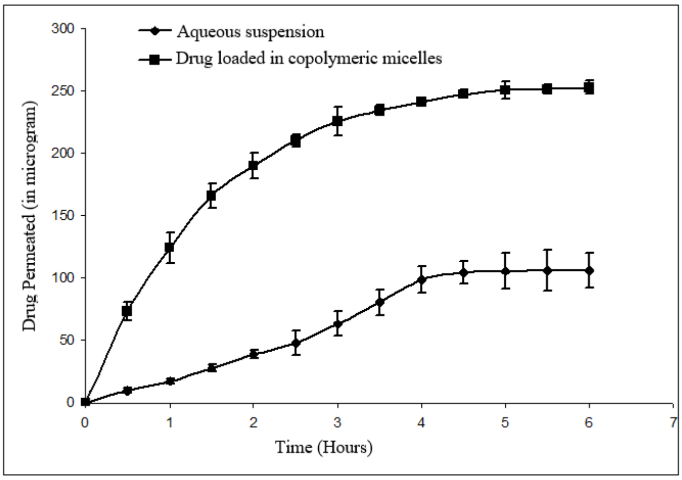


Figure 5. Comparison of permeation of pilocarpine prodrug from copoly(NIPAAM-VP-AA) micelle formulation with aqueous suspension of drug of the same concentration (2.0 mg/mL) through goat cornea. Results are represented as mean ±S.D. for N=5 ($p<0.05$).

High water solubility of pilocarpine hydrochloride contributes to the rapid loss of the drug from the precorneal area. On the other hand, the pilocarpine ester is relatively more hydrophobic. Since the epithelium layer of the corneal membrane can retain the hydrophobic drugs for longer periods of time, a greater penetration across the cornea is achieved. Another factor contributing to the enhanced permeation of the prodrug from the micellar solution is that the micelles are very small (35 nm) at 25°C. So, the corneal uptake of the ultra-low-sized drug-loaded micelles is also possible [23]. After penetrating the corneal membrane, the drug-containing micelles release the drug in a controlled manner over a long period.

3.5. Corneal hydration studies.

The corneal hydration levels after the permeation experiment were determined to assess whether the applied drug formulation causes membrane damage. It is already known that whenever there is any damage to the topmost epithelium layer, the water uptake of the stroma increases. As a result, the hydration level of the cornea increases enormously from its normal level of hydration. The corneal hydration levels were found between 79-80%, indicating the absence of any corneal damage upon topical application of these formulations [1].

3.6. Ocular irritation studies.

After instillation of eye drops, eye irritation was measured as eyelid closure time (eyes closed and half-open), and the results are shown in Figure 6. Pilocarpine caused slight ocular irritation, and the eyes were closed for 1 ± 0.5 min and half closed for 2 ± 1.0 min. The prodrug of pilocarpine showed an even stronger irritation with the eye closed for 2 ± 1.0 min and half closed for 5 ± 1.0 min. The copolymeric micelle solution was found to have very little irritation, and the eyes were half closed for only 0.2 ± 0.1 min. Administration of the hydrophobic prodrug resulted in a higher concentration of the drug in the corneal epithelium and, therefore, caused irritation. When a micelle solution is applied, the prodrug is not in direct contact with the corneal epithelium immediately, and the eye is not directly exposed to the high prodrug concentration, as in the case of the free prodrug.

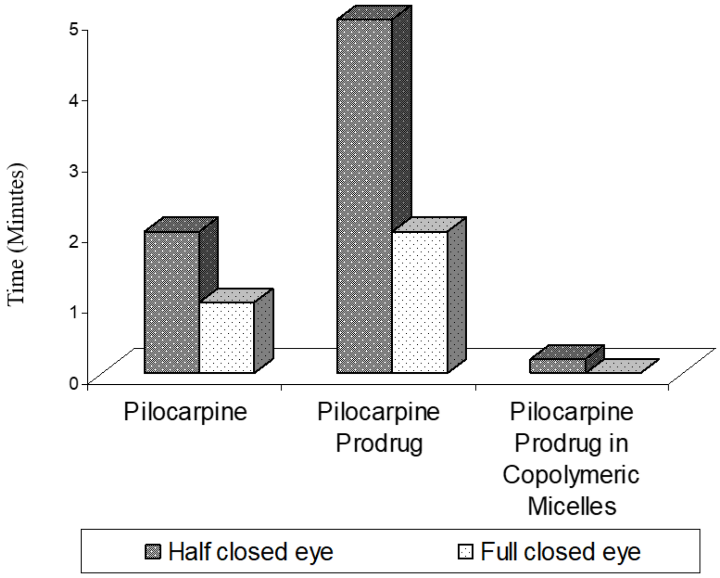


Figure 6. Duration of eyelid closure after ocular administration of pilocarpine solutions. The copolymeric micellar solution of the drug showed very little eye irritation compared to the free drug. Results are represented as mean ± S.D. for N=5 ($p<0.05$).

