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ORIGINAL RESEARCH

Reduced administration frequency for the treatment of fungal keratitis: a sustained natamycin release from a micellar solution

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ABSTRACT

Background: Natamycin is the only topical ophthalmic antifungal drug approved by the Food and Drug Administration (FDA) of the United States, but has unsatisfactory factors such as high dosing frequency.
Methods: We report the synthesis and preparation of self-assembled poly(ethylene glycol)-block-poly (glycidyl methacrylate) (PEG-*b*-PGMA) micelles. These nanoparticles exhibit sustained delivery of a hydrophobic natamycin by topical administration on eye due to the hydrolysable properties of PGMA segments of micelle. Hydrolysis of glycidyl groups within a physiologically relevant environment provides an additional driving force for drug release by generation of hydrophilic hydroxyl groups to 'push' the encapsulated hydrophobic drug away from the resultant hydrophilic domains and into surrounding environment.
Results: *In vitro and in vivo* results revealed that the self-assembled micelles and the encapsulated natamycin were not cytotoxic and the released drug have strong antifungal ability to *Candida albicans*. Importantly, sustained natamycin release from micelles leads to the reduced administration frequency of natamycin from 8 times per day to 3 times per day in rabbits suffering from fungal keratitis (FK).
Conclusion: This study demonstrates a facile method that can greatly reduce dosing frequency of natamycin administration and thus improve long-term patient compliance.

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Micelle; natamycin; eye drop; sustained release; fungal keratitis

1. Introduction

Fungal keratitis (FK) is one of the most devastating microbial keratitis with the worst visual prognosis [1]. Over 40% of microbial keratitis are caused by fungal infection in several tropical and subtropical countries [2,3]. Difficulties in early differentiating it from other kinds of microbial keratitis lead to severe complications such as hypopyon, corneal blindness, corneal perforation, and endophthalmitis [4]. *Aspergillus, Candida*, and *Fusarium* species are the three major groups of fungus that cause FK [5]. These fungi species usually produce serious pathological changes including immune rings, satellite lesions, pseudopods, hypha moss, hypopyon and endothelial plaques [2].

Natamycin, the only commercially available agent approved by the Food and Drug Administration (FDA) for treating FK [6] has been considered as one of the most valuable agents for experimental research. In comparison with other antifungal drugs such as fluconazole and amphotericin B, natamycin has the advantages of good antifungal effect and less adverse reactions [7,8]. Natamycin is a hydrophobic drug with low water solubility. Clinically, 5% *w/v* of natamycin suspension (50 mg mL^{-1}) is prescribed as one drop instilled in the conjunctival sac at hourly or two-hourly intervals for 3–4 days and at 6–8 times daily for 14–21 days [9] due to aforementioned reasons and tear wash. The high dosing frequency leads patient noncompliance and cited as the leading problem in treating FK. Hence, there is an urgent clinical need to design a corneal targeted prolonged release delivery system to reduce the dosing frequency of natamycin.

A variety of drug carriers including cyclodextrin and cell-penetrating peptide have been explored for the sustained release of natamycin [10–13]. Anjali *et al*. reported the cyclodextrins can improve the aqueous stability of natamycin and hence reduce local irritation [10]. Jain *et al*. reported that the conjugation between natamycin and cell-penetrating peptide leads to an enhanced solubility of the drug in aqueous medium [12]. However, issues have emerged due to the instability, incomplete inclusion, non-specificity, and rapid intracellular degradation [11,12]. Recently, great effort has been made to design and fabricate polymeric nanoparticles owing to their ability to encapsulate and release drugs on demand under specific conditions [14,15]. Micelles have been demonstrated as a good drug delivery carrier due to their sustained release capacity and high aqueous stability [16]. Micelles which usually can be prepared by the self-assembly of block

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Supplemental data for this article can be accessed here.

co-polymers is an amphipathic nano-sized carrier with a core-shell structure. The hydrophobic core offers the entrapment of the hydrophobic drugs serving as a drug reservoir and the hydrophilic shell forms a steric barrier, which prevents aggregation and ensures the solubility of micelles in an aqueous or biological environment. Therefore, the use of micelles for the hydrophobic drug encapsulation could directly increase the water-solubility of hydrophobic drugs in aqueous media and hence enhance the drug delivery efficiency [17–20]. In addition, micelles can be tailored to achieve a sustained or on-demand drug release pattern by controlling the composition, functionality, size, and overall morphology [21].

In the present study, we report a straightforward method to initiate drug release where the environment itself is used to initiate intrinsic changes to the chemical structure of the micelles. As a result, these changes can enhance the drug release into the outer environment without entire particle degradation. In order to achieve this, we developed a micellar delivery system by synthesizing a biocompatible poly(ethylene glycol)-block-poly(glycidyl methacrylate) (PEG-*b*-PGMA). Of particular note, the hydrolysis of hydrophobic segments usually occurred at a non-neutral pH in previous study. The glycidyl methacrylate units have been explored for post-polymerization modifications through ring opening reactions of the epoxy group by a nucleophilic agent at neutral pH [22,23]. Taking this advantage, the incorporation of PGMA into micellar carrier can intrinsically alter overall structural hydrophilicity at neutral pH, inducing drug release in a sustained manner for topical administration on eye. For the first time, we reported the preparation of the micelles via self-assembly of the block copolymers where partial of the epoxy groups (approximately 15 units) were used for cross-linking to covalently stabilize the micelles and the remaining epoxy groups (approximately units) can undergo sustained hydrolysis at neutral pH to enhance self-initiated drug release over prolonged time periods. Additionally, PEGylated shell of the micelles can enhance the contact time with the mucus layer of the tear film, act as mucoadhesion promoters and enhance the corneal penetration of the drug-loaded micelles [24,25]. This system was used as a new delivery platform for the sustained and controlled long-term release of hydrophobic natamycin *in vitro* and *in vivo* to treat fungal keratitis.

2. Methods

2.1. Materials

4-Cyano-4-(((dodecylthio)carbonothioyl)thio)-pentanoic acid (Boron Molecular Pty Ltd). 4-dimethylaminopyridine (DMAP, 99%, Sigma-Aldrich), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 99%, Sigma-Aldrich) were used as received. Glycidyl methacrylate (GMA, Sigma-Aldrich) was passed over the basic alumina columns (Scharlau) to remove the inhibitors and stored at 2–8°C. 2,2′-Azobis(2-methylpropionitrile) (AIBN, 98%, ACROS ORGANICS) was used after recrystallization from methanol. Methoxy-PEG-amine HCl salt (M_n = 5 kDa) was purchased from JenKem. Natamycin was purchased from Selleck Chemicals (Houston, USA). The *Candida albicans* (*C. albicans*, ATCC 10231) was a kind gift from Dr. Zhang (Department of Microbiology, Harbin Medical University, Harbin, China). The yeast extract peptone dextrose medium (YPD) broth was purchased from Meilunbio (Dalian, China). The human corneal epithelial (HCE-2) cell line was supplied by the American Type Culture Collection (ATCC, Manassas, USA). For the cell culture, the Dulbecco's Modified Eagle's Medium:nutrient mixture F12 (DMEM:F12) and phosphate-buffered saline (PBS) were purchased from Hyclone (Logan City, USA). The methyl thiazolyl tetrazolium (CCK-8) was purchased from Dojindo (Kumamoto Prefecture, Japan), while the pentobarbital sodium was obtained from Sigma-Chemical Co. (St. Louis, USA). The marketed tobramycin and dexamethasone eye ointment TobraDex®, levofloxacin eye drops Cravit® (Santen, Osaka, Japan) and oxybuprocaine hydrochloride eye drops Benoxil® (Santen, Osaka, Japan) were purchased from a local pharmacy store.

2.2. Polymer synthesis

2.2.1. Neutralization of methoxy-PEG-amine, HCL salt

Methoxy-PEG-amine, HCl salt (MeO-PEG-NH_2.HCl, 1 g, M_n = 5 k, JenKem) was dissolved in 20 mL of 0.1 M sodium hydroxide and stirred for 2 h. Then, the neutralized MeO-PEG-NH_2 was extracted by dichloromethane (DCM, 50 mL ×2), washed with water (40 mL ×1), and dried over $MgSO_4$. Afterward, the product was concentrated by removing the solvent under reduced pressure, precipitated in diethyl ether (DEE), and vacuum dried for over 2 days at R.T. (70–80% Yield).

2.2.2. Synthesis of macro-raft agent (macro-TTC)

The methoxy-PEG-amine (0.7 g, 0.14 mmol), 4-cyano-4-(((dodecylthio)carbonothioyl)thio)-pentanoic acid (67.82 mg, 0.168 mmol), 4-dimethylaminopyridine (25.65 mg, 0.21 mmol), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (40.26 mg, 0.21 mmol) were dissolved in 25 mL anhydrous DCM, and allowed to react overnight at room temperature. Then, the reaction mixture was diluted with DCM (25 mL), washed with deionized water (50 mL ×1) and hydrochloric acid (0.5 M, 50 mL ×1), saturated with $NaHCO_3$ (50 mL ×1) and deionized water (50 mL ×1), further saturated with NaCl (50 mL ×1). The organic phase was isolated and dried over $MgSO_4$. Afterward, the product (macro-TTC) was concentrated *via* rotary evaporation, precipitated in diethyl ether, and vacuum dried for over 2 days (yellow powder, 83% yield).

