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Novel pluronic-chitosan micelle as an ocular delivery system

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Abstract: Pluronic micelles were prepared for ophthalmic delivery by incorporation of ethyl acetate as a dispersion agent and their surfaces were modified by chitosan to improve their bioavailability. The micelles disperse well in the solution and have a core-shell like structure with a particle size ranging from 93 to 181 nm for drug unloaded, 123–232 nm for drug-loaded, and a zeta potential between 6.1 and 9.2 mV, indicating very suitable use as ophthalmic carrier. The *in vitro* serum stability tests indicate the particle size of the micelles was very stable during the serum absorption. The turbidity test reveals that the prepared micelles were very stable under phosphate buffered saline environment, which can prevent the blurred vision. The loading efficiency of metipranolol in micelles can be as high as 83%. Finally, the *in vitro* and *in vivo* studies indicate the pluronic micelles modified by chitosan have sustained release behavior and good pharmacological response. As the results, the pluroic-chitosan micelles system provides a potential opportunity in decreasing frequency of administration and improving patient compliance for ocular drug delivery. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2013.

Key Words: pluronic, chitosan, micelle, ocular delivery system

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INTRODUCTION

Ocular diseases are usually treated with topical application of eyedrops, however, this method is impeded by poor ocular bioavailability,[1,2] mainly due to the protective characteristics of the ocular biological barriers. These barriers include extensive precorneal drug loss by high tear fluid turnover, transient precorneal residence time, nonproductive absorption, drainage through the nasolacrimal duct, impermeability of the corneal epithelium, and metabolism of the drug by anterior segment enzymes. Typically, less than 5% of the drug applied penetrates the cornea and reaches the intraocular tissue.[3,4] As a result, a frequent dosing regimen is typically necessary to achieve the therapeutic effects.

Ophthalmic drug delivery, probably more than any other route of administration, may benefit from the characteristics of nanotechnology-based drug delivery systems,[5] such as microemulsions,[6,7] nanosuspensions,[8–10] nanoparticles,[11,12] micelles,[13,14] and liposomes.[15,16] These delivery systems have led to the solution of various solubility-related problems of poorly soluble drugs.[17] They can protect the encapsulated molecule while facilitating its transport to the different compartments of the eye.

Polymeric micelle formulation is one of the strategies currently used to improve drug absorption and increase ocular bioavailability.[18,19] Pluronic is one of the best known polymers used to prepare the micelles. Pluronic is an amphiphilic block copolymer, which is biodegradable and biocompatible. This block copolymer consists of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) blocks arranged in PEO-PPO-PEO tri-block structure.[20] In aqueous solutions, these copolymers self-assemble into micelles at concentrations above critical micelle concentration. The core of the micelles consists of hydrophobic PPO blocks that are separated from the aqueous exterior by the shell, which is hydrated of hydrophilic PEO chains. The core can be used to incorporate various compartments. This is the reason that it has been used extensively in nanomicelle drug delivery systems.[21] However, a major disadvantage of Pluronic is its low mucoadhesive activity. Some Pluronic-based ophthalmic formulations have been improved by adding mucoadhesive polymers, such as sodium hyaluronate[22,23] and Carbopol.[22,24]

Chitosan, the cationic polysaccharide derived from chitin, has been widely used in biomedical and pharmaceutical applications.[25,26] Chitosan has the specific bioadhesive property which can improve the residing time of vehicle on the ocular surface for a prolonged period. The electrostatic attraction between the negative charge of cornea and positive charge of chitosan appears to be the major driving force for mucoadhesion.[27] Apart from its mucoadhesive character, there are other favorable biological properties of chitosan, such as the possibility of additional intracellular pathways which may contribute to the enhancement of the cellular permeability. Based on literature, the chitosan-coated-poly-ε-caprolactone

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nanocapsules developed by De Campos et al.[28] were able to penetrate the cornea by a transcellular pathway. Yuan et al.[29] found that chitosan–cholesterol-coated polylactic acid nanoparticles showing a good retention onto the eye surface. The studies from Felt et al.[30] proved chitosan has good stability and ocular tolerance.

Metipranolol is a nonselective β_1- and β_2-adrenergic antagonist with a receptor selectivity similar to that of timolol and levobunolol.[31] After several successful trials in Europe, metipranolol effectively reduces intraocular pressure (IOP) by suppressing aqueous outflow in ocular hypertensive eyes.[32–34] However, its poor water soluble property is difficult to develop as a conventional ocular drug delivery system.

Hence, in our current work we developed a new nanomicelle system based on pluronic solution. The hydrophobic core of pluronic is beneficial for incorporating poor water soluble drug, such as metipranolol. The surface of Pluronic nanomicelle was modified by chitosan solution to improve its ocular bioavailability. The physicochemical properties of Pluronic-Chitosan nanomicelle system including diameter, surface charge, morphology, turbidity, and stability were characterized. In addition, the *in vitro* drug release behavior and the *in vivo* pharmacological response were evaluated.

EXPERIMENTAL

Materials

Pluronic (Plu, F-127, 12,600 Da) for micelles preparation and bovine serum albumin (BSA) for stability analysis were obtained from Sigma. Water-soluble Chitosan (Ch, degree of deacetylation 85%; viscosity: five CPS, 20°C; 50–60 kDa) for surface modification was supplied by Charming&Beauty, Taiwan. Poly(vinyl alcohol) (PVA, 20,000 Da), glutaraldehyde (GA), pyrene, and acetone were purchased from Merck, Germany. Ethanol was supplied by Fullin Niho Shiyaku, Bio., Taiwan. Span 80 and acetic acid were obtained from Island for a long time Pharmaceutical Co., Ltd., Japan. Model drug, metipranolol was purchased from British Pharmacopoeia, UK. Commercial eye drop (OptiPranolol®, metipranolol 0.3%, Bausch&Lomb) was obtained from local pharmacy.

reparation of metipranolol loaded pluronic-chitosan (Plu-Ch) micelles

Three solutions (pluronic, metipranolol-loaded ethyl acetate, and PVA-chitosan solutions) were prepared first prior to the preparation of drug-loaded Plu-Ch micelles. Pluronic F127 (28 wt. %) was slowly added to the distilled water with continuous agitation. To dissolve the Pluronic solution completely, it was left at 4°C until it formed a clear solution. An appropriate amount of metipranolol was added to acetic acid and was magnetic stirred to dissolve metipranolol completely, and then ethanol with a same volume as acetic acid was added to the solution to obtain ethyl acetate solution containing metipranolol. PVA (0.025 g) was added to the 10 mL deionized water and the solution was heated to 80°C until PVA was dissolved completely, then various concentration of chitosan (0.3, 0.5, and 0.8 wt. %) was added with 100 μL of GA (as a cross-linker), respectively, to the solution and was stirred continuously for 1 h to completely solubilize the chitosan. To prepare pluronic micelles, two drops of Span 80 (as a surfactant) were added to pluronic solution with fast agitation, and then ethyl acetate solution with metipranolol was slowly added with continuous magnetic stirring for 2 h. Finally, PVA-chitosan solutions with various concentration of chitosan were added to micelle solution, respectively, with continuous magnetic stirring for 8 h to perform the surface modification. Then, it was freeze-dried (EYELA, FDU-1200, Japan) to evaporate the ethyl acetate and obtain Plu-Ch micelle particles.

