ELSEVIER
Nanomedicine: Nanotechnology, Biology, and Medicine
xx (2017) xxx–xxx
nanomedicine
Nanotechnology, Biology, and Medicine
nanomedjournal.com

# Ion-paired pirenzepine-loaded micelles as an ophthalmic delivery system for the treatment of myopia

Yanan Li[a,1], Yong Zhang[b,1], Pengmei Li[c], Gujie Mi[d], Jiasheng Tu[a], Linlin Sun[d], Thomas J. Webster[d,*], Yan Shen[a,d,**]

[a]State Key Laboratory of Natural Medicines, Department of Pharmaceutics, China Pharmaceutical University, Nanjing, China
[b]Children's Hospital of Nanjing Medical University, Nanjing, China
[c]Department of Pharmacy, China-Japan Friendship Hospital, Beijing, China
[d]Department of Chemical Engineering, Northeastern University, Boston, MA, United States

Received 16 December 2016; accepted 1 May 2017

**Abstract**

Myopia is one of the most common ocular disorders for which standard treatments, such as refractive surgery, often involve invasive procedures. Pirenzepine (PRZ), a muscarinic receptor antagonist, has been recognized as a promising candidate for the treatment of myopia, but possesses poor ocular bioavailability. The overall objective of this study was to prepare PRZ-sorbic acid complexes suitable to be encapsulated into micelles with high efficiency for optimal ophthalmic delivery. The results demonstrated that sorbic acid, used as the counter ion, had the most significant effects in increasing octanol–water distribution coefficient of PRZ as well as improving its corneal permeability *in vitro* among various counter ions tested. *In vivo* absorption results showed that a 1.5 times higher bioavailability was achieved by the addition of sorbic acid at 1:1 ratio. Cytotoxicity study *in vitro* and the biocompatibility study *in vivo* indicated that the micelles did not cause significant toxicities on the eyes.


*Key words:* PRZ; Ion-pair formation; Micelles; And ophthalmic delivery

Myopia is one of the most common ocular disorders around the world and is becoming more prevalent among younger generations. Asia, in particular, has seen a rapid growth of occurrence with prevalence reaching a whopping 60% in recent years.[1] The progression of myopia could lead to some complications including maculopathy, cataract, glaucoma and retinal detachment.[2] More importantly, high myopia is one of the leading causes of blindness in developed countries.[3] Although traditional eyeglasses and refractive surgeries are able to correct the visual abnormalities caused by myopia, these treatments are still far from satisfactory for complete recovery of myopia in the long term.[4] Therefore, it is utterly important to identify proper treatments for children with myopia.[1–3] Recently, several controlled clinical trials have provided evidence that atropine, a classic muscarinic antagonist that binds potently to both $M_3$ (accommodation and mydriasis) and $M_1$ muscarinic receptors (putative myopia),[5,6] can slow down myopia progression in children. However, the clinical use of atropine as a therapeutic has been limited due to serious ocular side effects such as mydriasis and cycloplegia because of undesirable binding to the $M_3$ receptor.[7] Alternatively, pirenzepine (PRZ, Figure 1) is a muscarinic receptor antagonist that is selective only to the $M_1$ receptor,[8,9] and thus is less likely to cause mydriasis and

Funding sources: This work was funded by the Ministry of Science and Technology of China (NO. 2014ZX09507001006), the National Natural Science Foundation of China (NO. 81501579), the Natural Science Foundation of Jiangsu Province (NO. BK20150702), the Science and Technology Development Fund of Nanjing Medical University (2016NJMU105), and the Priority Academic Program Development of Jiangsu Higher Education Institutions.
Conflict of interest: The authors declare no competing financial interests.
*Corresponding author.
**Correspondence to: Y. Shen, State Key Laboratory of Natural Medicines, Department of Pharmaceutics, China Pharmaceutical University, Nanjing, China.
*E-mail addresses:* th.webster@neu.edu (T.J. Webster), shenyan19820801@126.com (Y. Shen).
[1] These authors contribute equally to this work.

http://dx.doi.org/10.1016/j.nano.2017.05.001
1549-9634/

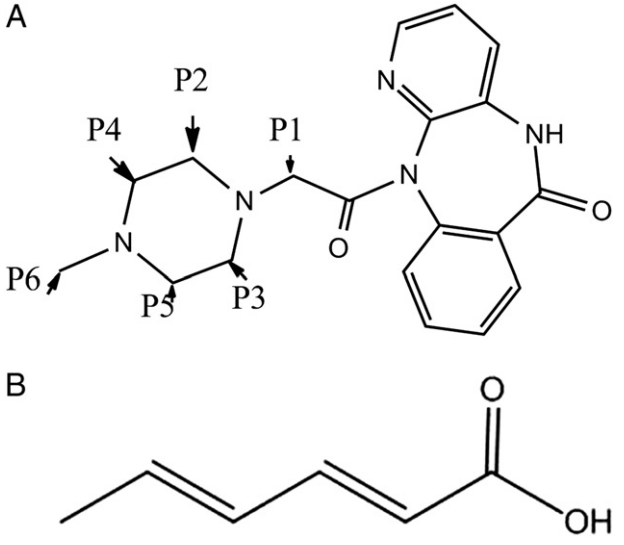


Figure 1. Structure of (**A**) PRZ and (**B**) sorbic acid.

cycloplegia than atropine. PRZ has also been shown to inhibit the development of deprivation-induced myopia and the axial elongation of eyes,[10,11] and PRZ solutions of up to 2% did not elicit any systemic side effects in adult volunteers according to previous phase-I trials on safety and tolerability.[12] As a hydrophilic compound, however, PRZ often suffers from very low transcorneal permeability and poor ocular bioavailability, which will decrease its anti-myopia effect.[13]

To overcome this delivery challenge, micellar systems had been widely investigated as carriers for PRZ to facilitate the internalization process *via* endocytosis and endosomal permeation and have been reported to increase ocular availability of PRZ by two-fold without causing any corneal damage, which is typically associated with free suspension of the drug. A material that is of particular interest in the current study is the amphiphilic block co-polymer mPEG-PDLLA. It has been reported that micelles self-assembled from mPEG-PDLLA display minimal cytotoxicity to both tumor and healthy mammalian cells[14–17] and are characterized by a unique core-shell structure with uniform size distribution. The spatial distribution of the drugs within the micelles depends on their polarities. In an aqueous environment, nonpolar molecules will be entrapped in the core, polar molecules will be adsorbed onto the surface, and substances with intermediate polarity will be distributed in certain intermediate positions.[18] However, the loading efficiency of PRZ into the micelles will be limited by its hydrophilicity, which makes it harder to be encapsulated into the hydrophobic core of micelles. Fortunately, many techniques can be utilized to further improve loading capacity as well as the corneal permeation of therapeutics.[19]

Ion pair formation, especially the organic acid ion pair formation, is one of the most promising strategies for improving loading capacity. An ion-pair is a pair of oppositely charged ions interacting with each other *via* Coulombic attractions instead of forming a covalent bond. As a result, they will behave like a single unit. Kato et al[19] reported that the ion pair formation between the drug and the organic acid could significantly increase the hydrophobicity of the drug and therefore effectively improve loading efficiency as well as eventually, bioavailability in the eyes.

Among the organic acids, sorbic acid (SA, Figure 1), an unsaturated fatty acid with six carbon atoms, might have the potential to help increase the hydrophobicity of the drug while maintaining suitable water solubility. Higashiyama et al discovered that SA could increase the oil–water distribution coefficient of timolol and its permeability across the cornea. At the optimal molar ratio of 2:1 (SA:timolol), the maximum concentration ($C_{max}$) and the area under the curve (AUC) were found to increase by 3.15 and 2.17-fold, respectively, as compared to the reference group, meaning significant enhanced permeability across the cornea.[20] In addition, the safety of SA for oral and ophthalmic use has been evaluated extensively, which is included in the China Pharmacopeia (volume IV) and approved by the State Food and Drug Administration (SFDA) for use.[21] Therefore, SA could be a promising candidate to be used as the counter ions to optimize lipophilicity as well as to enhance safety of the drug.