2.2.3. Synthesis of PEG-b-PGMA copolymer

The synthesis of PEG-*b*-PGMA copolymer was achieved via reversible addition-fragmentation chain transfer (RAFT) polymerization [26,27]. Briefly, Macro-TTC (500 mg, 0.092 mmol), glycidyl methacrylate (GMA, 392.34 mg, 2.76 mmol), and an initiator (AIBN, 3.02 mg, 0.0184 mmol) were dissolved in 2.7 mL of DMSO (1:3 *w/v* ratio of macro-TTC and GMA to solvent). Then, the reaction mixture was transferred to a Schlenk flask with a stirrer bar and degassed by three freeze-pump-thaw cycles. Afterward, the flask was back-filled with argon and stirred at 70°C for 19 h. Subsequently, the polymerization was quenched by opening to air and cooling to room temperature. Then, the product was purified by precipitation in acetone:DEE (1:9 *v/v*) twice, dried under vacuum overnight, and stored at room temperature.

2.3. Hydrolysis of the glycidyl groups

The hydrolysis of glycidyl groups was conducted under mild physiological conditions. Briefly, 100 mg of PEG-*b*-PGMA copolymer was dissolved in 5 mL of PBS buffer (pH 7.4), and the solution was incubated in a glass vial at 37°C for over 8 days. The purification of the resulted polymer was achieved by removing the water, re-dissolving in ACN (5 mL), and precipitating into cold DEE (50 mL ×2). Then, the product was collected by centrifugation and dried *in-vacuo* (0.1 mbar) for ^{1}H NMR spectroscopic analysis.

2.4. Micelle preparation

2.4.1. Self-assembly of PEG-b-PGMA into micelle

PEG-*b*-PGMA (100 mg, 0.01 mmol) was dissolved in 1 mL of tetrahydrofuran (THF), and 10 mL of non-solvent ethanol (EtOH) was added dropwise to the PEG-*b*-PGMA solution while continuously stirring. A turbid suspension was resulted, and the mixture was left stirring overnight at room temperature. Then, the THF was removed from the system by dialysis (ThermoFisher Scientific, MWCO: 3.5 kDa) against EtOH.

2.4.2. Covalent stabilization of self-assembled PEG-b-PGMA into cross-linked micelle

The obtained PEG-*b*-PGMA micelle solution (100 mg, 0.01 mmol) was concentrated to obtain a total volume of 3 mL. A solution of 1,7-diaminoheptane (9.27 mg, 0.079 mmol, 50 mol% of PGMA) was dissolved in 100 µL of THF and added dropwise to the continuously stirred solution of micelle suspension. The reaction mixture was left mixing for 3 days, and subsequently dialyzed against EtOH to remove any unreacted cross-linker and THF.

2.5. Drug loading of cross-linked micelle

Next, 20 mg of natamycin was dissolved in 500 µL of DMSO and added dropwise to 3 mL of cross-linked micelle (100 mg, 0.01 mmol) in ethanol while continuously stirring. After 2 h, the mixture was dialyzed against PBS (pH 7.4, 30 mL ×3) to remove any organic solvents and unloaded drug in micelle. UV-Vis spectroscopy was used to calculate the amount of unloaded drug in the solution using standard calibration curves generated from known drug concentrations at λ_{max} = 306 nm (a 1:1 ratio of PBS-to-DMSO was used for both calibration curves and samples) [28,29]. Drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following equations [30,31]:

$$\mathrm{DLC} = \frac{\text{weight of loaded drug}}{\text{weight of carrier} + \text{weight of drug}} \times 100\%$$

$$\mathrm{DLE} = \frac{\text{weight of loaded drug}}{\text{weight of total drug used}} \times 100\%$$

2.6. In vitro release of drug from drug-loaded micelle

In vitro drug release was performed using the dialysis method. Briefly, the drug-loaded micelle (i.e. containing 10 mg of micelle + 1.88 mg of natamycin) were suspended in 5 mL of PBS at pH 7.4. Then, the solutions were transferred into a dialysis bag (MWCO 3.5 kDa) and dialyzed against PBS pH 7.4 (200 mL). Afterward, the mixture was left stirring at 37°C to mimic the physiological conditions. At various intervals, 1-mL aliquots were taken from the outside of the dialysis bag and diluted with 1 mL of DMSO for UV-Vis spectroscopy [29,32]. The drug concentration was calculated according to the standard curve at λ_{max} = 306 nm.

2.7. Characterization

2.7.1. Nuclear Magnetic Resonance (NMR) Spectroscopy

Proton NMR (^{1}H NMR) analysis was conducted using a Varian Unity Plus 400 MHz spectrometer operating at 400 MHz. All samples were analyzed at a concentration of approx. 10 mg mL^{-1}, and deuterated chloroform was used as a reference.

2.7.2. Gel Permeation Chromatography (GPC)

The GPC characterization of polymers was carried out using THF as the eluent. GPC analysis was performed using a modular Shimadzu system that comprised of a Shimadzu RID-10A interferometric refractometer (λ = 633 nm) using three Agilent MIXED-C columns (5-µm bead size) operating at 45°C.

2.7.3. Transmission Electron Microscopy (TEM)

TEM images were taken using a Philips CM120 BioTWIN TEM at an operating voltage of 120 kV. The samples of the particles were air-dried on a carbon-coated Formvar film mounted on 300 mesh copper grids (ProSciTech, Australia).

2.7.4. Dynamic Light Scattering (DLS)

Dynamic light scattering measurements were performed on a Zetasizer Nano ZS (Malvern Instruments). Analysis was performed at a constant temperature of 25°C.

2.7.5. UV-visible spectroscopy

UV-Vis analysis was performed on a Shimadzu UV-1800 spectrometer using quartz cuvettes, with a 1-cm path length.

2.8. In-vitro studies

2.8.1. In-vitro cytotoxicity evaluation

2.8.1.1. Corneal epithelial cell culture. Human corneal epithelial (HCE-2) cells were maintained in DMEM/F-12 medium supplemented with 10% (*v/v*) fetal bovine serum (FBS), insulin (4 mg mL^{-1}), EGF (10 ng mL^{-1}), and penicillin–streptomycin (1%). Then, these cells were incubated in a humidified 5% CO_2 atmosphere at 37°C and sub-cultured at approximately 80% confluency using trypsin (0.05%). Afterward, cells from passages 2–6 were used to perform the *in vitro* assays.

2.8.1.2. Cytotoxicity assay. HCE-2 cells were seeded into 96-well plates at an initial cell density of 8,000 cells/well and incubated at 37°C. The natamycin-loaded micelle solution was diluted with cell culture medium at various concentrations of 10, 20, 40, 80, 160, 320, and 640 µg mL^{-1}, with natamycin concentrations of 2, 4, 8, 16, 32, 64, and 128 µg mL^{-1}, respectively. Pure natamycin with different concentrations (2, 4, 8, 16, 32, 64, and 128 µg mL^{-1}) was used as the

control experiment. After 24 h of cell culture, the medium was replaced with diluted drug-loaded micelle solution and pure natamycin solution, and further incubated for 24 and 72 h. Then, these cells were washed twice with PBS, and further incubated with 110 μL of DMEM/F-12 containing 10 μL of CCK-8 solution for 2 h. Finally, the medium was removed, and the absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Japan). All experiments were performed in triplicate. The cell viability (%) was calculated using the following formula, where [*A*] is the average absorbance:

$$\text{Cell viability}(\%) = \frac{[A]450(\text{sample}) - [A]450(\text{blank})}{[A]450(\text{control}) - [A]450(\text{blank})}$$

2.8.2. In vitro antifungal activity

2.8.2.1. Fungal cell culture (method of inoculation).

C. albicans was cultured on YPD plates and stored at 4°C for use. For amplification, a small inoculum from an isolated colony was selected with a sterile inoculating loop and suspended in a test tube containing YPD culture media by rubbing the inoculated loop against the wall of the test tube. This helps to dilute the pasty colonies by giving the media a turbid appearance. Then, the fungus was incubated in a humidified 5% CO_2 atmosphere at 37°C.

2.8.2.2. Minimum inhibitory concentration (MIC). The MIC of natamycin-loaded micelle was determined following the guidelines of the Clinical and Laboratory Standards Institute [CLSI 2014] and a previously reported method [33], with slight modifications. Briefly, pure natamycin (64.00–0.25 μg mL^{-1}) and natamycin-loaded micelle (320.00–1.25 μg mL^{-1}, containing natamycin with concentrations within 64.00–0.25 μg mL^{-1}) were diluted with YPD using the double broth dilution method in the wells of 96-well microtiter plates. Then, a suspension ($[A]_{600}$ = 0.001) of *C. albicans* (10^4 CFU/mL) in YPD was added, with the appropriate negative (Ctrl 1 = media only, must clear) and positive (Ctrl 2 = *C. albicans* only in media, must contaminative) controls. Afterward, these microtiter plates were incubated for 24 h, and the white turbidity ($[A]_{600}$) were recorded using a microplate reader (Bio-Rad, Japan). The endpoint was defined as the lowest concentration of the compound that resulted in total inhibition of growth, when compared to the growth in negative control wells. All experiments were performed in triplicate. The fungus numbers (%) were calculated using the following formula, where [*A*] is the average absorbance:

$$\text{Fungal numbers}(\%) = \frac{[A]600(\text{sample}) - [A]600(\text{blank})}{[A]600(\text{control}) - [A]600(\text{blank})}$$

2.8.2.3. Zone of inhibition. Drug sensitivity to antifungal drugs was determined by disc diffusion assay. The *Candida* cells grew for 24 h and five colonies were selected. Then, these cells were counted and adjusted to a density of 10^6 CFU/mL. In order to obtain a confluent lawn of growth, 400 μL of this suspension was added and spread onto a YPD plate. Then, antibiotic disks containing natamycin (25 μg and 50 μg), natamycin-loaded micelle (125 μg and 250 μg containing 25 μg and 50 μg natamycin, respectively) and PBS were prepared and placed on the YPD plates. Afterward, these plates were incubated for 24 h at 37°C and the inhibition zone was measured using a Vernier caliper.

