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ORIGINAL ARTICLE

A sustained release cysteamine microsphere/thermoresponsive gel eyedrop for corneal cystinosis improves drug stability

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Abstract

Cystinosis is a rare, metabolic, recessive genetic disease in which the intralysosomal accumulation of cystine leads to system wide organ and tissue damage. In the eye, cystine accumulates in the cornea as corneal cystine crystals and severely impacts vision. Corneal cystine crystals are treated with cysteamine eyedrops when administrated 6 to 12 times day and used within 1 week. The strict dosing regimen and poor stability are inconvenient and add to the burden of therapy. To reduce the dosing frequency and improve the stability, we present reformulation of cysteamine into a novel controlled release eyedrop. In this work, we characterize and evaluate a topical drug delivery system comprised of encapsulated cysteamine in polymer microspheres with a thermoresponsive gel carrier. Spray-dried encapsulation of cysteamine was performed. In vitro cysteamine release, stability, and ocular irritation and corneal permeation were evaluated. The data suggest that encapsulated cysteamine improves the stability to 7 weeks when compared with 1-week aqueous cysteamine eyedrops. Release studies from one drop of our system show that cysteamine release was present for 24 h and above the minimum cysteamine eyedrop amount (6 drops). Cysteamine from our system also resulted in negligible irritation and enhanced permeation when compared with traditional cysteamine eyedrops. In vivo studies were implemented to support ease of administration, tolerability, and retention for 24 h. These studies suggest that our controlled release delivery system may provide stable cysteamine from a safe, once daily gel eyedrop.

Keywords Ocular drug delivery · Cornea · Rare disease · PLGA microspheres · Thermoresponsive gel

Introduction

The cornea is a transparent, avascular tissue that covers the outermost anterior portion of the human eye. It is composed of five layers: the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium [1]. These layers play vital roles in the passage and refraction of light. The cornea's main function is to refract (bend) and focus light with the aid of the human lens to the back of the eye for visual processing. Loss of transparency, or corneal clarity, by damage or corneal disease can lead to progressive visual impairment. Such is the case for rare, genetic, lysosomal storage diseases that result in the accumulation of biological waste products within the lysosomes of cells, leading to system wide tissue and organ complications [2]. In autosomal recessive cystinosis, there is intracellular accumulation of these molecules in the cornea resulting in deposition of corneal crystals and corneal erosions severely impacting vision [3, 4].

In cystinosis the dysfunction or absence of cystinosin, a protein transporter of cystine, results in high accumulation of intralysosmal cystine [3]. Cystine crystal formation occurs intracellularly, causing cell apoptosis and subsequent kidney, liver, bone, connective tissue, thyroid, and ocular complications. In the eye, cystine

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crystals will aggregate in all tissues, but is most notably present as refractile, spindle-like crystals in the cornea [4, 5]. These hyperreflective corneal cystine crystals cause severe light sensitivity, foreign body sensation, and recurrent epithelial erosions, all of which are associated with visual impairment if left untreated [6].

Current treatments aim to manage the systemic complications through administration of cysteamine, which is capable of depleting, or reducing intracellular cystine [7]. Mechanistically, cysteamine enters the cell where a disulfide interchange reaction with cystine occurs, resulting in two species: cysteine (the reduced form of cystine) and a cysteine-cysteamine mixed disulfide [8]. These compounds exit the lysosome via metabolic processes, without requiring cystinosin (cystine protein transporter). The orally administered drug cysteamine (Cystagon®; and its sustained-release counterpart Procysbi®) is effective in removing cystine from many tissues in the body but has limited effect on the eye, likely due to its poor biodistribution to the eye and avascular nature of the cornea. Thus, a cysteamine eyedrop formulation (Cystaran™; 6.5-mg/mL cysteamine hydrochloride) was developed to target corneal cystine crystals. Topical cysteamine has proven to be safe and effective in reducing and clearing corneal cystine crystals, resulting in clear corneal layers and reduction in light sensitivity [3]. However, cysteamine eyedrops require frequent administration—up to once per waking hour (6 to 12 times per day) and are highly susceptible to oxidative degradation, therefore requiring that the eyedrops are frozen until opening, stored in the refrigerator, and disposed of within 1 week [9]. Furthermore, ocular irritation upon administration associated with corneal epithelial erosions [10] leads to high levels of noncompliance with the prescribed drop regimen. This inconvenient therapy and its associated complications, particularly in the context of a disease as complex and multi-faceted as cystinosis, severely impact the quality of life of patients.

To address the issues with ocular cysteamine delivery, including frequency of dosing and poor aqueous stability, cysteamine reformulation into various drug delivery systems has been investigated. Controlled release technologies including viscous gels [11, 12], hydrogels [13], contact lenses [14], and nanowafer discs [15] have recently been developed to increase the ocular retention and prolong the release of cysteamine. Of these studies, the nanowafer disc in particular has shown promise for extended release of cysteamine and moderately increased stability [15]. Despite these advances, development in this area, especially given the far-reaching implications for other rare diseases affecting vision [2] and the many new approaches for ocular drug delivery [16, 17], remains under investigated.

The work presented herein focuses on the development of a multicomponent extended release drug delivery system for treatment of corneal cystinosis. This system incorporates cysteamine-loaded poly(lactic-*co*-glycolic acid) (PLGA) microspheres within a thermoresponsive poly(*N*-isopropylacrylamide) (pNIPAAm) and poly(ethylene glycol) (PEG) gel matrix. The microsphere-gel suspension is administered similarly to a traditional eyedrop but forms a pliable depot after exposure to ocular surface temperatures when placed in the lower eyelid. The gel material is mixed with hydrolyzable PLGA microspheres, which are capable of delivering a range of ocular drugs for varying lengths of time, as with previous in vivo large animal studies demonstrating long-term efficacy, lower fornix retention and safety in glaucoma for 28 days [18], and bacterial endophthalmitis prophylaxis for 24 h [19].

Herein we describe the strategies for cysteamine encapsulation in PLGA microspheres and subsequent validation of microsphere morphology and in vitro drug release kinetics to achieve a minimum of 24 h of treatment, up to 12-fold reduction in dosing frequency. The resulting microsphere formulation demonstrated a dramatic increase in stability and ionic drug-surface tension interactions with the gel carrier for sustained release of drug. Additional characterization including ex vivo corneal permeability studies, ocular irritation evaluation with organotypic models, and in vivo topical administration and retention further supported the use of this novel system for conveniently and effectively treating corneal cystinosis.

Materials and methods

Materials: All materials and reagents were obtained from Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified.

Preparation and characterization of spray-dried cysteamine PLGA microspheres

Spray dried cysteamine microspheres (SD-CMS) were fabricated using a Büchi B290 Mini Spray Dryer with a B29F Inert Loop (Büchi New Castle, Delaware, USA). Approximately 2 g of cysteamine hydrochloride and 8 g of 75:25 poly(DL-lactide-*co*-glycolide) (IV 0.14–0.22 dl/g, Mw: 4000–15,000) (Evonik Maryland, USA) in a co-solvent consisting of a methanol:dichloromethane (10:90,v/v) solution was used to generate a 5% cysteamine liquid feed. Büchi spray dry process parameters were set as follows: compressed nitrogen, flow meter (40 mm), aspirator (100%), inlet temperature (45 °C), atomizing gas flow (473 L/hr), feed rate (10% ml/min), and outlet

temperature range (32–35 °C). Samples were collected using a Standard Cyclone and Product Collection Vessel. Cysteamine free microspheres (SD-BLANK-CMS) were produced using the same fabrication process without the addition of cysteamine hydrochloride.

The shape and morphology of SD-CMS and SD-BLANK-CMS were examined using scanning electron microscopy (SEM). Samples were gold sputter-coated and imaged using a JEOL 6335F Field Emission SEM (JEOL, Peabody, MA, USA). The zeta potential of SD-CMS and SD-BLANK-CMS was determined using electrophoretic light scattering equipped in the Zetasizer Nano Series (Malvern, Westborough, MA, USA). SD-CMS and SD-BLANK-CMS were suspended in 10-mM potassium chloride (KCL) to achieve a 1% (w/v) ratio. A disposable folded capillary cell was filled with 0.75 mL of each suspension and measured at 37 °C.

