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ORIGINAL ARTICLE

Rebaudioside A/TPGS mixed nanomicelles as promising nanocarriers for nimodipine ocular delivery

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Abstract

Nimodipine (NMD), a calcium channel blocker, has demonstrated benefits in treating glaucoma. However, its ocular therapeutic application remains limited due to its poor aqueous solubility, which restrains the development of an ophthalmic formulation. Thus, the present study aimed to formulate an NMD micelle ophthalmic solution to enhance the potential of NMD in an ocular topical formulation to treat glaucoma. The NMD micelle ophthalmic solution was formulated with nanocarriers composed of rebaudioside A and D-α-tocopheryl polyethylene glycol 1000 succinate. Spherical mixed micelles were optimized and obtained at a small micelle size 13.429 ± 0.181 nm with a narrow size distribution (polydispersity index 0.166 ± 0.023) and high encapsulation efficiency rate (99.59 ± 0.09%). Compared with free NMD, NMD in micelles had much greater in vitro membrane permeability and antioxidant activity. The NMD micelle ophthalmic solution was well tolerated in rabbit eyes. It profoundly improved the in vivo intraocular permeation of NMD, and in vivo intraocular pressure reduction and improved miosis were also observed. Accordingly, this NMD micelle ophthalmic solution might be a promising ocular formulation to treat glaucoma.

Keywords D-α-tocopheryl polyethylene glycol 1000 succinate · Rebaudioside A · Nimodipine · Mixed micelles · Ocular drug delivery

Introduction

Glaucoma is the second leading cause of blindness and the leading cause of irreversible vision loss worldwide [1]. Glaucoma is usually characterized by chronic, progressive neuropathy of the optic nerve and elevated intraocular pressure (IOP), and IOP is the main qualifiable risk factor of this disease [2]. Therefore, a common treatment strategy is reducing the IOP with ophthalmic drops [3].

However, the presently available antiglaucoma ophthalmic drops have limitations, such as low local bioavailability due to the tear film barrier and the corneal barrier to drug penetration and local and systemic adverse effects [4, 5]. Hence, the presently available commercial ophthalmic drops usually have a low patient compliance as they usually require frequent administration, which eventually leads to high drug accumulation in some ocular surface tissues, toxic ocular effects, especially corneal toxicity and conjunctival toxicity, and tissue damage [6, 7]. Micelles, nanoparticles, dendrimers, hydrogel, niosomes, liposomes, in situ gels, and many other novel drug delivery systems have been explored to address the current limitations, including improving the therapeutic efficacy and/or reducing the adverse drug reactions of antiglaucoma ophthalmic drops [8, 9].

Several kinds of antiglaucoma agents with different pharmacological mechanisms have been developed to decrease IOP, and the prostaglandin analogues, such as latanoprost and travoprost, are the most commonly used antiglaucoma agents in many countries [10]. However, prostaglandins, even those in preservative-free formulations, induce dry eye-like

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ocular surface damage [4] and other local ocular adverse effects, including irritation, ocular redness, and discoloration of the iris and surrounding skin [9]. β-blockers, another class of antiglaucoma agent in clinical practice, are β-adrenergic receptor antagonists that decrease IOP by inhibiting aqueous humor production in the eye [11]. Timolol, the first FDA-approved β-blocker for lowering IOP, became the first line drug for glaucoma treatment. However, β-blockers' one major limitation is bradycardia, and this adverse effect may harm patients with cardiac disease. Another limitation of β-blockers is their twice daily or more frequent application, which results in low patient adherence [9, 12]. Carbonic anhydrase inhibitors are also a commonly used antiglaucoma agent in ophthalmic drop solutions. However, several limitations, including eye irritation and low patient adherence due to multiple daily administrations, limit their clinical utility. Novel aqueous ophthalmic drop formulations, especially nanotechnological formulations, have better ocular bioavailability and less ocular adverse effects, which increase their clinical utility [13]. Therefore, new drugs and novel delivery systems are still desired for the treatment of glaucoma.

Calcium channel blockers (CCBs), such as verapamil and nimodipine (NMD), have been approved for glaucoma management due to their ability to improve ocular perfusion, prevent the progression of optic neuropathy, and lower IOP [14]. However, the treatment efficacy is almost negligible after systemic administration due to the photosensitivity, poor aqueous solubility, and severe first-pass metabolism of these agents. Some new formulations of NMD have been explored, including solid dispersion, nanoemulsion, formulation, chitosan nanoparticles, self-microemulsifying drug delivery systems, and micelle formulations [15–19]. However, all of these researched formulations were not for ocular delivery. As we know, almost all clinical antiglaucoma drugs are formulated as ophthalmic drops because ophthalmic drops result in high levels of patient adherence due to their convenience in the long-term treatment of glaucoma [20]. However, an ophthalmic drop formulation of NMD has not yet been market available. An ophthalmic formulation of NMD-cyclodextrin complexes was prepared and evaluated [21]. However, there was a dynamic balance between cyclodextrin inclusions and free drug, and stability was a challenge during storage and clinical application. Other ophthalmic formulations using different strategies should also be developed for further research.

Micelle solubilization has been widely explored to enhance the ocular bioavailability and efficacy of poorly soluble drugs. Single nanocarrier micelles were first explored, but some of their formulation parameters, such as micelle size, encapsulating capacity, and storage stability, struggled to meet the requirements of ophthalmic drops. Therefore, mixed micelles with two or more nanocarriers were formulated to synergistically improve the micelle ophthalmic drop characteristics [22]. Some kinds of micelle formulations of NMD have been explored, but these were not aimed to ocular administration. Some of these micelle formulations were in solid dispersion formulation [18, 23], or in freeze-dried formulation [24]. Some reported micelles were in aqueous formulations, but NMD concentration in solution was much low (< 1 mg/ml) [25, 26]. So, new micelle formulation of NMD for ocular delivery is still desired in ophthalmology and in pharmaceutics.

Rebaudioside A (RA) is a natural steviol glycoside extracted from the herb *Stevia rebaudiana* Bertoni. Inspired by the molecular structure of RA, by both its hydrophilic sugar side chain(s) and hydrophobic diterpene, which allow RA to self-assemble into micelles in aqueous solutions, researchers explored the use of RA as a promising agent in a nanocarrier system [27, 28]. It was reported that RA could self-assemble into nanomicelles with small particle sizes (i.e., < 4 nm) and a narrow size distribution (i.e., a polydispersity index [PDI] < 0.3) [27, 28]. RA's novel nanomicelles displayed substantial encapsulation of hydrophobic agents, such as curcumin and coumarin 6 (Cou6) [27, 28]. All previous results showed that micelle formulations of RA exhibited great potential to enhance the ocular bioavailability and efficacy of poorly soluble drugs, but this potential as a novel nanodrug delivery system was limited by the disadvantages of RA's aqueous solubility and low drug loading capacity [27]. These drawbacks limit the potential application of RA micelles in ocular drug delivery systems.

As a vitamin E derivative, D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) has been widely used in several commercial ophthalmic formulations [20]. TPGS can increase the hydrophobicity of a micelle core when mixed with other amphipathic materials.