3.7. In vivo measurement of intraocular pressure reduction: "BETAMETHASONE MODEL".

Figure 7 shows the reduction in IOP with respect to time achieved after instillation of 50 μL of copoly(NIPAAM-VP-AA) micelles and commercial eye drops (Pilocar, 4.0 % w/v pilocarpine eye drops). It is evident from Figure 7 that the reduction in IOP with pilocarpine-loaded micelles was much higher and longer-lasting than that observed with the commercial formulation containing an equivalent amount of drug. The time profiles of the IOP response in the micellar solution were significantly different from those of the commercial formulation, serving as the control. The duration and intensity of the effect were more pronounced after administration of the prodrug-loaded micellar solution. The prodrug-loaded micellar solution gave a maximum reduction in IOP from 33.0-35.0 mmHg to 20.0-26.0 mmHg after 3.0 hours and lasted for more than 9.0 hours, whereas with the commercial formulation, the maximum reduction in IOP from 33.0-35.0 mmHg to 24.0-26.0 mmHg took place after 1.0 hour, which lasted only for 2 hours.

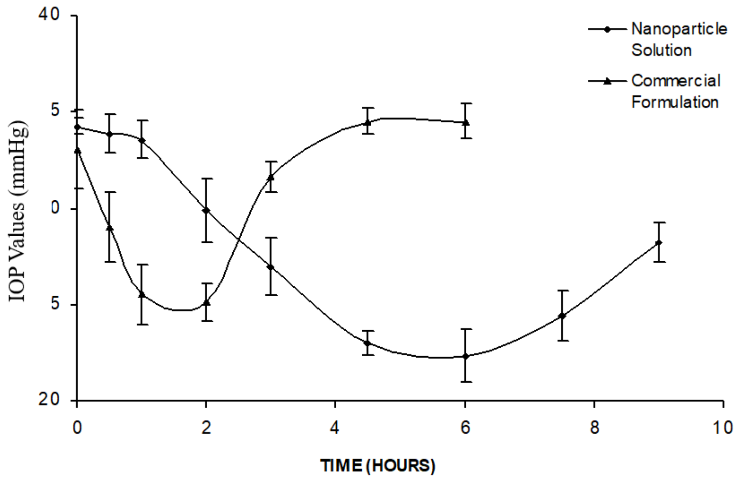


Figure 7. Reduction in IOP treated with pilocarpine nanoparticle solution and commercial eye drops of pilocarpine. Results are represented as mean ± S.D. for N=5 ($p<0.05$).

It is already known that anti-glaucoma drugs such as pilocarpine often exhibit IOP-lowering properties in hypertensive eyes. The prodrug, when encapsulated in copolymeric micelles, showed higher ocular bioavailability than free pilocarpine from the commercial formulation. Copolymeric micelles release the entrapped prodrug slowly in a controlled release fashion in the eye over a period of time. Subsequently, the ester groups of the prodrug get hydrolyzed, and pilocarpine is formed upon closure of the lactone ring. Sustained release of the prodrug can effectively reduce the IOP up to its normal values, as compared to the commercial formulation, which has an equivalent amount of drug that only partially reduces the IOP. These results suggest that ultrasmall micellar size and sustained drug release from the copolymeric micelles contribute to the enhanced therapeutic efficacy of pilocarpine.

4. Conclusions

The results indicated that the copoly(NIPAAM-VP-AA) micelles, with small size and controlled release properties, might serve as a suitable carrier for water-insoluble ocular prodrugs such as pilocarpine diester, whose clinical applications are limited due to their high eye-irritation potential. Pilocarpine prodrug-loaded copolymeric micelles achieved more efficient ocular delivery by reducing the drug's irritation potential. The pilocarpine prodrug-loaded micellar formulation demonstrated sustained therapeutic activity and enhanced the intraocular pressure-lowering response of the parent drug.