Gel fabrication and thermal characterization with differential scanning calorimetry

Free radical polymerization of *N*-isopropylacrylamide (NIPAAm) was performed by adding 2 mL of an 0.5-mg/mL solution of ammonium persulfate (APS) in MilliQ water to 100 mg of NIPAAm monomer. 5 µL of tetramethylethylenediamine (TEMED) initiator was added to the solution mixture and polymerization proceeded for 12 h at 4 °C. Residual TEMED and APS were removed from the synthesized poly(*N*-isopropylacrylamide) (pNIPAAm) via repeated phase transition cycling ($T > 37$ °C) in excess MilliQ water. The purified polymer was flash frozen in liquid nitrogen, lyophilized for 48 h, and stored at 4 °C prior to rehydration. A 9 wt% (m/v) gel was prepared via rehydration of ~ 470 mg of lyophilized pNIPAAm in 4.7 mL of MilliQ water with 470 µL of polyethylene glycol (PEG MW 200 kDa) as an additive. Hydration of the gels proceeded for 3 days with intermittent mixing and centrifugation at 4 °C, 1000 RPM (106 RCF). Samples were stored at 4 °C until use.

After preparation, SD-CMS gel suspensions (SD-CMS/Gel) and SD-Blank-CMS gel suspensions (SD-BLANK-CMS/Gel) were prepared by weighing out respective microspheres at a ratio of 1 mg: 100 µL gel. The lower critical solution temperature (LCST) of gel, SD-CMS/Gel, and SD-Blank-CMS/Gel were determined via differential scanning calorimetry (DSC). DSC analysis was performed using a Perkin Elmer Pyris 6 calorimeter. Samples were heated in hermetically sealed aluminum pans from 2 to 50 °C at a rate of 2 °C/min under a nitrogen atmosphere with a flow rate of 20 mL/min. LCST values were calculated as the endothermic peak in the resulting DSC curves.

Evaluation of in vitro drug release kinetics

Detection of cysteamine with pre-column derivatization high-performance liquid chromatography

Cysteamine was detected by derivatizing with 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) and analyzed with a modified high-performance liquid chromatography (HPLC) method [20]. CMQT was synthesized according to previously published methods [21]. Reduction of reduced cysteamine (cystamine) with tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was performed prior to CMQT derivatization. Standard cysteamine sample aliquots (100 µL) were added to 400 µL of a 0.1 M, pH 7.5 phosphate buffer solution. Approximately 20 µL of 0.1 M TCEP was added to the solution mixture, reacted for 15 min, followed by 20 µL of 0.1 M CMQT, which reacted for 5 min. The reaction mixture was then acidified with 50 µL of 3 M hydrochloric acid. Approximately 20 µL of reaction sample was injected into an autosampler on a 1220 Infinity Liquid Chromatography (Agilent Technologies, California, USA) attached with a 1220 DAD Liquid Chromatography UV detector (Agilent Technologies, California, USA). A reverse-phase Zorbax SD-C18 column (5 µm, 4.6 × 150 mm; Agilent Technologies, California, USA) was used to separate molecules undergoing a gradient elution. The gradient elution consisted of mobile phases acetonitrile (A) and trichloroacetic acid pH 2.0 (B) at ratios: 0–3 min (12% A, 88% B), 3–9 min (30%A, 70%B), and 9–12 (12%A, 88%B) for 15 min at a flow rate of 1.2 mL/min. The column temperature was held at 25 °C. UV detector was set at 355 nm. Retention times of cysteamine-derivative and excess CMQT were 10.5 min and 11.3 min, respectively. The column was equilibrated for 5 min after each injection. Peak height from cysteamine-CMQT derivative was used from standard aliquots to create a standard curve over the range of 1 to 50 µg/mL.

Release studies on SD-CMS and SD-CMS/Gel suspensions

Cysteamine loaded microspheres (SD-CMS) and cysteamine-free microspheres (SD-BLANK-CMS), along with their corresponding gel suspensions (SD-CMS/Gel, SD-BLANK-CMS/Gel), were further characterized for in vitro drug release kinetics over 24 h. Known masses of SD-CMS were suspended in 0.1 M, pH 7.5 phosphate buffer solution (500 µL) in a 1.5 mL Eppendorf tube. Samples were placed in a rotator and incubator at 37 °C. For each predetermined time point, samples were spun down at 3500 RPM (106 RCF)

for 5 min, and supernatant was removed for analysis. Fresh phosphate buffer solution was added to the remaining MS, vortexed, and placed back onto rotator to maintain sink-like conditions. Cysteamine concentrations in phosphate buffer solution were analyzed using the previously described HPLC method. Gel suspension release kinetics were determined similarly, by mixing MS at a ratio of 10 mg:100 µL (MS:Gel) in a 1.5 mL Eppendorf tube, sampling supernatant at 37 °C as not to disturb microsphere/gel conformation, replacing supernatant with fresh 37 °C phosphate buffer solution, and evaluating samples with HPLC.

Cysteamine microsphere stability studies

Quantification of cysteamine drug loading in PLGA microspheres

Cysteamine drug loading in PLGA microspheres was quantified using NMR spectroscopy. Reference spectra for PLGA quantification with NMR spectroscopy were implemented [22]. The ^{1}H-NMR spectra were obtained using a 500-MHz Bruker Avance III spectrometer at 293 K in deuterated dimethyl sulfoxide (DMSO-d6) with a sample concentration of 20 mg/mL. Spectra obtained from 32 scans were calibrated to the residual solvent peak at δ 2.50 ppm and processed with TOPSPIN™ software (Bruker, Billerica, Massachusetts, USA). Reference spectra of cysteamine, cystamine, and PLGA(Supplemental Fig. 1) were utilized as standards, where non-overlapping methylene proton resonances at δ 2.70 ppm (cysteamine), δ 3.10 ppm (cystamine), and δ 4.88 ppm (PLGA) were utilized to determine mass of cysteamine in MS, drug-loading, and % cysteamine according to Eqs. 1, 2., and 3, respectively.

$$\text{Mass of CYS in MS (mg)} = \left(\frac{\text{MM}_{\text{CYS}} \cdot \frac{\text{I}_{\text{CYS}}}{\text{P}_{\text{CYS}}}}{\left(\text{MM}_{\text{CYS}} \cdot \frac{\text{I}_{\text{CYS}}}{\text{P}_{\text{CYS}}}\right) + \left(\text{MM}_{\text{PLGA}} \cdot \frac{\text{I}_{\text{PLGA}}}{\text{P}_{\text{PLGA}}}\right)} \right) \times \text{Mass of MS (mg)} \quad (1)$$

where, MM_{CYS} is the molar mass of cysteamine, 77.15 g/mol; MM_{PLGA} is the molar mass of the PLGA repeat unit "LG," 126.0 g/mol; and I and P are the integral and number of protons, respectively.

$$\text{Drug} - \text{loading} \left(\frac{\text{ug}}{\text{mg}}\right) = \left(\frac{\text{Mass of CYS in MS (ug)}}{\text{Mass of MS (mg)}}\right) \quad (2)$$

$$\%\ \text{Cysteamine} = \left(\frac{\frac{I_{\text{cysteamine}}}{2}}{\frac{I_{\text{cysteamine}}}{2} + \frac{I_{\text{cystamine}}}{4}} \right) \times 100 \quad (3)$$

Cysteamine drug stability in MS and eyedrops

Cysteamine drug stability in SD-CMS and eyedrop formulations was monitored using NMR spectroscopy. SD-CMS formulations and a control cysteamine eyedrop solution [23] consisting of cysteamine hydrochloride (66 mg) in 15 mL deuterate water (D_2O) (Cabridge Isotope Laboratories Inc., MA, USA) with 0.01% benzalkonium chloride (1.5 mg) and 0.90% sodium chloride (135 mg) were evaluated at 4 °C and 25 °C, over a 7-week time period. The SD-CMS and eyedrop formulation samples were wrapped in aluminum foil and opened twice per day to simulate opening and closing of an eyedrop bottle. ^{1}H-NMR spectra were obtained bi-weekly using a 500-MHz Bruker Avance III spectrometer at 293 K. Approximately 1 mL aliquots of the D_2O-based eyedrop formulation were utilized for analysis, whereas 20 mg samples of SD-CMS were weighed out, dissolved in DMSO-d6, and analyzed within 10 min to capture the cysteamine:cystamine content within the SD-CMS. Spectra obtained from 32 scans were calibrated to the residual solvent peak at δ 2.50 ppm (DMSO-d6) and δ 4.80 ppm (D_2O) and processed with TOPSPIN™ software (Bruker, Billerica, Massachusetts, USA). Cysteamine content within each sample was determined by monitoring the non-overlapping methylene resonance of cysteamine and cystamine at δ 2.70 ppm (DMSO-d_6, Fig. 3a) or 2.85 ppm (D_2O; Fig. 3b) and δ 3.10 ppm (DMSO-d_6, Fig. 3a) or 3.05 ppm (D_2O; Fig. 3b), respectively. Percent cysteamine (%) was determined according to Eq. 3.