The current literature describes severely limited data for ocular topical formulations of NMD, and there is limited literature on RA in ocular drug delivery systems, especially its feasibility of forming mixed micelles with other amphipathic materials. Therefore, this work primarily aims to improve NMD's aqueous solubility by encapsulating it in mixed micelles composed of RA and TPGS to formulate an ophthalmic preparation. This work also aims to explore the potential feasibility of RA to form mixed micelles with other amphipathic materials such as TPGS to cover the drawbacks of RA micelles in ocular drug delivery systems. NMD micelles were evaluated by a series of physicochemical properties, such as the particle size, zeta potential, and morphology characterization. Then, further evaluations, including in vitro membrane permeation, in vitro antioxidant activity, in vivo ocular tolerance, in vivo ocular absorption, and in vivo IOP-lowering and miosis efficacy, were also performed (some experimental information listed in Table S1).

Materials and methods

Materials and animals

NMD and TPGS were obtained from Aladdin Shanghai Biochemical Technology Co., Ltd. (Shanghai, China) with a purity of ≥ 98% and used as received. RA was purchased from Jining Aoxing Stevia Products Co., Ltd. (Jining, China) with a purity of more than 98%, and it was used as received. Cou6 and benzalkonium chloride were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). Commercial timolol maleate ophthalmic drops (5 mg/ml, Wuhan Wujing Pharmaceutical Co., Ltd.) were purchased from the Qingdao Eye Hospital (Qingdao, China). Methanol was of HPLC grade, and all other reagents were of analytical grade.

New Zealand rabbits were purchased from Qingdao Kangda Foodstuffs Co., Ltd. (Qingdao, China). C57BL/6 mice were obtained from Jinan Pengyue Experimental Animal Breeding Co., Ltd. (Jinan, China). All animals were healthy and lacked clinically observable ocular abnormalities. Animal experiments were performed according to the Association for Research in Vision and Ophthalmology (ARVO) Statement in Ophthalmic and Vision Research and were approved by the Qingdao University of Science and Technology Ethics Committee for Animal Experimentation (permit no. 2017-1, Qingdao, China).

Fabrication of the NMD micelle ophthalmic solution

A micelle ophthalmic solution of NMD was fabricated using thin-film hydration [29]. Briefly, the NMD used during the preparation was fixed with 30 mg, while the weights of RA and TPGS were depended on their weight ratios. For example, NMD, RA, and TPGS were 30, 150, and 300 mg, respectively, to prepare the mixed micelles with an RA/TPGS weight ratio fixed of 1:2 and with an NMD/carrier weight ratio of 1:15. NMD, RA, and TPGS were dissolved in approximately 20 ml anhydrous ethanol. Then, the ethanol was removed by evaporation at 40 °C under reduced pressure to obtain a uniform thin film. Approximately 9 ml phosphate buffer solution (PBS) was added to hydrate the film at 40 °C and 100 rpm for 10 min. Then, the micelle solution was filtered through a 0.22-μm filter (SLGP033RB, Millipore, USA) to remove the non-encapsulated NMD. After a drug content analysis, and encapsulating efficiency and micelle loading efficiency calculation as the method reported [30], the solution was then further diluted with PBS and adjusted to an NMD concentration of 3.0 mg/ml, pH of 6.5 ± 0.1, and osmotic pressure of ~ 300 mOsmol/kg. Then, a second filtration through a 0.22-μm filter was performed to obtain a sterile ophthalmic solution. The NMD micelle ophthalmic solution was tightly packed into 10-ml colorless glass vials using a sterile procedure and packaged in aluminum foil to protect it from light. Then, it was stored at 4 °C for further use.

Cou6 was added to fabricate Cou6-labeled NMD micelles by the same fabrication method to investigate the in vivo corneal permeability [30]. Briefly, the micelles were fabricated using 29.5 mg NMD and 0.5 mg Cou6, resulting in 50 μg/ml Cou6 in the NMD micelle solution. The free Cou6 solution was prepared by a reported method and used as a control in the corneal permeability study in mouse eyes [31].

Physicochemical characterization of the NMD micelle ophthalmic solution

The particle size, the PDI, and the zeta potential of the NMD micelles were detected with a Zetasizer Nano ZS90 equipment (Malvern Instruments, Worcestershire, UK). The solution tested was diluted to an NMD concentration of 1.0 mg/ml. The surface morphology of the micelles was observed with transmission electron microscopy (TEM, JEM-1200EX, JEOL Ltd., Tokyo, Japan). The encapsulating efficiency of NMD in micelles was quantified with high-performance liquid chromatography (HPLC) as previously reported [32, 33]. Briefly, an HPLC system with a G1314A UV detector at a maximum detection wavelength of 236 nm, a G1311A Quat Pump, and a G1367A Injector (Agilent, USA) and a ZORBAX SB-C_{18} chromatographic column (250 mm × 4.60 mm, 5 μm, Agilent, USA) with a column temperature of 30 °C were used for detection. The mobile phase was composed of 40% acetonitrile, 30% methanol, and 30% water, and the flow rate was 1.0 ml/min. The standard curve was acquired for NMD over the concentration range of 20–2000 ng/ml with $r = 0.9995$. The RSD of NMD for within-day precision at low, medium, and high centrations (40, 200, and 1000 ng/ml, respectively) was below 3.00%, and the recoveries of NMD at these concentration levels were 96.14%, 99.29%, and 105.24%, respectively. The injection volume was 20 μl. NMD had a retention time 4.6~4.9 min, and nifedipine (internal standard) had a retention time 7.8~8.2 min.

Antioxidant activity

The antioxidant activity of the encapsulated NMD was evaluated with a 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) free radical scavenging assay, as described in previous reports [31, 34]. The final NMD concentrations in this ABTS assay were 250, 300, 350, 400, 450, and 500 μg/ml, and the incubation time range was 5–120 min. The corresponding mixed nanomicelle composed of RA and TPGS (i.e., 2.50, 3.00, 3.50, 4.00, 4.50, and 5.00 mg/ml TPGS

and 1.25, 1.50, 1.75, 2.00, 2.25, and 2.50 mg/ml RA) with an NMD/RA/TPGS weight ratio of 1:5:10 was also evaluated.

In vitro PAMPA

Free NMD was dissolved in dimethyl sulfoxide (DMSO) and then further diluted with artificial tear solution to a concentration of 1.0 mg/ml. NMD from micelle ophthalmic solution was diluted to 1.0 mg/ml with DMSO containing artificial tear solution, and the final DMSO concentration of these two samples was 5.0% (v/v). Blank artificial tear solution was added to acceptor wells at a volume of 0.5 ml. The donor wells were filled with 0.5 ml NMD micelle ophthalmic solution, and both the donor wells and the acceptor wells were incubated in a 25 °C water bath for 2.5 h. During incubation, solution was removed from the acceptor wells at predetermined time points, and these samples were analyzed with HPLC according to the method described above.

In vivo ocular tolerance

A modified Draize test was performed to evaluate the ocular tolerance of the NMD micelle ophthalmic solution, as previously reported [27, 31]. The original NMD micelle ophthalmic solution (containing 3.0 mg/ml NMD) was tested. PBS, BAC in PBS (0.1 mg/ml), and SDS in PBS (5 mg/ml) were used as controls. Pathological detection was performed 24 h after the last instillation, as previously reported [27].

Ocular biodistribution of the NMD micelle ophthalmic solution

To quantitatively determine the ocular biodistribution, the free NMD suspension solution or 3.0 mg/ml NMD micelle ophthalmic solution was administered in 4 doses (50 μl/dose 10 min apart). Three rabbits were euthanized 30, 60, and 90 min after the last instillation. The corresponding 6 eyes were collected and dissected quickly, and tissue samples were pre-frozen with liquid nitrogen and stored at − 80 °C until analyzed. Tissue samples (i.e., corneas, irises and ciliary bodies [ICB], and retinas) were weighed and homogenized with ice cold acetonitrile (1 ml acetonitrile per 50 mg tissue). The mixture was sonicated for 10 min, centrifuged, and analyzed by HPLC. The calibration curve was linear in the range of 20–2000 ng/ml for corneas, ICB, and retinas. All the standard curves generated R^2 values greater than 0.99. Average recovery values were determined in cornea (92.25%), ICB (95.50%), and retina (98.11%).

