Poly(N-isopropylacrylamide)—chitosan as thermosensitive in situ gel-forming system for ocular drug delivery

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Received 24 October 2006; accepted 5 May 2007
Available online 17 May 2007

Abstract

A novel copolymer, poly(N-isopropylacrylamide)—chitosan (PNIPAAm—CS), was investigated for its thermosensitive in situ gel-forming properties and potential utilization for ocular drug delivery. The thermal sensitivity and low critical solution temperature (LCST) were determined by the cloud point method. PNIPAAm—CS had a LCST of 32 °C, which is close to the surface temperature of the eye. The in vivo ocular pharmacokinetics of timolol maleate in PNIPAAm—CS solution were evaluated and compared to that in conventional eye drop solution by using rabbits according to the microdialysis method. The \(C_{\text{max}}\) of timolol maleate in aqueous fluid for the PNIPAAm—CS solution was 11.2 \(\mu\text{g/ml}\), which is two-fold higher than that of the conventional eye drop, along with greater AUC. Furthermore, the PNIPAAm—CS gel-forming solution of timolol maleate had a stronger capacity to reduce the intra-ocular pressure (IOP) than that of the conventional eye drop of same concentration over a period of 12 h. In addition, the MTT assay showed that there is little cytotoxicity of PNIPAAm—CS at concentration range of 0.5—400 \(\mu\text{g/ml}\). These results suggest that PNIPAAm—CS is a potential thermosensitive in situ gel-forming material for ocular drug delivery, and it may improve the bio-availability, efficacy, and compliance of some eye drugs.

Keywords: Thermosensitive polymer; Chitosan; Poly(N-isopropylacrylamide); In situ gel-forming; Timolol maleate

1. Introduction

Topical delivery of drugs into the lower cul-de-sac using eye drops is a conventional approach for treatment and diagnosis of ocular diseases [1–3]. The cornea is the primary location for these drugs to penetrate into eyes. Human cornea generally consists of three layers: the epithelium, stroma and endothelium. Epithelium, the most external layer, is composed of a number of well-organized and tightly packed cells and serves as a selective barrier for the penetration of ophthalmic drugs. It is noted that a high drug concentration at the cornea membrane surface is required for most of the hydrophilic drugs to ensure their essential delivery through the ocular barrier [4]. Unfortunately, the dropped drug solution is immediately diluted by the tear fluid, followed by rapid elimination from the pre-cornea area because of the lacrimal secretion and nasolacrimal drainage. This leads to a very short period of time for the drugs contacting with the cornea, and less than 5% of the applied drugs penetrate through the cornea and reach the intra-ocular tissues. Many efforts have been made to enhance the efficacy of eye drops [5,6]. These include but are not limited to increasing drug residential time in the cul-de-sac of the eye, prolonging intra-ocular exposure, slowing drug release from the delivery system, and minimizing pro-corneal drug loss [5,6]. Novel delivery concepts and approaches are in high demand for improving the effectiveness, safety, and convenience of eye drops.

Recently, in situ gel-forming systems, especially the thermosensitive ones, have showed their potential in increasing the
residential time and possible controlled release of drug molecules for eye diseases because of their capacity to improve bioadhesiveness of ophthalmic solutions [7–10]. A thermosensitive polymer solution behaves as a liquid below its low critical solution temperature (LCST) and forms gel when the environmental temperature reaches or is above the LCST. Thermosensitive in situ gel-forming solution has shown its possible utilization in enhancing ocular absorption of Timolol [11].

Poly(N-isopropylacrylamide) (PNIPAAm) is a well-known thermosensitive polymer with a thermoresponsive phase transition temperature of 32 °C, which is close to human body surface temperature. This polymer and its derivatives have been widely investigated and utilized in bio-medical, pharmaceutical and other fields [12–15]. However, previous studies have indicated that a homopolymer solution of PNIPAAm derivatives forms a rigid and uncomfortable hydrogel on the cornea. This property limits its utilization for ocular application. However, this limitation also presents an opportunity for further developing the improved thermosensitive in situ gel-forming polymer(s) for ocular drugs delivery.