Ocular irritation studies

Conjunctival irritation study on the hen's egg test on chorioallantoic membrane

Fertilized white leghorn hen eggs (Moyer's Chicks, Quakertown, PA) were obtained and incubated for 9 days at 37 °C and 60% humidity. Eggs were placed pointy end facing down

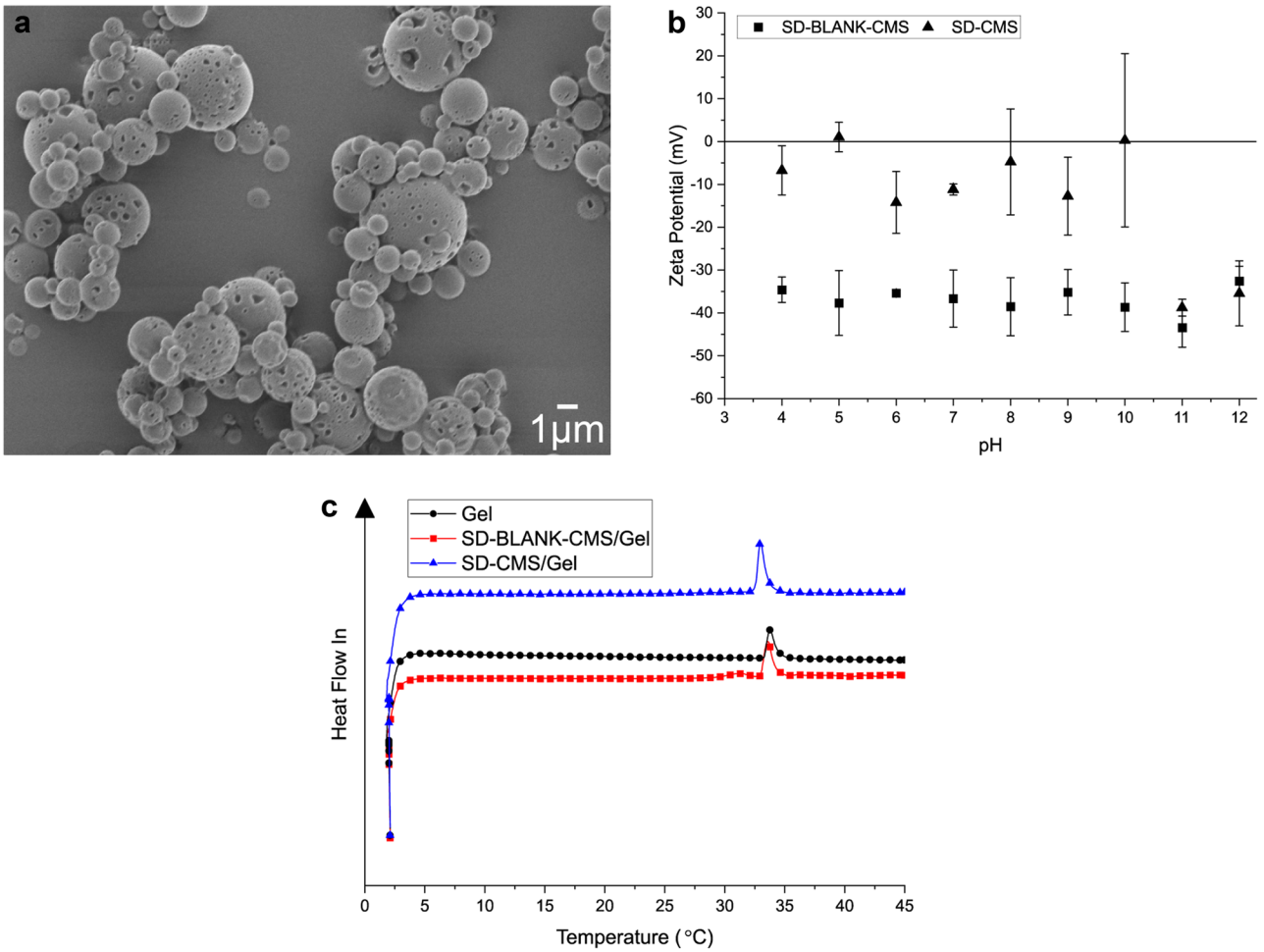

Fig. 1 **a** Scanning electron microscopy (SEM) of SD-CMS, **b** Zeta potential of microsphere formulations, and **c** determination of lower critical solution temperature (LCST) via differential scanning calorimetry (DSC). LCST of SD-CMS/Gel decreased to 32 °C from the base Gel at 34 °C

on egg holding racks and rotated twice a day manually. On the 6th day, eggs were candled using a candlar to observe embryo formation. Underdeveloped eggs were discarded. On the 9th day, eggs were removed from the incubator and were allowed to equilibrate to room temperature. A 1 cm radius circle was drawn on the top of the egg. A rotator tool (WEN Model #2305, Elgin, Illinois) with a cut-off wheel attachment was used to carefully cut through the eggshell, exposing a thin white membrane. Approximately 500 μL of 0.9% saline was added to the white membrane for 5 min. Curved forceps were used to remove the white membrane and expose the chorioallantoic membrane (CAM). The CAM was noted of any defects, and egg shell debris was removed carefully without damaging the CAM. An initial image of the CAM was taken using an inverted slit lamp (Eyephotodoc Fullerton, CA). For liquid controls, 300 μL of test material was placed on the CAM. For microspheres controls, 10 mg of material to 300 μL of 0.9% saline was placed on the CAM. For gel controls, 100 μL of material was placed. Similarly, SD-CMS/Gel and SD-BLANK/Gel suspensions were applied at a ratio of 10 mg:100 μL SD-CMS:Gel. Images at 30 s, 2 min, and 5 min were taken for each test material. Any signs of vascular hemorrhage, coagulation, and/or lysis (Supplemental Fig. 3) were recorded based on Eichenbaum et al. [24]. Endpoint effects were scored based on in vitro toxicology studies [25] and summarized in Supplemental Table 1.

Corneal irritation on the bovine corneal opacity test

Freshly enucleated bovine whole eyes were delivered on ice from Pel-freez Biologicals (Rogers, AR, USA) overnight, within 24 h of harvesting. Samples were inspected for corneal damage (scratches, cloudiness) or severe cataracts and were discarded from study. Viable bovine eyes were placed on aluminum dishes on top of 100 mL beakers filled with MilliQ water and placed in a 37 °C water bath. A silicon O-ring (RtDygert, Burnsville Minnesota) was placed on the

center of the cornea. Prior to application of testing materials, a 150 µL drop of 0.9% (w/v) saline was added to fill the O-ring. A Kim whip was used to blot off the saline and replaced with testing materials. Controls and test material masses and volumes were placed similar to that of the hen's egg test (HET)-CAM test. Corneas were exposed to materials for 30 s and subsequently rinsed off with 0.9% saline. Corneas post-exposure were incubated for 10 min and scored for corneal opacification and epithelial detachment. Epithelial integrity was determined by applying ophthalmic fluorescein strips (FluGlo, Akron Lakeforest,IL) and observed under blue cobalt light. Irritation scores were determined and categorized based on overall cumulative scoring [25], summarized in Supplemental Table 1 and Supplemental Fig. 3. These methods followed the OECD Test No. 437 guidelines for the testing of chemical eye irritants [26], which suggest a range of immediate exposure (30 s) to 4 h with longer exposure times suggested on case-by-case bases. As such, separate bovine eyes were observed for histology with H&E staining for 1, 3, and 8 h (Supplemental Figs. 4-6) following similar exposure times to procedures in a water bath [25].