For fluorescent microscope observation, experiments included a free Cou6 solution group with 6 mice, Cou6-loaded NMD micelle ophthalmic solution group with 6 mice, and blank control group with 2 mice. Free Cou6 solution or Cou6-loaded NMD micelle ophthalmic solution was administered in 4 doses (5 μl/dose 10 min apart). The mice in the blank control group did not receive any medication. Two mice in each group were euthanized 30, 60, and 90 min after the last dose. Subsequently, the corneas of the corresponding 4 eyes were carefully removed and fixed with 4% paraformaldehyde. Then, the corneas were carefully flat-mounted and observed with fluorescence microscopy.

Pharmacodynamic studies

IOP evaluations

A single dose-response design was conducted in this test. Mice were well sedated with inhaled isoflurane. The tested NMD micelle ophthalmic solutions (i.e., 3.0, 1.0, and 0.3 mg/ml), free NMD suspension solution (3.0 mg/ml), commercial timolol maleate ophthalmic drops, and PBS were instilled into the inferior conjunctival sac of the eye as 4 ophthalmic drops, 5 μl each, 10 min apart. The IOP for both eyes was measured with a rebound tonometer (TonoLab, Poland) before the initial administration (baseline) and at different intervals after administration until the IOP returned to baseline. The tonometer internally averages six valid readings and produces an averaged value for each individual reading. Three independent readings of the IOP were measured and then averaged as the final reading for each test for each mouse. Then, the average IOP reduction values for each group were plotted with time, as reported previously [35].

Miosis

Eighteen healthy New Zealand rabbits weighing 2.0–3.0 kg each were randomly divided into 6 groups for the IOP evaluations above with three rabbits in each group. Baseline measurements of the pupil diameter were recorded as d0. For each rabbit, 4 ophthalmic drops, 5 μl each, of the tested solution were instilled into each eye every 10 min. The pupil diameters 30, 60, 90, 120, 150, 180, 240, and 300 min after the last dose were recorded as dt, where t is the amount of time elapsed after the last dose. The miotic response (M) at each time point was further calculated by $M = (d0 - dt)/d0 \times 100\%$ and further plotted with time, as reported previously [35].

Statistical analysis

The data were expressed as means ± SDs. All data were analyzed with SPSS software, version 11.5 (SPSS, Chicago, IL, USA). An independent-samples t test was used to compare NMD in the parallel artificial membrane permeability assay (PAMPA) and in vivo ocular absorption tests. A multiple comparison with an ANOVA compared the results of the

in vivo pharmacodynamic tests. The significance level was set at $P < 0.05$.

Results

Characterization of the NMD micelle ophthalmic solution

NMD could be easily encapsulated in the mixed micelles of RA and TPGS using the simple procedure of thin-film hydration, but the weight ratio of RA/TPGS substantially affected the encapsulation efficiency of NMD (Fig. 1). When the RA/TPGS weight ratio was fixed at 2:1, an encapsulation efficiency rate of 85.64% was obtained with a weight ratio of 1:15 for NMD and the carrier, and the encapsulation efficiency rate climbed to 98.74% with a ratio of 1:21. However, NMD failed to be highly encapsulated in the mixed micelles with an RA/TPGS weight ratio of 1:1. NMD could be encapsulated in the mixed micelles with an RA/TPGS fixed weight ratio of 1:2, but a large difference in the encapsulating profiles was observed. Specifically, an encapsulation efficiency rate of 99.50% was obtained with an NMD to carrier weight ratio of 1:15, but the encapsulation efficiency rate decreased to 87.16% with a ratio of 1:21.

As the highest drug loading efficiency in the tested formulation during the optimizing process, the mixed micelles with an RA/TPGS weight ratio fixed of 1:2 and with an NMD/carrier weight ratio of 1:15 were further investigated. As shown in Fig. 2a, the NMD micelle ophthalmic solution was transparent and colorless and visually did not differ from water. However, the same concentration of NMD could not be dissolved, only suspended, in water. TEM observation revealed that the NMD micelles were spherical or aspherical with good dispersion (Fig. 2b). The NMD micelles had an average micelle size of 13.429 ± 0.181 nm, a PDI of 0.166 ± 0.023, and a zeta potential of − 1.007 ± 0.093 mV (Fig. 2c, d). Their encapsulation efficiency was 99.59 ± 0.09%, and the loading efficiency of NMD was 6.23% ± 0.0053%.

Determination of antioxidant activity

According to the results presented in Fig. 3, free NMD and the nanocarrier demonstrated weak scavenging activities for the various concentrations and time points. However, for each concentration, NMD micelles showed much stronger scavenging activity than those of free NMD ($P < 0.05$). Concentration-dependent and incubation time-dependent activity was observed for all three kinds of the solutions. For example, free NMD showed no scavenging activity at a concentration of 250 μg/ml with a 5-min incubation, while NMD micelles showed a 41.01% scavenging activity at a concentration of 250 μg/ml with a 5-min incubation and an increase to 50.99% scavenging activity at a concentration of 500 μg/ml NMD in micelles. If the incubation time increased to 120 min, 250 μg/ml free NMD showed a 3.03% scavenging activity, and 500 μg/ml free NMD increased the scavenging activity to 10.58%. In comparison, 100 μg/ml NMD micelles showed a 52.97% scavenging activity, and 500 μg/ml NMD micelles increased the scavenging activity to 61.67%. RA/TPGS also showed some antioxidant activity in this test, and its antioxidant activity was much weaker than that of NMD micelles but stronger than that of free NMD.

PAMPA

The PAMPA enables quick evaluation of the compound/formulation's trend to permeate membranes by passive diffusion and is widely used to evaluate potential drugs. As shown in Fig. 4, the results showed a marked increase in NMD permeation when compared with free NMD. The permeation of NMD micelles was as high as 98.82 ± 10.84 μg after 2.5 h, while the permeation of free NMD was only 0.22 ± 0.01 μg, which suggests that micelles could promote greater membrane permeation of NMD.

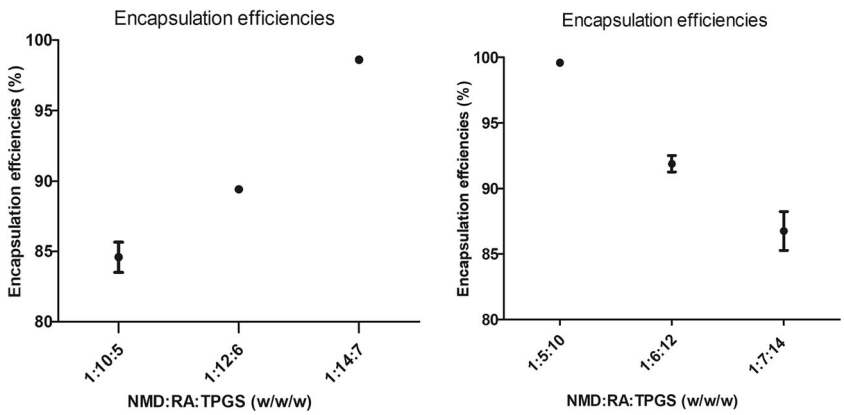

Fig. 1 Encapsulation efficiency profiles. Encapsulation efficiencies were detected with different weight ratios of RA and TPGS to NMD ($n = 3$). RA, rebaudioside A; TPGS, D-α-tocopheryl polyethylene glycol 1000 succinate; NMD, nimodipine