Chitosan (CS) is a deacetylated derivative of chitin, a natural polymer. It has a cation-intensified contact with the mucosa. In addition, chitosan also has several favorable biological properties such as penetration enhancing effect, drug loading and concentration gradient increasing, bio-degradability, non-toxicity, bio-compatibility and excellent ocular tolerance [16,17]. These characteristics make it very attractive for ophthalmic formulations.

A few recent studies discuss the creation of chitosan-PNIPAAm derivatives by different methods with the hope that these novel polymers may take the advantages of both chitosan and PNIPAAm and have improved in situ gel-forming properties [18–21]. These polymers include chitosan-graft-poly(N-isopropylacrylamide) injectable hydrogel with potential utilization for culturing of chondrocytes and meniscus cells [18], acrylic acid and poly(N-isopropylamidamide) graft chitosan which are used in the controlled delivery of coenzyme A [19], and chitosan-g-poly(N-isopropylacrylamide) which exhibits a thermoassociative behavior in which its elastic response dramatically increases when temperature is above the critical temperature or the association temperature [21]. In our previous report, NIPAAm monomers were polymerized with MPA to introduce the polymer with an end-group acid, followed by grafting with a chitosan molecule. The structure of the PNIPAAm–CS was confirmed by IR, 1H NMR, 13C NMR and elemental analysis [22]. To investigate the potential application of the new thermosensitive polymer as an ocular delivery system, an in situ gelling system of timolol maleate was developed using PNIPAAm–CS and compared with the conventional eye drop; the feasibility of this system as a potential glaucoma medication was investigated. Timolol maleate is a non-selective beta-adrenergic receptor blocking agent. It is a white, odorless, crystalline powder, which is soluble in water, methanol, and alcohol. Timolol maleate ophthalmic solution was selected in the present study because it has been successfully utilized to investigate the in vitro release behavior of an in situ gelling polymer system [23]. Moreover, timolol maleate is one of the primary drugs for treatment of open angle glaucoma as both the ophthalmal drop and gel-forming solution containing gelrite gellen gum (Timoptic-XE, Merck & Co., Inc). It is a challenge to enhance its bio-availability and safety through different pharmaceutical approaches [24].

2. Materials and methods

2.1. Materials

Chitosan was provided by Nantong Shuanglin Biochemical Co. Ltd (Nantong, China). The chitosan has a 90% of deacetylation degree, and an average molecular weight of 70 kD. N-isopropylacrylamide (IPN), 2,2′-Azo-bis-isobutylonitrile (AIBN), and 3-mercaptopropionic acid (MPA) were purchased from J&K Chemical. N,N,N′-terramethylethylene diamine (TEMED) was purchased from Acros. Dulbecco’s Modified Eagle’s Medium (DMEM), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma (St. Louis, USA). All solvents and reagents were used without further purification. Timolol maleate was from Tianjin Central Pharmaceutical Co., Ltd, China. The New Zealand white rabbits weighing 3.0–4.0 kg were obtained from the Animal Center of China Pharmaceutical University. All the treatments followed the recommendation of the regulation for the Administration of Affairs Concerning Experimental Animals.

2.2. Synthesis of thermosensitive chitosan derivative

PNIPAAm–COOH was prepared according to a previously reported procedure shown in Fig. 1 [22]. Briefly, AIBN (0.02 g, 0.12 mmol) was added into the reaction mixture containing IPN (1 g, 8.9 mmol) and MPA (0.2 ml, 0.11 mmol) in methanol (10 ml) at 60 °C under nitrogen while stirring. The reaction was continued at 60 °C for total of 24 h, and the products were collected and dissolved in 5 ml of acetone, and precipitated out

![Fig. 1. PNIPAAm–CS Synthesis outline.](image)
again by adding 2 ml of n-hexane. The precipitate was dried under vacuum at 40 °C overnight and 980 mg of white PNIPAAm–COOH was obtained. The number average molecular weight of PNIPAAm–COOH was determined by the endgroup titration method, and this molecular weight was used to calculate the molar ratio of PNIPAAm–COOH and chitosan in the following synthetic step.