2.9. In vivo studies

2.9.1. Ocular irritation tests

Healthy, 8-week-old New Zealand white rabbits (weighting 1.5–2.0 kg) were selected from the Animal Center of the First Affiliated Hospital of Harbin Medical University. Each animal had a corneal thickness of greater than 500 μm. These animals were fed for 1 week before model preparation for adaptation. The animal room reached the specific pathogen free (SPF) standard. All procedures were approved by the Ethics Committee of Harbin Medical University and conducted according to the guidelines of the ARVO statement for the Use of Animals in Ophthalmic and Vision Research (ethic approval letter number: KY2018-306). Ocular irritation was evaluated according to the modified Draize test [34]. Three rabbits were used in this experiment Solutions that consisted of 246 mg mL^{-1} of natamycin-loaded micelle were instilled into the left eyes of rabbits, while the right eyes were as controls. A single instillation of the sample (50 μl) every 1 h at eight times per day lasted for a period of 3 days. The ocular tissues were observed at 0 and 72 h after the last instillation by slit-lamp biomicroscopy. The corneal opacity was graded on a scale of 0–4 and iris hyperemia was graded on a scale of 0–2, while conjunctival congestion, swelling, and discharge was graded on scales of 0–3, 0–4, and 0–3, respectively. Then, the total mean scores for each treatment group were calculated. The evaluation criteria considered the solutions as nonirritant for values ranging 0–3.9, slightly irritant for values ranging 4.0–8.9, moderately irritant for values ranging 9.0–12.9, and seriously irritant for values ranging 13–16 [35].

2.9.2. Establishment of the FK rabbit model

Twenty Rabbits were kept under observation for 72 h to exclude any local or systemic diseases. General anesthesia was induced in these rabbits by intravenous injection of 1 mL kg^{-1} of 3% sodium pentobarbital. Topical anesthesia was achieved with two drops of oxybuprocaine hydrochloride (Benoxil). A 30-gauge insulin needle was used to create a corneal incision at approximately 0.1 mm in depth in the right eye of each rabbit. A 100-μL aliquot of the inoculums, which contained 400 × 10^4 CFU/mL of *C. albicans* was circularly injected into the superficial stroma of rabbit eyes [36,37]. Antibiotics (tobramycin and dexamethasone eye ointment, and levofloxacin eye drops) were administered to the eyes after surgery for three times a day for 15 days in order to prevent bacterial growth.

At 48 h after inoculation, these animals were divided into four treatment groups, with five rabbit eyes in each group: control (Ctrl) group, treated with saline solution for eight times a day (8 am to 4 pm, one drop per hour) up to 15 days; Low frequency of Nat group, treated with topical natamycin (50.00 mg mL^{-1}, 5% w/v) for three times a day (8 am to 4 pm, one drop every 4 h) up to 15 days; Nat group, treated with topical natamycin (50.00 mg mL^{-1}, 5% w/v) for eight times a day (8 am to 4 pm, one drop per hour) up to

15 days; natamycin-loaded micelle (Nat-Mi) group, treated with natamycin-loaded micelle (246.34 ± 8.34 mg mL^{-1}, containing 50.00 mg mL^{-1} natamycin) for three times a day (8 am to 4 pm, one drop every 4 h) up to 15 days. The concentration of natamycin-loaded micelle was calculated by DLC (detailed values in the Results). One hour after the last eye drop (day 15), animals were euthanized. Corneas and aqueous humor were collected immediately.

The general changes of rabbit eyes were dynamically observed using a slit lamp at several time intervals (day 5, 10, and 15). The fungal changes were recorded by direct smear and confocal microscopy on day 5 and 15, and fungal culture on day 15. The corneal changes were observed by optical coherence tomography (OCT) on day 5 and 15 and hematoxylin and eosin (H&E) staining on day 15. The drug extraction was analyzed by LC/MS-MS on day 15.

2.9.3. Slit lamp observation

The SLM-8E digital slit lamp analysis system (KANGHUA, Chongqing, China) was used to observe the conjunctival hyperemia, corneal clouding, corneal infiltration (measured as the size of the epithelial defect in mm), corneal neovascularization, and hypopyon level. The rabbits were anesthetized using sodium pentobarbital and oxybuprocaine hydrochloride. Then, the eyes were evaluated at baseline and random time points after injection by slit-lamp biomicroscopy.

The clinical scoring was performed after slit lamp examination. Parameters such as conjunctival hyperemia, corneal clouding, corneal infiltration (measured as the size of the epithelial defect in mm), corneal neovascularization, and hypopyon level were evaluated and noted in each rabbit. The clinical score was determined with a brief modification through the following Schreiber scoring system. In brief, the conjunctival hyperemia was graded as follow: 0, no; 1, mild; 2, medium; 3, high grade hyperemia. The corneal clouding was graded as follow: 0, no clouding or clear cornea; 1, mild clouding; 2, corneal clouding in two quadrants of the cornea; 3, total corneal clouding. Corneal neovascularization was graded as 0, no; 1, mild (≤2 mm from the limbus); 2, medium (>2 mm from the limbus); 3, high (until the center of the cornea) grade neovascularization. The corneal infiltrate and hypopyon diameter were measured using the largest diameter in millimeters [38].

2.9.4. Direct smear

The corneal specimens were scrapped from the damaged corneal surface using an ophthalmic scraper, placed on the clean slide, and stained with H&E dye. Then, the slide was gently warmed and directly examined under a microscope to check for the presence of fungus.

2.9.5. Confocal microscopy observation

Confocal laser microscopy (Heidelberg Engineering GmbH, Heidelberg, Germany) was used to directly observe fungus. Each rabbit eyelid was opened using an eye speculum and an assistant fixed the rabbit head in position. All camera settings were maintained constant throughout the experiment. For each examination, the repeated through-focus data sets were obtained from the corneal region to identify the presence of fungus.

2.9.6. Anterior segment optical coherence tomography examination

The anterior segment optical coherence tomography (AS-OCT) system (Optovue, CA, USA) was used to assess the corneal thickness. The rabbits were anesthetized, and the pupils were dilated with 1% tropicamide solution. The corneas were frequently lubricated during the imaging session. Afterward, these rabbits were placed in a restrainer for stability and a pediatric probe was used for optical coherence tomography (OCT) analysis. Then, 6-mm horizontal line scans in the inferior retina below the optic disk were used to capture the damaged corneal cross-sections.

2.9.7. H&E staining

Histological examination was used to observe the fungal hyphae, integrity and regularity of the cornea, inflammatory cells, and neovascularization. The corneas were fixed in buffered formalin, embedded in paraffin wax, cut at 5-µm sections, and stained with H&E dye. The damaged corneal section was cut for observation with microscope.

2.9.8. Fungal culture of cornea

The *C. albicans* colony was scrapped from several corneas and was observed by *in-vitro* culture. Briefly, the corneas were homogenized in sterile PBS using a homogenizer. Then, the supernatant was collected by centrifugation and diluted for 10 times. Afterward, a series of dilutions of samples were spread on YPD plates and incubated for 48 h at 37°C.

2.10. LC-MS/MS analysis

Aqueous humor was collected using 30-gauge insulin needles before euthanized and the corneas were tailored by ophthalmic scissors after euthanized on an ice bath. Then, these corneas were washed with PBS and homogenized using a hand homogenizer in cold methanol (1:10 *w/v*). 10 µL of 10 µg mL^{-1} of amphotericin B (internal standard) was added in the cornea's homogenate. Afterward, the homogenate was vortexed for 1 min, placed in a sonic oscillator for 10 min, and subjected to centrifugation at 4,000 × g for 10 min to separate the supernatant. Then, the separated supernatant evaporated under nitrogen and the residue after the evaporation was reconstituted in 100 µL of the mobile phase and subjected for LC-MS/MS analysis. Subsequently, 10 µL of 10 µg mL^{-1} of amphotericin B was added to 50 µL of aqueous humor and 200 µL of acetonitrile was used to extract the analyte from the samples. The supernatant was subjected for analysis after 4,000 × g of centrifugation for 5 min. All steps were tried to perform on ice as much as possible.

The concentration of natamycin in the study samples was measured through the LC-MS/MS. LC-MS/MS is a technique in which a component is separated from a mixture of components using the liquid chromatograph (LC) at first and then the ions are broken into different fragments using the mass spectrometry (MS) and the mass of components are detected by MS. The AB SCIEX QTRAP® 4500 triple-quadrupole mass-spectrometric

system (AB Sciex, MA, USA) coupled with ultrafast liquid chromatography (AB Sciex, MA, USA) was used. The analytes were separated on the Bridge® BEH C18 column (4.6 × 150 mm, 2.5 µm) using 10 mM of ammonium formate in water and acetonitrile as the mobile phase at a ratio of 60:40 (*v/v*) and pumped at 0.6 mL min^{-1}. The multiple reaction monitoring (MRM) of natamycin was analyzed using an electrospray in positive ion mode. The MRM transition was 666.3 → 503.4 and 924.5 → 906.6 (IS). The source was operated at 400°C with an electrospray voltage of 5,500 V. The ion source parameters were as follow: curtain gas 35 L min^{-1}, GS1 35, GS2 50, and CAD gas 6 L min^{-1}. The standard curve ranged from 10 ng mL^{-1} to 5,000 ng mL^{-1}.