Thus, for all of the above reasons, the objective of the current *in vitro* and *in vivo* study was to design, characterize and optimize an SA/PRZ encapsulated micellar system made from an amphiphilic block co-polymer for ophthalmic delivery. In our previous work,[18] PRZ alone was adsorbed onto the mPEG corona of the micelles and PRZ was only present on the outer surface of the micelles, which limited drug loading efficiency. The addition of SA in the current study increased the hydrophobicity of PRZ, which led to a higher drug loading efficiency in the hydrophobic core of the micelles. Additionally, the complexation between SA and PRZ in mPEG-PDLLA micelles could lower the polarity of the resulting complexes and lead to marked alterations to both ocular penetration and the bioavailability of PRZ *in vivo*. Specifically, the impact of different amounts of SA on PRZ, including their effects on ocular permeability as well as on the octanol–water distribution coefficient ($DC_{app}$) of PRZ in the micelle systems was extensively investigated. To demonstrate its applicability *in vivo*, the ocular pharmacokinetics of PRZ micelles using SA as the counter ion were also evaluated.

## Methods

### *Materials*

Pirenzepine dihydrochloride (purity >99.5%) was obtained from Wanlian Pharmaceutical Co. (Ningbo, China). Methoxyl poly (ethylene glycol)-poly(D,L-lactic acid) (mPEG-PDLLA, Mw = 5000, molar ratio of mPEG/PDLLA = 40/60) was purchased from Xi'an ruixi Biological Technology Co. Hydroxypropyl methylcellulose (HPMC) (Methocel K100 M) was obtained from Colorcon (Shanghai, China). All organic acids were purchased from WanQing Chemical Glassware Instrument (Nanjing, China). All the reagents were analytical grade and used without further purification.

### *Corneal epithelial cell culture*

Human corneal epithelial (HCE-2) cells purchased from the American Type Culture Collection (ATCC®number CRL-11135) were maintained in 175 $cm^2$ flasks in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Invitrogen, Carlsbad, Calif., USA) containing 10% fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate in a 37 °C, humidified, 5%

$CO_2$/95% air environment until 85–90% confluence was reached. HCE-2 cells were then trypsinized (0.25% trypsin/EDTA; Gibco, Invitrogen) for 3 min and the cell suspensions ($1 \times 10^5$ cells/ml, $1 \times 10^4$ cells/well) were seeded onto 96-well tissue culture plates. Cells at passage numbers of 3–5 were used in these experiments.

*Animals*

Rabbits weighing 2 ~ 2.5 kg were purchased from Qinglongshan farms (Nanjing, China). All animal experiments were conducted in full compliance with the National Institute of Health Guide for Care.

*Degradation kinetics of pirenzepin hydrochloride*

To determine the degradation kinetics of PRZ, 2% PRZ was diluted using different phosphate buffers with pH values ranging from 1 to 10 to a concentration of 2.26 M (1 mg/mL), packed into the ampoule, and incubated in water at 85 °C. At predetermined intervals (2, 4, 6, 12, and 24 h), a 0.5 mL aliquot of the solution was taken and was further diluted to a total volume of 10 mL. The pH of each solution was recorded and the amount of PRZ in solution (C) at each time point was determined by HPLC (Thermo Scientific Chromeleon 3000) using a Luna $RP_{18}$ 5 mm 4.6 × 150 mm column (Phenomenex Sci-Tech Co. Ltd., CA) and a guard column (Huaiyin Hangbang Sci-Tech Co. Ltd., China.) with methanol/0.02 M $KH_2PO_4$/sodium 1-pentanesulfonate (350/650/1, v/v/m, pH was adjusted to 8.0 by adding 1 M NaOH) as the mobile phase. The column temperature was at 35 °C, the flow rate was 1 mL/min and the UV detector was set at 280 nm. In order to determine the kinetic order of degradation reactions and the apparent hydrolysis rate constant (K) in different buffers, lnC (determined by HPLC) was plotted against time (t) in the degradation curve. 1nK ~ pH curve were then plotted to determine the pH where PRZ showed the best stability ($pH_m$), which can be found as the lowest point on the curve.

*Preparation of PRZ/SA ophthalmic micelles*

To evaluate the effects of the organic acid on the ocular permeability of PRZ loaded mPEG-PDLLA micelles, PRZ/SA loaded mPEG-PDLLA micelles were prepared as previously reported.[18] Briefly, HPMC (1%, 2%, 4% and 6%) was added into 100 mL of water (Part I) at 80–90 °C and were mixed until it was uniformly dispersed. 4% mPEG-PDLLA (40/60) and organic acid/PRZ (4%) were dissolved in 100 mL of a phosphate buffer solution (pH = 5.1) and the pH of the mixture was adjusted to 5.1 using a sodium hydroxide solution (part II). Part-II was then aseptically mixed with the 100 mL gel of Part-I. The mixture was autoclaved at 121 °C for 30 min. A homogeneous solution was obtained upon cooling the mixture to room temperature.

*Characterization of PRZ/SA micelles*

The morphologies of the micelles were observed under transmission electron microscopy (TEM, H-600, Hitachi, Japan). The size distribution of PRZ micelles and PRZ-SA/mPEG-PDLLA (40/60) micelles were determined by a dynamic light scattering (DLS) system (Malvern 3000 HSA). The viscosity and the osmotic pressure were measured by an ubbelohde viscometer (50,100, SCHOTT). To determine the loading efficiency of the PRZ in the micelles, free PRZ was first removed by dialysis (3000 Da) and the amount of encapsulated PRZ was then determined by the HPLC method [18] at 280 nm by dissolving the micelles in ethanol. Encapsulation efficiency (EE%) was estimated using the following Eq. (1):

$$EE(\%) = \frac{PRZ_{encapsulated}}{PRZ_{added}} \times 100 \quad (1)$$

*$^{13}C$- and $^{1}H$- nuclear magnetic resonance spectroscopy (NMR)*

PRZ and sorbic acid were dissolved at a molar ratio of 1:1 in a phosphate buffer of pH 5.1. The solution was then freeze-dried for 48 h to obtain white solid powders. The PRZ and SA were prepared following the same respective procedure used as controls. The $^{13}C$–NMR and $^{1}H$–NMR spectra of PRZ, sorbic acid, and their lyophilized complexes (molar ratio, 1:1) were recorded in dimethylsulfoxide-d6(DMSO-d6) using a Bruker(AVACE) AV-500 spectrometer ($^{13}C$ at 75 MHz and $^{1}H$ at 300 MHz). Tetramethylsilane (TMS) was used as an internal standard in DMSO-d6. The chemical shifts were relative to the DMSO signal at 39.7 ppm for $^{13}C$ NMR and the TMS signal at 0 ppm for $^{1}H$ NMR in DMSO-$d_6$.

*Effect of various organic acids on the apparent octanol–water distribution coefficients ($DC_{app}$) of PRZ*

PRZ (1 μM) and organic acid solutions (such as sorbic acid, maleic acid, fumaric acid, oxalic acid and citric acid) were prepared at different molar ratios in water saturated with n-octyl alcohol. The pH of these solutions was adjusted to 5.1 with either HCl or NaOH. The molar ratios of organic acid to PRZ were 0:1, 0.5:1, 1:1, 2:1, 4:1, 6:1 and 8:1. The solutions were placed in a thermostatic water bath at 34 ± 0.5 °C and were shaken at 100 rpm/min for 24 h on an orbital shaker (Germany IKA). The solutions were then centrifuged for 15 min to separate the two phases. Concentrations of PRZ in the water were determined before and after shaking ($C_b$ and $C_a$) with HPLC following the procedure described above. The apparent octanol–water distribution coefficient of PRZ ($DC_{app}$) was calculated by the following Formula (2):

$$DC_{app} = \frac{C_b - C_a}{C_a} \quad (2)$$

*Effect of various organic acids on the permeation coefficient of PRZ/SA micelles*

mPEG-PDLLA, PRZ and SA (molar ratio of PRZ to SA at 1:1) were dissolved in a phosphate buffer solution to make the concentration of PRZ 25.2 mg/ml. The pH of the solution was adjusted to 5.1 using 0.1 M NaOH and the solutions were also made isotonic by adding NaCl. Solutions with different organic acids were prepared according to a similar procedure. To harvest corneas, rabbits were sacrificed by injecting sodium pentobarbital intravenously in a lethal dose. The corneas of both eyes were then excised and were mounted onto a diffusion chamber (Figure S1). 5.00 mL of a receptor solution (Ringer solution) was added to the endothelial side, and 0.5 mL of PRZ-organic acid/mPEG-PDLLA micelles were applied to the epithelial side. The diffusion chamber was maintained at 34 ± 0.5 °C in a thermostatic water bath. Receptor buffers were taken out at predetermined time intervals