PNIPAAm–CS was prepared by coupling chitosan with PNIPAAm–COOH using \( N,N' \)-dicyclohexyl carbodiimide (DCC) as a coupling reagent. Chitosan (0.25 g, 1.55 mmol) was dissolved in 2% HCl (10 ml, v/v) with stirring, whereas PNIPAAm–COOH (980 mg, 0.392 mmol) was dissolved in water (10 ml). The DCC (0.2 g, 1.24 mmol) and TEMED (0.02 ml, 0.268 mmol) were dropped consecutively into the chitosan solution, and then PNIPAAm–COOH solution was dropped into the activated chitosan solution. The reaction mixture was kept at ambient temperature for 72 h, filtered, dialyzed (molecular weight cut off 10000) against distilled water for 4 days, and lyophilized. Finally, 141 mg of brown powder of PNIPAAm–CS was obtained.

The structure of PNIPAAm–CS was confirmed using IR, \(^1\)H NMR, \(^{13}\)C NMR and elemental analysis.

### 2.3. Determination of phase transition temperature of PNIPAAm–CS

The thermal behavior or LCST of PNIPAAm–CS was observed at 480 nm using a UV–VIS spectrophotometer [25]. To examine the reversible thermoresponse, optical transmittance was measured using a controlled temperature circular programmed, increasing from 20 to 40 °C followed by decreasing from 40 °C to 20 °C at 0.1 °C/min. LCST of the polymer solutions at different concentrations were defined as the temperature at which the optical transmittance of the solution reduced to 50% of its original value. The morphology change of the heated polymer solution was examined using an optical microscope at 80 kV with JEM-1230 (Jeol, Japan).

### 2.4. Viscosity measurements

The properties of PNIPAAm–CS at the concentrations of 1.5, 1.2, 0.8, 0.6, and 0.4 mg/ml have been studied by using the Ubbelohde viscometer at temperatures of 10–40 °C. Solvent flow time should exceed 100 s. The time lag to pass two menisci on the viscometer was recorded and converted to the specific viscosity, \( \eta_sp \), which is defined by \( \eta_sp = (\eta - \eta_0) / \eta_0 = t / t_0 - 1 \), where \( \eta \) and \( \eta_0 \) are the solution and solvent viscosities, respectively. Measurements were repeated at least three times so as to achieve a relative error of less than 0.1%. Intrinsic viscosity data were analyzed using the Huggins Equation [26]:

\[
\eta_sp/\eta_0 = k [\eta]^2 C.
\]

\([\eta]\) can be illustrated as a linear plot in which the intercept is the intrinsic viscosity. The Huggins constant, \( k \), is a dimensionless parameter related to solvent–polymer interactions. Both parameters \([\eta]\) and \( k \) are indirect means of the ability of the solvent to solvate a polymer, in particular the intrinsic viscosity is an expression of the hydrodynamic interference between the polymer and the solvent.

### 2.5. MTT assay

MTT assay was conducted following a previously described protocol [27]. Two male adult rabbits were sacrificed by rapid intravenously injecting pentobarbital, then the eyes were immediately removed and placed in the sterilized phosphate buffered saline (PBS) containing 1000 U/ml penicillin and 1000 μg/ml streptomycin. The corneal endothelium and the descemet membrane were separated under an anatomic microscope. The remnant membrane was transferred into a 35-mm dish containing 0.125% (w/v) trypsin and 0.125% (w/v) EDTA with the epithelium side facing the bottom, and cultured in a CO\(_2\) incubator. After 30 min, fetal calf serum (FCS) was added in the culture medium and the membrane was reversed. The epithelium was rinsed with DMEM culture medium, and the solution was collected and centrifuged at 3000 g for 5 min. The pellet was suspended and cultured in the DMEM/F12 (1:1, v/v) culture medium containing 10 mmol/l HEPES, 20% FCS, 200 U/ml penicillin and 200 μg/ml streptomycin.