2.11. Statistical analysis

The values were presented as mean ± standard deviation (SD). The differences were analyzed using one-way analysis of variance (ANOVA) followed by *t*-test for the pairwise comparison of subgroups. These statistical analyzes were performed using the SPSS software (v13.0; SPSS, Inc., Chicago, IL, USA) and a *P*-value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Synthesis and characterization of the PEG-b-PGMA copolymer

A general strategy for the synthesize of PEG-*b*-PGMA is illustrated in Figure 1. The macro-TTC was first synthesized via the EDC/DMAP coupling reaction. The preparation of PEG-based chain transfer agent (macro-TTC) was verified by analyzing the corresponding peaks in the ^{1}H NMR spectra (Figure S1). A new proton resonance e′ (in Figure S1B) at 2 ppm assigned to the proton of amide group (-N***H***CO-) was observed, suggesting the successful preparation of macro-TTC. The number-average molecular weight (M_n^{GPC}) of 6.9 kDa with a dispersity (*Đ*) of 1.07 was determined for macro-TTC by GPC (Figure S2). Then, the block copolymer PEG-*b*-PGMA was synthesized *via* the RAFT polymerization of glycidyl methacrylate (GMA) using the prepared macro-TTC. After 19 h of polymerization, near quantitative conversion of GMA (*ca*. 99%, 30 GMA units) was achieved through ^{1}H NMR analysis (Figure S1). Characteristic peaks m″ and i″ derived from methacrylate were observed after the RAFT polymerization (Figure S1C), which confirms the preparation of block copolymer PEG-*b*-PGMA. In addition, the GPC curve revealed a monomodal peak (M_n^{GPC} of 11.9 kDa) and a narrow dispersity (*Đ* = 1.23), suggesting a controlled polymerization (Figure S2).

3.2. Self-assembly and characterization of micelle

The PEG-*b*-PGMA copolymer comprised of hydrophilic and hydrophobic segments. The PEG segments provide solubility within aqueous environments, and PGMA segments offer drug loading capacity for hydrophobic drugs. The self-assembly of PEG-*b*-PGMA was achieved through the solvation of the block copolymer in THF, followed by the dropwise addition of EtOH as a non-solvent for the PGMA segment (Figure 2). The subsequent removal of THF was achieved by dialysis (MWCO = 3.5 k) against EtOH. The resultant nanoparticles (*NP*) were assembled in EtOH and displayed a hydrodynamic diameter (D_H) of approximately 200 nm by DLS measurement (Figure S3). The self-assembled structures tended to rapidly disassemble at concentrations below the critical association concentration and/or under external stimuli (e.g. temperature, pH and light) [39]. For drug delivery applications, it is often necessary to stabilize the structures to prevent the undesired burst release of the encapsulated cargo/drug. Therefore, in order to stabilize the micelle in the present study, 1,7-diaminoheptane was used as a cross-linker to covalently stabilize the assembled structures through the nucleophilic attack of 50 mol% of the total epoxy rings available (Figure 2). After stabilization, the cross-linked nanoparticle (*XL-NP*) was dialyzed (MWCO = 3,500 g mol^{-1}) against EtOH to remove any unreacted cross-linker. Then, DLS measurements were conducted to present the micelle size after cross-linking. As shown in Figure 3(a), the non-cross-linked micelle dissolved in THF (no Tyndall effect, insert-i) and recovered the block polymers with a D_H of 2.5 nm, which agrees well with the measured size of the PEG-*b*-PGMA coil in THF. In contrast, cross-linked micelle (*XL-NP*) exhibited an enlarged D_H of 294 nm in THF, when compared to the value obtained in EtOH (D_H of 190 nm with Tyndall effect, insert-ii), suggesting that the covalent cross-linking of micelle was successful.

In order to visualize the morphology of the cross-linked micelle, TEM was utilized (Figure 3(b)). From the TEM image, distinct spherical solid structures with average diameters ($Đ^{TEM}$) of approximately 160 nm could be observed, which

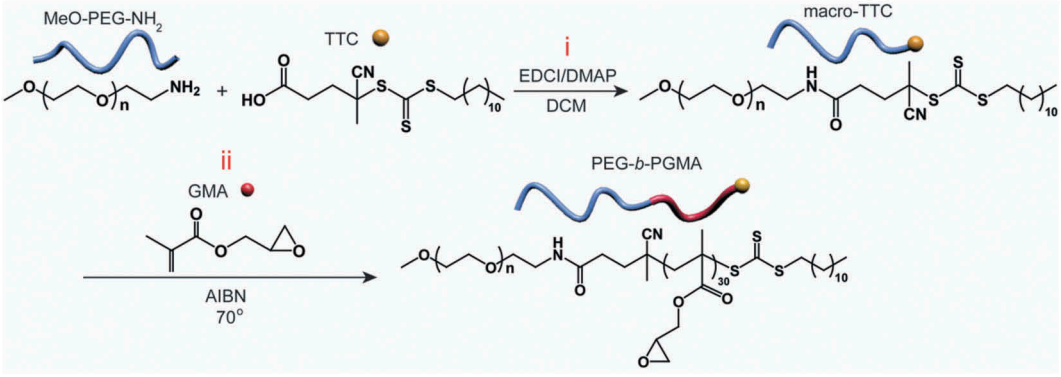


Figure 1. Schematic illustration of the synthesis of the PEG-*b*-PGMA copolymer.

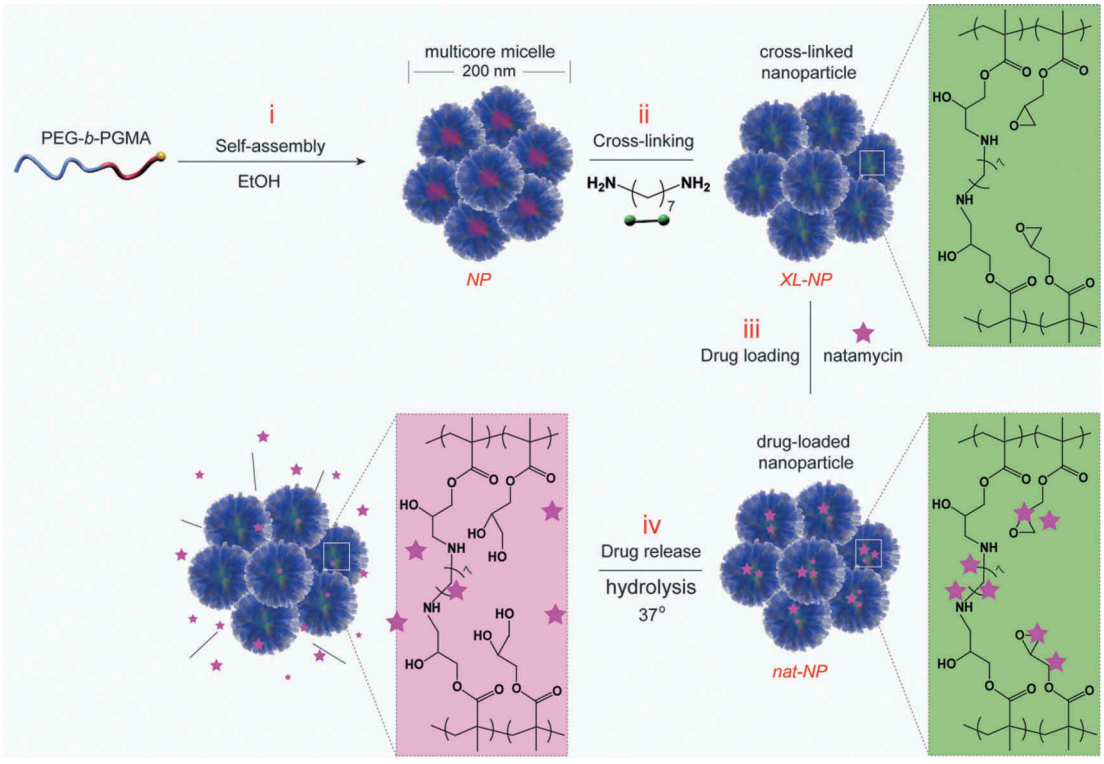


Figure 2. Schematic illustration of (i) the preparation of self-assembled micelle using EtOH as the non-solvent, (ii) the cross-linking of nanoparticles *NP*, (iii) the hydrophobic drug loading of nanoparticles (*nat-NP*), and (iv) the self-hydrolysis induced drug release.

is in good agreement with the size (190 nm) obtained from the DLS measurements (Figure 3(a)). Based on the literature, polymeric micelle is commonly known as *ca*. 20–70 nm in diameter. However, the sizes obtained in the present work were considerably larger (*ca*. 160–190 nm). It is likely that these particles represent 'crew-cut' multi-core micelle, in which the dimensions of the hydrophobic core are much larger than the corona [40].

