Topical, Aqueous, Clear Cyclosporine Formulation Design for Anterior and Posterior Ocular Delivery

Kishore Cholkar1, Brian C. Gilger2, and Ashim K. Mitra1

1 Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri-Kansas City, Kansas City, MO, USA
2 North Carolina State University, Raleigh, NC, USA

Correspondence: Ashim K. Mitra, Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri-Kansas City, 2464 Charlotte Street, Kansas City, MO, USA. e-mail: mitraa@umkc.edu

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Purpose: The main objective of this study was to optimize cyclosporine (CsA) nanomicellar solution and study in vivo ocular CsA tissue distribution with a topical drop.

Methods: An optimized blend of hydrogenated castor oil-40 and octoxynol-40 was prepared to entrap CsA within nanomicelles. In vivo studies were conducted in New Zealand White albino rabbits with topical drop instillation.

Results: Average size of CsA-loaded nanomicelles was approximately 22.4 nm. Ocular tissue CsA quantification with single and multiple dosing revealed that CsA levels followed as cornea → iris-ciliary body → aqueous humor → lens. Cyclosporine levels were also found to be in the following order: conjunctiva → sclera → retina/choroid → vitreous humor. High CsA level was detected in retina/choroid (53.7 ng/g tissue).

Conclusions: Ocular tissue CsA distribution studies revealed high CsA concentrations in anterior ocular tissues. Moreover, it appears that nanomicelles are transported through a conjunctival–scleral pathway and deliver CsA to the retina/choroid. Results suggest polymeric blend to be a safe carrier for anterior and posterior ocular tissues.

Translational Relevance: This study has significant translational relevance, disclosing results that suggest that aqueous nanomicellar approach can provide high corneal and conjunctival CsA concentrations. Aqueous nanomicelles can deliver high drug concentrations not only to anterior but also to back of the eye tissues, including retina. This article provides a platform for noninvasive back of the eye drug delivery with topical eye drops. Aqueous CsA nanomicelles have no perceptible toxicity such as cell membrane damage or cytotoxicity to corneal and retinal pigment epithelial cells. Clear aqueous nanomicellar solution can be translated to human conditions for keratoconjunctivitis sicca and other anti-inflammatory conditions.

Introduction

Cyclosporine (CsA) is a fungus-derived (Tolypocladium inflatum) cyclic decapeptide drug. Cyclosporine is a highly lipophilic drug with a poor aqueous solubility of 12 ng/mL.1 It exerts its mechanism of action by inhibiting T-cell activation and thereby inhibits inflammatory cytokine production. Also, CsA blocks the opening of mitochondrial permeability transition pores and inhibits apoptosis.2 Additionally, CsA has been found to be important in treating allergic inflammation because of its direct inhibitory effects on eosinophil and mast cell activation and release of mediators.3 It has been shown that CsA increases the conjunctival goblet cell density.4 Two decades ago, the potential of CsA in treating human dry eye (keratoconjunctivitis sicca) was evidenced in several small clinical trials.5,6 Despite several advantages, preparation of concentrated CsA aqueous solution was difficult because of its poor aqueous solubility. The CsA emulsion has been shown to be an effective therapeutic agent to treat moderate to severe dry eye disease in clinical trials, leading to US Food and Drug Administration (FDA) approval of the drug in 2003. The exact mechanism of enhanced tear production is not well delineated, but it is hypothesized that it may be related to CsA immunomodulatory activity, which reduces local inflammation.7

Cyclosporine 0.05% emulsion (Restasis) has been studied for various other ocular conditions such as...
uveitis, vernal and atopic keratoconjunctivitis, and posterior blepharitis. However, Restasis induces a burning sensation and redness following topical instillation. Cationic, anionic, and oily CsA emulsions were studied to reduce inflammation and stinging sensation and to improve safety and tolerability. Cationic CsA emulsions were found to be more tolerable relative to anionic and CsA oil; however, inflammation was observed with topical application of cationic CsA emulsion in rabbits. Despite tremendous research in ocular drug delivery, only one topical formulation, Restasis® (Allergan, Irvine, CA) has been commercialized in the United States. To minimize local adverse effects and improve patient’s acceptability, an aqueous, clear topical CsA formulation that overcomes ocular barriers and deliver high drug levels to the anterior ocular tissues was developed.

Our work is focused on development of a novel aqueous, clear topical CsA nanomicellar formulation composed of a blend of polymers, polyoxyethylene hydrogenated castor oil 40 (HCO-40) and octoxynol 40 (Oc-40). In the present study, our aim was to optimize CsA nanomicellar formulation and study CsA bioavailability with single and multiple topical drop administration. Both polymers are FDA approved for ocular use. A blend of polymers was used to encapsulate CsA and to deliver therapeutic levels to ocular tissues after topical drop instillation. Nanomicellar formulation (NMF) development, characterization, in vitro evaluation, and ocular tissue distribution studies (single drop and multiple dosing) were conducted with topical ocular dosing. Amphiphilic polymers were combined in different ratios to identify the blend of polymers that generate low critical micellar concentration (CMC). The combination of polymers with a particular ratio with lowest CMC was selected to prepare CsA-loaded NMF. Further, formulations were characterized for their size, polydispersity, shape, surface morphology, encapsulation, and loading efficiency. In vivo ocular tissue distribution studies were conducted in New Zealand White (NZW) albino rabbits. Hence, the objective of this study was to develop a clear, aqueous topical NMF drop and to evaluate in vitro ocular biocompatibility/toxicity and in vivo ocular CsA tissue distribution. Nanomicellar formulation consisting of a blend of polymers can be considered as a novel carrier system to deliver CsA. Such aqueous CsA nanomicellar formulation may be further indicated in the treatment of ocular diseases such as dry eye syndrome and uveitis after preclinical evaluation in animal models.

Methods and Materials

Critical micellar concentration of the individual polymers, that is, HCO-40 (Barnet Product Corp., Englewood Cliffs, NJ) and a blend of polymer surfactant selected for the preparation of NMF was determined following slight modification of a previously described procedure. Critical micellar concentration was determined using iodine as a probe. The ratios (weight percent [wt%]) of HCO-40 to Oc-40 were varied (see Table 1). Iodine solution (1:2 ratio of I2 and KI, respectively) was added to each polymer solution and was incubated overnight. Absorbance of hydrophobic iodine, I2, entrapped in the core of nanomicelle, was measured at 460 nm with the help of a microplate reader (DDX 880; Beckman Coulter, Inc., Jersey City, NJ) and multimode detection software (version 2.0.012).