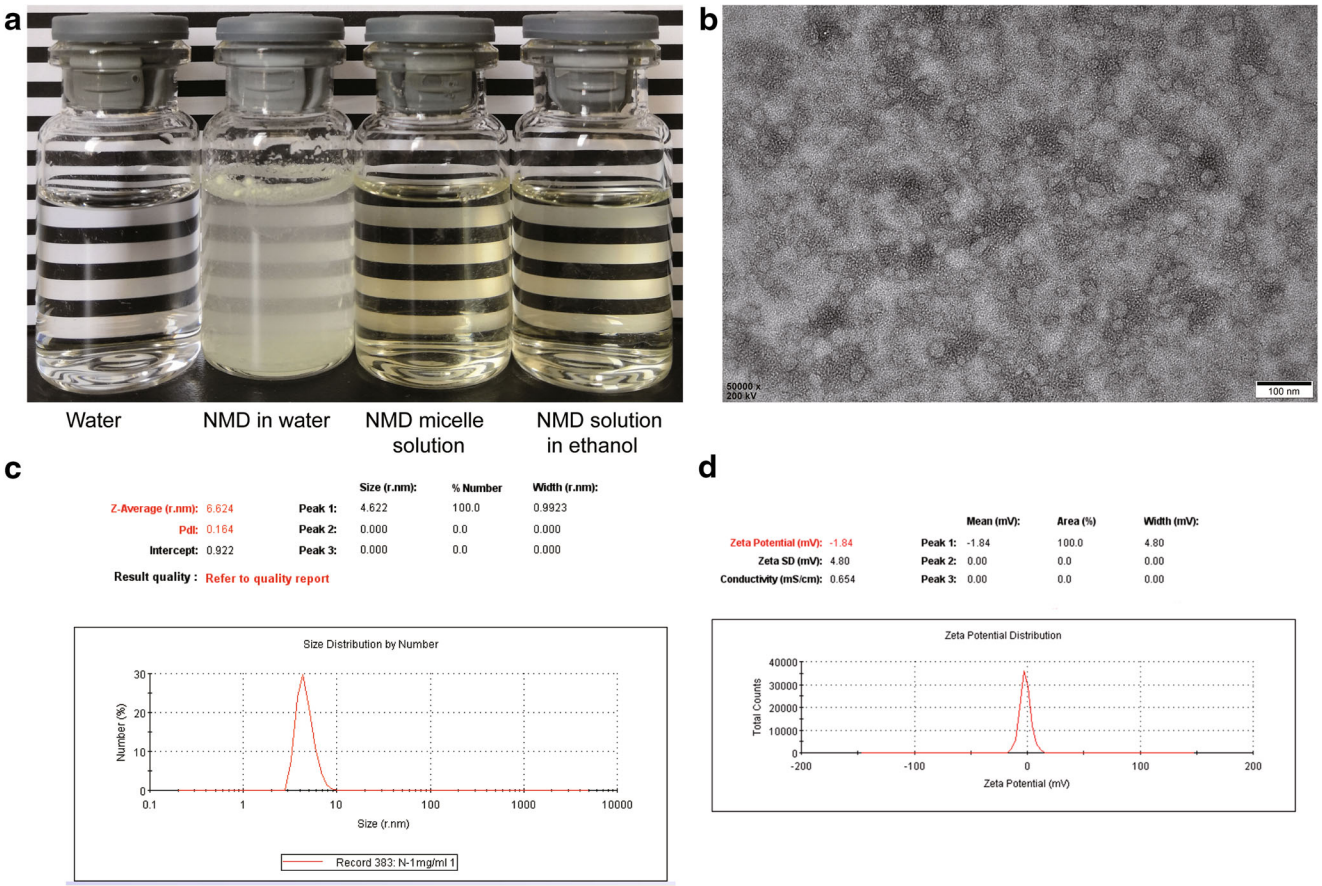

Fig. 2 NMD micelle ophthalmic solution characterization for an NMD/RA/TPGS weight ratio of 1:5:10. **a** The NMD micelle ophthalmic solution's appearance. **b** Morphology of NMD micelles observed by TEM (×50 k magnification, bar = 100 nm). **c** Micelle size and size distribution in the NMD micelle ophthalmic solution. **d** Zeta potential of the NMD micelle ophthalmic solution. NMD, nimodipine; TEM, transmission electron microscopy

In vivo ocular tolerability

Ocular abnormalities on an in vivo ocular tissue level, namely, irritation and toxicity, were examined by clinical symptom observation and histopathological examination, respectively. The results of the irritation test showed good ocular tolerance to the NMD micelle ophthalmic solution (Fig. 5). Even mild conjunctival redness, the most frequent symptom of ocular irritation, was absent during the entire observation period. PBS showed similar results to those for the NMD micelle ophthalmic solution. BAC also displayed good ocular tolerance, although mild conjunctival redness could be observed in this group, but the clinical score ranged from 0 to 2 and could be interpreted as no irritation. The SDS group, set as a positive irritation group, demonstrated severe conjunctival congestion, chemosis, and mucopurulent secretion that even obscured the eye with lid closure in the treated eye.

Histological examinations further revealed no tissue damage and no inflammation in the rabbit eyes exposed to the NMD micelle ophthalmic solution. The normal histological structure of the cornea epithelium, the lamellar stroma, and the endothelium were detected, and no edema or inflammation was detected. Other ocular tissues (i.e., the conjunctivae, irises, and retinas) were also observed without histopathological changes in the investigation of the NMD micelle ophthalmic solution, as shown in Fig. 6. Similar results were also observed in the PBS group. The BAC group demonstrated similar results to those in the PBS group, except for some inflammatory cells observed in the conjunctiva. However, in the SDS group, the cornea epithelium became thin, and only one layer of epithelial cells was observed in most areas of the cornea. Many inflammatory cells were observed in the conjunctiva tissue; the goblet cells decreased in number and became scattered; and slight retinal detachment was observed.

In vivo ocular permeation

As shown in Fig. 7, RA/TPGS micelles significantly improved NMD permeation into the cornea, which is the main barrier in topical drug administration. Specifically, the NMD concentrations in the corneas for the NMD micelle group were 4.99-, 6.76-, and 4.76-fold higher than those for the free NMD