Ex vivo corneal permeation studies

Trans-corneal permeation was studied using excised rabbit corneas (Pel-freez Biologics, Rogers, AR, USA) and Franz-type diffusion cells as previously described [23]. Rabbit whole eyes were shipped in Dulbecco's modified Eagle's medium with antibiotic and antimycotic per Pel-freez Biologics formulation, shipped overnight on ice within 24 h of harvesting. Whole eyes were evaluated for corneal damage with slit lamp imaging and discarded if present with superficial damage. Selected corneas were excised with iris type dissecting scissors with 1–2 mm of surrounding sclera tissue and were placed epithelium side down in 0.1 M, pH 7.5 phosphate buffer solution. The corneal medium and storage conditions have been previously reported [27] to support epithelial cell survival and reduce the likelihood of ultrastructure changes required for corneal transplants in humans [28]. Corneas were fitted on Franz cells (9 mm OD spherical joint interfaced with sclera, 5 mL receptor volume) with a 0.64 cm^2 corneal permeation area (PermeGear, Hellertown, PA, USA). The donor chamber was filled with 0.3 mL of cysteamine eyedrops (0.44% cysteamine hydrochloride, 0.01% benzalkonium chloride (1.5 mg) and 0.90% sodium chloride (135 mg), adjusted to pH 4.1–4.5 with 0.01 M hydrochloric acid), and 0.3 mL of SD-CMS/Gel at a ratio of 10 mg:100 µL of SD-CMS:Gel. The receptor chamber was filled with 0.1 M, pH 7.5 phosphate buffer solution (approximately 5 mL volume). The receptor chamber was continuously stirred at 600 RPM on a magnetic stir plate and kept at 37 °C. Approximately 200 µL of receptor solution was sampled hourly for a 5 h period and analyzed using previously described HPLC ($N = 3$). Blank assays were conducted similarly to test assays to verify interferences due to biological tissues in HPLC. Data are presented as mass of cysteamine permeated across a 64 cm^2 permeation area (µg/cm^2) as a function of time.

In vivo topical administration, retention, and preliminary safety in a rabbit model

Our study conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC Protocol #19096014, "Controlled release ocular cysteamine delivery"). New Zealand white rabbits (0.95–1.4 kg) were purchased from Envigo (formerly Covance Research, Somerset, NJ, USA).

The right eye of each subject ($N = 3$) was selected to undergo surgical resection of the nictiating membrane, a tissue that partially covers the surface of the rabbit eye, prior to treatment. This procedure has been previously described to be minimally invasive and safe with a healing time of 7 days [18, 19]. The nictitating membranes of the rabbit eyes were removed with a scalpel and cauterizing tool to excise the tissue and minimize bleeding. The rabbits were anesthetized intravenously via marginal ear vein by applying topical 5% lidocaine ointment (Amneal Pharmaceuticals LLC Bridgewater, NJ, USA) to the injection site 10 min prior to injection of 10 mg/kg of ketamine (Ketathesia; Henry Schein Animal Health, Dublin, OH, USA) and 1 mg/kg of xylazine (AnaSed Injection; Lloyd Laboratories, Shenandoah, IA, USA). To the eye, one topical drop of 0.5% proparacaine (Baush + Lomb, Bridgewater, NJ, USA) was administered before removal of the nictitating membrane. Following the procedure, one topical eyedrop of 0.3% tobramycin (Bausch + Lomb, Bridgewater, NJ, USA), and one topical eyedrop 1% prednisolone acetate (Pacific Pharmaceticals INC, Rancho Cucamonga, CA, USA) was immediately administered and repeated once daily for the following 4 days to prevent infection and limit inflammation.

The eyes of the rabbits were examined using slit-lamp photography (Eyephotodoc, Fullerton, CA, USA) throughout the study. After 7 days, one drop of 2% topical fluorescein ophthalmic suspension (Baush + Lomb, Bridgewater, NJ, USA) was administered and observed under cobalt light. After which, one 100 µL drop of SD-CMS/Gel (ratio 10 mg SD-CMS/100 µL Gel) was topically administered using a 1 mL syringe capped with a 200 µL pipette tip (see Fig. 6a), and placed in the inferior conjunctival cul-de-sac (see Fig. 6b) without local or systemic anesthesia. After 24 h, the rabbits were evaluated for eye health and gel retention via fluorescein drops and cobalt light imaging.

Statistical analysis

All in vitro characterization studies were completed in triplicate unless specified otherwise. For corneal permeation studies, a Mann-Whitney *U* test was used to compare the amount of cysteamine permeated through rabbit corneas from cysteamine formulations (alpha = 0.05) reported from Minitab 19 software (State College, PA).

Results

Microspheres and gel characterization

Characterization of microspheres using scanning electron microscopy (SEM) was performed to evaluate their outer pore morphology and approximate size. The zeta potential (ZP) of microspheres were determined as indicative evidence towards the surface potential and nature of surface charge (positive/negative). Upon fabrication, SEM, and ZP characterization, microspheres were combined into gel (pNIPAAm and PEG) and analyzed for thermal properties with differential scanning calorimetry (DSC) to ensure the lower critical solution temperature (LCST) of the combined system was similar to ocular surface temperature ranges (32–34 °C) [29]. These measurements were taken prior to drug release assays to verify that the fabrication methods resulted in thermal properties compatible with the intended clinical use.

Figure 1a shows representative SEM images of spray-dried cysteamine loaded microspheres (SD-CMS). SEM images represented spherical microspheres with a relative size of 2 µm, supporting microsphere fabrication with no appearance of free, unloaded cysteamine crystals. The ZP of SD-CMS and SD-BLANK-CMS was compared in Fig. 1b. Samples were measured at a range of pH (4–12) to compare surface potentials. SD-CMS ZPs were determined at pH 4–10 (between − 14.21 ± 7.235 mV and 0.29 ± 20.213 mV) and pH 11–12 (− 38.76 ± 1.939 mV and − 35.43 ± 7.569 mV). SD-BLANK-CMS ZPs were determined at pH 4–12 (between − 43.46 ± 4.556 mV and − 32.6 ± 3.469 mV). The differences in ZPs between SD-CMS and SD-BLANK-CMS support the inclusion of cysteamine ions within the fabrication process of SD-CMS. Having confirmed microsphere fabrication and characterization, the next step was to determine the LCST of our microsphere formulations and gel. Figure 1c presents differential scanning calorimetry thermograms of gel (no microspheres), gel with unloaded, cysteamine-free microspheres (SD-BLANK-CMS/Gel), and gel with cysteamine loaded microspheres (SD-CMS/Gel). The thermograms confirm the LCST (°C) as endothermic transitions observed during the heating phase. The LCST for the gel formulation was determined to be 33.9 °C, whereas the LCST of the gels with cysteamine and cysteamine-free microspheres was 32.0 and 33.6 °C, respectively. These measures confirm the ability for our drug delivery system to be administered below their LCST as an aqueous gel, and when applied to the ocular surface and eyelid will transition to a pliable drug delivery system.