HPLC Analysis

In vitro analysis of CsA (Xenos Bioresources, Inc., Santa Barbara, CA) was performed by a reversed-phase HPLC (RP-HPLC) method with a Shimadzu HPLC pump (Shimadzu Scientific Instruments, Columbia, MD). An Alcott autosampler (model 718 AL; Alcott, Norcross, GA), Shimadzu UV/visible detector (SPD-20A/20AV; Shimadzu Scientific Instruments, Columbia, MD), and multimode detection software (version 2.0.012). A reversed-phase liquid chromatography tandem mass spectroscopy (LC-MS/MS) method with cyclosporine-d₄ as the internal standard (IS). The LC-MS/MS comprised a triple-quadrupole mass spectrometer with SCIEX API 4000 (API 4000; Applied Biosystems/MDI SCIEX, Foster City, CA) coupled to a
liquid chromatography system (LC-10 AD; Shimadzu Scientific Instruments) and reversed-phase ACE 5 phenyl column, 50 × 2.1 mm × 5 μm (Advanced Chromatography Technologies, Ltd., Aberdeen Scotland, UK), and a guard column (ACE 5 phenyl, 10 × 2.1 mm, 5 μm; Thermo-Hypersil Keystone, City, ST). Column temperature was maintained at 70°C (Eppendorf CH-30 column heater; Sigma Aldrich, St. Louis, MO). A gradient mobile phase comprising 20 mM NH₄COOH with 0.1% formic acid as mobile phase A and ACN/Methanol/0.4 M NH₄COOH (90:7.5:2.5, vol/vol/vol, pH = 3.5) as mobile phase B was selected. The mobile phase was pumped at a flow rate of 0.75 mL/min. A sample volume of 25 μL was injected onto HPLC, and the analysis run time was 6 minutes. Analyst version 1.4.2 software (Applied Biosystems-MDS SCIEX), operated with Windows (Microsoft Corp., Redmond, WA), was utilized for data acquisition and peak integrations.

### Nanomicellar Formulation

Cyclosporine-loaded NMF was prepared following the solvent evaporation and film rehydration method as described previously. Briefly, 0.025%, 0.050%, 0.075%, and 0.10%-loaded CsA NMF (100 mL) was prepared in two steps: (1) preparation of basic formulation and (2) rehydration. In step one, cyclosporine (0.025%, 0.050%, 0.075%, and 0.10%), HCO-40 (1.0 wt%), and Oc-40 (0.05 wt%) were dissolved separately in ethanol. HCO-40 solution was added with calculated volumes of Oc-40 (diluted in ethanol). This solution was stirred to generate a homogenous solution. To this solution, calculated volume of cyclosporine solution was added drop by drop. Each volume was made up to 15 mL with ethanol. The solvent was removed with high-speed vacuum (Genevac, Ipswich, Suffolk, UK) evaporation overnight (~10 hours) to obtain a solid thin film. In step two, the resultant thin film was hydrated with 40 mL of double-distilled deionized water (pH = 7.0) and resuspended. The rehydrated formulation was added to 10 mL of water, and the volume was made up with 2× phosphate buffer solution containing 1.2% of povidone K90 (BASF SE, Ludwigshafen, Germany). Finally, the formulation was filtered with a 0.2-μm nylon filter membrane to remove unentrapped drug aggregates and other foreign particulates.

### Characterization of Nanomicellar Formulations

All the formulations were characterized for optical clarity, osmolarity, and thermal stability (dissociation temperature [DT] and regeneration time) following previously described protocols. Mean particle size, size distribution, and polydispersity index (PDI) of the NMF were measured by dynamic light scattering (DLS; Malvern Zetasizer Nano, Westborough, MA). Shapes and surface morphology of CsA-loaded NMF were determined with transmission electron microscopy (TEM) (JEOL JEM 1200 EX II Electron Microscope; Peabody, MA) using negative staining with uranyl acetate (UA). Also, all the prepared formulations were examined for entrapment and loading efficiency and calculated with Equations 1 and 2, respectively.

**Entrapment efficiency**

\[
\text{Entrapment efficiency} = \frac{(\text{amount of CsA quantified in NMF}) \times 100}{(\text{amount of CsA added in the NMF})} \tag{1}
\]

**Loading efficiency**

\[
\text{Loading efficiency} = \frac{(\text{amount of CsA quantified in NMF})}{(\text{amount of CsA added} + \text{amount of polymers used})} \times 100. \tag{2}
\]

All the formulations were subjected to dilution test with phosphate buffer. The effect on the micelle size from no dilution to 500 times dilution was studied. The NMF size and polydispersity indices were obtained from Malvern Zetasizer Nano and recorded. Nuclear magnetic resonance (NMR) analysis was performed for CsA-loaded HCO-40/Oc-40 nanomicelles. The $^1$H NMR spectra were recorded on a Varian 400 MHz spectrometer (Varian Medical Systems, Palo Alto, CA) in deuterated water (D₂O) or deuterated chloroform (CDCl₃) at room tempera-
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...ture. The prepared blank/placebo and 0.10% CsA NMFs (600 µL) were subjected to lyophilization to obtain dry pellets. The obtained pellets were resuspended in equal volumes (600 µL) of deuterated aqueous and organic solvents as follows: (1) Placebo NMF was resuspended in CDCl3; (2) 0.10% CsA NMF was resuspended in CDCl3 and D2O; and (3) CsA was dissolved in CDCl3.

All the solution and formulation viscosity was determined with a conventional glass viscometer (Ostwald-Cannon-Fenske; Fisher Scientific, Suwanne, GA). Equation 3 was used to calculate the viscosity:

\[
\text{Vis}_{\text{liq}} = \frac{\text{density}_{\text{liq}} \times \text{time}_{\text{liq}} \times \text{vis}_{\text{water}}}{\text{density}_{\text{water}} \times \text{time}_{\text{water}}}.
\]

Where \(\text{vis}_{\text{liq}}\) represents viscosity of the sample, \(\text{density}_{\text{liq}}\) represents density of the sample, \(\text{time}_{\text{liq}}\) denotes flow time for sample, \(\text{vis}_{\text{water}}\) shows viscosity of water (0.89 centipoise [cP], 25°C), \(\text{density}_{\text{water}}\) represents density of water (1 g/mL), \(\text{time}_{\text{water}}\) denotes flow time for water.