3.3. Investigation of the hydrolysis of the glycidyl groups

Due to the presence of the remaining glycidyl groups (50 mol%, 15 repeat units) in the hydrophobic core, the micelles are designed to undergo hydrolysis with the presence of H_2O as a nucleophile. Figure 4 presents the ^{1}H NMR spectroscopic analysis of *in situ* hydrolysis of glycidyl units under mild physiological conditions (PBS pH 7.4, 37°C) for over 8 days. After 8 days of incubation, the proton resonance peak ***c***, which corresponds to the glycidyl units of PGMA, underwent a change in resolution (Figure 4(a) *vs*. 4(b)), suggesting that partial hydrolysis, as peaks ***b*** and ***c***, were still present in the spectrum (consistent with the reports in the literature) [41]. Upon integration of the newly formed resonance ***c'*** of the hydrolyzed PEG-*b*-PGMA (methylene group, 2H, -CHC***H***$_2$OH-; Figure 4(b)) *vs*. resonance *c* of the PEG-*b*-PGMA (methylene group, 2H, -OCH_2CH_2-; Figure 4(a)), it could be

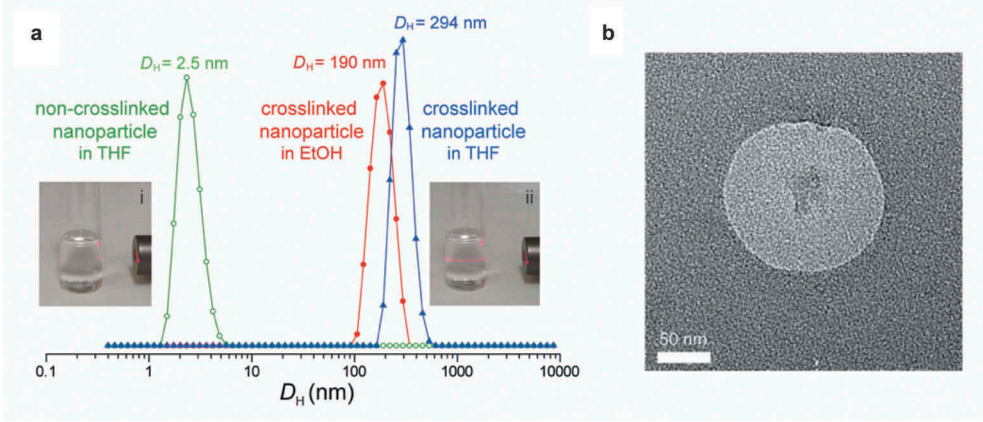


Figure 3. (a) DLS intensity average (D_H) measurements of non-cross-linked micelle in THF (green) and cross-linked micelle in EtOH (red) and THF (blue). The inset (ii) clearly represents the Tyndall effect of the cross-linked nanoparticles in THF; (b) TEM images of the cross-linked nanoparticles (*XL-NP*, multi-core micelle) (*XL-NP* was drop-casted from EtOH).

measured that ca. 53% of the total available PGMA groups have been hydrolyzed after 8 days. These results clearly show that the hydrolysis of the glycidyl groups occurs in physiological conditions, and causes the structural changes, in which two-hydroxyl groups are produced from the nucleophilic attack of the epoxy ring. This would increase the inherent hydrophilicity of the hydrophobic core regions of the micelle, which could be utilized for drug delivery applications. The hydrolysis of the glycidyl groups has been widely used to gain extra functionality [42,43], but to the best of our knowledge the application of this simple process has not been explored as an endogenous stimulus for sustained drug release. In addition, the hydrolysis of the glycidyl groups is commonly achieved under harsh conditions (i.e. 80°C in H_2SO_4), which does not mimic the physiological conditions [44]. However, this study revealed that the hydrolysis of the glycidyl groups could occur under mild physiological conditions (PBS pH 7.4, 37°C) over a longer period of time, which makes it suitable for the sustained release of hydrophobic drugs at physiological conditions.

3.4. In vitro drug loading and drug release

Natamycin, which is a hydrophobic antifungal drug, was encapsulated inside the cross-linked micelle (*nat-NP*, Figure 2). Briefly, natamycin was dissolved in DMSO (a good solvent for both drug and micelle), added dropwise into the cross-linked micelle suspension in ethanol, and left mixing. This helps the drug to be trapped inside the hydrophobic core of the cross-linked micelle. Then, the solution was dialyzed against PBS to further induce drug entrapment and remove any organic solvent. UV-Vis spectroscopy was used to calculate the amount of free drug in the solution using standard curves generated from known drug concentrations. The DLE and DLC were 85.02 ± 3.63% and 20.32 ± 0.69%, respectively. The high drug loadings of micelle can be attributed to the large size of the cross-linked micelle and the multicore structure (Figure 5(a)). Drug-loaded micelle exhibited a size of $Đ^{TEM}$ of approximately 160 nm in the TEM image (Figure 5(a)), which is in close agreement with the size of micelle observed in the DLS and TEM before drug loading (Figure 3). Besides, after encapsulation into micelles, the solubility of natamycin increased in PBS (pH 7.4) at the same drug concentration (Figure 5(b)). The *in vitro* release of natamycin from the micelle was subsequently investigated under physiological conditions (PBS pH 7.4, 37°C), which mimics the ocular environment. A rapid initial release (approximately 18%) was observed within 20 h, followed by a plateau (Figure 5(c)). After 8 days, a maximum of *ca.* 34% drug released from the micelle. The burst release within the first 20 h can be attributed to the release of attached or loosely trapped free drugs. The release of the other 16% of natamycin over 8 days could be attributed to the hydrolysis of the hydrophobic glycidyl groups, resulting in hydroxyl groups. This could increase the hydrophilicity of the micelle core and thus 'pushes' the encapsulated hydrophobic cargo out of the assemblies. Natamycin release in this study could occur due to two driving forces: (i) the concentration driven release (ii), and the change in inherent hydrophilicity of the assemblies.

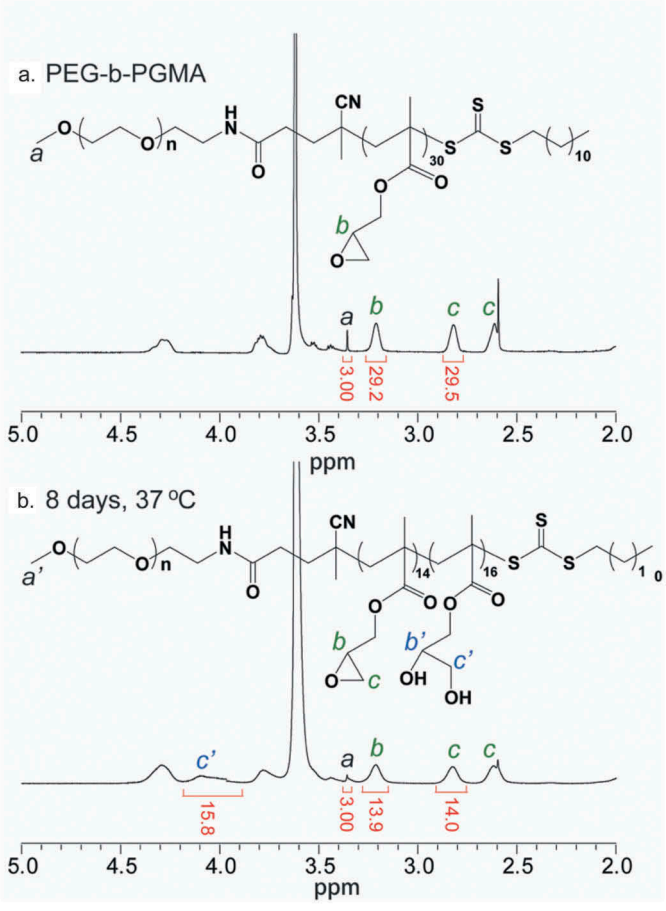


Figure 4. The ^{1}H NMR spectra of PEG-*b*-PGMA (a) before hydrolysis and (b) after 8 days of hydrolysis in PBS at 37°C.

3.5. Cytotoxicity of natamycin-loaded micelle in vitro

In order to study the biocompatibility of drug-loaded micelle (*nat-NP*), the cytotoxicity was investigated by CCK-8 assay. The toxicity of natamycin-loaded micelle (micellar formulation, Nat-Mi group) were compared with that of the pure natamycin in PBS (PBS formulation, Nat group). The concentration of natamycin in the Nat-Mi group was consistently adjusted with that in the Nat group. From *in vitro* drug release curve, a *ca.* 18% rapid release can be observed within 20 h, followed by a plateau with a total *ca.* 34% cumulative release in 8 days (Figure 5(c)). However, 8-day cell culture was too long due to the cell contact inhibition, while 72 h (*ca.* 31% release) was more suitable to perform cytotoxicity experiments *in vitro*. Hence, 24 h (rapid release phase) and 72 h (slow release phase) were chosen to perform short-term and long-term toxicity test, respectively. As shown in Figure 6, after 24 and 72 h of exposure to cells, the cell viability in the Nat-Mi group and Nat group was very high and was not statistically different when compared to the control group ($P > 0.05$). In addition, at the same concentration of natamycin, the viability of these two groups was not different ($P > 0.05$). These results imply that all substances including natamycin, micelle, and natamycin-loaded micelle were nontoxic to epithelium at all tested concentrations. Thus, the micellar formulation was a biocompatible carrier for ocular drug transportation.