FT-IR spectra analysis of Plu-Ch nanomicelles

The freeze-dried micelles were ground with potassium bromide (KBr) in the ratio 1:99 in an agate mortar, and they were pressed to a disk. Each KBr disk was scanned by Fourier transform infrared spectroscopy (FT-IR, Perkin Elmer, Spectrum One). Two hundred and fifty-six scans at 4 cm^{-1} resolution were used to obtain each spectrum. The spectrum was compared with spectra of chitoan and Pluronic to investigate whether the nanomicelles were prepared successfully. Conversely, the spectrum was also compared with ethyl acetate to examine whether the residual solvent was removed completely.

Measurement of micelle diameter

The micelles obtained through lyophilization were put in deionized water and sonicated for 10 min to full dispersion of micelles in deionized water and filtrated through a membrane filter (0.22 μm pore size, SLGV013NKP.1566, Merck Millipore, Billerica, MA) before the measurements. Dynamic light-scattering (DLS) method on a Zetasizer 3000 HAS (Malvern, UK) at 25°C with a wavelength of 633 nm and a detection angle of 90° was used to determine the average particle size. The results obtained were the mean (±SD) of 10 determinations.

Measurement of micelle zeta potential

The micelles obtained through lyophilization were put in deionized water and sonicated for 10 min to full dispersion of micelles in deionized water. The charge of the particle (known as the zeta potential) was determined using a Zetasizer 3000. The electrophoretic mobility of the micelles was measured using the laser Doppler anemometer of this instrument. Zeta potentials were calculated from the mean electrophoretic mobility using the Smoluchowski equation available in the instrument software, and the results obtained were the mean (±SD) of 10 determinations.

Morphology observation by transmission electron microscope (TEM)

The micelles obtained through lyophilization were put in alcohol solution and sonicated for 10 min to full dispersion of micelles in alcohol solution. The well-dispersed micelle solution was then dropped onto 400 mesh carbon-coated copper grids and was placed on oven to remove residual alcohol through vaporization. The morphological examination of the micelles was performed with a transmission electron microscope (FEI TECNA1 G2 F20 FEG-TEM, Japan).

Turbidity test of micelles

Various samples were placed in a quartz curette containing 100 mL distilled phosphate buffered saline (PBS) solution, setting at 34°C water bath. Transmittance was measured at 380 nm for different time periods by UV-Vis spectrometer (Lambda 25; Perkin Elmer) and turbidity was calculated using the following formula:

$$\text{Turbidity} = (100 - \%\text{Transmittance})/100 \quad (1)$$

All measurements were performed in triplicate.

Stability test of micelles

The *in vitro* serum stability of the metipranolol (0.3 wt. %)-loaded nanomicelles was evaluated after storage for 12 h under 0.1 wt. % BSA solution. Samples were stored in closed amber glass bottles at 34°C. Nanomicelles stability was characterized in terms of particle size of micelles. Particle size of micelles after being exposed to BSA solution was filtrated through a membrane filter (0.22 μm pore size, SLGV013NKP.1566, Merck Millipore, Billerica, MA) before the measurements by DLS at different time periods. All measurements were performed in triplicate.

Thermal analysis of micelles

Samples (metipranolol powder, Plu-Ch micelle powder, physical mixture of metipranolol and Plu-Ch micelle powders, and metipranolol-loaded Plu-Ch micelle powder) were separately sealed in aluminum cells and scanned by a Differential scanning calorimetry (DSC, Perkin Elmer DSC7) from 25 to 130°C. Thermal analysis was performed at a heating rate maintained at 10°C per minute in an air atmosphere. Alumina was used as the reference substance.

Drug loading efficiency

Freeze-dried metipranolol-loaded nanomicelles were put in simulated tear fluid (STF, compositions: 2 g $NaHCO_3$, 6.7 g NaCl, 0.08 g $CaCl_2{\cdot}2H_2O$, and deionized water was added up to 1 L) and were placed in a mechanical shaking bath for 3 days to assure completely release of drug from micelles. Before sampling, the beaker was sonicated for 1 min to precipitate the nanomicelles. Then the supernatant of aliquots (1 mL) containing metipranolol were withdrawn from the release medium and was measured by UV–vis spectrometer (Lambda 25; Perkin Elmer) at 380 nm. Drug incorporation efficiency was expressed as drug loading efficiency (%); represented by Eq. (2):

$$\text{Drug loading efficiency}(\%) = \text{Mass of drug in nanomicelle}/\text{Mass of drug used in formulation} \quad (2)$$

All measurements were performed in triplicate.

Solubility measurements

Excess amount of metipranolol was added to 15 mL acetic acid and was magnetic stirred to dissolve metipranolol completely, and then 15 mL ethanol was added to the solution to form ethyl acetate solution containing metipranolol. This solution was magnetic stirred continuously for 24 h at 25°C and allowed to stand for a further 2 h. Preliminary experiment showed that solubility equilibrium was reached in less than 24 h. The solution was then filtered and diluted appropriately and the drug concentration was determined by a UV–vis spectrometer (Lambda 25; Perkin Elmer) at a wavelength of 380 nm. All the solubility experiments were performed in triplicate. Conversely, for the purpose of comparison, the solubility of metipranolol in the continuous nanodispersion phase containing ethyl acetate was also measured. The same aforementioned procedure was applied excepting extra 0.25 g dried micelles were added to the solution.

***In vitro* drug release**

Metipranolol (0.3 wt. %)-loaded nanomicelle suspensions with various concentration of chitosan and commercial eye drops (0.3 wt. % metipranolol) were put in a 10-mL tube filled with STF, respectively. The tube was placed in a circulating water bath and the temperature was maintained at 34°C without shaking. Before sampling, the tube was sonicated for 1 min to precipitate the nanomicelles. Then the supernatant of aliquots (1 mL) were withdrawn from the release medium at each sampling time and replenished with 1 mL of fresh STF. The aliquots were analyzed on a UV–vis spectrometer (Lambda 25; Perkin Elmer) at a wavelength of 380 nm to determine the amount of metipranolol released. All the drug-release experiments were performed in triplicate.