To improve the viscosity of NMF, povidone K90 was selected. Earlier studies in our laboratory identified that povidone K90 improved NMF properties (small diameter of NMF and bioadhesion) in comparison to other viscosity enhancers such as PVP-K30, hydroxypropyl methyl cellulose, hydroxyethyl cellulose, or polycarbophil.15

**Cell Culture**

Rabbit corneal epithelial cells (rPCEC) and human retinal pigment epithelial cells (D407) were cultured according to a previously published protocol from our laboratory.16,17 Both rPCEC and D407 cells were cultured in flasks, harvested at 80% to 90% confluence with TrypLE Express (a superior replacement for trypsin) (Invitrogen Corp., Carlsbad, CA). Cells were then plated in 96-well plates at a density of 10,000 cells per well and utilized for studies. The NMFs (blank and CsA loaded) were resuspended in half the volume of endotoxin-free cell culture water (Hyclone Laboratories, Inc., Logan, UT), and volume was made up with 2× Dulbecco’s modified Eagle’s medium.

**Cytotoxicity Studies**

To evaluate the toxicity of NMFs, lactate dehydrogenase (LDH) release was conducted on rPCECs for 2 hours. Also, the toxicity was evaluated on rPCEC and D407 with WST-1 cell proliferation reagent assay. The CsA-loaded and blank NMFs were prepared and evaluated for topical ocular delivery. Following topical drop administration, nanomicelles primarily come in contact with corneal epithelial cells. Therefore, we aimed to test the effect of NMF on rPCEC cells.

**Cell Proliferation Assay**

Cell viability assay was performed to determine the toxicity of CsA NMF on rPCEC and D407 cells. Briefly, 10,000 cells per well were plated into 96-well plates. The NMFs (blank and CsA loaded) were prepared in serum-free cell culture media and filtered with 0.22-µm sterile nylon membrane filters under laminar flow. Cells were exposed to 100 µL of placebo-loaded (blank/empty) and CsA-loaded (0.025%, 0.05%, 0.075%, and 0.1%) NMFs for 1 hour. In these experiments cell culture medium and 10% Triton X-100 served as negative and positive controls, respectively. Percent of viable cells were calculated using the Premixed WST-1 cell proliferation reagent according to the manufacturer’s protocol (Clontech Laboratories, Inc., Mountain View, CA). Percent of cytotoxicity of the placebo- and CsA-loaded NMFs was calculated.

**Lactate Dehydrogenase Assay**

In vitro plasma membrane damage assay for blank and CsA-loaded NMFs was conducted on rPCEC cells and quantitatively measured with Takara LDH cytoxicity detection Kit (Takara Bio, Inc., Mountain View, CA). The rPCEC cells were grown on 96-well plates and added to 100 µL of serum-free cell culture medium and 100 µL of blank and CsA-loaded NMFs. Cells were incubated for 2 hours at 37°C. In this assay, cell culture media and 10% Triton X-100 served as negative and positive controls, respectively. After a 2-hour incubation, the plate was centrifuged, and the amount of formazan formed in supernatant was measured using a 96-well microtiter plate reader with absorbance set at 490 nm.

**In Vivo Studies**

Studies were conducted in adult female NZW albino rabbits (Oryctolagus cuniculus) to determine ocular tissue drug concentrations and whole blood CsA levels after topical ocular instillation. Placebo and 0.1% CsA-loaded NMF were selected for in vivo studies. In these studies, balanced salt solution (BSS; Alcon Laboratories, Fort Worth, TX) served as negative control.
Animals

Adult female NZW albino rabbits weighing between 2.0 and 3.0 kg were obtained from Charles River Laboratories (Durham, NC). Use of animals in this study adhered to the ARVO statement for the use of animals in ophthalmic and vision research. Animals were acclimated for 7 days under the photoperiod 12 hours light/12 hours dark and at a temperature of 68° ± 2°F. Rabbits were allowed water ad libitum and were provided with Hi Fiber Rabbit Diet (Purina, St. Louis, MO). Protocol for performing the surgical procedure was also approved by Institutional Animal Care and Use Committee (IACUC) of North Carolina State University.

Single Dosing

To study CsA concentrations in the individual ocular tissues or fluids with single topical drop administration, animals were divided into three groups (BSS, placebo, and 0.1% CsA NMF), with two animals per group. Rabbits were manually restrained, and the upper eyelid was gently elevated to expose the cornea. Both eyes were dosed with BSS, placebo, and 0.1% CsA NMF (35 μL) without contacting the cornea. The rabbit was then allowed to blink several times to distribute the applied NMF/solution over the eye prior to returning the animal to the cage. After dosing, rabbits were left undisturbed in the cage for 60 minutes. After topical dosing (1 hour), approximately 3 mL of whole blood was collected. Following blood collection, animals were euthanized with an intravenous injection of an AVMA-approved barbiturate-based euthanasia agent (Fatal-Plus; Vortech Pharmaceuticals, Dearborn, MI). Eyes were enucleated and immediately frozen until further processing.

Multiple Dosing

Animals were divided into two groups (placebo and 0.1% CsA NMF), with two animals per group. The right eye of the animal was treated with NMF (placebo or 0.1% CsA) and the contralateral eye with BSS. This procedure was repeated four times per day at 2-hour intervals for 5 days. After the last dosing on day 5, rabbits were tranquilized, and 3 mL of whole blood was collected from the jugular vein. Animals were euthanized; eyes were enucleated and immediately frozen until further processing.

Eyes were dissected while frozen to isolate ocular tissues and to minimize drug diffusion to adjacent tissues during dissection. Dissection was performed on a cooled ceramic tile that was placed on dry ice/isopentane bath to avoid any thawing of the eye during dissection. Care was taken to avoid cross-contamination. The globe was initially separated in half (dorsal and ventral halves). The frozen aqueous humor was removed first and placed into preweighed vials. The cornea sections were removed next, followed by lens and vitreous. The iris and ciliary body were removed together, followed by the retina and choroid. The remaining sclera sections were then collected. Blood and tissues samples were stored at –80°C until further processing.