For MTT assay, the cells with 5 × 10\(^4\)/well in a 96-well plate (Corning, USA) were treated with different concentrations of PNIPAAm–CS (0–400 μg/ml) for 24 h and with 400 μg/ml of PNIPAAm–CS for 0–24 h. Then 0.5% MTT solution was added to the medium and incubated for 4 h at 37 °C. After discarding the culture medium, 0.1 ml of DMSO was added to dissolve the precipitates, and the resulting DMSO was measured for absorbance at 570 nm (Molecular Devices, USA). Statistical analysis was performed by one-way analysis of variance followed by the Newman–Keuls test. Differences with a \( P \) value less than 0.05 were considered statistically significant.

### 2.6. Cell morphology examination

The obtained cornea epithelial cells were seeded in 6-cm dishes and cultured for 72 h. The cells were treated with different concentrations of PNIPAAm–CS for 24 h. The morphology of the cells was examined by fluorescent microscopy (1X81, Olympus, Japan).

### 2.7. Ocular pharmacokinetics of timolol maleate from PNIPAAm–CS in situ gel-forming system by using microdialysis technology [28,29]

#### 2.7.1. Probe characterization

The theory of probe characterization was described previously [30,31]. In brief, microdialysis sampling analyses are removed from the extra-cellular space by a diffusion gradient (across the dialysis membrane) established via the continuous perfusion of medium through the probe. Thus, at a typical perfusion rate, the continuous flow of perfusion fluid through the probe inhibits the concentration equilibrium between solutions inside and outside of the probe. Under the non-equilibrium conditions, the testing sample concentration in the dialysate is less than the actual concentration in the extra-cellular fluid surrounding the probe. The ratio between the recovered fraction and the actual concentration is usually expressed as a percentage, and referred to recovery percentage.
The probe (LM-10 Linear Microdialysis Probes, BASi, USA) was perfused with the balanced salt solution (BSS), while its dialysis membrane was immersed in 30 ml saline containing 400 ng/ml timolol maleate \( (C_m) \). After equilibrating for 30 min, the microdialysis sample was collected and subjected to HPLC measurement to determine the drug recovery and delivery in vitro. The gradient and recovery of a particular compound depend not only on the difference in the concentration between the perfusate and the extra-cellular fluid, but also on the velocity of flow inside the microdialysis probe. Flow rate may alter the recovery, thus, when the flow is slowed down to certain level, the solutions outside and inside of the membrane may be equilibrated and lose concentration gradient. This may lead to a high recovery value close to 100% as the flow rate approaches zero, whereas decrease in an exponential fashion may be observed with increased flow rate. Conversely, the recovery is zero at zero flow rate, increasing and reaching a plateau at a higher flow rate when the tissue is unable to deliver more substance to the perfusion medium. The rate of perfusion was 2, 2.5, 3, 4, 5 \( \mu \text{ml/min} \), respectively. Linearity between the perfusate concentration \( (C_p) \) and the net increase \( (C_d-C_p) \) of timolol maleate concentration in the dialysate \( (C_d) \) was estimated, and the slope of the calibration line, corresponding to the recovery \( (R) \), was calculated using the equation: 

\[
R = \frac{(C_d - C_p)}{C_m - C_p} \times \text{100%}
\]

The recovery in vivo was evaluated by the retrodialysis method after probe implantation [32].