Author Contributions

Investigation, A.K.G. and M.G.; Conceptualization, A.K.G.; Methodology, M.G.. and A.K.G.; Data curation M.G. and A.K.G.; Formal analysis, M.G. and A.K.G.; Validation, M.G. and A.K.G.; Visualization, M.G. and A.K.G.; Writing – original draft, M.G..; Writing – review & editing, A.K.G. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

All Animal studies were conducted after obtaining approval from the Institutional Animal Ethics Committee (IAEC) of Ganpat University, Gujarat, India, in compliance with CPCSEA guidelines.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

Funding

This research received no external funding.

Acknowledgements

The authors are thankful to the late Professor A. N. Maitra, Department of Chemistry, University of Delhi, for his research guidance in conducting this research work.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Kapourani, A.; Kontogiannopoulos, K.N.; Barmpalexis, P. A Review on the Role of Pilocarpine on the Management of Xerostomia and the Importance of the Topical Administration Systems Development. *Pharmaceuticals* **2022**, *15*, 762, https://doi.org/10.3390/ph15060762.
2. Jackson, M.A.; Giyanani, J.; Shabaik, Y.; Penzner, J.; Gore, A.V.; Robinson, M.R.; Waring, G.O. *In vitro* and In-Eye Comparison of Commercial Pilocarpine Ophthalmic Solution and an Optimized, Reformulated Pilocarpine for Presbyopia Treatment. *Ophthalmol. Ther.* **2022**, *11*, 869-879, https://doi.org/10.1007/s40123-022-00482-2.
3. Wiseman, L.R.; Faulds, D. Oral Pilocarpine: A Review of its Pharmacological Properties and Clinical Potential in Xerostomia. *Drugs* **1995**, *49*, 143-155, https://doi.org/10.2165/00003495-199549010-00010.
4. Owodeha-Ashaka, K.; Ilomuanya, M.O.; Iyire, A. Evaluation of sonication on stability-indicating properties of optimized pilocarpine hydrochloride-loaded niosomes in ocular drug delivery. *Prog. Biomater.* **2021**, *10*, 207-220, https://doi.org/10.1007/s40204-021-00164-5.
5. Kour, J.; Kumari, N.; Sapra, B. Ocular prodrugs: Attributes and challenges. *Asian J. Pharm. Sci.* **2021**, *16*, 175-191, https://doi.org/10.1016/j.ajps.2020.08.002.
6. Cardoso, A.M.L.; Oliveira, E.E.; Machado, B.A.S.; Marcelino, H.R. Eudragit®-based nanoparticles for controlled release through topical use. *J. Nanoparticle Res.* **2023**, *25*, 32, https://doi.org/10.1007/s11051-023-05678-6.
7. Pawar, A.; Kamdi, V.; Alaspure, A.; Gangane, P. Recent Updates on Polymeric Micelles: A Review. *Int. J. Pharm. Sci. Rev. Res.* **2022**, *73*, 37–52, https://doi.org/10.47583/ijpsrr.2022.v73i01.010.
8. Jafernik, K.; Ładniak, A.; Blicharska, E.; Czarnek, K.; Ekiert, H.; Wiącek, A.E.; Szopa, A. Chitosan-Based Nanoparticles as Effective Drug Delivery Systems—A review. *Molecules* **2023**, *28*, 1963, https://doi.org/10.3390/molecules28041963.
9. Ho, K.C.; Stapleton, F.; Wiles, L.; Hibbert, P.; Alkhawajah, S.; White, A.; Jalbert, I. Systematic review of the appropriateness of eye care delivery in eye care practice. *BMC Health Serv. Res.* **2019**, *19*, 646, https://doi.org/10.1186/s12913-019-4493-3.
10. Arabpour, Z.; Salehi, M.; An, S.; Moghtader, A.; Anwar, K.N.; Baharnoori, S.M.; Shah, R.J.; Abedi, F.; Djalilian, A.R. Exploring Hydrogel Nanoparticle Systems for Enhanced Ocular Drug Delivery. *Gels* **2024**, *10*, 589, https://doi.org/10.3390/gels10090589.
11. Pawar, P.K.; Rathod, R.D.; Jagadale, S.R. A review on topical ophthalmic drug delivery system: reference to viscosity enhancer. *Polim. Med* **2024**, *54*, 71-84, https://doi.org/10.17219/pim/166413.