***In vivo* pharmacological response**

Ne1010w Zealand albino rabbits were used as the model animals in the *in vivo* experiments. All animal experiments were performed in compliance with guidelines approved by the Institutional Animal Care and Use Committee at the Southern Taiwan University, Tainan, Taiwan. Rabbits of either sex, free of gross ocular defects and weighing 2.5–3 kg, were positioned into restraining boxes and placed in an isolated room with 50 W of controlled lighting. The IOP, which was used to evaluate the pharmacological response of metipranolol, was measured using a tonometer (iCare, TV01, Finland). All rabbits were acclimatized to laboratory testing conditions for 30 min before initiating the study. After the 30-min period, the left and right IOPs were alternatively measured four times within 30 min to establish baseline values for both eyes. For each pair of readings, the changes in IOP (control minus test eye) were determined. These predosing differences were averaged, and the mean was used to convert post-administration data to the baseline-corrected values. This process minimized both animal and day variation.

All the samples intend for the *in vivo* ocular testing were subjected to sterile filtration procedures in the laminar flow cabinet. All the samples were adjusted to pH 4.0 and prefiltrated by a 2 μm glass fiber filter (Ultipor GF Plus U 220Z, PALL, St Germain en Laye, France) and followed by a 0.45 μm cellulose acetate filter (Ministart NML, Sartorius, Palaiseau, France). Finally, they were filtrated by a 0.20 μm cellulose acetate filter (Ministart NML, Sartorius, Palaiseau, France).

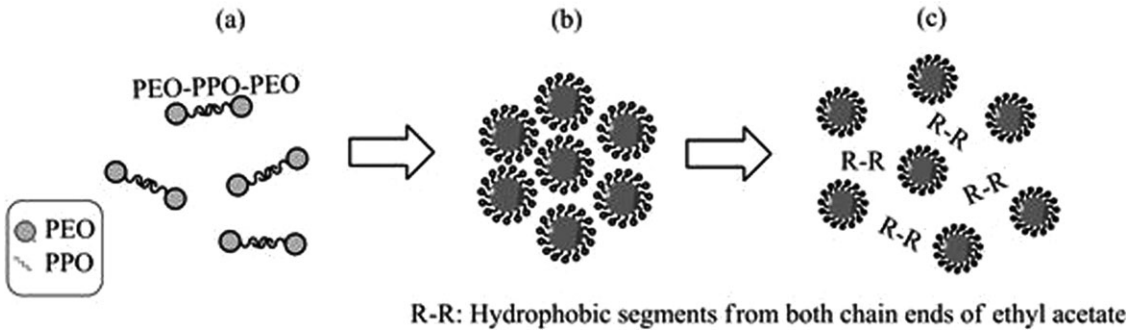

FIGURE 1. Mechanism of Pluronic micelles formation (a) Pluronic segments; (b) aggregated Pluronic micelles; and (c) well dispersive Pluronic micelles using ethyl acetate as a dispersion agent.

As the drug loading efficiency of Pluronic micelles and Plu-Ch 0.5% micelles differs greatly (12.6 *vs.* 84.6%), to achieve the same dosage (0.3 wt. % metipranolol) at *in vivo* experiments, the desired weight of micelles was determined by taking drug loading efficiency into account. That is, the lower the drug loading efficiency, the higher the weight of micelles is required. The requested weight of micelles was then dissolved in the same volume of STF. With that respect, the same dosage of drug can be obtained.

Fifty microliters of pluronic, Plu-Ch 0.5% nanomicelle suspensions, and commercial eye drops, each with 0.3 wt. % of metipranolol, were dosed from a micropipette, respectively. The drug-containing formulations were administered at room temperature and were placed in the lower conjunctival sac, approximately midway between the inner and outer canthus. In order to avoid experimental bias, the left eye of each rabbit was first administered with the control vehicle (formulation with no drug), followed by the application of drug-containing vehicle (formulation with drug) to the right eye. After administration of each formulation, IOPs of both eyes were measured, according to the following time schedule: 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 min. For each time point, the change in IOP (control minus test eye) were calculated; the data were then converted to baseline-corrected value (i.e., the pharmacological response of metipranolol) by subtracting the average baseline change in IOP for each experiment on the basis of the readings obtained before dosing. To assess the extent of total pharmacological response of the various formulations, areas under the change in IOP (after baseline correction) *versus* time profiles in 360 min ($AUC_{0\text{-}360}$) were calculated using the trapezoidal rule.

Statistical analyses

All statistical analyses were undertaken using the T-test with a SPSS® statistical software.

RESULTS AND DISCUSSION

Mechanism of formation of chitosan-coated Pluronic micelles

In this study, Pluronic micelles to be used as ophthalmic carrier were prepared first then their surfaces were modified by chitosan to improve their bioavailability. Span 80 was used as a surfactant in Pluronic micelles preparation. During the preparation, the long term of stirring may cause the aggregation of nanomicelles due to the cohesive force induced by the agitation. The addition of Span 80 can prevent the nanomicelles from aggregation. The gelation mechanism of Pluronic can be explained by the change in micelle structure as a function of concentration and temperature.[22,35] However, at room temperature, Pluronic micelles may aggregate, as shown in Figure 1(b). In this study, ethyl acetate was used as a dispersion agent; the hydrophobic segments from both chain ends of ethyl acetate can repel the hydrophilic domains from micelle surface and attain well dispersive micelles, as shown in Figure 1(c).

The well dispersive Pluronic micelles were then subjected to surface modification by water soluble chitosan. Prior to the modification, PVA solution was used to dissolve chitosan and form PVA-chitosan complex through interactions between hydroxyl groups of PVA and hydroxyl or amino groups of chitosan. As chitosan is less hydrophilic than PVA, the formation of PVA-chitosan complex not only improves the hydrophilicity but also enhances the adhesion property of chitosan, which helps the attachment of chitosan to the Pluronic nanomicelles.[36] In the early stage of modification, hydrophilic chains from chitosan can adhere to the hydrophilic shell of Pluronic micelles and thicken the shell, as shown in Figure 2(b). After that, the linear chitosan chains may entangle and creates lots of voids, in which nanomicelles may interpenetrate, as shown in Figure 2(c). The surface modification by chitosan not only stabilizes the micelles but also improves the mucoadhesive property and provides the positive charge on the micelle surface.