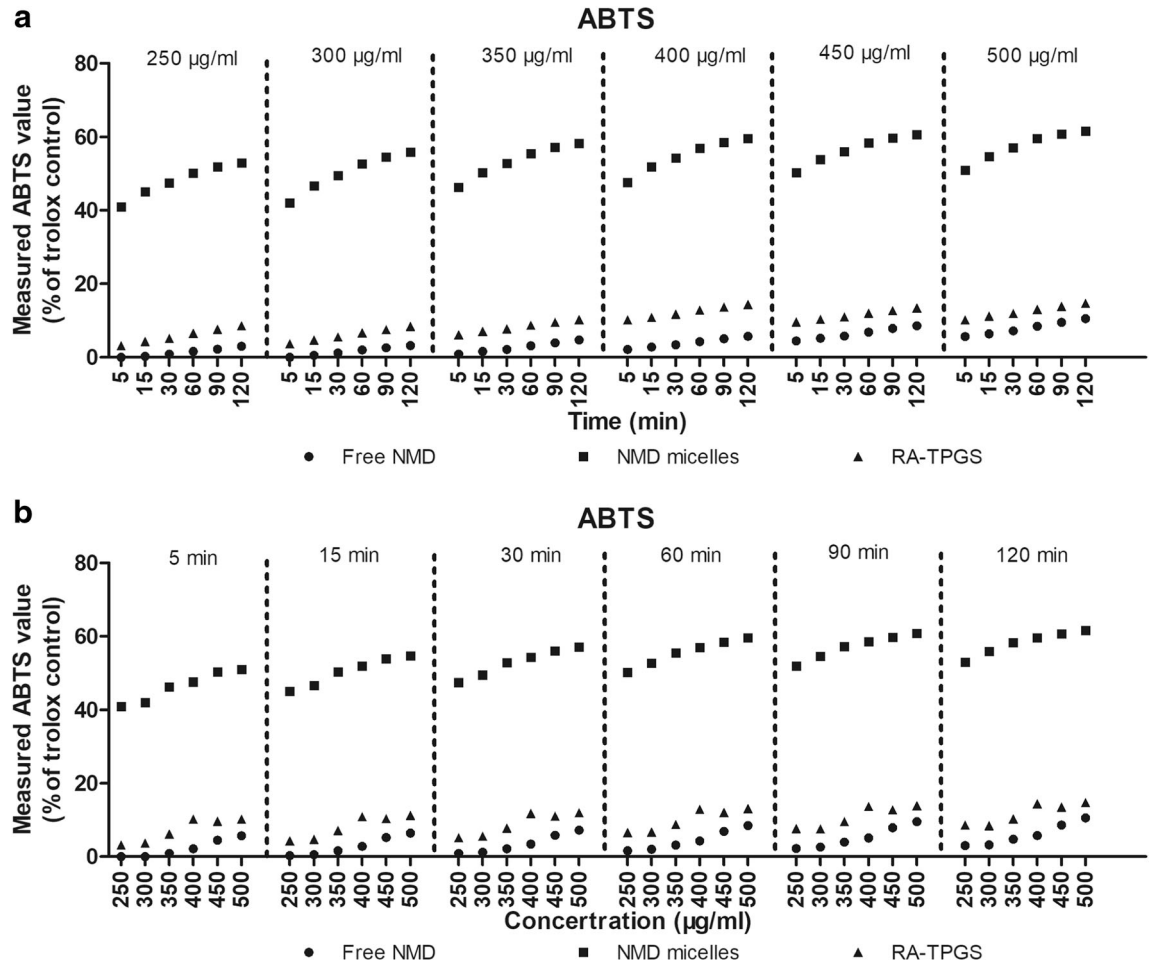

Fig. 3 In vitro antioxidant characterizations. Measured ABTS values as an indicator of the antioxidant activity of free NMD and the NMD micelles with different concentrations as a function of time (**a**) and different incubation times as a function of concentration (**b**). RA, rebaudioside A; TPGS, D-α-tocopheryl polyethylene glycol 1000 succinate; NMD, nimodipine

group (Fig. 7a). As shown in Fig. 7b of the fluorescence microscope results of corneal permeation, the corneas from the Cou6-loaded NMD micelle ophthalmic solution group displayed much stronger fluorescence, but the fluorescence intensity became weaker with time. However, corneas from the free Cou6 solution group revealed severely weaker fluorescence than those from the Cou6-loaded NMD micelle group at the same time point.

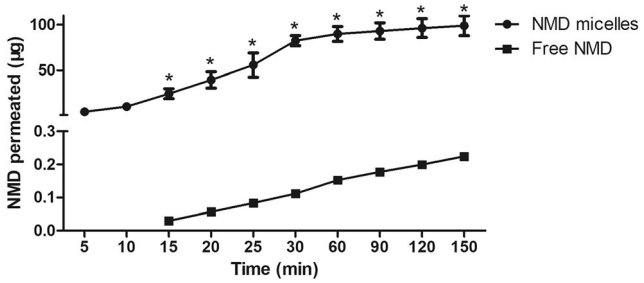

Fig. 4 Characterizations by the parallel artificial membrane permeability assay (PAMPA). In vitro PAMPA using the transwell method (*$P < 0.05$ when compared with the free NMD group). $n = 3$. NMD, nimodipine

Significantly improved concentrations of NMD were also determined in the ICB and retina groups (Fig. 7a). Specifically, the NMD concentrations for the NMD micelle group were 6.11-, 4.17-, and 1.01-fold higher in the ICB and 4.86-, 7.36-, and 4.17-fold higher in the retina than those for the free NMD group.

In vivo pharmacodynamic evaluation

IOP reduction

Figure 8a shows the IOP reduction from ophthalmic administration of the NMD micelle ophthalmic solution (0.3, 1.0, and 3.0 mg/ml) compared with that of the free NMD suspension solution (3.0 mg/ml) and the commercial timolol maleate ophthalmic drops (5.0 mg/ml). The commercial timolol maleate ophthalmic drops were observed to have a maximum IOP reduction of 10.55 ± 5.96% after 1 h ($P < 0.05$ when compared with the PBS group). Then, the IOP lowering effect gradually weakened and returned to the baseline values less than 4 h

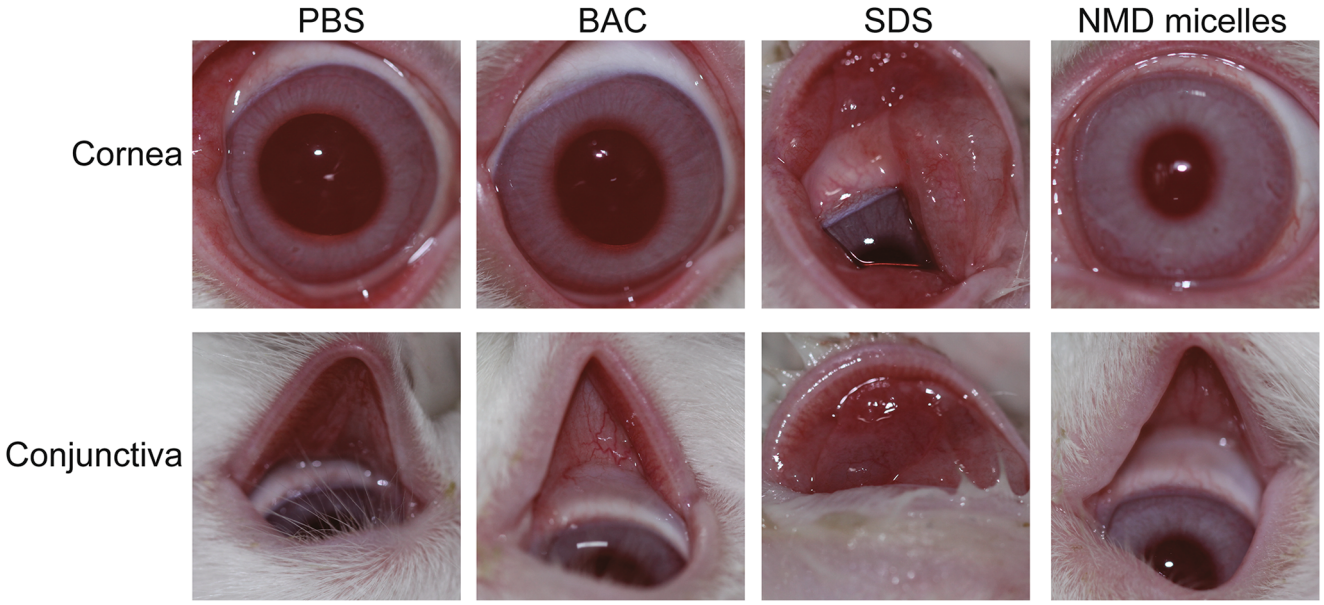

Fig. 5 Ocular tolerance evaluation. Slit-lamp biomicroscopic observation of rabbit eyes 24 h after the last topical instillation. PBS, 0.1 mg/ml BAC in PBS solution, 5 mg/ml SDS in PBS solution, and 0.3 mg/ml NMD micelle ophthalmic solution were tested in this experiment. BAC, benzalkonium chloride; SDS, sodium dodecyl sulfate; NMD, nimodipine

after ocular administration. Data demonstrated that application of the NMD micelle ophthalmic solution reduced the IOP in a dose-dependent manner. The 3.0-mg/ml NMD micelle ophthalmic solution demonstrated a maximum IOP