Rabbit ocular tissues (conjunctiva, cornea, eyelid, iris-ciliary body, lacrimal gland, lens, retina/choroid, and sclera) were homogenized in extraction buffer (20:80 ACN, 50 mM NH₄OAc, pH 4.4). Tissue homogenates were subjected to a protein precipitation CsA extraction method with acetonitrile. The supernatant was further extracted using a Waters Ostro 96-well plate (Waters Corporation, Milford, MA). Similarly, aqueous and vitreous humors were subjected to protein precipitation CsA extraction method. Methanol was added to aqueous and vitreous humor samples (3:1) and vortex mixed. Samples were then centrifuged and supernatant was transferred to HPLC vials for analysis. Calibration curve was prepared in 3:1 methanol/rabbit aqueous or vitreous humor. Cyclosporine and IS were extracted from 250 μL of rabbit whole blood (K₂EDTA) by liquid–liquid extraction using methyl-t-butyl ether (MTBE) afteracidification with HCl. The organic extract was then washed with an alkaline solution. After evaporation to dryness and reconstitution, the extracts were analyzed by LC-MS/MS.

Data and Statistical Analysis

Data for in vitro experiments (N = 8) are expressed as mean ± SD. Statistical comparison of mean values was performed with Student’s t-test. P < 0.05 was considered to be statistically significant.

Results

Critical Micellar Concentration

Critical micellar concentration was determined for HCO-40 and polymer blends with iodine as a probe. The CMC of HCO-40 was found to be approximately 0.03 wt%. The other surfactant, Oc-40, generated a CMC value of 0.107 wt%, which is greater than the CMC of HCO-40. A combination of the surfactants in varying ratios lowered the CMC value from 0.03 wt% to 0.00707 wt%. The results are presented in
Table 1. A combination of HCO-40 and Oc-40 at 1.0 wt% and 0.05 wt% resulted in reduced CMC.

Size, Polydispersity Indices, and Surface Charge

Placebo- and CsA-loaded nanomicelles presented a size range of 10 nm to 80 nm (Figs. 1A and 1B) when measured with DLS. The average 0.1% CsA-loaded nanomicellar size was approximately 22.4 ± 0.411 nm. All the size determinations for increasing payload of CsA are provided in Table 2. Similarly, all the NMFs were found to carry a slight surface negative charge (Table 3).

Visual Appearance

Visual appearance for all the formulations was found to be clear, transparent, and devoid of any particulate matter. To determine the presence of any particulate matter presence in the formulation, all the prepared NMFs were subjected to a UV absorbance test at 400 nm. The results show NMF to exhibit no or negligible absorbance (Table 3).
Transmission Electron Microscopy

The prepared aqueous 0.10% CsA nanomicellar formulations was clear, extremely small (Fig. 2), and devoid of any visible particles to naked eye (Fig. 2A, right vial). The formulation appeared to be transparently clear, similar to water (Fig. 2A, left vial), and free flowing. The TEM images for 0.10% CsA nanomicelles appeared to show smooth surface morphology, with spherical shape and no aggregation (Fig. 2B). The size distribution was visualized at a scale bar of 500 nm (LS3600, 100.0 kV, ×60). The TEM image is in agreement with the particle size measurement, and the size distribution is between 10 and 80 nm.

Thermal Dissociation

In general, thermal DT of the nanomicellar formulations is about 20° to 40°C higher than formulation containing drug. Low DT of the drug-containing NMFs indicates that drug molecules are incorporated into nanomicelles and thereby solubilized. With temperature rising above DT, nanomicelles appear to dissociate into individual monomer units. This disruption of nanomicelle structures causes drug release in surrounding aqueous medium. Such destabilization results in the formation of a cloudy or milky white solution. The DT of the formulation appears to be high (>45°C), which provides stability to the formulation at room temperature (Table 3).

Nanomicelle Regeneration Time

After attaining the DT, NMFs were allowed to cool to room temperature under ambient room temperature. The time taken for the disappearance of the cloudiness and regeneration of transparent, clear formulation was recorded. This time was recorded as nanomicelle regeneration time.

Osmolarity and pH

Tonicity was adjusted with sodium chloride solution. The pH of the formulations was adjusted similar to the tear pH of ~7.0 ± 0.1 with 0.1 N sodium hydroxide or hydrochloric acid solution. The results are summarized in Table 3.

Nanomicelle Entrapment and Loading Efficiency

The prepared NMFs were subjected to entrapment and loading efficiency tests by determining CsA concentrations in the nanomicelles after breaking or reverse opening the nanomicelles. The results demonstrated a high entrapment efficiency of >95%, and the

<table>
<thead>
<tr>
<th>Table 2. Nanomicellar Average Size (nm) and Polydispersity Indices</th>
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<tbody>
<tr>
<td><strong>Formulation (wt%)</strong></td>
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<tr>
<td>----------------------</td>
</tr>
<tr>
<td>Placebo</td>
</tr>
<tr>
<td>0.025</td>
</tr>
<tr>
<td>0.050</td>
</tr>
<tr>
<td>0.075</td>
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<td>0.100</td>
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<table>
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<tr>
<th>Table 3. Characterization of CsA NMFs</th>
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<tbody>
<tr>
<td><strong>CsA Formulations (wt%)</strong></td>
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<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>Placebo</td>
</tr>
<tr>
<td>0.025</td>
</tr>
<tr>
<td>0.050</td>
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<tr>
<td>0.075</td>
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http://tvstjournal.org/doi/full/10.1167/tvst.4.3.1
loading CsA was improved to 8.85%. The results are summarized in Table 3.

**Dilution Effect**

The effect of dilution on the NMF was studied following the size and polydispersity indices. There is no significant effect of size upon dilution with phosphate buffer. The polydispersity index was found to be increasing with dilution up to 500 times. Results indicate that the nanomicelles alter size distribution in the formulation. The results are summarized in Table 4.