FT-IR spectra analysis of Plu-Ch nanomicelles

To investigate whether nanomicelles of Plu-Ch-Metipranolol were prepared successfully and whether there was any residue of ethyl acetate, FT-IR analysis was employed to verify the results. Figure 3(a) displays that chitosan has characteristic absorption peaks of O—H at 3460 cm^{-1}, stretch vibration of amide I at 1657 cm^{-1}, bending of N—H at 1530 cm^{-1}, and C—O—C at 1082 cm^{-1}. Pluronic has absorption peak caused by characteristic saturated CH_2 stretching vibration at 2880–3000 cm^{-1} and C—O—C stretching vibration at 1100–1300 cm^{-1} [Figure 3(b)]. Metipranolol has

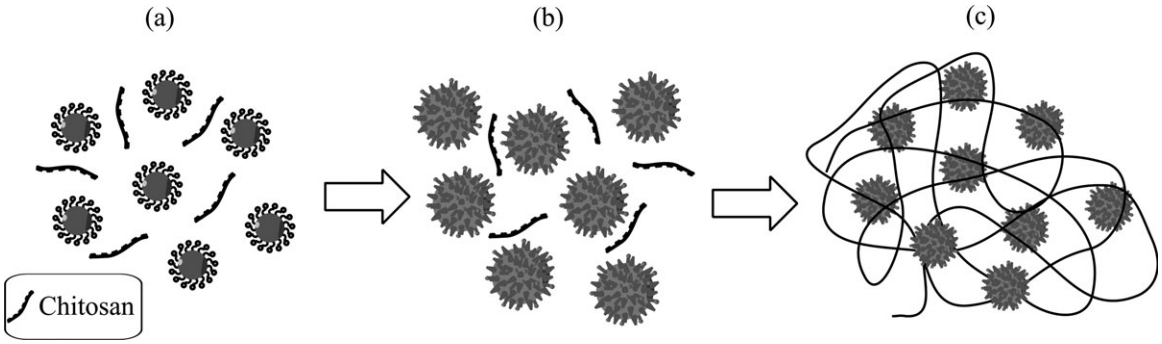

FIGURE 2. Mechanism of chitosan-coated Pluronic micelles formation, (a) adding chitosan solution into Pluronic micelle solution; (b) surface modification by chitosan; and (c) micelles penetrating into voids created by entangled chitosan molecular chains.

characteristic absorption peak of C=C from benzene ring at 1605 cm^{-1} and C=O from benzene ring's ester group at 1750 cm^{-1} [Figure 3(c)]. In the Plu-Ch-Metipranolol nanomicelles [Figure 3(d)], characteristic absorption peak at 1657 cm^{-1} was assigned to stretch vibration of amide I from chitosan, the peak at 1100 cm^{-1} was belonged to C—O—C stretching vibration from Pluronic, and the peak at 1750 cm^{-1} was contributed from C=O from benzene ring's ester group of Metipranolol, indicating nanomicelles were prepared successfully. Besides, Figure 3(e) shows ethyl acetate solution has significant characteristic absorption peak of O—H at 1715 cm^{-1}. As ethyl acetate was used as dispersion solvent in the process of the micelle preparation and such solvent may irritate to the eyes, the process of freeze and drying to remove the solvent is required. In comparison of Figure 3(d,e), the former is powders of micelles after freeze and drying and characteristic absorption peak was not found at 1715 cm^{-1}. Therefore we validate that ethyl acetate was successfully eliminated from the micelle after freeze and drying, which avoids irritation of the prepared micelle to the eyes.

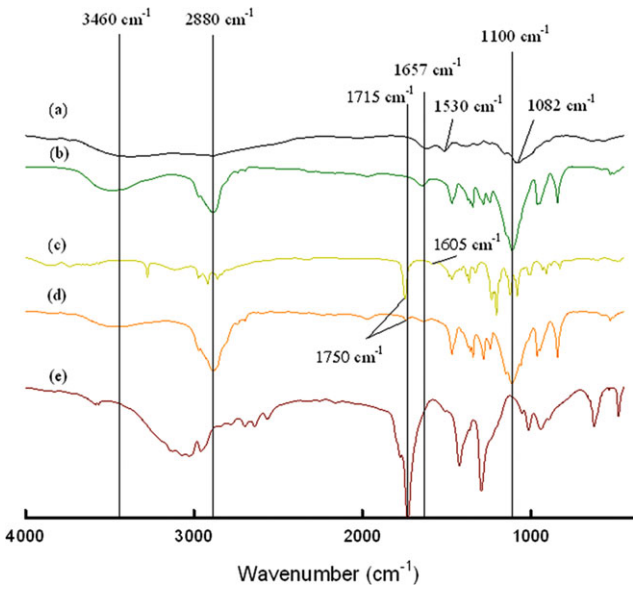

FIGURE 3. FTIR spectra analysis of (a) chitosan; (b) Pluronic; (c) Metipranolol; (d) Plu-Ch-Metipranolol nanomicelles; and (e) ethyl acetate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Measurement of micelle diameter and surface charge

To investigate the size of nanomicelles prepared in this study, DLS was used for diameter analysis. Micelles were dispersed in deionized water and shaken evenly before measurement. The diameter of the swollen nanomicelles was determined. Table I lists the diameter of drug unloaded micelles with various compositions of chitosan. The average diameter of Pluronic nanomicelles was 85 nm with a polydispersity index (PDI) of 0.275. When its surface was modified by chitosan, its diameter enlarged to about 93–181 nm with PDI varied from 0.117 to 0.157. As listed in Table I, the size increased with increasing chitosan concentration because the shell of micelles was thicken through surface modification. PDI of surfaced modified micelles are lower than that of unmodified micelle, suggesting more uniform of particle size was obtained after surface modified by chitosan solution. Conversely, particle size and PDI of unmodified and modified metipranolol-loaded Pluronic micelles were listed in Table II. The size of all the micelles (varied from 99 to 232 nm) increases about 14-51 nm after drug loaded. Most of PDI (varied from 0.193 to 0.433) were higher than those of drug unloaded micelles, indicating drug loading affects the uniformity of particle size. For instance, the particle size and PDI of Plu-Ch 0.8% micelle increased from 181 to 232 nm and 0.146 to 0.433, respectively, after drug loaded, speculating that the entanglement between molecule chains of chitosan molecules increased due to relatively higher concentration and caused difficulty for drug to enter micelles and just allowed it adhering on the micelle surface. The particle size prepared in this study is larger than methoxy poly(ethylene glycol)-hexylsubstituted poly(lactide) micelle[14] (37.4 ± 0.1 nm with a PDI of 0.4 for unloaded micelles and 51.4 ± 0.4 nm with a PDI of 0.3 for Cyclosporin A-loaded micelles); the authors claimed the nanosize leads to transparent solutions and may facilitate good corneal penetration, which will increase the bioavailability of the incorporated drug. Nevertheless, the particle size prepared in this study is comparable to other nanoparticle or micelle systems.[11,12,37,38] Literatures indicate that among physical properties of micelles, particle size and PDI are of