In vitro release studies

Having confirmed the fabrication of SD-CMS and thermal properties of our gel-microsphere delivery system, the next

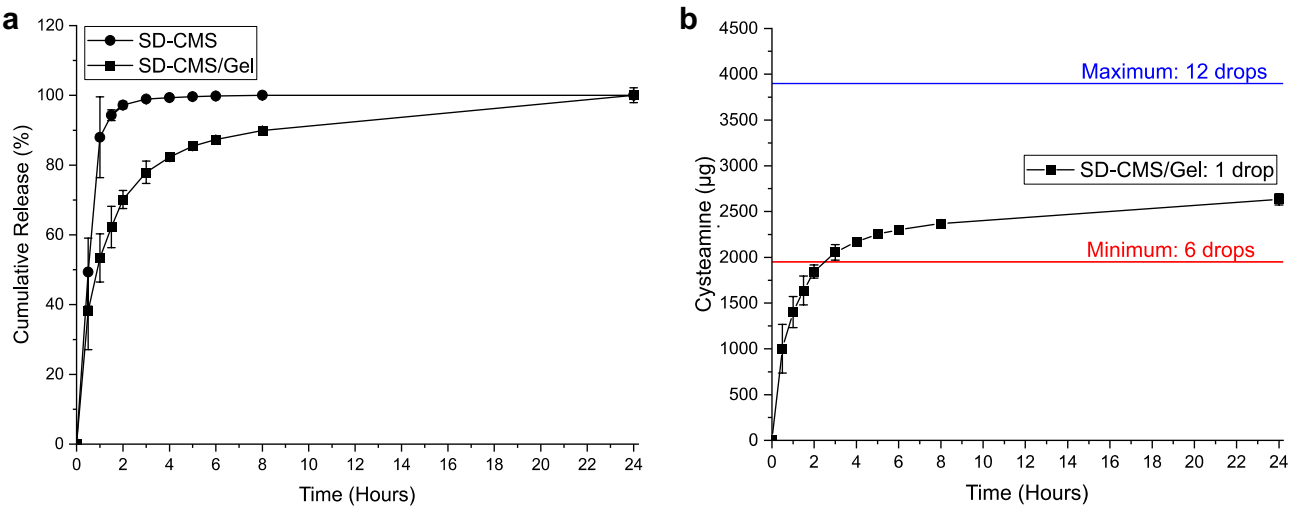

Fig. 2 **a** Cumulative in vitro release of cysteamine from PLGA microspheres ($n = 3$) and microspheres in gel ($n = 3$). **b** Cysteamine release total from microsphere in gel system ($n = 3$) relative to maximum and minimum clinically recommended cysteamine eyedrop [31]

step was to determine the release profile of cysteamine from the SD-CMS and SD-CMS/Gel. In vitro cumulative release of cysteamine from a known mass of microspheres alone and microspheres within our gel for 24 h was compared in Fig. 2a. Analysis of percentage of total amount of cysteamine encapsulated was reported with the goal of verifying potential changes in release kinetics from our gel. Figure 2b present cysteamine release totals from our combined system relative to the amount of cysteamine delivered to the eye at the clinically recommended frequency, with a minimum 6 drops (1950 µg cysteamine) and maximum 12 drops (3900 µg cysteamine) daily (see Supplemental Table 2 for mass calculation) [9, 30]. As expected, cysteamine release from our system within 24 h was above the lower limit of cysteamine delivery with an average cumulative release of 2633.08 ± 61.797 µg/day released over 24 h. Depletion of cysteamine from microspheres over 24 h was also verified using differential scanning calorimetry (Supplemental Fig. 2) by evaluating the melting point of cysteamine (66.7 °C) after 5 days of release.

Stability profile of cysteamine eyedrops and cysteamine microspheres

Cysteamine in eyedrop formulations has been well reported to be chemically unstable as it readily oxidizes to therapeutically inactive cystamine, the dimer of cystamine [3, 9, 10]. Therefore, stability analysis of cysteamine in eyedrops and microspheres was evaluated at 25 °C and 4 °C with repeated opening and closing to introduce oxygen, much like the clinical use of the current cysteamine eyedrops. Figure 3 shows the stability of cysteamine (%) in an eyedrop formulation ($n = 3$) at 1 week at 4 °C with 92.5% ± 1.84% cysteamine, and 25 °C with

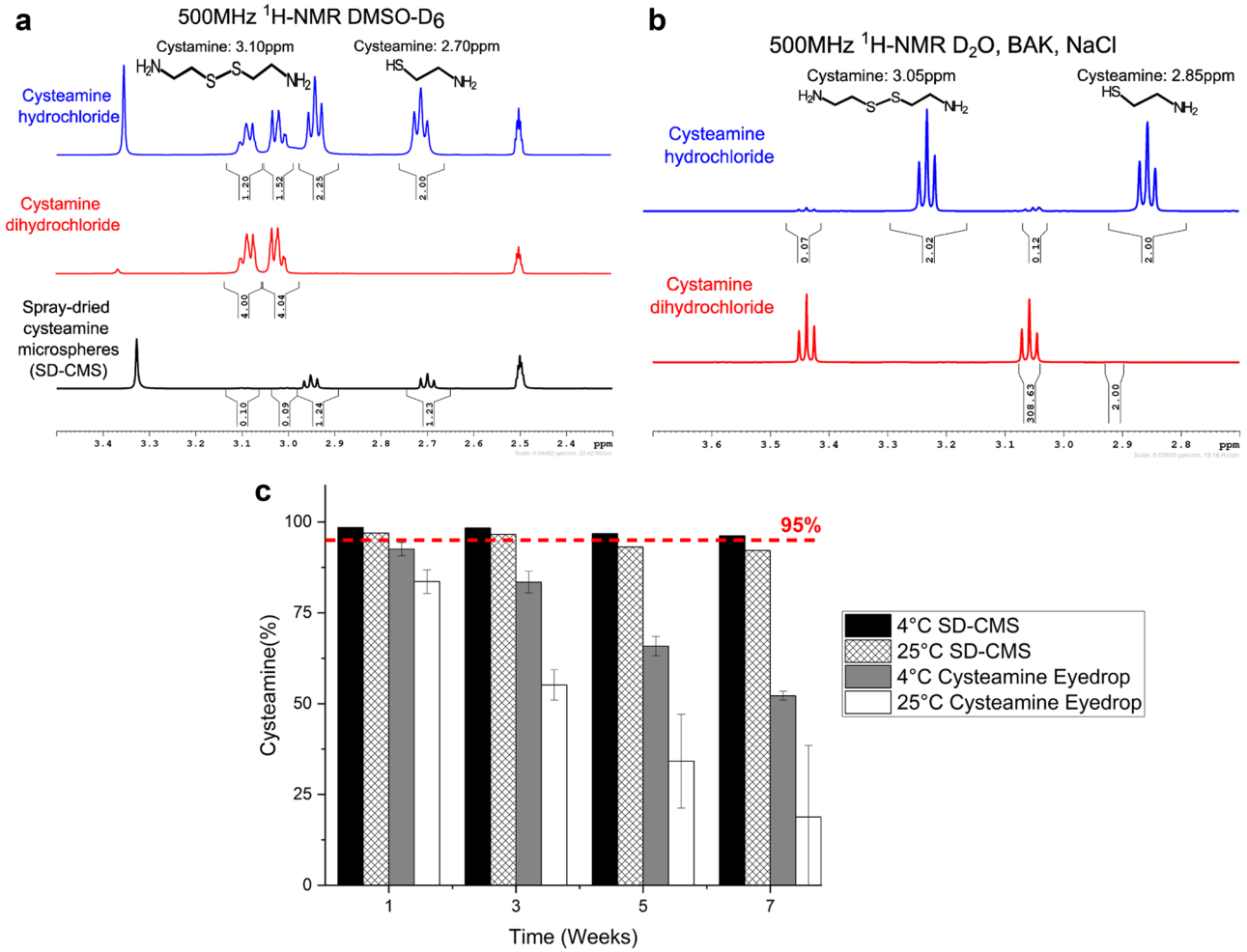

Fig. 3 **a** ^{1}H-NMR of cysteamine microspheres (SD-CMS) in deuterated dimethyl-sulfoxide (DMSO-D_6) with cysteamine (2.70 ppm) and cystamine (3.10 ppm) reference peaks. **b** ^{1}H-NMR of cysteamine eyedrops with benzalkonium chloride (BAK) and sodium chloride (NaCl) in deuterated water (D_2O) with cysteamine (2.85 ppm) and cystamine (3.05 ppm) reference peaks. **c** Stability profile of cysteamine eyedrops and SD-CMS over 7 weeks as quantified by ^{1}H-NMR for active cysteamine (%). Cysteamine eyedrops ($n = 3$) fell below 95% (indicated by red dashed line) at 1 week, 4 °C to 92.5% ± 1.84% cysteamine