Table 1
Observed rate constants (K) for the degradation of PRZ in aqueous solution at different pH values (1–10).

| pH | K ($h^{-1}$) | ln K | R |
|---|---|---|---|
| 1.1 | $4.73 \times 10^{-2}$ | −3.05 | 0.9874 |
| 2.2 | $4.20 \times 10^{-3}$ | −5.47 | 0.9948 |
| 3.0 | $3.50 \times 10^{-3}$ | −5.65 | 0.9933 |
| 4.0 | $2.80 \times 10^{-3}$ | −5.88 | 0.9861 |
| 5.1 | $1.60 \times 10^{-3}$ | −6.81 | 0.9940 |
| 6.0 | $4.00 \times 10^{-3}$ | −5.52 | 0.9857 |
| 7.0 | $1.35 \times 10^{-2}$ | −4.31 | 0.9914 |
| 8.1 | $3.18 \times 10^{-2}$ | −3.45 | 0.9960 |
| 10.0 | $4.67 \times 10^{-1}$ | −0.76 | 0.9999 |

Temperature was 85 °C, ionic strength (I) = 0.3, and R is the linear correlation coefficient.

(0.5, 1, 1.5, 2, 3, 4 and 6 h), and were then promptly replaced with the same amount of fresh aerated receptor buffer. The solutions were filtered through 0.45 μm microporous membranes and were quantified for PRZ concentration by HPLC.

The cumulative penetration Q was calculated by the following Formula (3):

$$Q_n = V_0\left(C_n + \frac{V}{V_0}\sum_{i=1}^{n-1} C_i\right) = V_0 C_0 + V\sum_{i=1}^{n-1} C_i \quad (3)$$

where $C_n$ is the concentration of PRZ in the receptor solution at the $n^{th}$ sampling point; $C_i$ is the concentration of PRZ before the $n^{th}$ sampling point; $V_0$ is the volume of the receptor solution; and $V$ is the sample volume.

The apparent permeation coefficient ($P_{app}$, cm/s) of PRZ was calculated as follows:

$$P_{app} = \frac{\Delta Q}{\Delta t \cdot C_0 \cdot A \cdot 3600} \quad (4)$$

where $C_n$ is the concentration of PRZ in the receptor solution at the $n^{th}$ sampling point; $C_i$ is the concentration of PRZ before the nth sampling point; $V_0$ is the volume of receptor solution, and $V$ is the sample volume. A is the efficient cross-section area. The steady-state flux ($J_{ss}$) was determined by Formula (5):

$$J_{ss} = C_0 P_{app} \quad (5)$$

### *In vivo pharmacokinetics and biocompatibility study*

All *in vivo* experiments were carried out on healthy New Zealand albino rabbits. All experimental protocols *in vivo* were peer-reviewed and approved by the China Pharmaceutical University Animal Experiment Center. The detailed procedures for both the *in vivo* pharmacokinetics and biocompatibility studies were included in the supplementary materials. PRZ pharmacokinetic parameters, including $C_{max}$, $T_{max}$ and $AUC_{0\text{-}t}$, were calculated using standard noncompartmental pharmacokinetic methods by WinNonlin software.

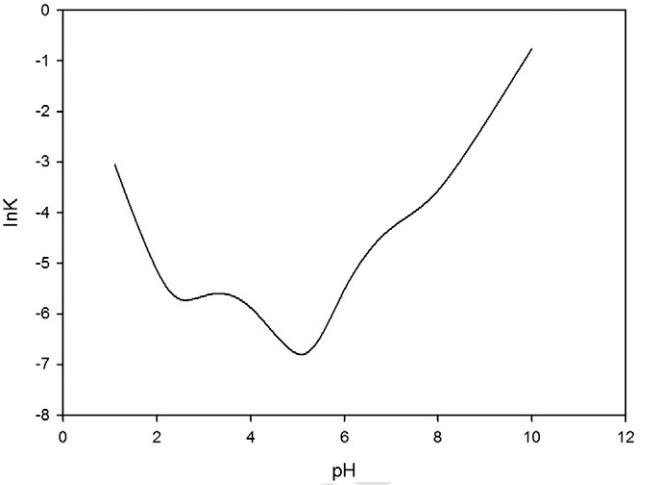


Figure 2. The hydrolysis of PRZ at various pH, 85 °C and I = 0.3.

### *Statistical analysis*

Statistical analysis was performed by SPSS using a standard Student's *t* test (comparing only two individual groups) with a minimum confidence level of 0.05 for the significant statistical difference. All values are reported as mean ± standard deviation (SD).

## Results

### *Degradation kinetics of pirenzepine*

PRZ is ionic in nature and is not stable in aqueous solution. In order to obtain a relatively stable formulation, the degradation kinetics and acid-alkali ionization constant of PRZ were studied in aqueous solution. ln C *vs*. time (t) at predetermined intervals was plotted as shown in Figure S2. It was found that the plots were linear at all pH values tested indicating that the hydrolysis followed first-order kinetics (Eq. (6)). K values (Table 1) at different pH were calculated using the slope of the line (Figure S2) as follows:

$$\mathrm{InC_A} = \mathrm{InC_{A,0}} - \mathrm{Kt_A} \quad (6)$$

where $C_A$ is the concentration of PRZ at the $t_A$(h) and $C_{A,0}$ is the initial concentration of PRZ.

lnK values were plotted against pH values as shown in Figure 2. The curve took a sigmoidal turn between pH values of 2.2 ~ 6.0, which indicated that PRZ was deprotonated to the free alkali form. PRZ showed the highest stability at pH 5.1 ($pH_m$) where lnK was the smallest. While above pH 6.0, the degradation of PRZ started to increase and the hydrolysis rate of PRZ increased significantly with the increase of pH as demonstrated by a sharp increase in lnK value. We hypothesized that the increase in degradation is probably due to the alkali-catalyzed hydrolysis in more alkaline conditions. As a result, pH 5.1 was selected for the following preparations, which is also a pH value within the tolerance limits of 5.0 ~ 9.0 for ophthalmic preparation.[22]

### *Characterization of PRZ/SA ophthalmic micelles*

The size distribution of the polymeric micelles was analyzed by dynamic light scattering (DLS) (Figure 3). For the PRZ/