12. Kolawole, O.M.; Cook, M.T. In situ gelling drug delivery systems for topical drug delivery. *Eur. J. Pharm. Biopharm.* **2023**, *184*, 36-49, https://doi.org/10.1016/j.ejpb.2023.01.007.
13. Mostafa, M.; Al Fatease, A.; Alany, R.G.; Abdelkader, H. Recent Advances of Ocular Drug Delivery Systems: Prominence of Ocular Implants for Chronic Eye Diseases. *Pharmaceutics* **2023**, *15*, 1746, https://doi.org/10.3390/pharmaceutics15061746.
14. Fazal, T.; Murtaza, B.N.; Shah, M.; Iqbal, S.; Rehman, M.-u.; Jaber, F.; Dera, A.A.; Awwad, N.S.; Ibrahium, H.A. Recent developments in natural biopolymer based drug delivery systems. *RSC Adv.* **2023**, *13*, 23087-23121, https://doi.org/10.1039%2Fd3ra03369d.
15. Weissberger D, Stenzel MH, Hunter L. Precious Cargo: The Role of Polymeric Nanoparticles in the Delivery of Covalent Drugs. *Molecules*. **2024** Oct *19;29(20), 4949.* doi: 10.3390/molecules29204949. Li,
16. S.; Chen, L.; Fu, Y. Nanotechnology-based ocular drug delivery systems: recent advances and future prospects. *J. Nanobiotechnol.* **2023**, *21*, 232, https://doi.org/10.1186/s12951-023-01992-2.
17. Bisen, A.C.; Biswas, A.; Dubey, A.; Sanap, S.N.; Agrawal, S.; Yadav, K.S.; Singh, V.; Rawat, P.; Sagar, S.; Mugale, M.N.; Bhatta, R.S. A review on polymers in ocular drug delivery systems. *MedComm - Biomater. Appl.* **2024**, *3*, e77, https://doi.org/10.1002%2Fmba2.77.
18. Vaneev, A.; Tikhomirova, V.; Chesnokova, N.; Popova, E.; Beznos, O.; Kost, O.; Klyachko, N. Nanotechnology for Topical Drug Delivery to the Anterior Segment of the Eye. *Int. J. Mol. Sci.* **2021**, *22*, 12368, https://doi.org/10.3390/ijms222212368.
19. Singh, J.; Nayak, P. pH-responsive polymers for drug delivery: Trends and opportunities. *J. Polym. Sci.* **2023**, *61*, 2828–2850, https://doi.org/10.1002/pol.20230403.
20. Bhattacharjee, A.; Das, P.J.; Adhikari, P.; Marbaniang, D.; Pal, P.; Ray, S.; Mazumder, B. Novel drug delivery systems for ocular therapy: With special reference to liposomal ocular delivery. *Eur. J. Ophthalmol.* **2019**, *29*, 113-126, https://doi.org/10.1177/1120672118769776.
21. Ruiz-Virgen, L.; Hernandez-Martinez, M.A.; Martínez-Mejía, G.; Caro-Briones, R.; Herbert-Pucheta, E.; Río, J.M.d.; Corea, M. Analysis of Structural Changes of pH–Thermo-Responsive Nanoparticles in Polymeric Hydrogels. *Gels* **2024**, *10*, 541, https://doi.org/10.3390/gels10080541.
22. Aslam, A.; Ashraf, M.U.; Barkat, K.; Mahmood, A.; Hussain, M.A.; Farid-ul-Haq, M.; Lashkar, M.O.; Gad, H.A. Fabrication of Stimuli-Responsive Quince/Mucin Co-Poly (Methacrylate) Hydrogel Matrices for the Controlled Delivery of Acyclovir Sodium: Design, Characterization and Toxicity Evaluation. *Pharmaceutics* **2023**, *15*, 650, https://doi.org/10.3390/pharmaceutics15020650.
23. Chauhan, A.; Khan, T. Prodrugs - Current development and applications in ocular drug delivery. *J. Drug Deliv. Sci. Technol.* **2021**, *66*, 102836, https://doi.org/10.1016/j.jddst.2021.102836.
24. Bundgaard H, Falch E, Larsen C, Mikkelson TJ. Pilocarpine prodrugs I. Synthesis, physicochemical properties and kinetics of lactonization of pilocarpic acid esters. *J Pharm Sci.* **1986** *75(1),* 36-43. doi: 10.1002/jps.2600750109.
25. Gupta, A.K.; Madan, S.; Majumdar, D.K.; Maitra, A. Ketorolac entrapped in polymeric micelles: preparation, characterisation and ocular anti-inflammatory studies. *Int. J. Pharm.* **2000**, *209*, 1-14, https://doi.org/10.1016/S0378-5173(00)00508-1.

Publisher's Note & Disclaimer