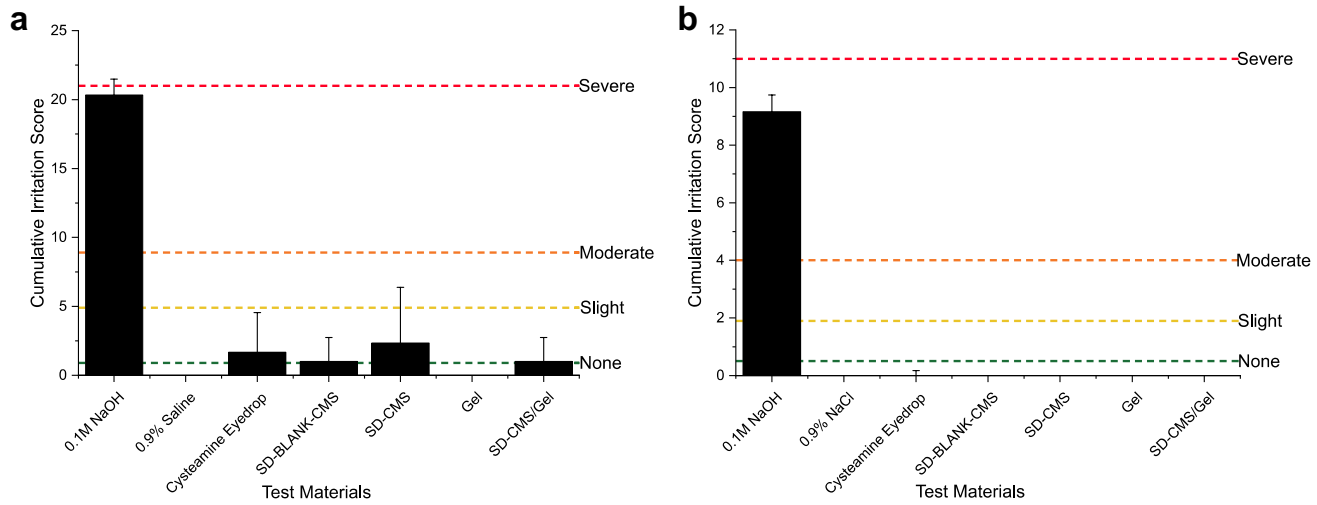

Fig. 4 **a** Cumulative HET-CAM scores of controls, cysteamine microsphere formulations, and gel suspensions ($N = 3$). **b** Cumulative BCOP scores of controls, cysteamine microsphere formulations, and gel suspensions ($N = 3$)

83.58% ± 3.25% cysteamine. Stability of cysteamine in microspheres ($n = 1$) at 7 weeks at 4 °C with 96.2% cysteamine and at 5 weeks at 25 °C with 93.1% cysteamine. Cysteamine encapsulated in PLGA microspheres remained in its therapeutic, effective form for 7 weeks, an improvement from cysteamine eyedrops, in which is stable for 1 week. Further, as seen in Supplemental Fig. 1, we can track the ratio of degradation by independently quantifying unique resonance for cysteamine ($\delta = 2.70$ ppm) and cystamine ($\delta = 3.10$ ppm) via ^{1}H-NMR.

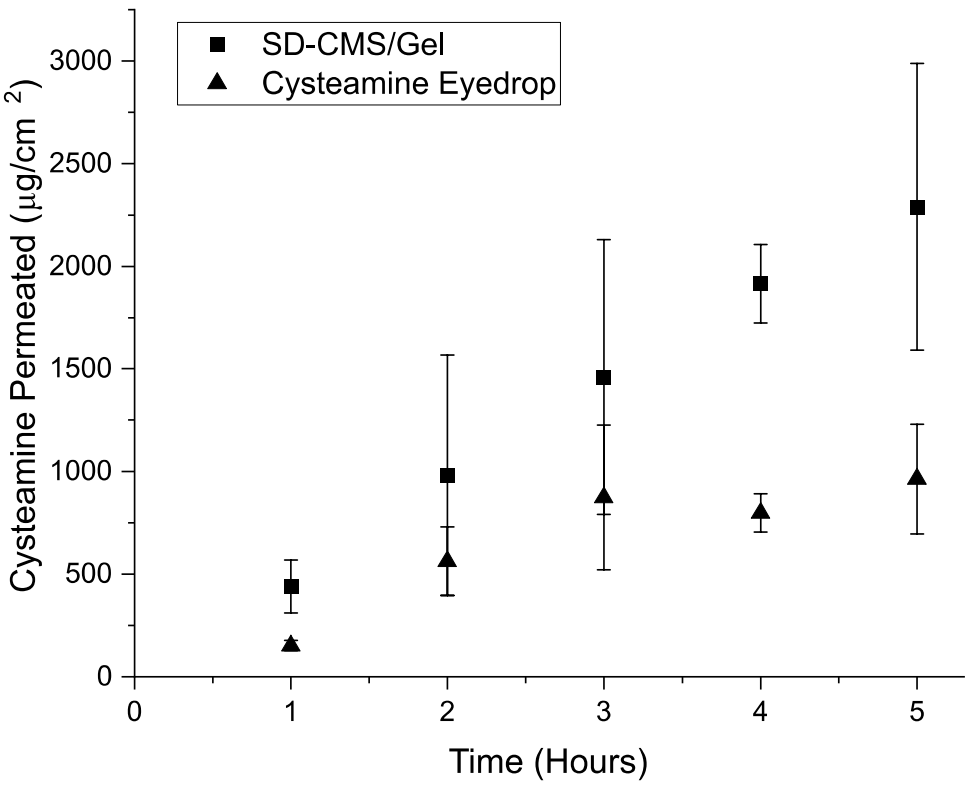

Fig. 5 Comparison of cysteamine permeation from cysteamine eyedrop and cysteamine microspheres in gel (SD-CMS/Gel) in a Franz diffusion cell mounted with a freshly excised rabbit cornea. Cysteamine was quantified using high-performance liquid chromatography. A Mann-Whitney *U* test was performed for each timepoint comparing formulations ($p = 0.05$, no statistical significance reported)