TABLE I. Particle Size, Polydispersity Index, Zeta Potential, and Drug Loading Efficiency of Unmodified and Modified Pluronic Micelles

Formulation[a]	Particle Size (nm)	Polydispersity Index (PDI)	Zeta Potential (mV)	Drug Loading Efficiency (%)
Pluronic	85 ± 3	0.275 ± 0.171	−1.2 ± 0.4	12.6 ± 0.9
Plu-Ch 0.3%	93 ± 1	0.157 ± 0.069	6.1 ± 0.4	37.8 ± 3.0
Plu-Ch 0.5%	117 ± 2	0.117 ± 0.076	7.8 ± 0.2	84.6 ± 1.4
Plu-Ch 0.8%	181 ± 7	0.146 ± 0.020	9.2 ± 0.2	54.5 ± 3.6

[a] The composition of Pluronic in Plu-Ch micelles is kept constant as 28 wt. %.

great importance for bioavailability.[11,39,40] A particle size below 250 nm[41] with a PDI near 0.25[42] was considered optimum for ocular administration. Diameter of all the drug-loaded and unloaded Plu-Ch nanomicelles prepared in this study is under 250 nm with most of PDI lower than 0.25, therefore, they are very suitable for being used as carrier of ophthalmic drug.

Surface charge of nanomicelles can affect the retention time of micelles on the anterior cornea. Hence, DLS was applied to analyze surface potential of nanomicelles. Table I lists surface potential charge (known as the zeta potential) of nanomicelles, where unmodified and modified Pluronic micelles were measured. Pluronic is a kind of amphiphilic polymer without charge in nature, but when micelle polymer is dispersed in deionized water, it has somewhat negative charge, listed as Table I, which might be caused by ionization of peripheral hydrophilic groups in water. As chitosan is a natural polymer with positive charge, Plu-Ch micelles have significant positive charge after being modified by chitosan solution. The zeta potential of Plu-Ch micelles increases from 6.1 to 9.2 mV as chitosan concentration increases from 0.3 to 0.8 wt. %. By coating positive-charged chitosan solution on surface of Pluronic micelles, electrostatic interactions between surface of micelles and anterior cornea occurs. Literature[43] indicates that anterior cornea has negative charge, through attraction between surface of micelles and anterior cornea, particles adhere to surface of cornea easily, preventing micelles from leaving the anterior cornea due to ophthalmic barrier. By this way, bioavailability is increased so as to be beneficial for slow release effect on the cornea.

Morphology observation by transmission electron microscope (TEM)

Nanomicelles in alcohol prepared in this study were observed for their morphology by TEM. Figure 4 shows morphology images of nanomicelles observed by different magnifications. Of which Figure 4(a–d) were obtained by a setting of 12,000× magnification, such smaller magnification allows dispersion of micelles be observed. Figure 4(a) shows Pluronic micelles which have the largest quantity and are more aggregated among others. Surface of Pluronic micelles were modified by different proportion of chitosan, where micelles modified by 0.3% of chitosan [Figure 4(b)] are found lighter in color and are dispersive; ones modified by 0.5% of chitosan [Figure 4(c)] are darker and closer to round in morphology and not aggregated; ones modified by 0.8% of chitosan [Figure 4(d)] are less uniform in sizes, indicating excessive concentration of chitosan tends to make adhesion on micelles more difficult.

Figure 4(e–h) was obtained by a setting of 120,000× magnification. In Figure 4(e), the sizes of Pluronic micelles are less uniform and smaller; in Figure 4(f)–(h), the surface-modified nanomicelles are more compact and larger in diameter, speculating they are formed by coating plenty of entangled chitosan molecular chains on micelles. TEM images illustrate that nanomicelles modified with 0.5% chitosan are evenly dispersive in diameter and not aggregated, indicating it is more suitable for serving as a carrier of ophthalmic drugs.

TABLE II. Particle Size and Polydispersity Index of Unmodified and Modified Metipranolol-Loaded Pluronic Micelles

Formulation[a]	Particle Size (nm)	Polydispersity Index (PDI)
Pluronic	99 ± 5	0.244 ± 0.067
Plu-Ch 0.3%	123 ± 3	0.193 ± 0.135
Plu-Ch 0.5%	138 ± 7	0.261 ± 0.091
Plu-Ch 0.8%	232 ± 35	0.433 ± 0.123

[a] The composition of Pluronic in Plu-Ch micelles is kept constant as 28 wt. % and the concentration of metipranolol is kept constant as 0.3 wt. %.

Turbidity test of micelles

In the process of ophthalmic drug delivery using nanomicelles, suspension with excessive concentration might cause blur in eyes. In order to prevent from impairment of vision without lowering concentration of drugs in the process of the therapy,[44] a turbidity experiment was conducted in an environment simulating the eyes. When soaking micelles in PBS solution, turbidity of nanomicelles increases with concentration of chitosan as shown in Figure 5, which not only increases particle diameter but also turbidity; furthermore, it is found that turbidity shows a upward trend with time, which is attributed to the great cohesion in nanoscale objects as nano particles in the suspension solution tend to gather over time. Such gathering enlarges particle diameter and lower light penetration, leading to increasing turbidity.