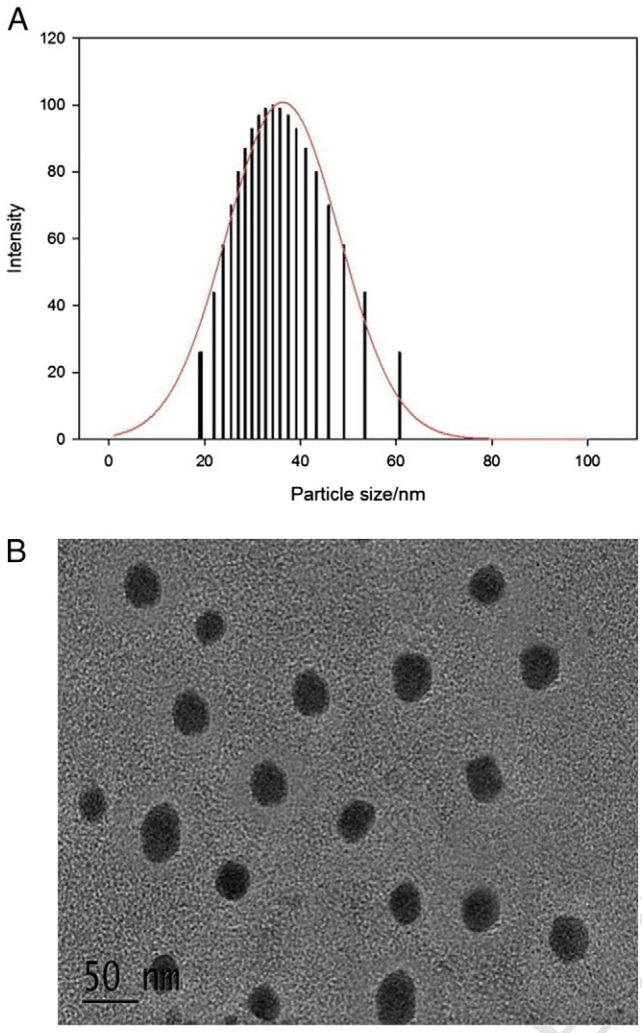


Figure 3. **(A)** The size distribution and **(B)** morphology of the PRZ/SA ophthalmic micelles.

mPEG-PDLLA ophthalmic micelles, the average hydrodynamic diameter was about 60 nm and the dispersion index was less than 0.2, which indicated a relatively uniform distribution. The average size of polymeric micelles increased slightly to 62 nm after the addition of organic acid, which is in accordance with a previous report[18] that the adsorption of the hydrophilic PRZ onto the mPEG corona of the polymeric micelles may result in increases in the size of the micelles. The TEM micrograph showed that the PRZ/SA micelles exhibited a relatively uniform spherical shape. More importantly, the addition of SA can also increase the hydrophobicity of PRZ, which leads to a higher drug loading efficiency (78%) compared to the ones without SA (46%). The reason might be that the formation of ion pairs between PRZ and SA could shield the charges of PRZ and therefore increase the hydrophobicity of PRZ/SA complexes as compared to their ion-form counterparts. Methoxy poly(ethylene glycol)-polylactide copolymer (mPEG-PDLLA) is an amphiphilic block polymer which is known to self-assemble into polymeric micelles.[23–25] The micelles are characterized by their unique core-shell structures, in which hydrophobic segments(PDLLA) are segregated from the aqueous exterior to form an inner core

Table 2 t2.1
The effect of HPMC on the viscosity and osmotic pressure of the PRZ ophthalmic solution. t2.2

| HPMC concentration (%) | 0.5 | 1.0 | 2.0 | 3.0 | 4.0 | t2.3 |
|---|---|---|---|---|---|---|
| Viscosity(cP) | 0.51 | 4.89 | 20.40 | 80.90 | 132.85 | t2.4 |
| Osmotic pressure (osmol/kg) | 297 | 295 | 294 | 294 | 334 | t2.5 |

surrounded by hydrophilic segments (mPEG). Based on previous studies,[26–27] these micelles are known to have an anisotropic water distribution within their structures and often demonstrate a polarity gradient from highly hydrated surface (corona) to the hydrophobic core. As a result, the spatial position of certain solubilized substances (drugs of interest) within micelles will depend on its hydrophobicity. Thus, more PRZ was entrapped into the hydrophobic core of the micelles. Additionally, as the PRZ/SA complexes still retains sufficient water solubility and can interact with the mPEG corona (Figure S3), a certain amount of the drug was allowed to absorb onto the surface. Altogether, the drug loading efficiency was greatly improved.

The viscosity and osmotic pressure of the PRZ ophthalmic solution at different HPMC concentrations were determined and the results are shown in Table 2. The viscosity increased with the increase of HPMC concentrations while the osmotic pressure maintained pretty much the same value except for a small increase observed with the 4% HPMC. Researchers have shown that increasing the viscosity to around 10 ~ 20 cP could greatly improve the bioavailability of the ophthalmic solution due to the enhancement of the drug retention time in the eyes,[28] yet hyper-viscosity could cause discomfort to the eyes. Therefore, the 2% HPMC formulation with a viscosity of around 20 cP might be an optimal concentration for the preparation of ophthalmic micelles, which might have the potential to enhance bioavailability while minimizing irritation to the eyes.

### *$^{13}C$ and $^{1}H$- nuclear magnetic resonance spectroscopy (NMR)*

The $^{1}H$ NMR and $^{13}C$ NMR spectra of PRZ with and without the presence of sorbic acid were recorded and representative spectra are shown in Figure 4. Upon the addition of SA into PRZ, negligible changes in SA signals were observed in the PRZ/SA complex when compared to the $^{1}H$ NMR spectra of SA alone. The germinal protons of P1 (shown in Figure 1) in PRZ had a signal at 3.34 ppm in its free form and split into a doublet of doublets (dd) signal (P1a and P1b) due to the spin–spin coupling phenomenon. We hypothesized that this could be because of the complexation of SA at the tertiary amine group (the one next to P1) in PRZ, and two distinguishable signals were produced from both the vicinal and germinal coupling (Figure 4, *A*). Additionally, the chemical shifts of carbon atoms in the free form of PRZ and in the PRZ/SA complex were recorded (Figure 4, *B*) and are summarized in Table 3. It was demonstrated that the signals of P1, P2, P3, P4 and P5 in the PRZ/SA complex changed by about 0.2 ppm, indicating that the complexation of SA with PRZ had an impact on the carbon atoms in close vicinity to the tertiary amine group of PRZ. Based on the evidence shown above, it was proposed that SA was able to form a stable complex with PRZ and complexation occurred between the tertiary amine group of PRZ and the carboxyl group of the SA.