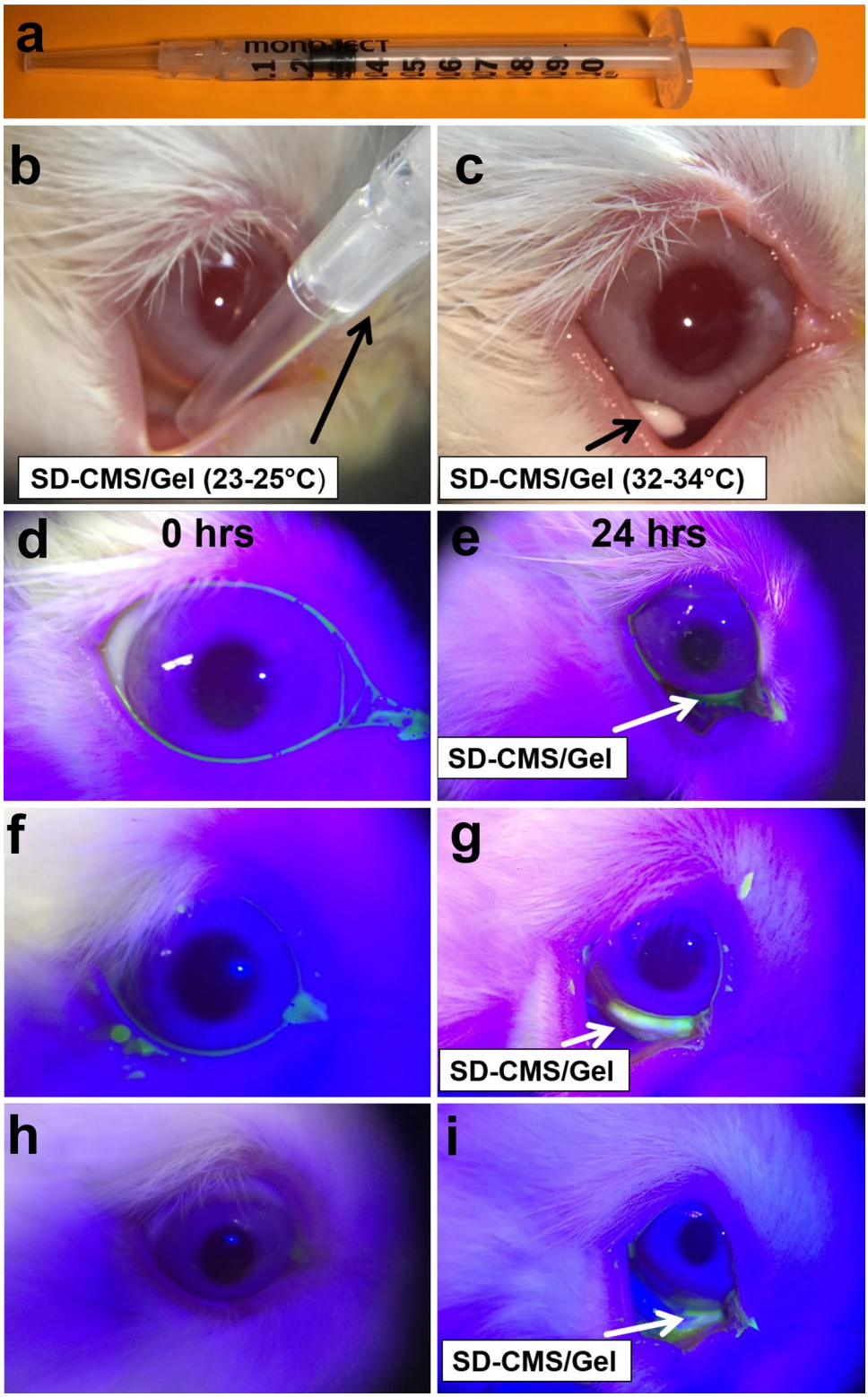

Fig. 6 **a** Administration of SD-CMS/Gel was achieved by utilizing a 1-mL syringe containing SD-CMS/Gel with a 200-µL pipette tip. **b** The delivery system was administered at room temperature (23–25 °C) in the lower eyelid. **c** The delivery system transitioned into an opaque gel at ocular surface temperatures (32–34 °C). Preliminary safety exhibited no corneal damage as recorded by cobalt light imaging and fluorescein staining prior to administration **d**, **f**, **h** and after the gel was retained for 24 h **e**, **g**, **i**

In vitro ocular irritation studies

Previously developed organotypic ocular irritation models [32, 33] that are sensitive to irritating chemicals materials were used to evaluate the potential irritation of our materials and cysteamine eyedrops. Figure 4a represents cumulative HET-CAM irritation scores indicating that cysteamine eyedrops (n = 3, 1.67 ± 2.86) and cysteamine loaded microspheres

in gel ($n = 3$, 1.00 ± 1.73) had no irritation when compared with positive controls with severe irritation (0.1 M NaOH, $n = 3$, 20.33 ± 1.15) and negative controls with no irritation (0.9% saline, $n = 3$, 0 ± 0 None). The microsphere formulations with no cysteamine (SD-BLANK-CMS, $n = 3$, 1 ± 1.73) and cysteamine loaded (SD-CMS, $n = 3$, 2.33 ± 4.04) when applied as dry powders were categorized as no irritation and slight, respectively. Bovine Corneal Opacity (BCOP) results presented in Fig. 4b show similar findings with all materials falling within the no irritation category when compared with severely irritating positive controls of 0.1 M NaOH. These findings along with histology evaluations over 1, 3, and 8 h (Supplemental Figs. 4-6) indicate that our materials have no irritation and the combined cysteamine loaded microspheres and gel is likely to be tolerated as an eyedrop when exposed to approximately 85% (8 h) of cumulative cysteamine release (Fig. 2a).

Franz cell corneal permeation studies

Cysteamine permeation through excised rabbit corneas in a Franz diffusion cell was investigated in our cysteamine microspheres/gel (SD-CMS/Gel) delivery system and compared with cysteamine eyedrops. We selected rabbit corneas for direct translation of our delivery system to our previous in vivo studies for glaucoma [18] and endophthalmitis [19], which suggest physiological and anatomical similarities to humans. Figure 5 presents cysteamine permeation data for each formulation for 5 h. We observed no significant difference in cysteamine permeation between cysteamine eyedrops (and SD-CMS/Gel). The resulting cysteamine permeation findings indicate cysteamine delivery from SD-CMS/Gel was readily absorbed through the cornea.

In vivo retention studies

The eyes of New Zealand white rabbits were surgically modified to anatomically resemble human eyes following safe surgical procedures and conventional after care. After monitoring eye health for 7 days, without anesthesia, our topical drug delivery system was administered to the lower eyelid at room temperature (Fig. 6b) and transitioned upon administration at ocular surface temperatures (Fig. 6c). For each subject ($N = 3$), one drop of 2% fluorescein was topically applied prior to SD-CMS/Gel administration (Fig. 6d, f, h) and 24 h after (Figs. 6e, g, i) to assess corneal health and retention. All subjects ($N = 3$) retained our drug delivery system at a volume of 100 µL (SD-CMS/Gel ratio 10 mg/100 µL) for 24 h. Cobalt light and fluorescein staining suggest no corneal or conjunctival damage.

Discussion

The high instability of cysteamine in aqueous solutions is one of the greatest challenges in development of therapies for corneal crystals in cystinosis. Due to its unstable nature, clinical data regarding safety and efficacy required hourly cysteamine eyedrops to reduce corneal crystals—leading to the acceptance of a clinical standard of 6–12 drops daily [3, 9]. Often, administration of cysteamine eyedrops requires caregivers for pediatric patients further adding to the burden of therapy. While caregivers become experts in the clinical management of cystinosis, the irritation associated with disease and cysteamine eyedrop administration can lead to patients tolerating one drop per day, resulting in noncompliance. For these reasons, the area of controlled release with encapsulated materials may afford localized delivery of cysteamine that effectively treats cystine crystals in cystinosis patients' eyes, with a reduction in frequency of administration.

As seen in studies on the reformulation of cysteamine eyedrops in to controlled release systems, cysteamine release was extended to a few hours [11, 12, 14, 15] to a maximum of 24 h [13]. The small size and hydrophilicity of cysteamine may contribute to the difficulties in controlling the rate of diffusion and drug loading of cysteamine from these systems. Such was the case when designing a PLGA microsphere with a drug loading equivalent to the range of cysteamine delivered to achieve crystal reduction in patients. Achieving the proposed amount of cysteamine in traditional emulsion (single and double emulsions) based techniques as recommended by the literature [34] proved to be challenging with an ultra-small, hydrophilic, chemically unstable thiol.

Extensive efforts in microsphere formulation development and fabrication were conducted to achieve drug release magnitudes at the recommended amount of cysteamine equivalent in the range of 6–12 eyedrops/day [9]. Total amount of cysteamine delivered from standard cysteamine eyedrops is the current clinical parameter for formulation development as the field lacks critical in vivo ocular pharmacokinetic data to determine efficacious cysteamine absorption amounts. Therefore, at a 0.05-mL dose volume of a 6.5-mg/mL cysteamine hydrochloride solution [30], a total of 0.325-mg (325 µg) cysteamine is delivered per drop. When scaled to the recommended frequency 6–12 drops/day, a minimum to maximum range is equivalent to 1950–3900 µg cysteamine (see Supplemental Table 2).

Several iterations of microsphere formulations were characterized and analyzed until ultimately, emulsion strategies were determined to be incompatible for encapsulation of cysteamine. As an alternative, removing potential interactions with larger volumes of aqueous solvents and thereby overcoming the hydrophilic nature of cysteamine, spray-dried

fabrication of cysteamine achieved spherical microspheres (Fig. 1a). Inclusion of cysteamine ions from fabrication was indicated by differences in zeta potential magnitude and nature of surface charge (positive/negative) between SD-CMS and cysteamine free SD-BLANK-CMS (Fig. 1b). The presence of these ions slightly lowered the LCST after mixing in topical pNIPAAm gel (Fig. 1c). We observed 100% cysteamine depletion from PLGA microspheres within 2 h and extension of release from gel (SD-CMS/Gel) to 8–24 h (80%-100%). During sampling, the SD-CMS/Gel supernatant was sampled in a 37 °C water bath to prevent reversible transition and disruption of the microsphere-gel conformation., Cysteamine loading and delivery within daily minimum and maximum cysteamine mass delivery were achieved following our methods (Fig. 2 a and b).