As shown in Figure 5, the turbidity of all the specimens increases with increasing soaking time; but their turbidities are all less than 0.2 after soaking in PBS for 6 h. Literature[45] indicates that light transmission $\geq$90% (i.e., turbidity $\leq$0.1) is defined as transparent, 10–90% is translucent, and one lower than 10% is opaque. Eighty percent of transparency (i.e., turbidity = 0.2) is the allowable limit for vision turbidity which does not make a blurry vision. It is found

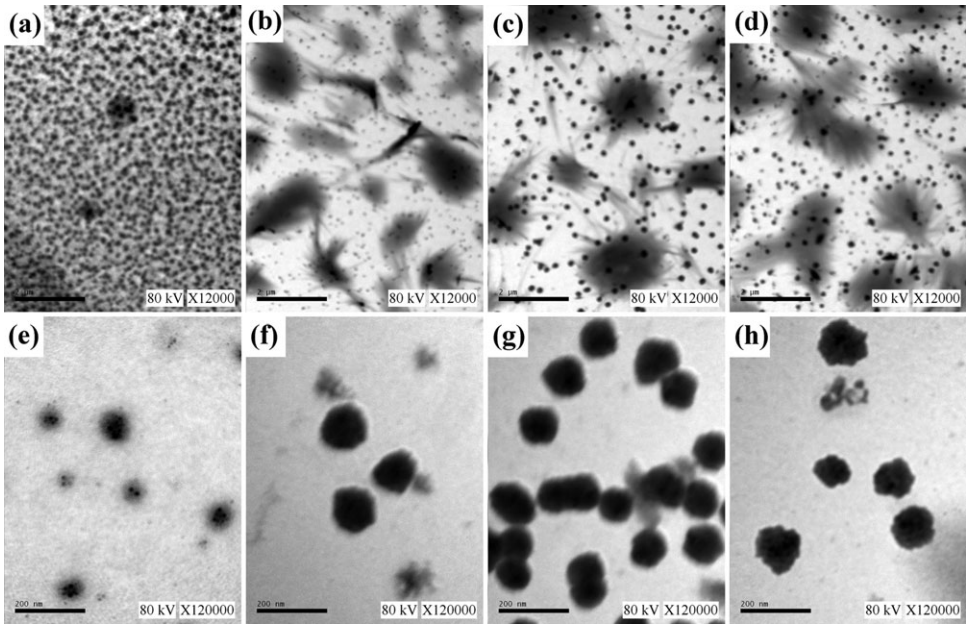

FIGURE 4. Morphologies of chitosan-coated Pluronic micelles observed by TEM, (a) and (e) Pluronic micelles; (b) and (f) Plu-Ch 0.3% micelles; (c) and (g) Plu-Ch 0.5% micelles; (d) and (h) Plu-Ch 0.8% micelles. Magnification of (a–d): 12,000; (e–h): 120,000.

that although the suspensions of nanomicelle prepared in this study may fail to maintain their original clarity when administered to the eyes, their transparency lies in the allowable range, which makes them a potential application in ophthalmic drug.

Stability test of micelles

To simulate whether drug-loaded nanomicelles become unstable after adhering to protein in eyes, micelles were dispersed in BSA (particle size: 21 nm) solution to analyze their stability by observing variation in their diameter. It is found in Figure 6 that the particle size of all the micelles during the 12 h exposure in BSA solution is very close to their controls (micelles not dispersed in BSA), and the diameter deviation between particles at different time point is less than 10%; supporting the fact that plenty of protein in the eye-simulated environment did not tend to attach to the micelles.

It is well known from literatures[46–48] that the tightly bound water layer between the protein and the self-assembled monolayers (SAMs) via hydrogen bonds is associated directly with the total force acting on the protein. This force becomes more repulsive as the number of hydrogen bonds between water molecules and SAMs increases. In this study, Pluronic micelles were modified by water-soluble chitosan, which produces a core-shell like structure. The core of the micelles consists of hydrophobic PPO blocks that are separated from the aqueous exterior by the shell, which is hydrated of hydrophilic PEO chains and entangled chitosan

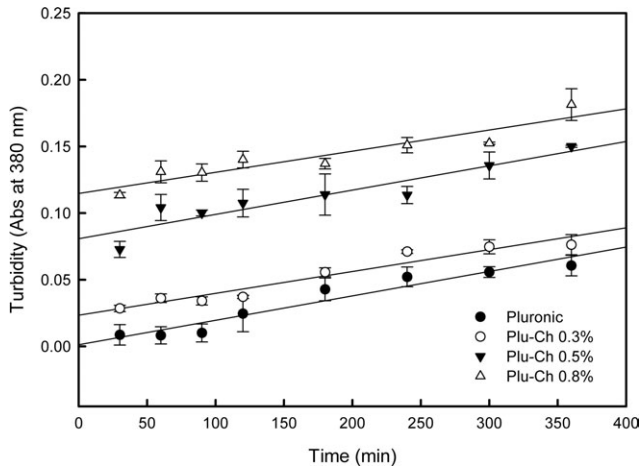

FIGURE 5. Turbidity test of unmodified and modified micelles as a function of time.

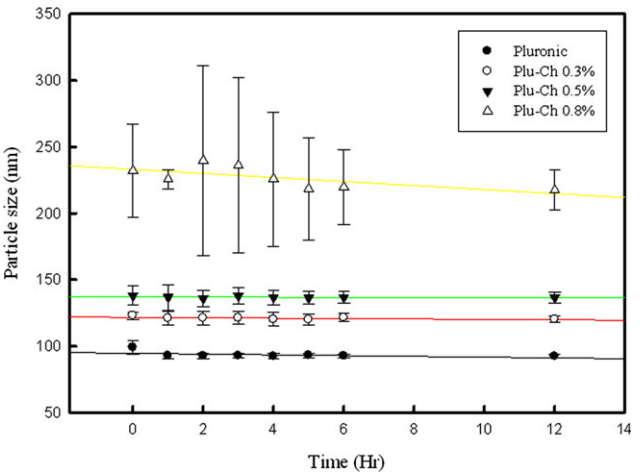

FIGURE 6. Particle size variation of unmodified and modified micelles after being dispersed in BSA solution for different time period. All the micelles were loaded with 0.3 wt. % of metipranolol. [Color figure can be viewed in the online issue, which is available at wileyonline library.com.]

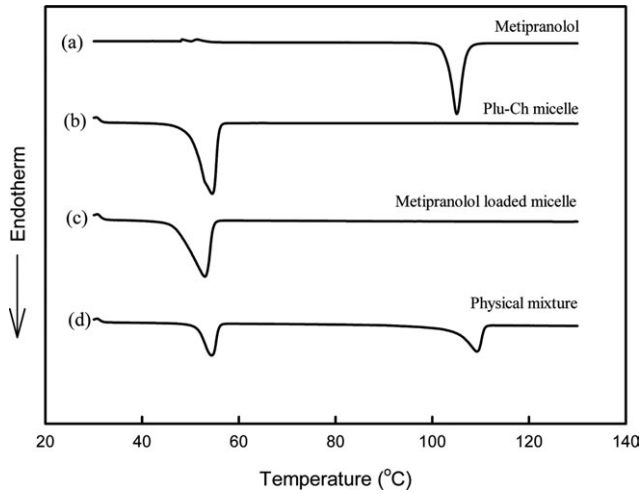

FIGURE 7. Thermal analysis of metipranolol powders, Plu-Ch micelle powders, physical mixture of metipranolol with Plu-Ch micelle powders, and metipranolol-loaded Plu-Ch micelle powders.

molecular chains. When micelles were dispersed in BSA solution, the water molecules form hydrogen bonds with the hydrophilic sites. Therefore, although there might be fewer congregations when the positively charged micelles contact with negatively charged BSA, the strong water-micelles interactions will prevent the protein from approaching the surface closely. The formation of the tightly bound hydration layer with low diffusivity leads to large repulsive hydration forces, which prevent the protein from direct contact with the micelle surface.