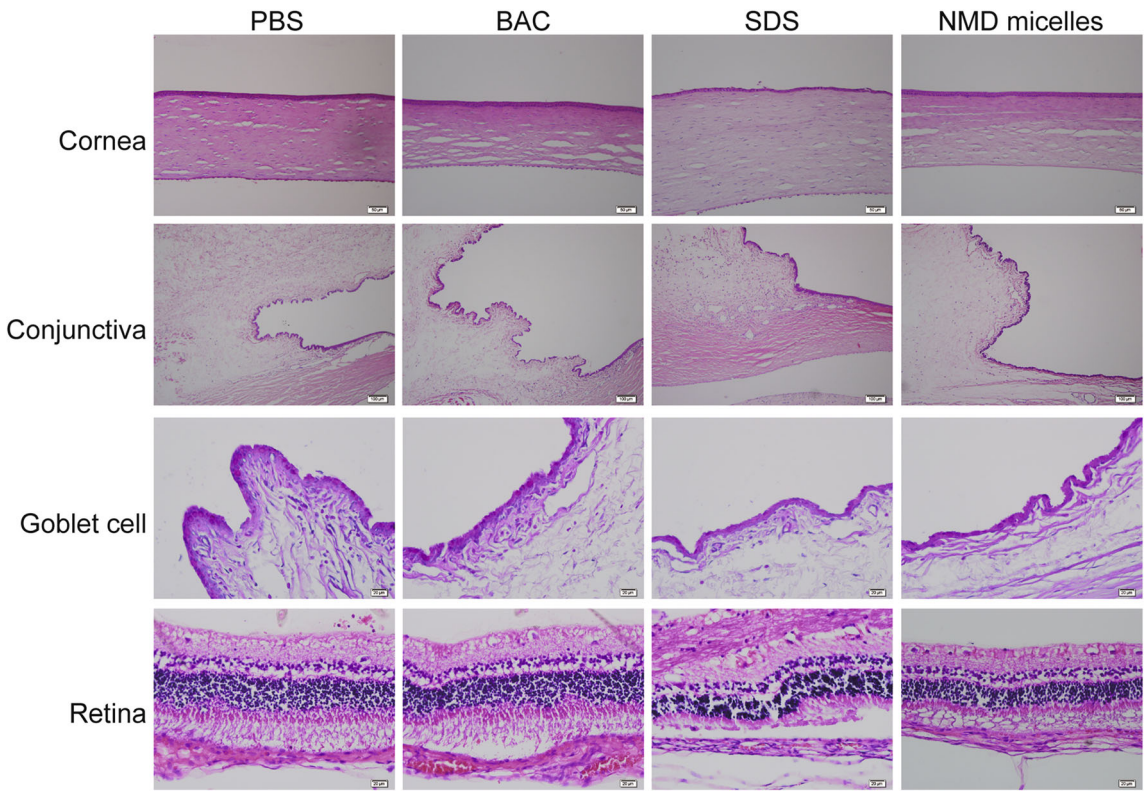

Fig. 6 Histopathologic evaluations of rabbit corneas**.** Histopathological evaluations of rabbit corneas, conjunctivae, goblet cells, and retinas 24 h after the last topical instillation. PBS, 0.1 mg/ml BAC in PBS solution, 5 mg/ml SDS in PBS solution, and 0.3 mg/ml NMD micelle ophthalmic solution were tested in this experiment. BAC, benzalkonium chloride; SDS, sodium dodecyl sulfate; NMD, nimodipine. Bar for cornea tissues = 50 μm; bar for conjunctiva tissues = 100 μm; bar for retina and goblet cell observation = 20 μm

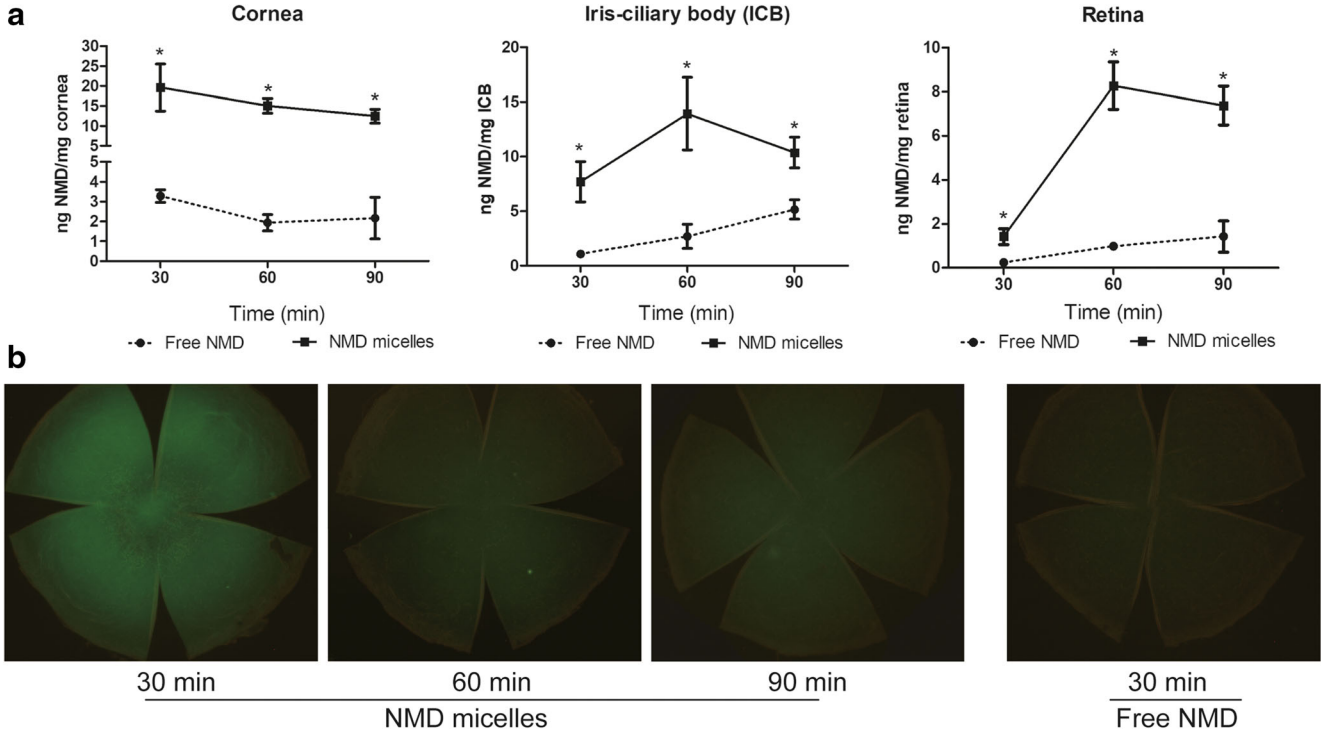

Fig. 7 The in vivo ocular permeation profiles for the NMD micelle ophthalmic solution. **a** The nimodipine (NMD) concentration in rabbit corneas, irises and ciliary bodies (ICB), and retinas after 4 doses (50 μl/dose 10 min apart) of either the NMD micelle ophthalmic solution or free NMD suspension solution (*$P < 0.05$ when compared with free NMD suspension solution, $n = 6$). **b** Fluorescence microscope observation of flat-mounted mice corneas after 4 doses (5 μl/dose 10 min apart). Images were taken at × 10 magnification