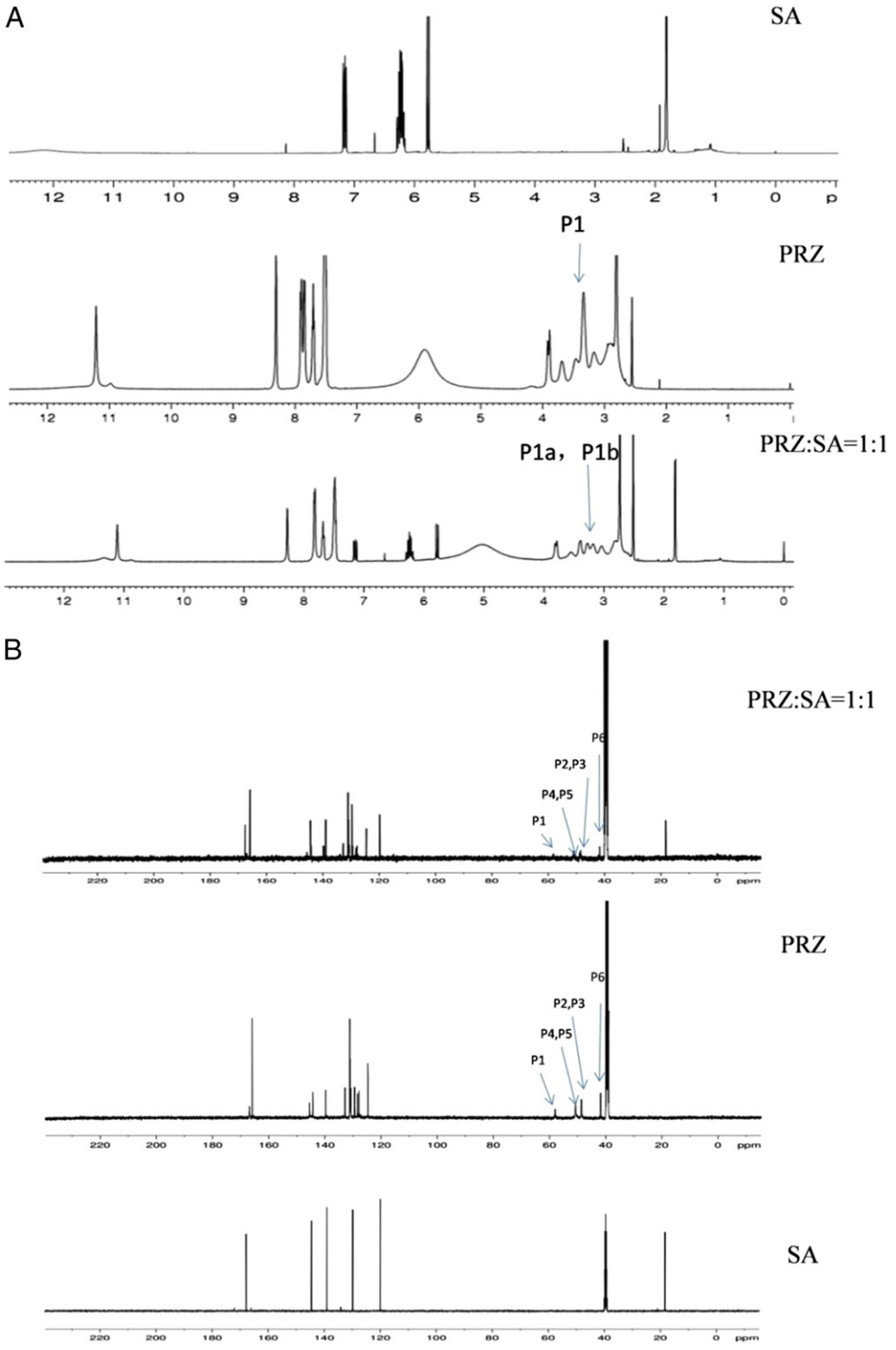


Figure 4. The $^{1}$H–NMR **(A)** and $^{13}$C–NMR **(B)** of sorbic acid, PRZ and lyophilized complexes of PRZ and sorbic acid (1:1 molar ratio).

*Effect of various organic acids on the apparent octanol–water distribution coefficients of PRZ*

To improve the low $DC_{app}$ of PRZ due to its poor hydrophobicity under physiological conditions, organic acids were added to facilitate ion pair formation, a process that would greatly increase the hydrophobicity of PRZ. It was demonstrated that there was almost a 9.6-fold ($p \leq 0.01$) increase in log($DC_{app}$) upon adding SA, which indicated that the SA might be able to form stable ion pairs with PRZ (Table 4). The formation of ion pairs between PRZ and organic acids, therefore, could shield the charge of PRZ and increase the hydrophobicity PRZ/organic acid complexes as compared to their

t3.1 Table 3
t3.2 Chemical shifts (δ, ppm) of PRZ obtained from $^{13}C$ NMR spectra in DMSO-$d_6$.

| t3.3 Compound | PRZ(ppm) | | | |
|---|---|---|---|---|
| t3.4 | P1 | P2, P3 | P4, P5 | P6 |
| t3.5 PRZ | 57.97 | 48.64 | 50.74 | 41.78 |
| t3.6 PRZ:SA (1:1) | 58.16 | 48.53 | 50.98 | 41.74 |

ion-form counterparts. It is hypothesized that PRZ is a slightly alkaline drug that carries positive ions in water, while organic acids dissociate, lose a hydrogen ion and act as the counter ion. Among all organic acids tested, sorbic acid, an unsaturated fatty acid with six carbons, exhibited the most improvement in distribution coefficients. Oxalic acid, citric acid, fumaric acid and maleic acid all have higher water solubility than SA, and therefore showed smaller hydrophobicity increments when complexing with PRZ (Figure 5, *A*). A proposed mechanism of the ion pair formation to increase loading efficiency is also shown in the schematic diagram in Figure 5, *C*. It is hypothesized that PRZ is a slightly alkaline drug that carries positive ions in water, while organic acids dissociate, lose a hydrogen ion and act as the counter ion. In addition, the ion pair formation with any fatty acids with more than 6 carbons, although improving hydrophobicity, is far less soluble than SA.[20] Due to the aforementioned reasons, SA was chosen as the counter ion in the ophthalmic preparation in this study. Additionally, the 1:1 molar ratio of PRZ:SA was chosen for subsequent studies due to following reasons: firstly, SA became more insoluble as the ratio exceeded 1:1. It will inevitably increase the difficulty in formulation preparation and could potentially hinder its future applications, in which easy preparation with high reproducibility is often preferable. Secondly, it has also been shown both in cytotoxicity assay in current study (Figure 6) and in the literature[26] that an increase in SA concentration could lead to a higher toxicity to healthy corneal cells. In this sense, a lower molar ratio (1:1) was chosen to minimize potential toxicity. Finally, according to Figure 5, *C*, which demonstrated the dissociation mechanism of PRZ, all SA might theoretically complex with PRZ completely at a 1:1 ratio and could therefore shield the charge to minimize the polarity, which ultimately led to marked alterations to both ocular penetration and the bioavailability of PRZ *in vivo*.

### *Effects of various organic acids on the permeation coefficient of PRZ*

*In vitro* experiments on corneal penetration were carried out to investigate the effect of SA on transcorneal absorption of PRZ. The results showed that PRZ permeated through the cornea at a constant rate in the presence of different organic acids, and the diffusion behaviors were in accord with zero-order kinetics (Figure 5, *B*). The results also indicated that the organic acids were able to increase the hydrophobicity of PRZ while not altering its diffusion behavior. Compared with other organic acids, the PRZ/SA formulation achieved the most significant increase in terms of steady-state flux. Hydrochloride salts of PRZ were used in this study as the reference, the corneal penetrating coefficient of PRZ/SA was found to be 1.93 ($p \leq 0.05$) times higher than that of the PRZ/hydrochloride, which indicated that SA might have the potential to significantly improve the corneal permeability of PRZ

t4.1 Table 4
t4.2 Effects of various organic acids on the octanol–water partition coefficients of PRZ (n = 3).

| t4.3 Molar ratio (acid:PRZ) | 0:1.0 | 0.5:1.0 | 1.0:1.0 | 2.0:1.0 | 4.0:1.0 | 6.0:1.0 | 8.0:1.0 |
|---|---|---|---|---|---|---|---|
| t4.4 SA | 0.230 | 0.316 | 0.358* | 0.472 | 0.663 | 0.743 | 0.861 |
| t4.5 Maleic acid | 0.220 | 0.235 | 0.264 | 0.295 | 0.35 | 0.352 | 0.373 |
| t4.6 Fumaric acid | 0.228 | 0.223 | 0.237 | 0.237 | 0.258 | 0.26 | 0.267 |
| t4.7 Citric acid | 0.241 | 0.251 | 0.267 | 0.284 | 0.312 | 0.362 | 0.393 |
| t4.8 Oxalic acid | 0.202 | 0.245 | 0.280 | 0.293 | 0.336 | 0.373 | 0.388 |

t4.9 Note: P* < 0.05, *vs.* SA: PRZ (0:1.0).

(Figure 5, *B*). The results shown here further suggested that SA used as the counter-ion can form a low polarity complex with PRZ and increase its hydrophobicity. Finally, it was illustrated (Figure S4) that the apparent permeation coefficient was consistent with the apparent octanol–water distribution coefficients, further suggesting that SA is the most suitable counter-ion for the preparation of PRZ micelles at a 1:1 molar ratio.

### *The determination of corneal hydration value*

The corneal hydration value is the most important index for detecting corneal injury after organic acid treatment. The detailed procedure for the corneal hydration value determination was included in the supplementary materials. It is reported that the normal corneal hydration value is between 76% and 80% while a hydration value higher than 83% indicates that the cornea might suffer from a certain degree of damage.[29] The results correlated with literature values very well and showed that the corneal hydration value of the normal cornea was 78.21 ± 1.21%. After the diffusion experiment *in vitro* for 4 h, the corneal hydration values were determined and the results are shown in Table 5. There was no significant difference among the control and the experimental groups. The results indicated that the addition of the organic did not cause significant damage to the integrity of the cornea.