**Proton NMR Studies**

The $^1$H NMR spectral analysis results are shown in Figures 3A to 3D. In CDCl$_3$, the resonance peaks corresponding to CsA and nanomicelles are observed. However, in D$_2$O, peaks corresponding to nanomicelles are only observed, and no peaks for CsA were evident. These results clearly indicate CsA was entrapped into the inner hydrophobic core of nanomicelles when CsA-loaded nanomicelles are suspended in D$_2$O.$^{13,18}$

**Formulation Viscosity**

The rehydrated NMF when resuspended in only 2× phosphate buffer generated viscosity nearly equal to water (0.9 cP). It was earlier reported that higher viscosity does not affect drainage rate unless viscosity exceeds a critical value of about 4.4 cP.$^{19}$ Therefore, 0.6 wt% of povidone K90 was added, which raised NMF viscosity to 2.0 cP and maintained the viscosity well below critical value. Addition of povidone K90 improved the viscosity, with negligible effect on flow property of the formulation.

**In Vitro Cytotoxicity**

Results with cell proliferation assay indicate that both cells, rPCEC and D407, displayed negligible cytotoxicity after NMF exposure (blank and CsA) and were comparable to negative control (culture medium) (Fig. 4A). On the other hand, Triton X-100 exposure significantly reduced rPCEC and D407 cell number to 24% and 25%, respectively, relative to blank culture medium. In another set of experiments (LDH assay) on rPCEC cells, NMF did not generate any significant release of LDH. The results were again comparable to negative control (culture medium), with negligible effect on cell plasma membrane. The positive control (Triton X-100) caused significant release of LDH by damaging the rPCEC cell membrane (Fig. 4B).

**Table 3.** Effect of Dilution on Nanomicellar Size

<table>
<thead>
<tr>
<th>Dilution Factor</th>
<th>Average Nanomicellar Size (nm)</th>
<th>Polydispersity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20.8</td>
<td>0.147</td>
</tr>
<tr>
<td>10</td>
<td>21.8</td>
<td>0.159</td>
</tr>
<tr>
<td>20</td>
<td>23.4</td>
<td>0.215</td>
</tr>
<tr>
<td>50</td>
<td>21.8</td>
<td>0.213</td>
</tr>
<tr>
<td>100</td>
<td>22.6</td>
<td>0.226</td>
</tr>
<tr>
<td>200</td>
<td>26.1</td>
<td>0.329</td>
</tr>
<tr>
<td>500</td>
<td>24.5</td>
<td>0.360</td>
</tr>
</tbody>
</table>
In Vivo Studies

LC-MS/MS Method

A sensitive LC-MS/MS method was developed and applied to quantify CsA concentrations in ocular tissues, fluids, and whole blood. The established analytical ranges for CsA are as follows: 0.125 to 30 ng (low range) and 1.00 to 2500 ng (high range) for ocular tissues, 2.00 to 2000 ng/mL for aqueous humor and vitreous humor, and 0.100 to 100 ng/mL for whole blood.

Ocular tissue concentrations of CsA, collected 1 hour after single dosing and 1 hour following the last dose on day 5 of multiple dosing are summarized in Tables 5 and 6. The results of the tissue analyses were converted to nanograms per gram by correcting for the amount of tissue analyzed. CsA ocular tissue distribution after single topical dosing showed higher drug concentrations in the anterior ocular tissues (cornea and conjunctiva) and tears. With single topical dosing, conjunctival CsA concentrations were 1.71 times higher than in cornea. Also, higher CsA levels were observed in sclera. All other ocular tissues produced low CsA concentrations. Whole blood CsA concentration was found to be 0.73 ng/mL. With repeat dosing, highest average CsA concentrations were observed in the treated eye. CsA levels in ocular tissues were found to be in the following order: cornea → conjunctiva → sclera → iris-ciliary body → aqueous humor. Lower CsA concentrations were observed in the lens → retina/choroid and → vitreous humor. CsA concentrations in the contralateral eye treated with BSS were extremely low, suggesting minimal systemic drug transfer. Concentration of CsA in whole blood with repeated topical dosing was found to be only 0.718 ± 0.295 ng/mL.

Discussion

Therapeutic delivery with no ocular side effects after topical dosing is a challenging task. Ocular static and dynamic barriers pose a challenge by impeding drug transport across cell membrane/tissues. After topical dosing, a large fraction of the dose is lost due to excessive tear production and/or drainage through nasolacrimal ducts. Further suboptimal physico-chemical properties of drugs will retard their aqueous solubility, thereby impeding drug delivery. In such a
scenario, clear aqueous NMFs appear to be highly promising. Nanomicelles can encapsulate hydrophobic drugs inside the hydrophobic core, improving aqueous solubility. Use of single polymer to encapsulate a drug may result in clear aqueous micelles, but may cause poor formulation stability. To improve the stability of micelles and lower their CMC, a second polymer may be added. In our studies, we included Oc-40 as the second polymer. A CMC is a point at which the monomer surfactants start to aggregate and form micelles. Lower CMC indicates better stability of the micelle formulation.21 For the blend of polymer surfactants, CMC was determined. In the present study, CMC for HCO-40 and blend of surfactants was determined with iodine as a probe. Pure I$_2$ is crystalline, fairly hydrophobic, and, particularly, not soluble in water. Iodine solubility in an aqueous environment is improved by adding its salt, potassium iodide, to the solution. Addition of potassium iodide solubilizes water-insoluble iodine by forming complexes, that is, KI, KI$_2$, KI$_3$, and KI$_4$. As the iodine solution is added to polymer solution, the solubilized iodine prefers to partition into the hydrophobic core of the nanomicelles. It prefers to interact with the hydrophobic nanoenvironment of nanomicelles, causing conversion of I$_3$ to I$_2$ from excess KI in the solution. The ether oxygen of PEG groups in the surfactant molecules donate electrons to the vacant $\sigma^*$ orbital of I$_2$, resulting in the formation of a surfactant-iodine donor-acceptor type complex.12 A rise in absorbance for iodine entrapment indicates increase in micelle concentration. As the concentra-

Table 5. Summary of Ocular Distribution of CsA in Rabbits After a Single Topical Drop Administration (35 $\mu$L) ($N = 4$ eyes)