2.7.2. Animal model

The rabbits were anesthetized with phenobarbital (50 mg/kg, i.p.), and the fornix-based conjunctival flap (FBCF) was removed with microscissors. A 20-ga needle was then introduced into the anterior chamber in an oblique fashion from 3 mm posterior to the limbus, and the microdialysis probe (Bioanalytical System Inc, USA) was gently inserted through the opening created by the removed needle until the probe tip was completely spanned in the anterior chamber. The opening was sealed at the base of the probe. The probe was sutured via its attached anchor to the upper eyelid with 2-0 silk. Saline was perfused through the probe at a rate of 2.5 ml/min for 20 min prior to drug administration. To prevent intramural fibrin formation, flunixin meglamine was injected (2.5 mg/kg, i.v.) 30 min before surgery as well as 4 h after surgery. After 5–7 days of recovery, the animals were used for experiments.

2.7.3. Topical administration

After implantation of the microdialysis probe and the recovery period, two drops of 0.5% (w/v) timolol maleate PNIPAAm–CS thermosensitive gel-forming solution was applied into the cul-de-sac. The probe was continuously perfused at a constant flow rate of 2.5 \( \mu \text{ml/min} \) and the perfusates were collected at 0, 15, 30, 45, and 60 min of perfusion. The concentration of timolol maleate in each dialysate was directly measured using high performance liquid chromatography (HPLC) with a spectrometric detector at the 295 nm wavelength. The mobile phase consisted of 50 parts of methanol and 50 parts of deionized water and 0.1 parts triethylamine, and its pH was adjusted to 3.0 with acetic acid. The flow rate was 1.0 ml/min. An Alltima C18 column (5 \( \mu \text{m} \), 200 x 4.6 mm) was used at 25 °C. The dialysate (20 \( \mu \text{l} \)) was injected into the chromatographic system without any pre-treatment. The retention time of timolol maleate was 3.4 min under the experimental conditions.

2.8. In vivo pharmacodynamic test

In vivo pharmacodynamic study was performed to measure reduction of intra-ocular pressure (IOP) using four rabbits (2.0–2.5 kg each) with alpha-chymotrypsin-induced glaucoma [33,34]. The basal IOP was measured using a Schiotz indentation tonometer (YZ-TA, Suzhou Medical Instrument Factory, China). After being adapted to the experimental settings in a week, the rabbits were lightly anesthetized with 0.5% alcaine proparacine hydrochloride, and alpha-chymotrypsin was injected into the posterior chamber of their eyes. The IOP was measured daily. When the IOPs of the rabbits reach 50 mm Hg and last for 3 days, the glaucoma model is ready for treatment. Twenty-five microliters of the 0.5% (w/v) timolol maleate PNIPAAm–CS thermosensitive gel-forming solution and the 0.5% timolol maleate eye drops were applied into the right and left eyes of the model rabbits, respectively. The IOP was then determined after 12 h of treatment. Reduction of IOP for each eye was calculated as follows: Reduction of IOP = IOP\(_{\text{time}~0} - \text{IOP}_{\text{time}~n}\), and reported as the mean±S.E.M. \((n=4)\). Statistical analysis was performed using the Student’s t-test. Differences with a \( P \) value less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Preparation of PNIPAAm–CS

PNIPAAm–CS was prepared by coupling chitosan with PNIPAAm with an introduced carboxylic group (Fig. 1). AIBN acted as an initiator in the free radical polymerization reaction, whereas 3-mercaptopropionic acid was used as a chain transfer agent. Degree of grafting by PNIPAAm–CS was confirmed by comparing its IR, 1H NMR, 13C NMR, and elemental analysis data with that previously reported [22].

![Fig. 2. The determination of LCST as shown in the transmittance alternation of the copolymer aqueous solutions when temperature increasing or decreasing.](image-url)
3.2. Phase transition temperature of PNIPAAm–CS polymers