The mechanism of release from our combined delivery system (SD-CMS/Gel) is based on diffusion from PLGA microspheres and extended in our pNIPAAm/PEG gel by potential molecular interactions of cysteamine ions between polymer-microsphere networks, attributed to a change in LCST. The LCST property of pNIPAAm has been tailored to achieve different temperature ranges, with studies exploring the lowering effect on LCST by adding sodium salt ions following the Hofmeister series [35, 36]. One study confirmed three mechanisms: surface tension effect, direct anion binding, and polarization of water molecules directly to hydrophobic portions of pNIPAAm (isopropyl groups and hydrocarbon backbone) [36]. In our zeta potential characterization of SD-CMS and SD-BLANK-CMS (Fig. 1b), we determined a difference in ZP magnitude at pH 4–10 between microspheres with cysteamine (SD-CMS) and cysteamine-free microspheres (SD-BLANK-CMS). When included into pNIPAAm/PEG gel, SD-CMS had a lowering effect on LCST (from base pNIPAAm gel 34 °C to 32 °C). The changes in zeta potential and lowered LCST may be attributed to the presence of cysteamine ions during the spray-dried fabrication process of cysteamine hydrochloride and PLGA. The inclusion of cysteamine ions and a potential increase in surface tension from SD-CMS into pNIPAAm is likely to delay the release of cysteamine as increased molecular interactions may occur through the microsphere-polymer network. These experiments and our mechanistic insights represent the first cysteamine release kinetic profile from PLGA microspheres (Fig. 2a) and PLGA microspheres incorporated in thermoresponsive gels (Fig. 2a and b). Alone, SD-CMS release kinetics are similar to other spray-dried microsphere formulation [37]. While release occurs faster than what is typically noted in the literature for PLGA microspheres, [38] these results represent a 12-fold decrease in daily cysteamine eyedrop administration.

We further present the first long-term stability profile of encapsulated cysteamine in a direct comparison with traditional eyedrops using ^{1}H-NMR spectroscopy. This method is unique compared with other detection methods [15] because it can detect both cysteamine and cystamine. Further, our stability study took into account different storage conditions (4 °C and 25 °C), which was not incorporated in other studies [15, 23]. We also performed repeated opening and closing as suggested by a study comparing cysteamine eyedrop stability under inert gas [39]. The stability profile for standard cysteamine eyedrops (Fig. 3c) demonstrates a temperature dependency of degradation behavior and a level below 95% after 1 week, as expected [9, 23]. A possible explanation for the poor aqueous stability of cysteamine is the pH influence of degradation into cystamine [23]. This study compared cysteamine stability in aqueous solutions at pH 7.4 and pH 4.0–4.2 and found that values of pH 7 oxidized more quickly due to the presence of ionized thiol groups. By encapsulating the drug, the influence of aqueous pH within the microspheres is minimized. We do, however, observe a temperature dependence of cysteamine degradation in microspheres, which is similar to previous studies [23]. Still, cysteamine entrapped within microspheres is stable for up to 5 and 7 weeks at 25 °C and 4 °C, respectively. This result represents a significant improvement over current storage methods, which greatly contribute to the overall inconvenience of current cysteamine eyedrops. These results further suggest that the cysteamine loaded in our microspheres is likely to release from the hydrogel delivery system to the cornea in its active form upon administration. Ongoing studies regarding the stability of cysteamine after gamma sterilization suggest a change in 3% cysteamine ($N = 10$) after storage, packaging under a nitrogen gas glove box, and shipping (see Supplemental Table 3). Future studies will confirm the efficacy of stable cysteamine from ocular gel administration in a mouse model of cystinosis [40].

Confirmation of our target LCST via DSC (Fig. 1c) was performed and was comparable with previous LCST measurements of our gels administered in vivo [18, 19]. Our in vivo studies show the ease of administration, safety, and tolerability of our delivery system in a rabbit model (Fig. 6b–i) which is further supported in our previous studies [18, 19] for anti-glaucoma and antimicrobial ocular drug delivery. As with previous testing of the gel eyedrop, irritation testing on models for conjunctiva (HET-CAM; Fig. 4a) and cornea (BCOP; Fig. 4b) showed negligible to mild irritation levels. This agrees well with previous reports for cysteamine [23, 33]. These data suggest that the irritation upon administration in patients may be partially due to the underlying disease which causes corneal sensitivity and corneal epithelial erosion. Hourly administration of traditional eyedrops containing the preservative benzalkonium chloride (BAK) is likely a major contributing factor to ocular irritation, which can be mitigated with our preservative-free delivery system using 1 drop versus 12 drops. These organotypic models, current in vivo

studies, and previous long-term in vivo glaucoma study [18] support the potential use of our materials for cysteamine delivery as a non-irritating formulation. Repeated dosing and ocular pharmacokinetics will be investigated in future studies comparing our delivery system with cysteamine eyedrops in our large animal model. Data from previous human clinical trials [12, 31, 41] will guide our studies, which suggest no severe adverse effects (redness expanding 50% of the conjunctiva, pain affecting daily activities, and vision loss). We will focus on the local transient effects (stinging, burning, redness) observed in these studies that lasted less than 1 h from viscous gel formulations dependent on cysteamine concentration when administered 4 times daily in an open-label phase III 4-year study. Despite these local transient effects, patients expressed their preference for a 4 times daily formulation over an eyedrop after a *Comparisons of Ophthalmic Medications for Tolerability* questionnaire [12, 31].

These studies will be of particular importance as there is currently limited knowledge regarding cysteamine absorption through the cornea. The in vitro permeability studies presented herein demonstrated a trend of higher absorption after 5 h compared with standard drops. Pescina et al. [23] demonstrated that permeation enhancers were required to improve permeability compared with standard eyedrops, in contrast to our delivery system. The group further investigated pH dependence of cysteamine diffusion in aqueous formulations (without permeation enhancers) which resulted in a preference for higher permeation at pH 7.4 versus 4.2. This analysis offers an explanation for cysteamine permeation from our cysteamine delivery system, which has a pH 8.6 prior to thermal transition, compared with cysteamine eyedrops (pH 4.2). We aimed to limit variability in our samples by assessing the quality of corneas upon excision and using commercially manufactured Franz diffusion cells; however, validation studies on static Franz cells have indicated inadequate mixing in the side arm as influences to variability during sampling from receptor volumes [42], which may have contributed to our permeation variability. Overall, these data suggest that cysteamine is likely to be absorbed to the cornea upon administration and tolerated similar to alkaline hypromellose 0.3% lubricant eyedrops (pH 8.34) [43] for chronic dry eye, [44], which will be furthered explored in the aforementioned planned in vivo studies with improved tissue cysteamine detection via mass spec [45].

Conclusion

In this study we present the development and characterization of a thermoresponsive controlled release microsphere/gel cysteamine delivery system as an eyedrop formulation for corneal crystals in cystinosis. Spray-dried encapsulation of cysteamine into microspheres dramatically improved the stability of cysteamine and achieved drug loading and release from gel equivalent to a daily course of drug administration (representing a 12-fold decrease in dosing frequency). The formulation was shown to be non-irritating in vitro with more cysteamine permeated from our formulation compared with traditional eyedrops. A daily course of our therapy was administered topically without anesthesia and retained in vivo. Future studies of this delivery system will investigate the pharmacokinetic profile in large animals and efficacy of the delivery system in the only cystinosis animal model, a knockout of the CTNS gene in mice.

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Authors' contributions Conceptualization: Jorge Jimenez, Ken K. Nischal, Morgan V. Fedorchak; Methodology: Jorge Jimenez, Michael A. Washington; Validation: Jorge Jimenez, Michael A. Washington; Investigation: Jorge Jimenez, Michael A. Washington, Jayde L. Resnick; Formal Analysis: Jorge Jimenez; Writing – Original Drat: Jorge Jimenez; Writing—review and editing: Morgan V. Fedorchak; Supervision: Morgan V. Fedorchak; Funding acquisition: Morgan V. Fedorchak.

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Data availability There are no linked research data sets for this submission. The data are available on request due to privacy.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval All institutional and national guidelines for the care and use of laboratory animals were followed.

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