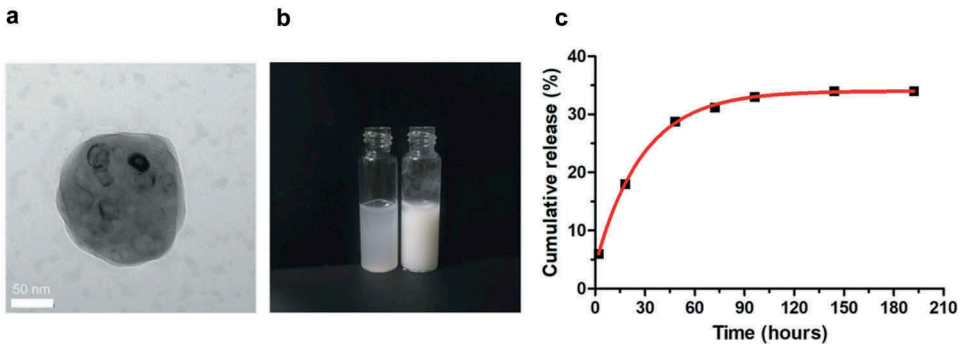


Figure 5. (a) TEM images of drug-loaded (or natamycin-loaded, *nat-NP*) multicore micelle (*nat-NP* was drop casted from PBS); (b) Solubility of drug-loaded multicore micelle and pure natamycin in PBS (pH 7.4), left for micellar formulations, right for dispersion formulations; (c) *In vitro* cumulative drug release percentage of natamycin with pH 7.4 at 37°C.

3.6. Anti-fungal effect of natamycin-loaded micelle in vitro

Since drug was loaded in the micelle and then released from it, the anti-fungal ability of the released natamycin needs to be determined to see whether the released drug was still effective or not. Prior to quantitative experiments, several qualitative studies were carried out to measure the minimal inhibition concentration (MIC) and the inhibition zone diameters of *Candida albicans*, which is the safest fungus for laboratory culture isolated from eyes with FK.

The MIC represents the lowest drug concentration that inhibits fungus growth after 18–24 h of incubation. The lower the MIC values, the stronger the anti-fungal ability. In the present study, the MIC values in the Nat-Mi group were compared with those in the Nat group and control group. The positive control contained the media and fungus, while the negative control contained only the media. In addition, it needs to be emphasized that the drug-loaded micelle was stored at 37°C to achieve maximal release prior to the MIC experiment. After 24 h of incubation, the white turbidity absorbance of each group at 600 nm were determined. As shown in Figure 7, the concentration where the number of fungus reduces to zero and inhibits the fungus growth is presented as the MIC values. The MIC values of micelle (20 mg mL^{-1}, containing 4 mg mL^{-1} of natamycin) were similar to that of pure natamycin (2 mg mL^{-1}), suggesting that the ability of micellar formulation was slightly weaker when compared to PBS formulation, although the drug was fully released from the micelle prior to performing experiment.

In addition, the inhibition diameter of 9.65 ± 0.65 mm and 14.19 ± 1.08 mm were observed for PBS formulation (Nat group) at dilutions of 25 µg and 50 µg, respectively. Slightly smaller zone of inhibitions 9.09 ± 0.66 mm and 13.85 ± 0.95 mm were found after 24 h of incubation with micellar formulation at natamycin dilutions of 25 µg and 50 µg in PBS, respectively (Figure 7(b)). From the obtained results of the MIC values and the diameter of inhibition zone, a similar but slightly lower anti-fungal activity of micellar formulation was observed when compared to PBS formulation. Micelle, as a drug carrier, has no anti-fungal ability to *Candida albicans*. Hence, it can be concluded that all fungal inhibitions resulted from the natamycin released from micelle. Slightly lower anti-fungal activity of micellar formulation may be attributed to the restriction by the micelle structure and the incomplete release of drug from the micelle.

3.7. Toxicity of natamycin-loaded micelle in vivo

In vitro experiments implied that the drug-loaded micelle was nontoxic to cells. *In vivo* eye irritation test was performed to detect the potential adverse reactions of the micellar formulations according to the scoring system of modified Draize test. Natamycin-loaded micelle were instilled into the left eyes of rabbits, while blank PBS were instilled into the right eyes,

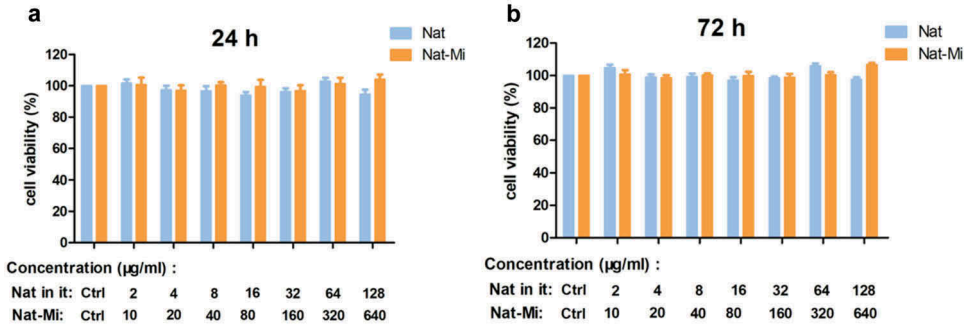
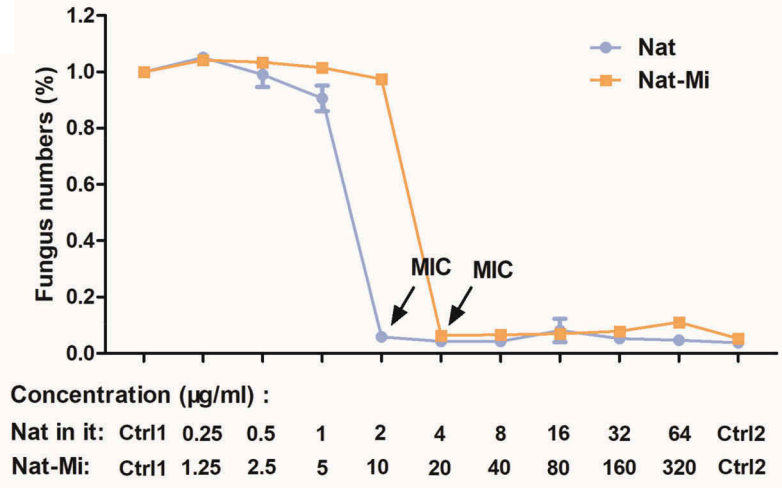


Figure 6. The natamycin-loaded micelle was nontoxic to corneal epithelial cells. The cell viability in the Nat group and Nat-Mi group was detected by CCK-8 after 24 h (a) and 72 h (b) of incubation. The data were expressed as mean ± standard deviation (SD), *n*= 3. Ctrl: control; Nat: natamycin; Nat-Mi: natamycin-loaded micelle.

a



b

Zone of inhibition (mm)	*Candida albicans*	
	25 μg	50 μg
PBS	-	-
Nat	9.65 ± 0.65	14.19 ± 1.08
Nat-Mi	9.09 ± 0.66	13.85 ± 0.95

Figure 7. Natamycin-loaded micelle inhibited *Candida* growth *in vitro*. Minimal inhibition concentration (a) and the diameter of zone of inhibition (b) in the Nat-Mi group and Nat group against *Candida* were measured after 24 h of incubation in/on YPD medium. The data were expressed as mean ± standard deviation (SD), n= 3. Ctrl1 = control 1, Ctrl2 = control 2, Nat: natamycin; Nat-Mi: natamycin-loaded micelle.

which was used as controls. After three days of instillation, no significant eye reaction (such as corneal opacity, iris hyperemi, conjunctival congestion, swelling, and discharge) was observed. Total clinical scores of the drug-loaded micelle were 0, thus it can be concluded that the prepared micellar formulations were nonirritant for eye and exhibited excellent cytocompatibility in eye (Figure S4).

3.8. Anti-fungal effects of natamycin-loaded micelle in vivo – improvements of clinical observation

In order to determine the therapeutic effect of natamycin-loaded micelle, general clinical evaluation was performed under a slit lamp. FK infected animal models were established through the injection of a *C. albicans* colony into the corneal stroma.

The representative images of infected cornea with *Candida albicans* which was treated with natamycin-loaded micelle (3 times/day), natamycin (normal frequency: 8 times/day, low frequency: 3 times/day) and untreated eyes over 15 days are presented in Figure 8. A slit lamp was used to observe the conjunctival hyperemia, corneal clouding, corneal infiltration, corneal neovascularization, and hypopyon level of the eyes. On the day of inoculation, all experimental eyes instantly exhibited the development of conjunctival hyperemia. Pertaining to the corneal clouding, moderate clouding was observed in the low frequency of Nat group, Nat group, and Nat-Mi group at day 5 after treatment (grade 2). In contrast, the rabbit eyes in the Ctrl group presented with severe clouding (grade 3) and invisible iris due to the diffuse of the corneal edema. At day 10, almost no clouding in the Nat group (grade 0) and mild clouding in the low frequency of Nat group (grade 1), and Nat-Mi group (only in the bottom right corner) (grade 1) was noted. At day 15, no clouding could be observed in the Nat group (grade 0) and Nat-Mi group (grade 0), and a small clouding part remained in the low frequency of Nat group (grade 1). However, more and more serious clouding appeared in the Ctrl group as the cornea became nontransparent at day 15 (grade 3).

For the corneal infiltration, rabbit eyes in the Nat group and Nat-Mi group presented with 2.118 ± 0.353 mm and 1.829 ± 0.198 mm corneal lesions at day 5, respectively. At day 10, 80% of the corneal lesions in the two groups regressed to ≤1 mm. And it was completely and clinically recovered by the end of the treatment (day 15). For the Ctrl group, the highest level of corneal lesion diameter (2.250 ± 0.375 mm, 2.723 ± 0.365 mm, and 5.143 ± 1.029 mm at day 5, 10, and 15, respectively) was observed during all examination times. Corneal lesions in the low frequency of Nat group shrunken gradually to 1.937 ± 0.379 mm and were not completely healed at day 15.