<table>
<thead>
<tr>
<th>Tissue/Fluid</th>
<th>CsA Concentration at 1 Hour Post Dosing ($N = 4$ eyes) (ng/mL or ng/g)$^a \pm$ SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous humor</td>
<td>1.53 $\pm$ 0.398</td>
</tr>
<tr>
<td>Conjunctiva</td>
<td>1417.50 $\pm$ 123</td>
</tr>
<tr>
<td>Cornea</td>
<td>828.25 $\pm$ 53.2</td>
</tr>
<tr>
<td>Eyelid</td>
<td>745.00 $\pm$ 190.6</td>
</tr>
<tr>
<td>Iris ciliary body</td>
<td>8.19 $\pm$ 1.917</td>
</tr>
<tr>
<td>Lacrimal gland</td>
<td>3.22 $\pm$ 0.406</td>
</tr>
<tr>
<td>lens</td>
<td>1.03 $\pm$ 0.063</td>
</tr>
<tr>
<td>Retina-choroid</td>
<td>13.03 $\pm$ 2.417</td>
</tr>
<tr>
<td>Sclera</td>
<td>216.25 $\pm$ 14.9</td>
</tr>
<tr>
<td>Tears</td>
<td>800.50 $\pm$ 98.7</td>
</tr>
<tr>
<td>Vitreous humor</td>
<td>2.09 $\pm$ 0.753</td>
</tr>
</tbody>
</table>

$^a$ CsA concentrations in ocular tissues are represented as nanogram per milliliter for ocular fluids and nanogram per gram for tissues.
A sudden rise in absorbance may be observed. Iodine intensity as a function of the logarithm of surfactant mass concentration was plotted. A combination of surfactants (HCO-40/Oc-40, 1.0:0.05) generated a lower CMC value, 0.00707 wt% (Table 1), than did the individual polymers. Such low CMC value indicates that this combination of surfactants, at very low concentrations, will be able to entrap the hydrophobic drugs and can provide adequate solubility and stability.

To prepare CsA-loaded NMF, the blend of polymers (HCO-40/Oc-40, 1.0:0.05) was selected due to their low CMC. An aqueous clear nanomicellar CsA formulation was successfully prepared following a novel solvent casting technique. In another set of experiments, we tried to encapsulate 1 mg of CsA following a direct dissolution method. Addition of CsA (0.1 wt%) to the blend of polymers in aqueous solution resulted in drug precipitation. Therefore, we selected a solvent casting method to prepare CsA-loaded NMF, which resulted in clear aqueous solution. The NMF was devoid of any particulate matter. The prepared NMF needs to be maintained in the physiological range for osmolarity and pH. In general, to adjust tonicity, agents such as glucose, xylitol, glycerol, boric acid, or sodium chloride are added. Tears have the tonicity equivalent to 0.9% solution of sodium chloride, which produces osmolarity of ~305 mOsm/kg. Hypertonicity or hypotonicity of the tear film can lead to overexpression or underexpression of the ocular surface epithelium.

Table 6. Summary of Ocular Tissue CsA Concentrations Following Topical Ocular Administration of 0.1% CsA HCO-40 (pH 7.0) Formulation or Placebo to the Eye (4 times/day at 2-hour intervals for 5 days) to NZW Rabbits

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Treatment</th>
<th>OD</th>
<th>Average</th>
<th>OS</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous humor</td>
<td>0.1% CsA NMF administered 4 times/day (0, 2, 4, and 6 h) for 5 d</td>
<td>133</td>
<td>134.5</td>
<td>5.51</td>
<td>6.36</td>
</tr>
<tr>
<td></td>
<td>Placebo NMF</td>
<td>136</td>
<td>7.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitreous humor</td>
<td>0.1% CsA NMF administered 4 times/day (0, 2, 4, and 6 h) for 5 d</td>
<td>11.3</td>
<td>8.4</td>
<td>1.89</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>Placebo NMF</td>
<td>5.44</td>
<td>6.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conjunctiva</td>
<td>0.1% CsA NMF administered 4 times/day (0, 2, 4, and 6 h) for 5 d</td>
<td>2700</td>
<td>2125</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>Placebo NMF</td>
<td>1550</td>
<td>NC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cornea</td>
<td>0.1% CsA NMF administered 4 times/day (0, 2, 4, and 6 h) for 5 d</td>
<td>8130</td>
<td>7805</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>Placebo NMF</td>
<td>7480</td>
<td>NC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lens</td>
<td>0.1% CsA NMF administered 4 times/day (0, 2, 4, and 6 h) for 5 d</td>
<td>73.4</td>
<td>68.6</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>Placebo NMF</td>
<td>63.8</td>
<td>NC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iris-ciliary body</td>
<td>0.1% CsA NMF administered 4 times/day (0, 2, 4, and 6 h) for 5 d</td>
<td>274</td>
<td>204</td>
<td>NC</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>Placebo NMF</td>
<td>134</td>
<td>15.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retina/choroid</td>
<td>0.1% CsA NMF administered 4 times/day (0, 2, 4, and 6 h) for 5 d</td>
<td>85.0</td>
<td>53.7</td>
<td>NC</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td>Placebo NMF</td>
<td>22.4</td>
<td>22.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sclera</td>
<td>0.1% CsA NMF administered 4 times/day (0, 2, 4, and 6 h) for 5 d</td>
<td>786</td>
<td>720</td>
<td>22.7</td>
<td>45.6</td>
</tr>
<tr>
<td></td>
<td>Placebo NMF</td>
<td>655</td>
<td>68.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.2</td>
<td>NA</td>
<td>42.2</td>
<td>NA</td>
</tr>
</tbody>
</table>

OD indicates right eye; OS, left eye; BQL, below quantitation limit; NA, not applicable; NC, not calculated. Concentration below quantitation limit. Average value calculated by assuming NC = 0.