Nanomicelles not adhering to protein can maintain their particle size without increasing turbidity and are prevented from being removed from eyes by lacrimal fluid, and so their retention period on the anterior cornea extends, resulting in enhancing the bioavalibility.[49]

Conversely, to investigate the stability of micelles once the drug is loaded, thermal property analysis was performed on various specimens. The pure drug (metipranolol) has a thermo absorption peak at melting point 105°C [Figure 7(a)], while nanomicelles prepared in this study has a thermo absorption peak at the melting point 55°C [Figure 7(b)]. However, by physically mixing a pure drug with nanomicelles, since not any drug is incorporated, two thermo absorption peaks are observed as shown in Figure 7(c). In Figure 7(d), it is found when the drug enters nanomicelles, only one thermo absorption peak is observed around 55°C, and the other absorption peak at 105°C disappears, suggesting that the incorporated drug was shielded by micelles and after the drug entering the micelles they have very good compatibility. Therefore, by reviewing Figure 7(c,d), the drug can be clearly judged of being incorporated or not. This experiment has illustrated that drugs incorporated in nanomicelle system has very good stability.[50]

Drug loading efficiency

The loading efficiency of metipranolol on nanomicelles was measured by a spectrophotometer, three measurements for each formulation. Table I lists the loading efficiency of metipranolol on different compositions, among which Pluronic micelle has the lowest, only 12.59%. It is supposed that unmodified nanomicelles tend to accumulate and make it difficult for drugs to enter the core of micelles.

However, notable raise of loading efficiency is shown for modified nanomicelles. As after surface modification by chitoan solution, the positive charges on micelle surface can induce electric repulsion between nanomicelles, that provides more space and opportunity for drugs to enter into the core of micelles. The highest value appears in the case of Plu-Ch 0.5% with a loading efficiency up to 84.6%. This value is higher than other nanoparticle or micelle systems[12,14,37,38] and comparable to Sparfloxacin-loaded PLGA nanoparticles.[11] As too much chitosan may lead to entanglement of excess polymers and cause difficulty for drug to enter micelles, the loading efficiency of Plu-Ch 0.8% drops to 54.52%.

Furthermore, to confirm that most of the drug is incorporated in the micelles, the solubility measurements of metipranolol were conducted, since the location of drug within micelle compartments depends on its solubility. The measured solubility of metipranolol in the continuous nanodispersion phase containing ethyl acetate (0.404 $\pm$ 0.06 g/L) was significantly lower than that in ethyl acetate solution (4.52 $\pm$ 0.12 g/L). This suggests that most of the drug was entrapped inside the micelles thus lowers its solubility in aqueous solution.

In vitro drug release

An *in vitro* release study on various micelles embedded of 0.3 wt. % metipranolol prepared in this study was conducted and the result was compared with the commercial eye drop (also with 0.3 wt. % metipranolol). The experiment was conducted at 34°C using STF as releasing medium.

Figure 8 shows *in vitro* drug release profiles of various formulations. The results show that the amount of

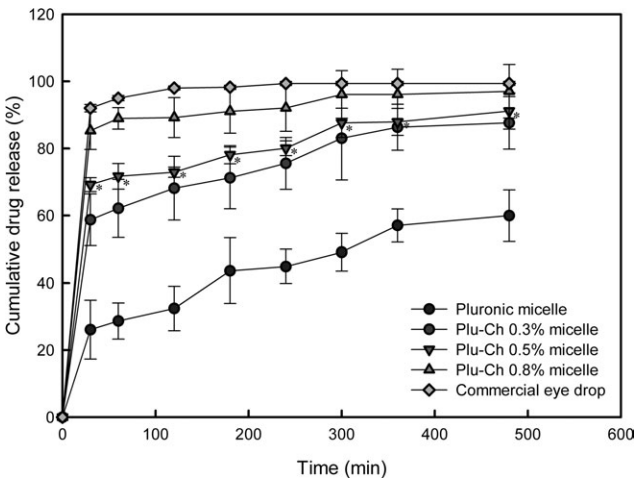

FIGURE 8. *In vitro* drug release behavior of unmodified and modified Pluronic micelles and commercial eye drops; all formulations contain 0.3 wt. % of metipranolol. *Comparison of Plu-Ch 0.5% micelles with commercial eye drops at the same time point with significant difference ($p < 0.05$).

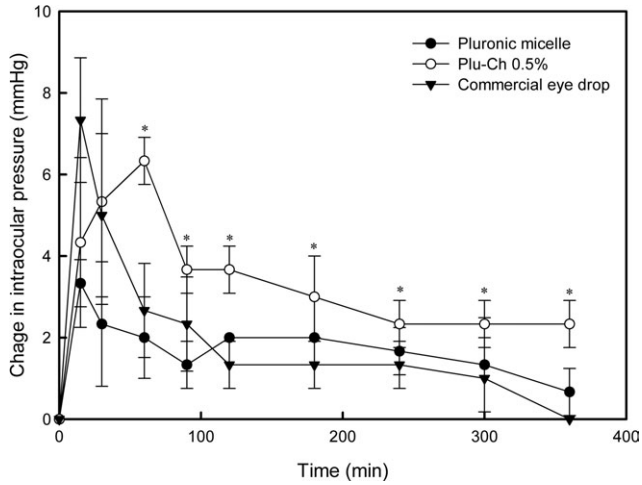

FIGURE 9. *In vivo* pharmacological response in terms of change in IOP for Pluronic and Plu-Ch 0.5% micelles and commercial eye drops; all formulations contain 0.3 wt. % of metipranolol. *Comparison of Plu-Ch 0.5% micelles with commercial eye drops at the same time point with significant difference ($p < 0.05$).