Phase transition temperature is an important parameter for in situ gel-forming polymer, which determines the potential utilization of the polymer in ocular drug delivery [35]. It is also well-noted that polymer concentration may alter the phase transition property of its solution. In the present study, PNIPAAm–CS solutions were examined for their phase transition behaviors using two temperature programs, increasing from 20 °C to 40 °C and decreasing from 40 °C to 20 °C. PNIPAAm–CS solutions at concentrations of 0.5 mg/ml and 0.25 mg/ml had LCST of 30 °C and 32 °C, respectively, regardless of the testing temperature program (Fig. 2). These LCST values were lower than that of 35 °C observed for 0.5 mg/ml PNIPAAm–COOH solution under the same testing conditions. Statistical analysis showed no significant difference between the LCST values of the two concentration PNIPAAm–CS solutions, suggesting less effect of concentration on its solution LCST. Also the transparency of PNIPAAm–CS solution was negatively associated with its concentration, although all PNIPAAm–CS solutions lost transparency at temperatures above their LCSTs (Fig. 2). In addition, the phase transition curves that were prepared using different temperature programs for the two PNIPAAm–CS solutions at either concentration were almost identical. The results suggest that PNIPAAm–CS is a thermoreversible polymer. This characteristic is important for the utility of polymeric solutions. At temperatures below the LCST, the enthalpy term of PNIPAAm–CS, which is mostly a result of hydrogen bonding between polymer polar groups and water molecules, leads to dissociation of the polymer molecules. Above the LCST, the entropy term (hydrophobic interactions) dominates the polymeric gel forming in water. This is caused by the coil-to-globule transition during dehydration of hydrophobic isopropyl groups. However, the carbonyl groups of PNIPAAm–CS still form hydrogen bonds with water molecules after gelling.

To examine the phase transition property of PNIPAAm–CS solution, a morphology assay was conducted by microscopy (1X81, Olympus, Japan). PNIPAAm–CS molecules were present as small particles with uniform size and shape in the solution. It was also noted that these particles possess colloidal dispersion gel structure and were slightly aggregated together. In contrast, irregular gel structure was observed when the temperature was brought up to its LCST (Fig. 3B). This phase transition may be explained by the thermal breakage of the polymer–water hydrogen bonds and the enhanced inter- and intra-molecular hydrophobic interactions. The diameters of the polymer particles were 800–900 nm below LCST and 2–4 μm above LCST, which were in agreement with that measured by using photo correlation study (Malvern Mastersizer 3000, UK).

3.3. Viscosity measurements

The phase transition of a thermosensitive polymer is a result of the change in its hydrophilic–hydrophobic balance. Below the LCST the hydrogen-bonding interactions of the polymer with water molecules determine its solubility. When raising the temperature these interactions are weakened and above the LCST the hydrophobic interactions become predominant. Changes in the structure of the polymer chain that increase its overall hydrophobicity result in decreasing of the LCST of the polymer solution. The relationship between the sol–gel transition intrinsic viscosity and temperature are shown in Fig. 4. This shows that the interaction is decreased between...
water molecules and PNIPAAm-CS with temperature increasing. A change in the appearance of the polymer solution is distinctly observable: a clear solution (below 24 °C), through a clear suspension phase (24 °C–32 °C), to a white shrunken gel (above 32 °C). Usually, the temperature-induced transition from a flexible coil polymer to small globule gel occurs at about 24 °C. With a further increase of the temperature (24 °C–32 °C), hydrogen bonding of intra- and inter-polymer chains and hydrophobic interactions between side-chains in PNIPAAm–CS takes place. Water then becomes a rather poor solvent that is expelled from the microgel network and the microgel further collapses to form aggregates of microgel particles. Graphical representation shows two significant convex peaks occurring at about 24 °C and 32 °C, and indicates a coil–globule, hydrophilic–hydrophobic two transformations process with increasing temperature. The latter process led to phase transformation. Apparently, its fluidity is due to a collapsed particle size and weakened water–polymer hydrogen bonding upon an increasing of the temperature.