Aqueous humor and vitreous humor concentrations are expressed in nanogram per milliliter. All other tissue concentrations are expressed in nanogram per gram.
nicity of the topical drops may cause irritation to the eye. The pH of the eye ranges from 6.5 to 7.6. Therefore, osmolarity and pH was maintained at ~300 mOsM/Kg and ~7.0, respectively. The NMF produced much higher drug entrapment (>95%) and loading efficiencies (8.85%). This NMF demonstrated a small particle size ranging between 10 and ~80 nm. These data were also in agreement with real-time TEM images. The NMF, after resuspending in phosphate buffer saline, produced viscosity similar to that of water (0.9 cP). Topical dosing results in immediate loss of the drug due to tear drainage, spill on the cheeks, or by drainage through the nasolacrimal ducts.21 One method to improve viscosity is with the addition of viscosity enhancer. Therefore, NMFs (placebo and CsA loaded) contained viscosity-enhancing agent, povidone K90. We selected povidone K90 because it imparts viscosity and bioadhesion to NMF.15 The dual nature of povidone K90, viscosity improvement and bioadhesive property, may aid the formulation to improve viscosity and keep NMF in contact with the precorneal pocket for a longer time period. The surface potential for the formulations is slightly negative. This negligible charge may help to reduce repulsion of NMF with negative cell surface. Furthermore, dilution studies of NMF were conducted to determine the stability of nanomicelles. In general, tears are produced at an average flow rate of 1.2 µL/min.21 An excessive tear production following NMF instillation should not cause NMF dissociation and drug release. Therefore, we examined the dilution effect on nanomicellar size and PDI up to 500 times (way above tear dilution that is expected in vivo). Dilution of NMF caused a very small increase in mean micelle size. Similarly, PDI of NMF was slightly higher with dilution (up to 500 times). The reason may be attributed to change in concentration of monomers in the NMF with dilution. The results obtained are similar and in agreement with the earlier results observed by Xu et al.22 Upon nanomicelle dilution, the outer hydrophilic polyethylene oxide segment may form large and loose complex clusters in an aqueous solution. These new larger aggregates that are developed with dilution exhibit thermodynamically reversible association. Hydrophobic effect and hydrogen bonding may be forcing the outer hydrophilic segment to cluster and form larger aggregates in aqueous solution. The clustering of outer hydrophilic segment may be the reason for increase in micelle size upon dilution. However, NMFs sustained the dilution effect up to 500 times, produced negligible effect on their size, and demonstrated high stability upon dilution. Thermal dissociation and regeneration time of NMFs suggest a fairly robust formulation. The results may help to select a formulation with a high thermal DT. Following thermal dissociation when NMFs are allowed to cool to room temperature under ambient conditions, nanomicelles regenerated within less than 10 minutes, resulting in optically clear solution. In general, nanomicelles are unstable structures possessing two characteristic relaxation times, that is, fast (τ1) and slow (τ2). The results suggest that time taken to regenerate the nanomicelles is in minutes, because both polymers are nonionic in nature and display longer nanomicellar relaxation times (τ2). This peculiar behavior of the formulation indicates that hydrophobic CsA is entrapped in the nanomicellar core even after being subjected to harsh conditions.

CsA has poor aqueous solubility of 12 ng/mL at room temperature (25°C). There is a possibility that a small amount/concentration of CsA, due to its aqueous solubility, may be present in the outer aqueous solution of NMF. We conducted qualitative proton nuclear magnetic resonance (1H NMR) studies that may determine the presence of free/unentrapped CsA molecules in solution at parts per million (ppm) levels. Formulations were prepared in different media, such as CDCl3 and D2O. The 1H NMR spectroscopy studies were conducted for CsA, a mixture of HCO-40/OC40 in CDCl3, and NMFs with entrapped CsA in CDCl3 and D2O. Excessive addition of CsA above its aqueous solubility results in drug precipitation. In NMF we did not observe any CsA precipitation indicating drug entrapment in NMF, at a level higher than its aqueous solubility. On the other hand, results show that in CDCl3, the resonance peaks corresponding to CsA and nanomicelles are present as evidenced from the spectra. However, in D2O, only peaks corresponding to nanomicelles are detected, whereas peaks for CsA were not evident (Fig. 3D). These results clearly indicate that CsA is entrapped in the inner hydrophobic core of nanomicelles in D2O. When D2O suspended micelles were dried and resuspended in CDCl3, resonance peaks corresponding to CsA were evident, and the spectra were similar to Fig. 3C. These results indicate that the nanomicelles entrapped CsA in the inner core, and when subjected to organic solvent as outer environment, NMF reversed and released CsA. Therefore, 1H NMR identified peaks corresponding to CsA in CDCl3. However, in aqueous solvent (D2O), NMF entrapped CsA inside the core, and the NMR signals were lost.
An important aspect of topical ocular drug delivery is to evaluate drug delivery vehicles for biocompatibility. Formulation components are expected to be safe, reliable, and effective for its intended use. Also, these components are to be biodegradable and biocompatible. In vitro toxicity evaluation and ocular tissue drug distribution are the primary requirements to determine the safety of the carrier systems and their ability to deliver therapeutic drug levels to ocular tissues. In vitro toxicity tests are often conducted in cells. Therefore, the selection of a cell line is crucially important because it should reproduce any toxic response that is similar to what occurs in vivo. Therefore, we selected rPCEC and D407 as in vitro cell culture models to study the toxicity of CsA NMF.

To evaluate the cytotoxic effect of NMFs (blank and CsA loaded), cell proliferation (rPCEC and D407) and LDH assays (rPCEC) were performed. Since eye drops are rapidly cleared from the precorneal pocket, cell proliferation assay was performed for 1 hour. It was assumed that a 1-hour incubation period would be sufficient to evaluate any toxicity. In the first set of experiments (cell viability assay), the percent of viable cells in NMFs was comparable to that of negative control (culture medium) (Fig. 4A). Triton X-100 served as positive control and reduced the percent of cell viability to ~25% of the total cell number (rPCEC and D407). In another set of studies, a cell plasma membrane damage test (LDH assay) was conducted. A 2-hour incubation time is sufficient because these formulations are to be delivered as topical eye drops into the precorneal pocket. After topical drop instillation, most of the formulation is lost within minutes. In these experiments, the amount of LDH released in the culture media directly correlates with cytotoxicity. Triton X-100 caused significant toxicity/membrane damage and released high amounts of LDH. Percentage of cytotoxicity to rPCEC cells with exposure to NMF (blank and CsA loaded) appeared to be negligible (Fig. 4B). Even with 2-hour continuous exposure, the formulations did not cause any cell membrane damage. Results from these assays clearly suggest that NMFs do not cause cell death and/or damage to plasma membrane. NMF appears to be safe for topical ocular application. Therefore, in vivo studies with 0.1% CsA-loaded NMF and placebo were conducted, where BSS served as negative control.