reduction of 23.76 ± 4.33% after 2 h ($P < 0.05$ when compared with the PBS group). Then, the IOP lowering effect gradually weakened and returned to the baseline values less than 6 h after ocular administration. However, the 1.0- and 0.3-mg/ml NMD micelle ophthalmic solutions and the 3.0-mg/ml free NMD suspension solution failed to lower the IOP of normal mice ($P > 0.05$ when compared with the PBS group).

Miosis

The alterations in pupil diameter were plotted with time and are shown in Fig. 8b. Timolol maleate ophthalmic drops caused a maximum miotic response 30 min after administration, with a maximum pupillary rate of − 9.29 ± 5.73%, and the duration of the pupillary effect was less than 150 min. Obviously, the 3.0-mg/ml NMD micelle ophthalmic solution produced stronger miosis than the other test solutions ($P < 0.05$ when compared with the PBS group), but the duration of the effect with the 3.0-mg/ml NMD micelle ophthalmic solution was similar to that of timolol maleate ophthalmic drops. However, the 3.0-mg/ml free NMD suspension solution and 1.0 and 0.3 mg/ml NMD micelle ophthalmic solution did not demonstrate pronounced miosis ($P > 0.05$ when compared with the PBS group). These miosis results were consistent with those of IOP reduction, and all these outcomes implied that the 3.0-mg/ml NMD micelle ophthalmic solution had better pharmacodynamics in contrast to the commercially available timolol maleate ophthalmic drops.

Discussion

RA has been reported to have a solubility of approximately 330 mg/ml in aqueous solution at 25 °C [36]. However, we found that RA could easily be dissolved in PBS with concentrations higher than 27 mg/ml, but RA precipitated during storage at 25 °C and 4 °C. Only when the RA concentration was less than approximately 25 mg/ml was no precipitation observed during storage (unpublished data). It was also found that an RA/drug weight ratio > 14 resulted in high encapsulation efficiency. Therefore, the single RA micelles struggled to meet the formulation requirements of high dosage ophthalmic drops. However, mixed micelles might fill this void. It has been widely accepted that mixed micelles are formulated using two or more nanocarrier materials to synergistically enhance the micelle characteristics [22], including improved stability and high drug loading capacity [37]. As an FDA-approved biomaterial, TPGS can self-assemble into nanomicelles in aqueous solution. TPGS micelles are regarded as an interesting drug delivery system, as they can also inhibit P-glycoprotein efflux biological activity [38]. Therefore, mixed micelles that combine TPGS with other

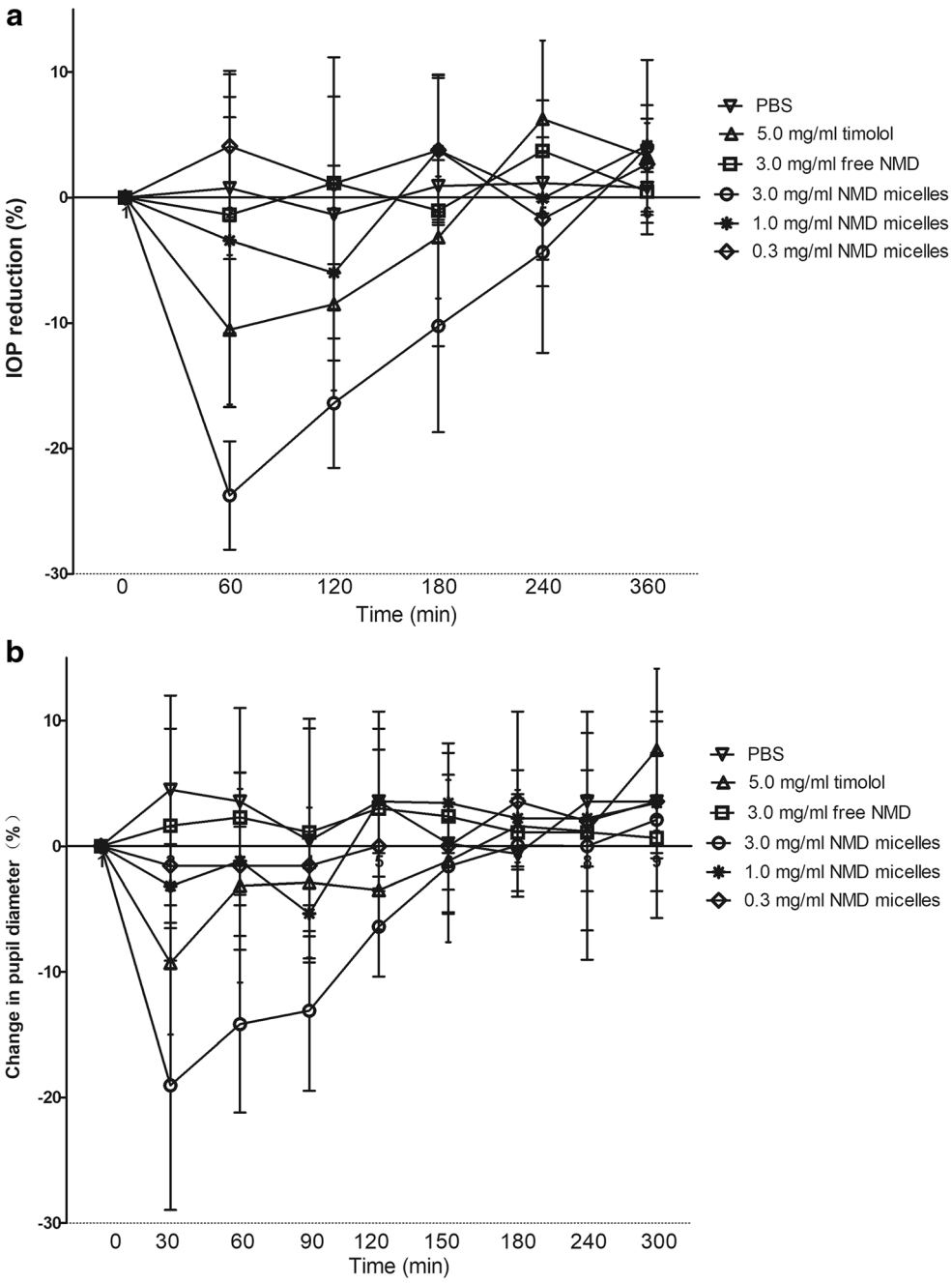

Fig. 8 In vivo pharmacodynamics ($n = 6$). **a** IOP lowering effect of ocular administration of the NMD micelle ophthalmic solution. **b** Miotic response profiles after ocular administration of the NMD micelle ophthalmic solution. NMD, nimodipine; IOP, intraocular pressure

materials have been reported in many studies as nanocarriers for poorly soluble drugs [39]. However, TPGS micelles combined with small natural amphiphilic molecules have not been studied. This manuscript was the first report on the use of TPGS mixed micelles with a small natural amphiphilic molecule, RA, to formulate an ocular drug delivery system.