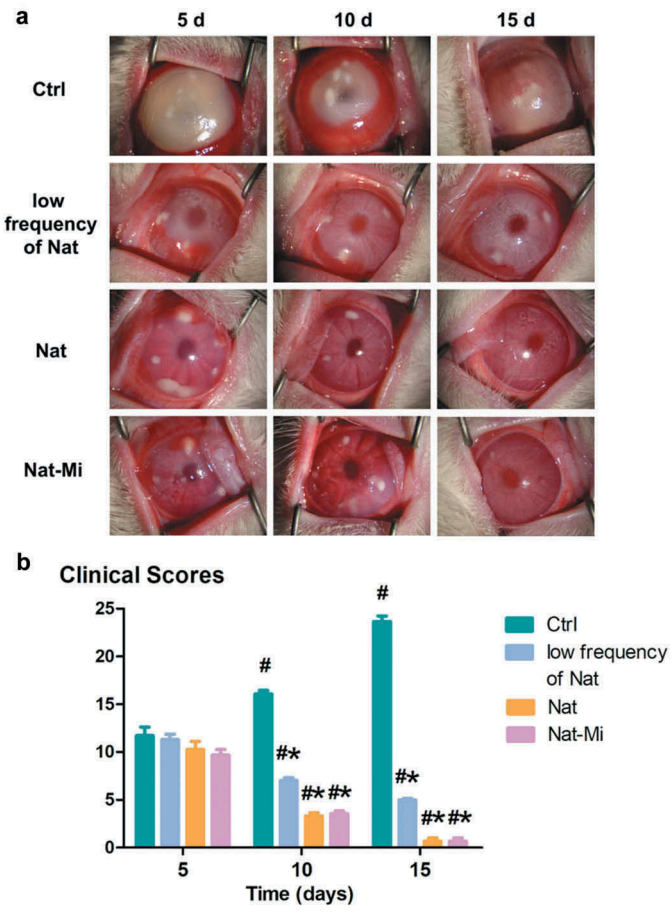


Figure 8. Natamycin-loaded micelle recovered the fungal keratitis in clinic. New Zealand white rabbits were infected with *Candida albicans* and were routinely and visually evaluated for corneal involvement for 15 days under a slit lamp. The control group comprised of untreated fungal keratitis rabbits, while the remaining rabbits were given anti-fungal drugs. A score was assigned for each of the following five criteria: conjunctival hyperemia, corneal clouding, corneal infiltration (measured as the size of epithelial defect in mm), corneal neovascularization and hypopyon level. For the 15 consecutive days of observation, the representative images (a) and clinical score measurements (b) are shown. The images indicate the disease progression at 5, 10 and 15 days. $^{*}P< 0.05$, compared with the Ctrl group at the same time point; $^{\#}P< 0.05$, compared with same group at day five; $n= 3$. Ctrl: control; Nat: natamycin; Nat-Mi: natamycin-loaded micelle.

At day 5, the Nat-Mi group had the narrowest corneal neovascularization ring followed by the Nat group < low frequency of Nat group < Ctrl group, but it was less than 2 mm from the limbus (grade 1) for all groups. At day 10, a remarkable increase of neovascularization was observed in the Ctrl group (grade 2, >2 mm from the limbus), while regression was displayed in the low frequency of Nat group (grade 1), Nat group (grade 0), and Nat-Mi group (grade 0). The neovascularization in the Ctrl group progressed to the center of the cornea and even covered the entire eyes (grade 3) at day 15. At low frequency of Nat group, a circle of new blood vessels still remained (grade 1).

For the height of hypopyon-fluid level in the Nat group and Nat-Mi group, it was no more than one-fifth of the rabbit eye diameter at day 5 (2.483 ± 0.717 mm and 2.519 ± 0.513 mm, respectively). The hypopyon in this two groups were disappeared from the beginning of day 10. Similarly, hypopyon in the low frequency of Nat group was also decreased but a small part remained at day 15 (0.280 ± 0.159 mm). Nevertheless, the hypopyon-fluid level in the Ctrl group increased from day 5 (3.143 ± 0.655 mm) to day 10 (5.333 ± 0.611 mm), and after 15 days it became full of pus in the anterior chamber (10.519 ± 0.679 mm) (Figure 8(a)).

Clinical score was measured by calculation of the total scores because the sum of five clinical parameters could better describe the process of clinical healing throughout the treatment period. As shown in Figure 8(b), no statistical difference of the clinical scores were observed between the four groups at day 5 ($P > 0.05$). At day 10, an increase of the mean clinical scores in the Ctrl group was observed ($P < 0.05$). In contrast, a significant decrease of scores were observed in the low frequency of Nat group, Nat group, and Nat-Mi group ($P < 0.05$) while scores in the low frequency of Nat group was significantly higher than the other two groups ($P < 0.05$). At the study endpoint (day 15), the difference in the clinical scores was more evident ($P < 0.05$), and the scores in the Nat group and Nat-Mi group were close to zero. These results revealed that the micellar formulation could achieve good clinical treatment effect with 3 times per day administration that could be replaced with 8 times per day administration of PBS formulation of natamycin. This suggests the success of sustained release of natamycin-loaded micelle on eye.

3.9. Anti-fungal effects of natamycin-loaded micelle in vivo – decrease of fungal numbers

Macro scale clinical observation in the previous section showed that FK could be treated by the application of natamycin-loaded micelle (3 times per day); however, the micro scale changes need to be determined. Hence, in order to observe the microscopic changes of fungus in the cornea, the methods of direct smear, confocal microscopy observation, and fungal culture were used. The use of direct smear method was the only qualitative experiment which provides a way to directly observe the appearance of the fungus. Corneal specimens were scrapped from the corneal surface and examined under a microscope after staining with the H&E dye. As shown in Figure 9, the shape of the mature spores is similar to beads in all groups at day 5, indicating infection of all groups by *Candida albicans* infection. At day 15, spores were observed in the Ctrl group and low frequency of Nat group, while no fungus was found in the Nat group and Nat-Mi group, indicating that the infection was controlled in the last two groups.

In order to quantitatively measure the numbers and distribution of fungus, confocal microscopy was used. Intravital confocal microscopy is the gold standard for the diagnosis of bacterial or fungal infection in clinic. Fungal structures in rabbit cornea were recorded at day 5 and 15 (Figure 9). The examination revealed highly reflective, linear, branching and bifurcating fungal structures in all groups at day 5. A significant amount of fungus was presented in the Ctrl group while less numbers were found in the low frequency of Nat group, followed by Nat group and Nat-Mi group (Figure 9). Due to the persistent antifungal treatment, fungal structures in the Nat group and Nat-Mi group were progressively reduced, and finally disappeared in the corneas at day

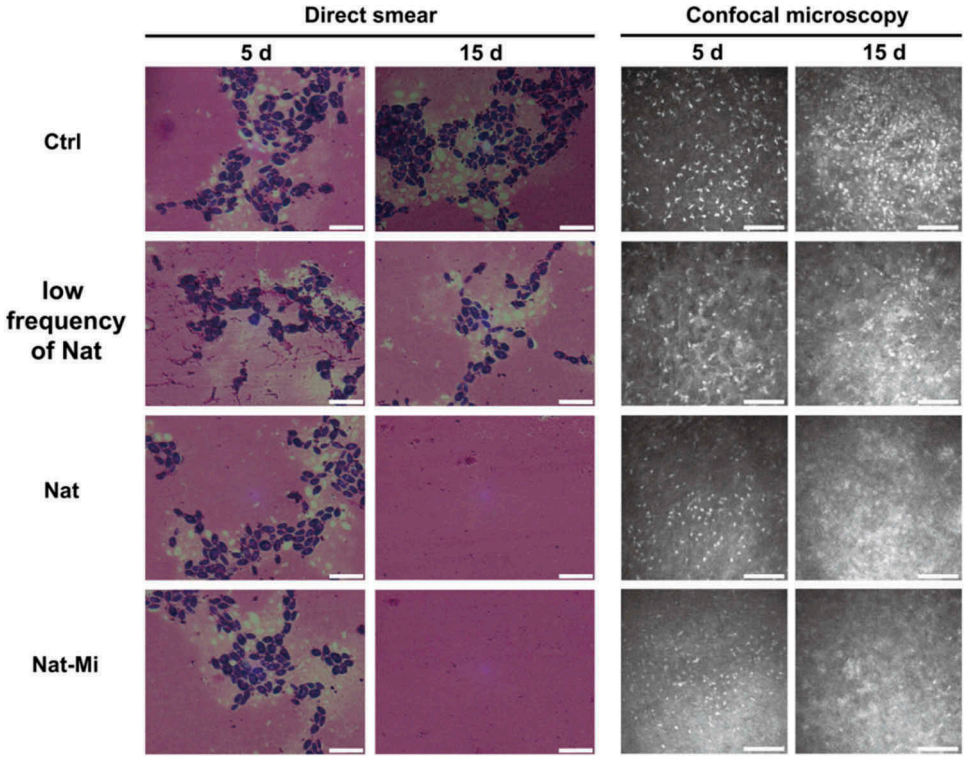


Figure 9. Natamycin-loaded micelle reduced the fungal numbers *in vivo*. The fungal morphology and numbers were observed with direct smear (left) and confocal microscopy (right) at 5 and 15 days. Scale bars: 10 μm for left, 100 μm for right, n= 3. Ctrl: control; Nat: natamycin; Nat-Mi: natamycin-loaded micelle.