3.4. Bio-compatibility and cytotoxicity of PNIPAAm–CS

MTT assay, first described by Mosmann in 1983 [36], is based on the ability of a mitochondrial dehydrogenation enzyme in viable cells to cleave to the tetrazolium rings of the pale yellow MTT and form formazane crystals with a dark blue color. Therefore, the number of surviving cells is directly proportional to the level of the formed formazane. MTT assay was performed by using a multi-well plate reader with a spectrophotometric detector (SpectraMax 190, Molecular Devices, USA). Compared with the control, although there was a slightly descending tendency as the concentration was increased or the time was prolonged, the growth rate of the primary cultured corneal epithelium cell was not significantly inhibited by PNIPAAm–CS at the concentration range of 5–400 μg/ml after 24 h cultivation (Fig. 5A) or at the concentration of 400 μg/ml in various cultivation times (Fig. 5B). These results were supported by morphology examinations. The cells treated with the polymeric solutions appeared in a normal polygon-shape.
with the round or elliptic nucleus as did those treated with saline (Fig. 6A). The nucleoli were also clearly visible in the cells. PNIPAAm–CS treatment at 200 or 400 μg/ml for 24 h showed no obvious influence on the morphology of the primary cultured cornea epithelial cells and no visible abnormal signs to cornea (Fig. 6B and C). These data suggested a good ocular tolerance of PNIPAAm–CS.

3.5. Ocular pharmacokinetics of timolol maleate

3.5.1. The probe recovery in vitro

It is well-known that some ocular drugs have rapid absorption and elimination rates, which results in relatively short period for the local concentration measurements. To fully utilize the microdialysis technique, the probe recovery, which is defined as estimation for the in vivo extractable fraction of the testing compound from the space, first needs to be determined. To determine the desired probe flow rate for in vivo pharmacokinetic study, the recovery in vitro as well as the functionality of the probe was tested. In the present study, the calculated recovery in vitro was close to 50% at a flow rate of 2.5 μl/min. In other words, the recovery and delivery ratios were almost identical at the flow rate, which provided an experimental proof for the recovery measurement in vivo by retrodialysis method.

3.5.2. The probe recovery of in vivo

After the recovery period of implantation of the microdialysis probe, the probe was perfused with a series of timolol maleate solutions ranging from 25 to 4000 ng/ml, with 2.5 μl/min of flow rate. After equilibrating for 30 min, the perfusate was collected and measured using HPLC to determine recovery in vitro. Linearity between the perfusate concentration (Cp) and the net increase (Cd−Cp) of timolol maleate concentration in the dialysate (Cd) was established, and the slope of the line corresponded with the recovery (R). A linear relationship was observed between Cd−Cp and Cp with a R2 value of 0.9943, indicating that the probe and determination systems are ready for ocular pharmacokinetic study of timolol maleate (Fig. 7). According to the definition of recovery, the following equation was obtained: C_d−C_p = −0.3477C_p + 12.505. The in vitro recovery was calculated as 34.77% (n=4) (Fig. 7). Compared with the recovery of 50% in vitro under agitated conditions, the decreased recovery in vivo may contribute to the stationary diffusion process around the dialysis membrane and the smaller concentration gradient in aqueous humor [37]. In addition, sticking to the iris and fibrin formation might also reduce the recovery in vivo.

3.5.3. Ocular pharmacokinetics of timolol maleate in thermosensitive gel-forming system

Microdialysis is a useful technique for continuously monitoring the trace substances in vivo [38–41]. Currently, there is a growing interest in using the technique for pharmacokinetic and bio-pharmaceutics studies. By means of the sampling technique, the ocular pharmacokinetic behaviors of timolol maleate were studied while the gel-forming solution and conventional drop were administrated. The concentration–time curves of timolol maleate in aqueous humor are illustrated in Fig. 8. The peak values in aqueous humor were obtained with the gel-forming solution, as shown in Table 1. The pharmacokinetic parameters of timolol maleate in situ gel-forming system and timolol maleate conventional drop solution in rabbit aqueous humor (n=5) are presented in Table 1.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Tmax (min)</th>
<th>Cmax (μg/l)</th>
<th>AUC (μg/l min)</th>
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<tbody>
<tr>
<td>Drop solution</td>
<td>15.21±1.52</td>
<td>5.58±1.20</td>
<td>312.90±44.49</td>
</tr>
<tr>
<td>Gel-forming solution</td>
<td>30.43±2.71</td>
<td>11.20±1.41</td>
<td>811.83±146.60</td>
</tr>
</tbody>
</table>

Data were represented as mean±SEM (n=5).