### *Cytotoxicity assay*

The cytotoxicity of SA/PRZ micelles toward human corneal epithelial cells *in vitro* was investigated *via* MTT assays. As illustrated in Figure 6, the results indicated that the blank micelle group showed minimal toxicity with an inhibition rate of around 6% even at the highest concentration tested (50 g/l). The cytotoxicity of the PRZ/SA solution significantly increased along with the increase in PRZ/SA concentration. It was hypothesized that the increase in toxicity was due to the addition of the cytotoxic sorbic acid, which was further supported by other studies where the addition of 2.0 g/L sorbic acid caused more than 95% corneal cell death in a period of 5 h.[30] Fortunately, the encapsulation of PRZ/SA complexes into the mPEG-PDLLA micelles significantly decreased its cytotoxic effect as exemplified in Figure 6, possibly due to the fact that cells would have less access to those PRZ/SA complexes within the hydrophobic core of the micelles. Additionally, the concentration of sorbic acid used in our formulation was 2.0 g/L (the corresponding concentration of mPEG-PDLLA was 20 mg/ml) and the cell inhibition rate (%) at this concentration was no more than 10%, which was within the limit of safety use. However, *in vivo* safety still needs further characterization.

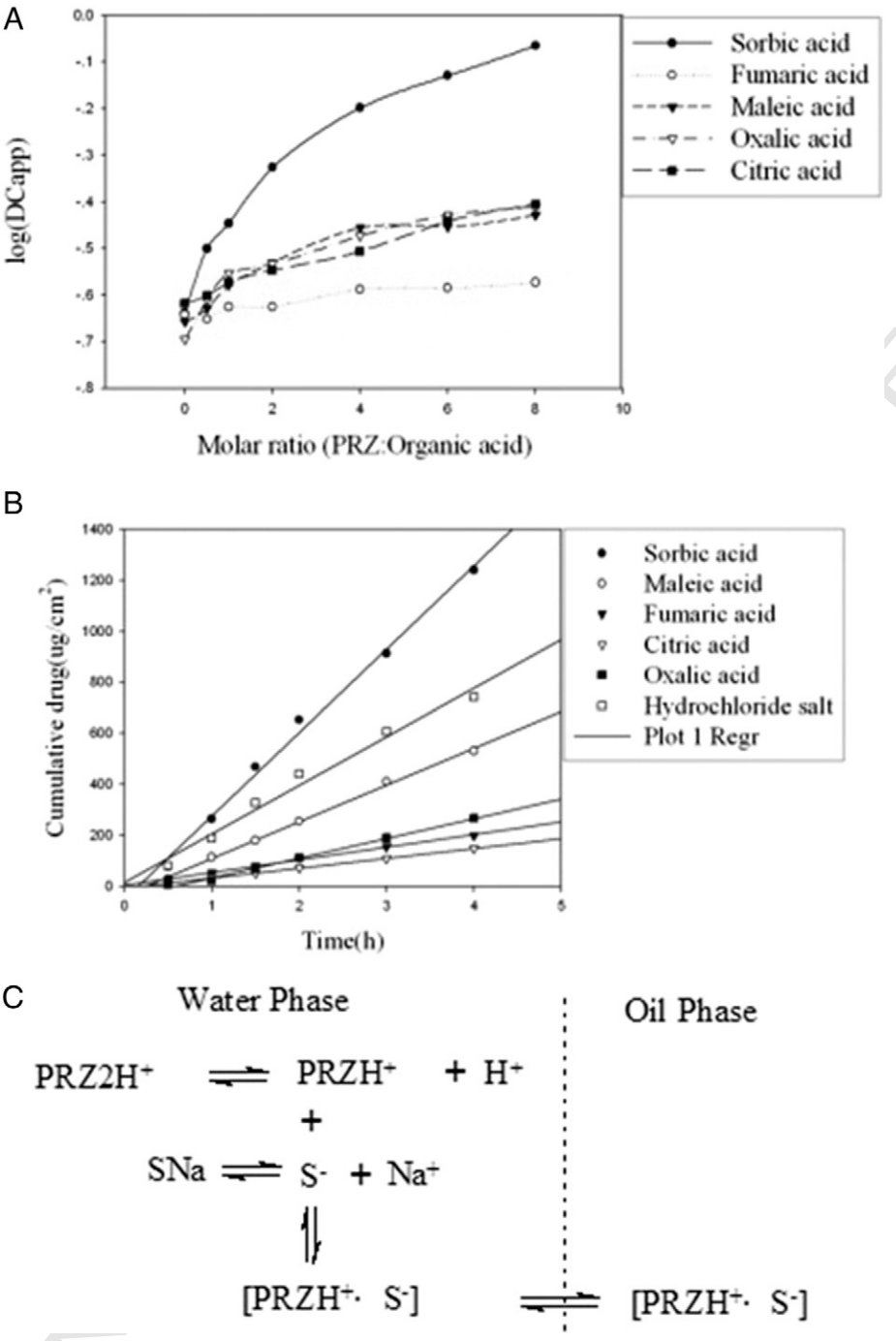


Figure 5. **(A)** Effects of various organic acids on the apparent octanol–water partition coefficients of PRZ at different molar ratios. **(B)** *In vitro* transcorneal permeation profiles of PRZ penetrated by various organic acids (n = 3, PRZ/organic acid (molar ratio = 1:1). **(C)** Schematic diagram of ion pair formation in water and octanol.

### *In vivo pharmacokinetics*

*In vivo* studies were conducted to compare the ocular pharmacokinetic behavior in the aqueous humor of the rabbits between PRZ ophthalmic micelles and PRZ/SA ophthalmic micelles. The concentration-time profiles of PRZ in the humor of conscious rabbits after instillation with either PRZ or PRZ/SA micelles are shown in Figure 7. Compared to our previously published results,[18] in which 2% of the PRZ ophthalmic solution was utilized, the PRZ/mPEG-PDLLA preparation in the current study achieved a significantly higher PRZ concentration in the aqueous humor after instillation. The bioavailability of the PRZ/mPEG-PDLLA preparation almost doubled when compared to the PRZ solution alone. However, it was identified upon closer examination that both formulations had similar release profiles with the Tmax around 2 h after instillation. The $C_{max}$ and $AUC_{0-48}$ for

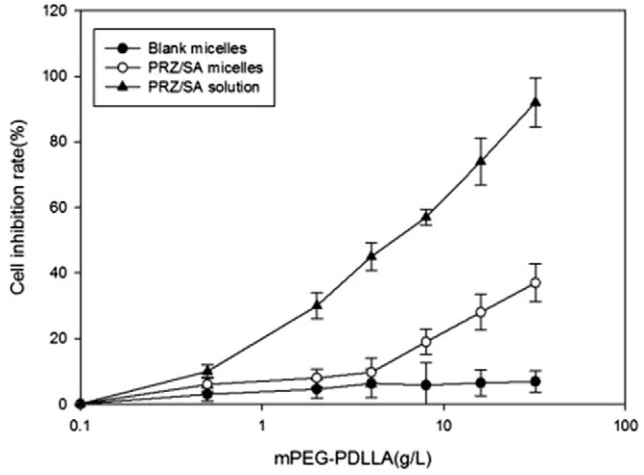


Figure 6. The cell inhibition rate (%) of the bank micelles, the PRZ/SA solution and the PRZ/SA micelles to HCE-2 cells at 37°C after incubation for 24 h.