Our preliminary evidence indicates that NMD failed to be encapsulated in the mixed micelles with an RA/TPGS weight ratio of 1:1, which had an encapsulation efficiency rate greater than 50% (unpublished data). However, high encapsulation efficiency was obtained for micelles with an RA/TPGS weight ratio of 2:1 or 1:2. Therefore, an RA/TPGS weight ratio of 2:1 and 1:2 were further evaluated. The CMC values of the mixed micelles with an RA/TPGS weight ratio fixed of 1:2 at 34 °C were 1.338 ± 0.321 and 1.347 ± 0.011 mg/ml in artificial tears and PBS, respectively, suggesting that mixed RA/TPGS with an weight ratio fixed of 1:2 has a strong tendency to form micelles in artificial tears and PBS. Completely different micelle encapsulation efficiency profiles were observed for 2:1 and 1:2 RA/TPGS. The encapsulation efficiency of 2:1 RA/TPGS was enhanced with an increasing weight ratio of RA and TPGS, while a decreasing trend was observed for 1:2 RA/

TPGS. The NMD aqueous apparent solubility in micelles was also affected by the RA/TPGS weight ratio, and as high as 30.21 ± 13.89 mg/ml of aqueous apparent solubility was observed to the mixed micelles with an RA/TPGS weight ratio fixed of 1:2 and with an NMD/carrier weight ratio of 1:15, the optimized formulation in this manuscript (Figure S1). The explanations for these phenomena need further exploration. Based on our preliminary evidence, which indicates that the concentration of NMD in ophthalmic solution should reach 3 mg/ml to substantially reduce the IOP, 1:2 RA/TPGS was more suitable to fabricate the NMD solution in this study than 2:1 RA/TPGS, as a low concentration of RA in the solution prevented the precipitation of RA during long storage periods. The mixed micelles with RA/TPGS exhibited a larger size than single RA micelles (13.429 ± 0.181 nm vs 3.96 ± 0.85 nm) [27]; the mixed micelles exhibited small micelle size [40]; and many reports confirmed that nanomedicine with small and uniform size distributions favor ocular tissue absorption [27]. The NMD ophthalmic solution exhibited well storage stability in 14-day short-term storage stability, as well as the chemical stability of NMD during this short-term storage (Figure S2). Long-term storage stability, as well as storage stability explored in more ambient storage conditions (for example, stored at 25 °C), was need further investigation.

Although our previous report confirmed the safety of the topical ophthalmic administration of RA [27] and many reports have confirmed the safety of the topical ophthalmic administration of TPGS [41, 42], ocular safety concerns were still respected in this manuscript. The results were consistent with the single test reports of RA and TPGS in that the NMD ophthalmic solution displayed good ocular tolerance. Rabbits from the BAC solution group also displayed good ocular tolerance, and these results seem to contradict previous reports and even common sense that many preservatives, including BAC, result in pronounced ocular surface toxicity [43, 44]. One explanation was that the BAC and its concentration tested in this experiment (0.1 mg/ml) was a widely used preservative and its concentration in marketed eye drops supported its well eye tolerance results. The other explanation was that the healthy rabbits were explored in this experiment, and the healthy eye might have well tolerance even to preservatives of eye drops. To preclude a potential false-negative, the SDS group was explored as a positive control, and the results confirmed that SDS caused severe ocular damage that even reached the posterior segment, such as the retina [45]. All these evaluations confirmed the ocular safety of the NMD ophthalmic solution.

NMD has already been confirmed to have antioxidant activity and significantly reduce oxidative stress as well as reduce the production of reactive oxygen species [46–48]. In this study, the antioxidant ability of NMD was evaluated through the scavenging activity of ABTS. The results showed that high antioxidant activity was observed for the NMD micelle solution. This pronounced enhancement of antioxidant activity could be due to the significant improvement in aqueous solubility, as the nanocarrier and the free NMD displayed low antioxidant activity.

The pronounced improvement in aqueous solubility and the nanosize effect of the fabricated micelles contributed to the in vitro PAMPA results and in vivo ocular permeation [49]. There were much higher NMD concentrations in ICB and the retina as well as the cornea. The mechanism of IOP reduction by NMD remains unclear, but the ciliary epithelium might be involved [14]. The high concentration of NMD in ICB might contribute to its IOP reduction efficacy. Glaucoma is associated with the loss of retinal ganglion cells [50]. NMD micelles prompted high NMD concentrations in the retina, and this activity might benefit glaucoma treatment.

In vivo pharmacodynamic evaluations widely use normal animals (including rabbits and mice) with normotensive eyes (not glaucomatous eyes) to evaluate new formulations for glaucoma therapy [21, 42, 51], and IOP reduction and miosis are the two most frequent parameters to evaluate novel formulations for glaucoma [21, 35]. Both these two evaluations revealed NMD micelle ophthalmic solution dose-dependent pharmacodynamics, and the 3.0-mg/ml NMD micelle ophthalmic solution displayed significant IOP reduction and miosis. The commercial timolol maleate ophthalmic drops, widely explored as a positive control in novel glaucoma formulation evaluation, showed a maximum IOP decrease of 10.55 ± 5.96%, and this result was similar to that in some reports that tested normotensive eyes [52, 53]. However, the 3.0-mg/ml NMD micelle ophthalmic solution displayed a maximum percentage decrease in IOP of 23.76 ± 4.33%. The miosis evaluation results resembled those of IOP reduction, which further confirms the improved efficacy of the NMD micelle ophthalmic solution. However, it should not be omitted that pharmacodynamic duration was not improved when compared with the commercial timolol maleate ophthalmic drops. Although the administration of the commercial timolol maleate ophthalmic drops was 1 drop 1–2 times daily, the duration of IOP reduction and miosis were both less than 180 min in the normal mice test. For the NMD micelle ophthalmic solution, the duration of IOP reduction and miosis in the normal mice test was less than 360 min and 180 min, respectively. The glaucoma dosage regimen for the NMD micelle ophthalmic solution needs further investigation, and further explorations are needed to develop the NMD micelle ophthalmic solution into hybrid formulations (such as a micelle in hydrogel formulation) [54, 55] with prolonged duration to promote their clinical application.

Conclusion

In this paper, an NMD micelle ophthalmic solution was developed for antiglaucoma drug delivery with a nanocarrier formulated with RA and TPGS. The NMD micelle ophthalmic solution was evaluated by various physicochemical characterizations and in vitro and in vivo studies. The NMD micelles displayed small micelle size and a narrow size distribution, and the ophthalmic solution showed significantly improved in vitro antioxidant activity and faster NMD membrane permeation. The NMD micelle ophthalmic solution was well tolerated in rabbit eyes. The topical NMD micelle ophthalmic solution significantly improved the in vivo ocular absorption of NMD and in vivo IOP reduction and miosis. In summary, this NMD micelle ophthalmic solution might be a promising ocular formulation to treat glaucoma.

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Compliance with ethical standards

Animal experiments were performed according to the Association for Research in Vision and Ophthalmology (ARVO) Statement in Ophthalmic and Vision Research and were approved by the Qingdao University of Science and Technology Ethics Committee for Animal Experimentation (permit no. 2017-1, Qingdao, China).

Conflict of interest The authors declare that they have no conflicts of interest.

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