In vivo studies conducted in female NZW rabbits with single and multiple dosing (4 times/day, at an interval of 2 hours, consecutively for 5 days) showed very high CsA levels in anterior ocular tissues. Whole blood concentrations of CsA were measurable (albeit at very low levels) in rabbits that received the 0.1% CsA NMF single and multiple doses for 5 days (Table 5 and 6). The mean CsA whole blood concentration (0.718 ng/mL) in this study was similar to the C\text{max} (0.745 ng eq/mL) value reported following a single-dose administration of an Allergan 0.2% 3H-CsA formulation in NZW albino rabbits. Anterior ocular CsA concentrations in the present study are higher than values reported following topical drop administration of CsA-loaded methoxy poly(ethylene glycol)-hexyl-substituted poly(lactide) (MPEG-hexPLA) copolymer micelles. High CsA concentrations were delivered in aqueous and humor iris-ciliary body when compared to MPEG-hexPLA micelles, indicating the better carrier ability of NMF. Considering that only a single time point (1 hour after the last dose) was sampled for analysis, the data were remarkably consistent between the two treated animals. For instance, the CsA concentrations observed in the cornea were 8130 and 7480 ng/g (Table 6). The ocular tissue concentrations of CsA observed in this study were similar to or higher than those observed following repeat dose administration (twice a day for 7 days) of an Allergan 0.2% 3H-CsA formulation to rabbits. From Tables 5 and 6, it is evident that CsA-loaded NMF is able to safely deliver high CsA concentrations in anterior and posterior ocular tissues. However, limitations for these studies include lack of comparison in tissue drug levels due to experimental conditions and study design. Moreover, this CsA aqueous NMF is indicated for the treatment of dry eye syndrome and uveitis, pending further investigations. Other in vivo toxicological evaluations with single and multiple dosing, pharmacokinetics (single and multiple dose), and pharmacodynamics are beyond the scope of this article. Therefore, as described earlier, in the current communication we report the development, optimization, and pharmacokinetics of CsA aqueous topical drop formulation. We will be reporting all in vivo toxicological evaluations and their results in future communications in a sequential manner.

Instillation of topical eye drops is the most preferred and convenient route of drug administration, resulting in higher patient compliance. This novel CsA formulation was able to translocate across the cornea and deliver high levels in the anterior ocular tissues. Interestingly, we also found high drug levels in the tissues at the back of the eye (retina/
choroid) with CsA aqueous topical drops. There are two potential pathways for a molecule to reach the back of the eye following topical administration: (1) the intraocular route, through cornea → aqueous humor → lens → vitreous humor, and finally → retina, and (2) the trans-scleral or conjunctival–scleral route, around the conjunctiva through sclera → choroid and to retina. The first pathway is often unsuccessful for hydrophobic molecules such as CsA with high hydrophobicity, since the hydrophilic stroma becomes the rate-limiting barrier for transcorneal absorption. Moreover, fluids in the anterior and posterior segments flow in opposite directions, hindering the passage of molecules from the aqueous humor to lens and through to the vitreous humor, thus making this an unfavorable pathway. The second pathway offers a viable strategy to reach the back of the eye for hydrophilic molecules by passive diffusion through the scleral aqueous channels/pores. But again, CsA, being a highly hydrophobic drug, will encounter aqueous sclera as a potential barrier for permeation through the conjunctival–scleral route. CsA hydrophobicity limits the feasibility of formulating it into a clear aqueous solution at sufficient concentration to achieve therapeutic levels in the ocular tissues. Hence, CsA is currently marketed as opthalmic emulsion (Restasis), which on chronic use may cause side effects. For CsA to reach the back

Figure 5. (A) CsA-loaded nanomicelles composed of HCO-40 and Oc-40. (B) Partial reversal of CsA-loaded nanomicelles. (C) Complete reversal of nanomicelles and CsA release upon contacting lipoidal ocular tissues.
of the eye following topical administration, it is imperative to utilize the aqueous channels/pores of sclera. Hence, highly hydrophobic CsA, when entrapped inside nanomicelle with a hydrophilic exterior/corona, results in highly hydrophilic outer corona. The scleral aqueous pore size ranges between 20 and 80 nm.32 Our CsA-loaded NMFs have small size (10–80 nm), with hydrophilic coronas. We hypothesize that these nanomicelles utilize the hydrophilic corona and carry the CsA payload to the back of the eye tissues following the conjunctival scleral pathway. Also, ocular tissue CsA concentrations (conjunctiva/sclera/retina/choroid) support our hypothesis of conjunctival–scleral route for CsA delivery. Moreover, these CsA nanomicelles, upon reaching back of the eye tissues such as Bruch’s membrane and retinal pigment epithelium (RPE) may release CsA cargo by nanomicellar reversal, as shown in Figures 5A–C. The lipid bilayer of RPE and Bruch’s membrane may aid in lipid–lipid interactions of nanomicellar core and lipid bilayer, causing CsA release into the cells. Furthermore, CsA permeation into the hydrophilic vitreous humor is prevented because of the hydrophobic nature of CsA, where it gets deposited into the highly lipophilic tissues (RPE, retina).

Conclusions

In summary, an aqueous, clear nanomicellar CsA-loaded formulation was successfully prepared with a blend of HCO-40/Oc-40 polymers. At a specific ratio of HCO-40 and Oc-40 (1.0:0.05), the CMC was found to be lowest relative to individual polymers. Prepared placebo and CsA NMF demonstrated absence of any particulate matter. The NMF showed improved drug entrapment (>95%), loading efficiencies (8.8%), and a small size range between 10 nm and ~80 nm, and it is spherical in shape with smooth surface architecture, which is evident from the TEM images. The surface potential for the formulations was found to have a small negative potential. In other words, the surface potential for NMF is negligible. The dilution had no significant effect on the micelle size, but the PDI was observed to increase with higher dilution (up to 500 times). Qualitative 1H NMR studies revealed absence of free CsA in NMF. In vitro cytotoxicity assays with cell proliferation and LDH release on rPCEC and D407 cells demonstrated the formulations to produce negligible toxicity on cell viability and plasma membrane. These results indicate that these formulations are safe and well-tolerated. In vivo studies with topical drop application of BSS, placebo, and 0.1% CsA-loaded NMF showed very high CsA levels in anterior ocular tissues. Also, CsA concentrations in tissues in the back of the eye (retina/choroid) were much higher than the therapeutic level, suggesting that NMF follows the conjunctival–scleral pathway to reach retina/choroid. A blend of HCO-40 and Oc-40 is a safe carrier to deliver drugs to tissues in the back of the eye in therapeutic concentrations with aqueous topical drop instillation.

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