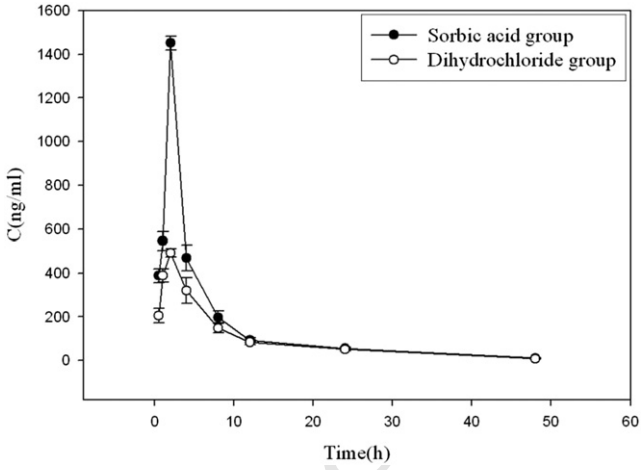


Figure 7. Concentration-time profiles of PRZ in the aqueous humor after instillation of 20 mg/ml PRZ/mPEG-PDLLA ophthalmic micelles and PRZ/SA ophthalmic micelles in a conscious rabbit (n = 5).

t5.1 Table 5
t5.2 Cornea hydration level at different conditions (n = 3).

| t5.3 Group | Hydration level (% ± SD) | Group | Hydration level (% ± SD) |
|---|---|---|---|
| t5.4 pH 5.1 | 82.99 ± 0.93 | SA | 82.54 ± 1.66 |
| t5.5 HCl | 80.92 ± 2.02 | Maleic acid | 81.31 ± 1.31 |
| t5.6 Citric acid | 82.17 ± 1.86 | Fumaric acid | 81.78 ± 0.77 |
| t5.7 Oxalic acid | 81.66 ± 0.45 | Negative control | 81.27 ± 0.76 |

t5.8 Values represent the mean ± SD, N = 3.

524 the PRZ/SA micelles were 840.65 ± 43.37 ng/mL and 7074.37 ± 525 230.02 ng h/mL, respectively, a significant increase when compared 526 to 165.18 ±26.91 ng/mL and 4544.89 ± 343.98 ng h/mL for the 527 PRZ micelles. We hypothesized that the PRZ/SA micelle achieved 528 significantly higher aqueous humor PRZ concentrations after 529 instillation than PRZ micelles due to the increase in permeation 530 abilities across the cornea *via* the ion-pair formation between PRZ 531 and SA. The AUC of the PRZ/SA micelles is 1.55 times higher than 532 that of the PRZ micelles, indicating the potential of PRZ/SA 533 micelles to increase *in vivo* bioavailability ($p \leq 0.05$), which could 534 be vital in improving drug efficacy while minimizing side effects.

535 The micelles have to mix with tears before they can be absorbed 536 through the cornea. According to the Akaike information criterion 537 (AIC) of different compartment models, the compartmental 538 analysis indicated that it fitted the two-compartment model 539 (weighting factor was 1/C, R > 0.9999) which is the lowest. The 540 results were consistent with the absorption and distribution 541 behavior. Based on the results above, the addition of SA 542 significantly enhanced the permeation of PRZ micelles *in vivo*, 543 which is consistent with the results observed *in vitro*.

544 *Biocompatibility study*

545 The safety of the ophthalmic solution was further evaluated to 546 ensure that it has low eye toxicity and causes negligible irritation 547 to the eyes. High irritation to the eyes would cause secretion of 548 tears, which will dilute drug concentration and affect its efficacy. 549 The results showed that all the tissue areas were intact and 550 smooth. Histopathological results (Figure 8) demonstrated that 551 the instillation of PRZ/SA micelles into rabbit eyes did not cause 552 any significant irritation compared to the saline group, indicating 553 that the PRZ/SA micelles were within the limit of the eye 554 tolerance and could be safe to use in ophthalmic applications.

## Discussion 555

556 Based on both *in vitro* and *in vivo* experiments, it is safe to 557 conclude that SA can enhance the transcorneal permeation of PRZ 558 mPEG-PDLLA micelles as well as increase the bioavailability of 559 PRZ *in vivo*. Lipophilization of ionic drugs that are highly 560 hydrophilic by ion-pair formation with appropriate counter-ions 561 have been shown to be promising for several applications.[19–20] In 562 this study, we demonstrated that the complexes formed ion-pair 563 formation between SA and PRZ and can be successfully formulated 564 into micelles to improve drug loading efficiencies for PRZ. Our 565 findings also indicated that the complexation between SA and PRZ 566 in mPEG-PDLLA micelles lowered the polarity of the resulting 567 complexes and led to marked alterations to both ocular penetration 568 and the bioavailability of PRZ *in vivo*. Additionally, PRZ/SA 569 micelles developed in this study do offer several unique advantages 570 over other delivery systems of PRZ, such as liposomal preparation, 571 iontophoresis and intravitreal/subconjunctival injection. PRZ 572 encapsulated liposomes were shown to increase bioavailability 573 *in vivo* and can partly inhibit the development of myopia, which will 574 have to be confirmed in further studies. Moreover, PRZ/SA micelles 575 represent a less complicated preparation process and improved 576 loading efficiency, reproducibility and stability, which currently 577 hampers myopia treatment in the clinic.[31] Chronic administration 578 of PRZ by iontophoresis,[32] although effectively preventing 579 experimentally induced myopia, requires special equipment and 580 operations and therefore suffers from poor patient compliance. 581 Similarly, intravitreal or subconjunctival injection of PRZ improves 582 delivery and distribution of PRZ across all ocular tissue, but needs 583 surgical operations. Therefore, ion-paired pirenzepine-loaded 584 mPEG-PLA based ophthalmic polymeric micelles developed in 585 this study could be a promising candidate for clinical applications to

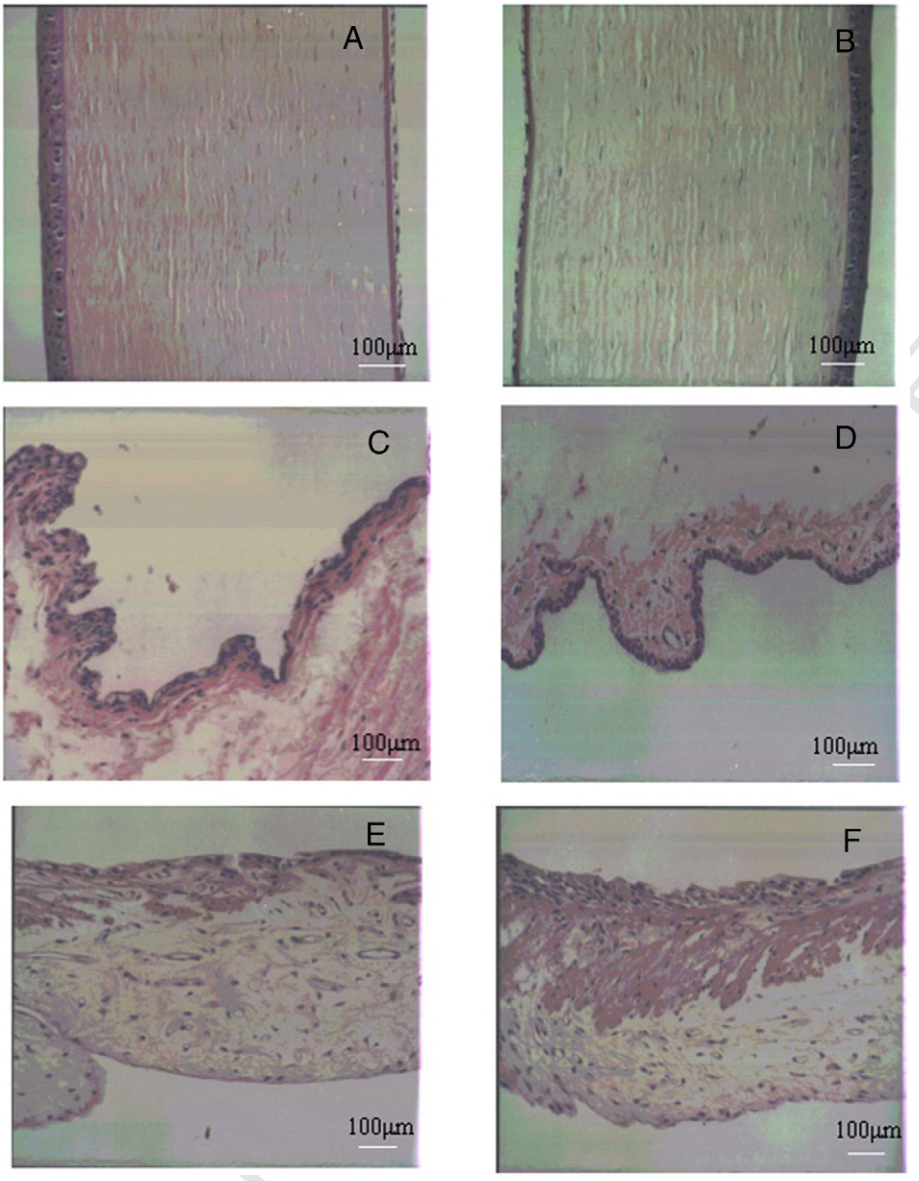


Figure 8. The histopathological results for different area of the eyes. (**A**) Saline group (cornea); (**B**) PRZ/SA micelle group (cornea); (**C**) saline group (conjunctiva); (**D**) PRZ/SA micelle group (conjunctiva); (**E**) saline group (iris); and (**F**) PRZ/SA micelle group (iris).

treat myopia due to its simple preparation procedures,[33] high drug loading efficiency, good reproducibility and minimal toxicity. Further experiments are still needed to fully characterize its distribution and transport mechanisms as well as to evaluate its efficacy in preventing myopia compared to existing treatments.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2017.05.001.