metipranolol release from commercial eye drop reaches up to 92% in 30 min, and its cumulative release is close to 100% within 2 h. This indicates that commercial eye drop does not have the effect to prolong drug release and the release process is completed in the shortest time. Comparing the release profile of Pluronic suspension with the commercial one, it is obvious that drug release from Pluronic micelle has better sustained release behavior than the commercial eye drop. Drug release from Pluronic micelle is 28.6% in the first hour and merely 57.2% after 6 h. The amount of metipranolol release is evidently bettered after chitosan modification; in the first hour, the release amount from Plu-Ch 0.3% and Plu-Ch 0.5% micelles are 59 and 69%, respectively, and they both reach 88% within 6 h. They both show sustained release behavior and the drug concentration for treatment is maintained effectively. Obviously, the amount of drug release from Pluronic micelles is enhanced at each time interval by surface modification with chitosan solution. The water soluble chitosan coated on the nanomicelles is susceptible to erode under STF, therefore faster release rate of drug is observed in surface modified nanomicelles. Through modification by chitosan, the micelles possess positive charges and are able to prolong their retaining on the precorneal via electrostatic force, thus it is expected that sustained release can be achieved and bioavailability of the drug is therefore enhanced.

However, Plu-Ch 0.8% micelle is less efficient to prolong its drug release as its release amount is as high as 88% in the first hour while it reaches 96% in the 5th hour. It is because that higher concentration of chitosan allows surplus chitosan polymer chains entwined to the outer layer of nanomicelles and make it difficult for drugs to enter cores of micelles as most of the drugs attach to the polymer chains of excess chitosan in the outer layer. Thus, the drug is quickly released under the physiological environment and allows a great amount of release in a short time.

Among the nanomicelles prepared in this study, the unmodified Pluronic micelle has the best ability in sustained release; however its release amount is only 57.2% after 6 h. As eye drop must reach the treatment level within the shortest time, it can not reach effective treatment concentration if the amount of release is too low. Such problem can be improved through modification by chitosan. Modified nanomicelles can release the drug to a sufficient concentration within short time and has significant ability in sustained release. However, as concentration of chitosan must not be too high, subsequent *in vivo* experiment is conducted by using Plu-Ch 0.5% nanomicelles.

In vivo pharmacological response

To investigate the pharmacological response of Plu-Ch nanomicelles, the *in vivo* study was conducted. Metipranolol is a nonselective β adrenergic blocking agent, it reduces secretion of aqueous humor to lower the IOP and causes miosis, thus it is a common agent for treating glaucoma.[51] In the experiment, nanomicelles suspension was dripped into the eyes of rabbit to allow the pupils shrank and the IOP reduced, through which pharmacological response of the drug could be evaluated.

Figure 9 shows variation profiles of IOP caused by *in vivo* drug release. It is found that commercial eye drop has the largest IOP variation 15 min after administration, suggesting very strong pharmacological effect occurs at very short time which is attributed to the burst release. However, the change in IOP displays declining trend 30 min after administration and almost no pharmacological effect after 120 min. So, do the Pluronic nanomicelles which show the greatest IOP variation magnitude 15 min after administration, but without good effect in reducing pressure. Its variation in IOP is 3.33 mmHg which is the smallest among three formulations, as list in Table III. At 120 min after administration, the variation of IOP lowers to about 2 mmHg. The extent of IOP variation illustrates that Pluronic micelles does not have strong resistance to the physiological barriers of the eyes and tends to be hindered. Although it has somewhat effect in sustained release, overall pharmacological effect is not significant.

The micelles modified with chitosan have the greatest IOP variation 60 min after administration. After that, the change in IOP drops gradually and has 4 mmHg magnitude at 120 min, and still displays pharmacological effect 360 min after administration, which suggests significant efficacy in sustained release. The area under the change in IOP *versus* time profiles in 360 min ($AUC_{0\text{-}360}$, list in Table III) shows that the nanomicelles of Plu-Ch have larger size than

TABLE III. Pharmacokinetic Parameters of Metipranolol-Loaded Plu-Ch Micelles in Rabbits' Lower Conjunctival Sac

Formulation	T_{max} (min)	ΔIOP_{max} (mmHg)	$AUC_{0\rightarrow 6h}$ (mmHg min)
Pluronic micelle	15	3.33 ± 0.58	552.5
Plu-Ch 0.5% micelle	60	5.33 ± 2.52	1040
Commercial eye drop	15	7.33 ± 1.53	622.5

others (1.67- and 1.88-fold greater than commercial eye drop and Pluronic micelle, respectively), indicating that Plu-Ch nanomicelles have long-term efficacy of sustained release and strong pharmacological effect.

The *in vivo* studies show that micelles modified with chitosan indeed prolong the residence of micelles on the precorneal, as the positive charge of the micelle surface and the negative charge of the precorneal generate an electrostatic force which endues the micelles with excellent ability in sustained release. As a result, the micelles sustain adequate pharmacological effect and treatment efficacy, and also avoid general side effects caused by frequent administrations. Thus it is more proper to serve to treat glaucoma compared with common commercial eye drops.

The results of *in vitro* drug release show the nanomicelles modified with chitosan appeared a trend of huge amount release in a short time, while ones not modified had a trend of sustained release. On the contrary, in the *in vivo* experiment, unmodified nanomicelles have shorter peak release time relative to modified ones, as in the *in vitro* study, nanomicelles were placed in a fixed STF solution, while in an *in vivo* setting they encountered dilution of plenty tear, which resulted in degradation of nanomicelles. The surface of the nanomicelle modified with chitosan had positive charge which generated electrostatic attraction force to allow it stayed longer on the precorneal and it degraded only when the chitosan molecular chains on the micelle surface disentangled, thus needed longer time to reach peak release. Therefore, modified nanomicelles had significant difference in pharmacological effect in comparison with other formulations. The assessment of two different experiments shows that the novel Plu-Ch nanomicelles prepared in this study have excellent performance in sustained release which can promote bioavailability of drugs applied for the ocular mucosa.

CONCLUSIONS

In this study, pluronic-based micelles were successfully prepared for ophthalmic delivery by incorporation of ethyl acetate as a dispersion agent and their surfaces were also modified by chitosan to improve their bioavailability. The physicochemical characterization of Pluronic-Chitosan nanomicelles including diameter, surface charge, morphology, turbidity, stability, and loading efficiency, demonstrates that they are very suitable use as ophthalmic carrier. Furthermore, the *in vitro* and *in vivo* studies indicate these micelles have sustained release behavior and good pharmaceutical response. As the results, the system provides a potential opportunity in decreasing frequency of administration and improving patient compliance for ocular drug delivery.

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