Fig. 7. The probe recovery of microdialysis in aqueous humor in vivo.

Fig. 8. Timolol maleate concentration in aqueous humor after instillation of 0.5% timolol maleate conventional and thermosensitive PNIPAAm–CS gel-forming solution (n=5).

Fig. 9. The IOP-lowering effect of timolol maleate in thermosensitive PNIPAAm–CS and conventional eye drop (n=4).
aqueous humor concentration ($C_{\text{max}}$) of 5.58 ng/ml was reached at about 15 min for the conventional drop, whereas the maximum concentration of 11.2 ng/ml was detected after 30 min for the thermosensitive gel-forming solution. It was also noted that the area under concentration–time curve (AUC) of the thermosensitive gel-forming system was two-fold greater than that obtained with the conventional drop solution. The concentration of timolol maleate in the aqueous humor after instillation of thermosensitive gel-forming solution is higher than that of the control at same time (Fig. 8). These data suggested that the thermosensitive gel-forming system may significantly improve ocular availability and lead to enhanced effectiveness of timolol maleate (Table 1). The thermosensitive gel-forming system kept in a liquid state before dropping, and formed a gel after dropping. This resulted in the sustained release and prolonged pre-corneal retention of timolol maleate because the water-soluble drug was readily embedded in the gel and its elimination from the aqueous humor by tear was consequently inhibited. In addition, the PNIPAAm–CS might enhance the corneal permeability and absorption of timolol maleate due to its positive charge and adhesive characteristics.

### 3.6. Pharmacodynamic test in vivo

The thermosensitive gel-forming solution was evaluated and compared with the conventional eye drop solution for their effects on pharmacodynamic properties of timolol maleate. The capacities of both timolol maleate formulations in reducing the intra-ocular pressure (IOP) were measured for 12 h. As shown in Fig. 8, the onset of action was observed at 0.5 h and lasted for 12 h for both formulations. At all time points, the thermosensitive gel-forming solution of timolol maleate exhibited stronger IOP reduction activity than that of its conventional eye drop solution (Fig. 9). Also the thermosensitive gel-forming solution containing timolol maleate resulted in the largest IOP decrease of 3.375 kPa at 2 h after the administration, whereas the conventional eye drop had the largest IOP decrease of 2.395 kPa (Fig. 9). These data suggested the potential of PNIPAAm–CS in enhancing the efficacy of timolol maleate. These data also support the observation that PNIPAAm–CS may improve the ocular bio-availability and pharmacokinetic properties of timolol maleate. PNIPAAm–CS is a polycationic bio-polymer, and has a static interaction with the mucus and cornea cell membrane with negative charges at physiological pH.

### 4. Conclusions

The present study demonstrated that a novel thermosensitive polymer PNIPAAm–CS may have possible utilization in improving the efficacy, bio-availability, and pharmacokinetic properties of water-soluble eye drugs such as timolol maleate. MTT assay in this research did not detect any cytotoxic effect of PNIPAAm–CS under the experimental conditions. Moreover, this research supports the possible role of thermosensitive polymer solutions or hydrogels in controlled release of therapeutic agents for treatment of glaucoma and other eye diseases. In addition, lower or non-cytotoxicity of the polymer suggests its potential use in the delivery of health beneficial factors.

### Acknowledgements

The authors wish to thank Dr. Zhao-Qiu Wu for his expert technical assistance and helpful discussion. This study is financially supported by the key program of international science and technology research cooperation (2005DFA30350) of the State Ministry of Science and Technology of China, and supported by the key program (2003, 03090) of Science and Technology of the State Education Ministry of China and the Natural Science Foundation (BK2006154) of Jiangsu Province, China.

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