## References

1. Foster P, Jiang Y. Epidemiology of myopia. *Eye* 2014;**28**:202-8.
2. Farbrother JE, Kirov G, Owen MJ, Guggenheim JA. Family aggregation of high myopia: estimation of the sibling recurrence risk ratio. *Invest Ophthalmol Vis Sci* 2004;**45**:2873-8.
3. Donovan L, Sankaridurg P, Ho A, Naduvilath T, Smith III EL, Holden BA. Myopia progression rates in urban children wearing single-vision spectacles. *Optom Vis Sci* 2012;**89**:27-32.
4. Bar Dayan Y, Levin A, Morad Y, Grotto I, Ben-David R, Goldberg A, et al. The changing prevalence of myopia in young adults: a 13-year series of population-based prevalence surveys. *Invest Ophthalmol Vis Sci* 2005;**46**:2760-5.
5. Schwahn HN, Kaymak H, Schaeffel F. Effects of atropine on refractive development, dopamine release, and slow retinal potentials in the chick. *Vis Neurosci* 2000;**17**:165-76.
6. Tong L, Huang XL, Koh AL, Zhang X, Tan DT, Chua W-H. Atropine for the treatment of childhood myopia: effect on myopia progression after cessation of atropine. *Ophthalmology* 2009;**116**:572-9.

7. Schmid K, Brinkworth D, Wallace K, Payor R, Fritsch C, Lambrou G. Inhibitory effect of atropine on myopia development in the chick: mode of application and unilateral versus bilateral lens wear. *Invest Ophthalmol Vis Sci* 2004;**45**:1239.
8. Dörje F, Wess J, Lambrecht G, Tacke R, Mutschler E, Brann M. Antagonist binding profiles of five cloned human muscarinic receptor subtypes. *J Pharmacol Exp Ther* 1991;**256**:727-33.
9. Buckley NJ, Bonner TI, Buckley CM, Brann MR. Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol Pharmacol* 1989;**35**:469-76.
10. Leech E, Cottriall C, McBrien N. Pirenzepine prevents form deprivation myopia in a dose dependent manner. *Ophthalmic Physiol Opt* 1995;**15**:351-6.
11. Tigges M, Iuvone PM, Fernandes A, Sugrue MF, Mallorga PJ, Laties AM, et al. Effects of muscarinic cholinergic receptor antagonists on postnatal eye growth of rhesus monkeys. *Ophthalmol Vis Sci* 1999;**76**:397-407.
12. Bartlett JD, Niemann K, Houde B, Allred T, Edmondson MJ, Crockett RS. A tolerability study of Pirenzepine ophthalmic gel in myopic children. *J Ocul Pharmacol Ther* 2003;**19**:271-9.
13. Dai Y, Chu R. The pharmacokinetic of pirenzipine in the aqueous humor of rabbits. *Chin J Optom Ophthalmol* 2002;**4**:228-9.
14. Phan QT, Le MH, Le TT, Tran TH, Xuan PN, Ha PT. Characteristics and cytotoxicity of folate-modified curcumin-loaded PLA-PEG micellar nano systems with various PLA:PEG ratios. *Int J Pharm* 2016;**507**:32-40.
15. Mundargi RC, Babu VR, Rangaswamy V, Patel P, Aminabhavi TM. Nano/micro technologies for delivering macromolecular therapeutics using poly(d,l-lactide-co-glycolide) and its derivatives. *J Control Release* 2008;**125**:193-209.
16. Ouahab A, Cheraga N, Onoja V, Shen Y, Tu J. Novel pH-sensitive charge-reversal cell penetrating peptide conjugated PEG-PLA micelles for docetaxel delivery: in vitro study. *Int J Pharm* 2014;**466**:233-45.
17. Essa S, Rabanel JM, Hildgen P. Characterization of rhodamine loaded PEG-g-PLA nanoparticles (NPs): effect of poly(ethylene glycol) grafting density. *Int J Pharm* 2011;**411**:178-87.
18. Tu J, Pang H, Yan Z, Li P. Ocular permeability of pirenzepine hydrochloride enhanced by methoxy poly (ethylene glycol)–poly (D, L-lactide) block copolymer. *Drug Dev Ind Pharm* 2007;**33**:1142-50.
19. Kato A, Iwata S. In vitro study on corneal permeability to bunazosin. *J Pharmacobiodyn* 1988;**11**:115-20.
20. Higashiyama M, Inada K, Ohtori A, Tojo K. Improvement of the ocular bioavailability of timolol by sorbic acid. *Int J Pharm* 2004;**272**:91-8.
21. *China Pharmacopoeia. volume IV*. 1st ed. Beijing: China Medical Science; 2015, p.471-2.
22. Zhang YJ, Li X, Lv S, Mo M, Chen JL. Influence of different conjunctiva sac flushing on eyes. *J Chin Mod Ophthalmol* 2005;**4**:324-5.
23. Kwon GS. *Polymeric Micelles for Multiple-Drug Delivery*. New York: Multifunctional Nanoparticles for Drug Delivery Applications; 2012, p.133-52.
24. Stirland DL, Nichols JW, Miura S, Bae YH. Mind the gap: a survey of how cancer drug carriers are susceptible to the gap between research and practice. *J Control Release* 2013;**172**:1045-64.
25. Torchilin VP. Structure and design of polymeric surfactant-based drug delivery systems. *J Control Release* 2001;**73**:137-72.
26. Torchilin VP. Polymeric micelles as pharmaceutical carriers. In: Uchegbu IF, editor. *Polymers in Drug Delivery*. Florida: CRC Press; 2006. p. 111-25.
27. Danafar H, Davaran S, Rostamizadeh K, Valizadeh H, Hamidi M. Biodegradable m-PEG/PCL core-shell micelles: preparation and characterization as a sustained release formulation for curcumin. *Adv Pharm Bull* 2014;**2**:501-10.
28. Chen JM, Wang WY, Zhuang SS. Research progress on improving the corneal permeability of ophthalmic preparations. *Chin J Hosp Pharm* 2014;**34**:1227-31.
29. Saettone MF, Chetoni P, Cerbai R, Mazzanti G, Braghiroli L. Evaluation of ocular permeation enhancers: in vitro effects on corneal transport of four β–blockers, and in vitro/in vivo toxic activity. *Int J Pharm* 1996;**142**:103-13.
30. Zhang C, Wang X, Deng SJ. Cellular toxicity of preservatives in corneal and conjunctive lepithelial cells and cytoprotective action of sodium hyaluronate. *Rec Adv Ophthalmol* 2008;**28**:85-9.
31. Dai SZ, Zeng JW. Pharmacokinetics of pirenzepine liposome in the rabbit eyes. *Chin Ophthal Res* 2006;**24**:132-5.
32. Dai YK, Wu W, Chu RY. Study of the pharmacodynamics of pirenzepine iontophoresis for experimental myopia. *Chin J Optom Ophthalmol* 2008;**10**:298-301.
33. Cottriall CL, McBrien NA, Annies R, Leech EM. Prevention of form-deprivation myopia with pirenzepine: a study of drug delivery and distribution. *Ophthalmic Physiol Opt* 1999;**19**:327-335.