15. Fungus in the low frequency of Nat group were also decreased but were not eliminated completely at day 15.

Fungal cells grow very fast and exponentially. A small amount of fungus can be ignored easily under microscopy but can be noticed easily by amplification test *in vitro*. Therefore, fungal culture was used to further confirm whether there were invisible fungal residues under microscope in the Nat group and Nat-Mi group or not. The supernatant from the homogenized cornea were cultured on YPD plates. After 48 h of culture, plates remained clean in the Nat group and Nat-Mi group. However, a number of white spherical fungus cells were observed in the Ctrl group and the low frequency of Nat group (data not shown).

The obtained results from the above three methods suggested that the improvement of keratitis attributes to the reduction in fungal cell numbers and proved that the anti-fungal effect of micellar formulation (3 times/day) was equal to PBS formulation (8 times/day).

3.10. The anti-fungal effect of natamycin-loaded micelle in vivo – recovery of corneal morphology

Since the fungal numbers were decreased after the natamycin-loaded micelle treatment, the corneal morphology was expected to be improved from the micro perspective as well. The corneal morphology was recorded through anterior segment optical coherence tomography (AS-OCT) and H&E staining. OCT is a new technology for detecting light scattering signals from different layers of eye tissues through the principle of a weakly coherent light interferometer. Keratitis could lead to corneal stromal edema and an increased in corneal thickness. The thickness of cornea, the depth of inflammatory infiltration, and the general appearance of each corneal layer could be recorded by AS-OCT non-invasively in rabbits. As shown in Figure 10, at day 5, deficient epitheliums, shallow basal layer, diffused high reflection signal in stroma, increased central corneal thickness (443.67 ± 13.20 μm, 465.82 ± 24.26 μm, 470.33 ± 20.13 μm, and 485.33 ± 21.08 μm, respectively), and *ca*. 50% corneal infiltration were observed in all groups. In particular, a small amount of gray-white substance which was assumed to be purulent secretion in the anterior chamber was observed in the Ctrl group, implying serious damage of the cornea. After 10 days of treatment (day 15), the corneal thickness in the Ctrl group was increased to 736.67 ± 14.36 μm, while in the low frequency of Nat group, Nat group, and Nat-Mi group were decreased to 406.84 ± 18.96 μm, 317.00 ± 14.11 μm, and 351.00 ± 9.54 μm, respectively. Depth of corneal infiltration was significantly decreased and no fungal lesions were observed in the Nat group and Nat-Mi group; however, a lesion with an infiltration depth of half of cornea was still existed in the low frequency of Nat group at day 15. Nevertheless, the depth of the lesion was enhanced to the full-thickness of cornea in the Ctrl group and a large number of purulent secretions was observed in the anterior chamber at day 15.

H&E staining was performed to record histopathological differences such as inflammatory reactions, fungal hyphae, and structure regularity in the cornea. Normal cornea consists of five layers included epithelial layer, Bowman's layer, stromal layer, Descemet's layer, and endothelial layer. As shown in Figure 10, mixed inflammatory reactions including

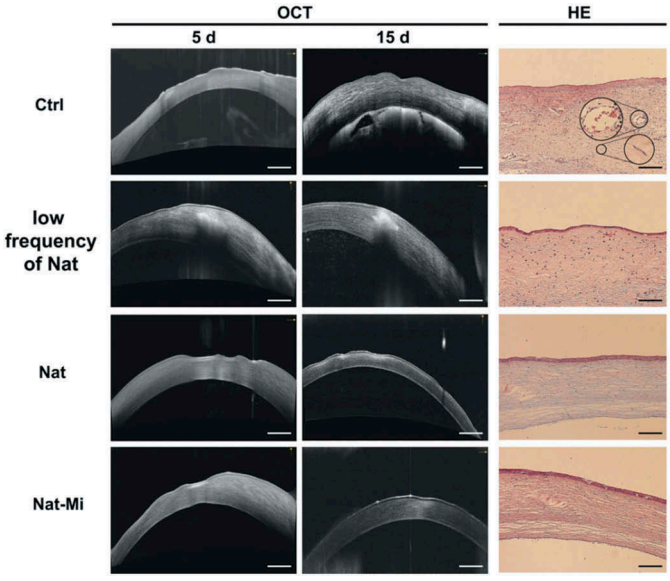


Figure 10. Natamycin-loaded micelle improved the corneal morphology *in vivo*. The corneal morphology was observed by anterior segment optical coherence tomography (AS-OCT) (left) and H&E staining (right). Scale bars: 500 μm for left, 100 μm for righ, *n*= 3. Ctrl: control; Nat: natamycin; Nat-Mi: natamycin-loaded micelle.

the presence of polymorphonuclear cells, lymphocytes, and plasma cells both inside and around the infiltrate were recorded in the control specimens at day 15. In addition, a numerous superficial and deep vascular reaction with endothelium on the vascular wall and erythrocytes inside the vascular wall could be observed in the Ctrl group. The presence of *Candida* pseudofilaments in the corneal epithelium and stroma implied severe fungal infections in rabbit eyes. Edematous and disorganized stromal layer in the Ctrl group suggested the direct damage to the corneal structure after fungal infection. However, lighter inflammatory reaction (including inflammatory cell infiltration and angiogenesis), less pseudofilaments, and more regular corneal structure were found in the low frequency of Nat group. This revealed the importance of early medical treatment in fungal keratitis even if the treatment dose is insufficient. More slight inflammatory reactions than low frequency of Nat group, no pseudofilaments, and almost normal corneal structure were observed in the Nat group and Nat-Mi group. These results implied that the micellar formulation could improve the corneal morphology of keratitis well, and have similar efficacy with PBS formulation of natamycin [45].

3.11. Natamycin-loaded micelle promote drug penetration in vivo

The present results revealed that the less frequency use of micellar formulation of natamycin could cure fungal keratitis within 15 days with a similar therapeutic effect as PBS formulation of natamycin. In fact, topically instilled drug does not remain in eye for periods exceeding few minutes because of tear wash and blinking. Literatures have reported that micelle nanoparticles could enhance drug penetration in the skin and tumor [46,47]. Hence, it was expected that the present PEG-*b*-PGMA micelle could increase natamycin penetration into the cornea. In order to verify this assumption, LC-MS/MS was employed to measure the concentration of natamycin in the cornea and aqueous humor (a kind of liquid behind the cornea) of several groups. Specific fragment peak of natamycin was detected in LC-MS/MS, indicating the presence of free and functional natamycin. In Figure 11, it could be observed that after 15 days of treatment, the concentration of natamycin in the Nat-Mi group was similar to that in the Nat group in both the cornea and aqueous humor ($P > 0.05$). In the low frequency of Nat group, the concentration of natamycin was much lower than the two groups ($P < 0.05$). These results exhibited the effectiveness of the FK treatment using natamycin-loaded micelles due to the detected free amount of natamycin in the cornea and aqueous humor. Additionally, it showed that the use of PEG-*b*-PGMA micelle could help to the solubility of the natamycin and its penetration into the cornea and aqueous humor. The reason could be the improved mucoadhesion of micelles which can enhance the contact time with the mucus layer of the tear film and increase the corneal penetration of micelle due to the PEGylated shell of micelles as it has been also shown by previous researchers [24,25,29].

4. Conclusion

A drug carrier with sustained release characteristic for the ocular delivery system was developed to reduce the frequent administration doses of natamycin for FK treatment. Micelles were prepared *via* the self-assembly of PEG-*b*-PGMA block copolymer consisting of a hydrophilic PEG segments and a hydrolysable PGMA

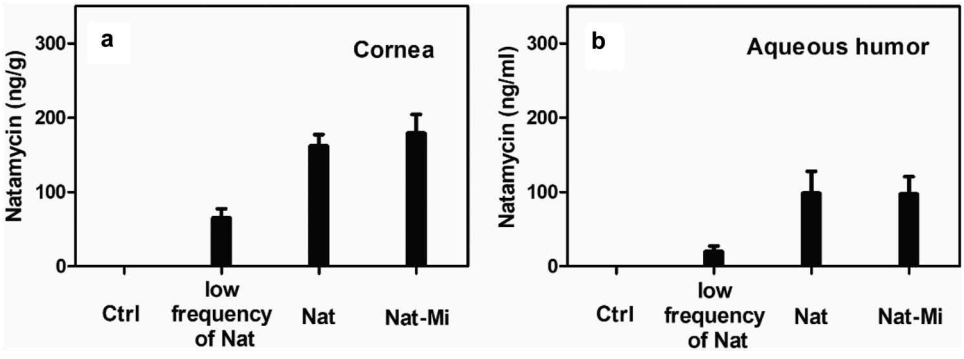


Figure 11. Natamycin-loaded micelle promoted the drug penetration *in vivo*. The drug levels in eye tissues including the cornea (a) and aqueous humor (b) were detected at the end of 15 days of post-dosing. *P< 0.05, compared with the Ctrl group; #P< 0.05, compared with the Nat group; *n*= 3. Ctrl: control; Nat: natamycin; Nat-Mi: natamycin-loaded micelle.

segments. Our results demonstrated that this new platform is capable of enhancing drug bioavailability and drug activity. The natamycin-loaded micelle system can significantly enhance the loading and prolong delivery efficiency of drug in aqueous humor and cornea, and finally reduce the administration frequency to improve long-term patient compliance.

Author contributions

Yiyuan Guo, Fatemeh Karimi, and Qiang Fu were involved in conception, design, performing experiments, analysis of data, and writing the manuscript. Hong Zhang and Greg. G. Qiao were involved in conception and guidance to perform experiments.

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Declaration of interest

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