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Synergistic Cysteamine Delivery Nanowafer as an Efficacious Treatment Modality for Corneal Cystinosis

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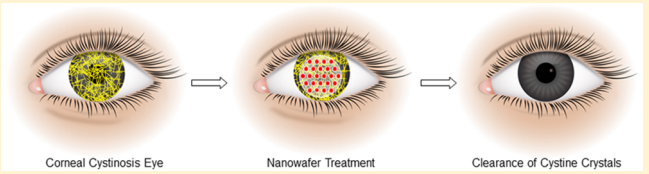
Supporting Information

ABSTRACT: A synergy between the polymer biomaterial and drug plays an important role in enhancing the therapeutic efficacy, improving the drug stability, and minimizing the local immune responses in the development of drug delivery systems. Particularly, in the case of ocular drug delivery, the need for the development of synergistic drug delivery system becomes more pronounced because of the wet ocular mucosal surface and highly innervated cornea, which elicit a strong inflammatory response to the instilled drug formulations. This article presents the development of a synergistic cysteamine delivery nanowafer to treat corneal cystinosis. Corneal cystinosis is a rare metabolic disease that causes the accumulation of cystine crystals in the cornea resulting in corneal opacity and loss of vision. It is treated with topical cysteamine (Cys) eye drops that need to be instilled 6−12 times a day throughout the patient's life, which causes side effects such as eye pain, redness, and ocular inflammation. As a result, compliance and treatment outcomes are severely compromised. To surmount these issues, we have developed a clinically translatable Cys nanowafer (Cys-NW) that can be simply applied on the eye with a fingertip. During the course of the drug release, Cys-NW slowly dissolves and fades away. The *in vivo* studies in cystinosin knockout mice demonstrated twice the therapeutic efficacy of Cys-NW containing 10 μg of Cys administered once a day, compared to 44 μg of Cys as topical eye drops administered twice a day. Furthermore, Cys-NW stabilizes Cys for up to four months at room temperature compared to topical Cys eye drops that need to be frozen or refrigerated and still remain active for only 1 week. The Cys-NW, because of its enhanced therapeutic efficacy, safety profile, and extended drug stability at room temperature, can be rapidly translated to the clinic for human trials.



KEYWORDS: *Nanowafer, drug delivery, corneal cystinosis, cysteamine*

INTRODUCTION

Polymer biomaterials play an important role in the development of drug delivery systems, implants, and matrixes for tissue repair and regeneration.[1−3] In particular, a synergy between the polymer biomaterial and the drug will enhance the therapeutic efficacy of the drug delivery system in disease treatment.[4] In ocular drug delivery, because of the wet mucosal surface and highly innervated cornea eye is very sensitive and the ocular surface generates a rapid and strong inflammatory response to the foreign materials invasion.[5,6] Hence, choosing the right polymer and drug combination that elicit negligible immunological and inflammatory responses is crucial for the development of synergistic drug delivery systems.[7−9] In this article, we describe the development of a cysteamine delivery nanowafer (Cys-NW) to treat corneal cystinosis in a cystinosin knockout mouse model ($Ctns^{-/-}$). In Cys-NW, the drug and the polymer are in synergy to induce minimal inflammatory responses, improve drug stability, and enhance the therapeutic efficacy.

Corneal cystinosis is a rare metabolic disease that causes cystine to accumulate in cells because of the defective transport across the lysosomal membrane into the cytoplasm.[10,11] Thus, accumulated cystine, a disulfide amino acid, crystallizes in the cornea.[12] It begins in infancy, and by the time the patient reaches 6−8 years of age, the symptoms begin to appear, such as corneal lesions, ocular inflammation, and photophobia, which affect the patient's quality of life to such an extent that a slight glimmer of sunlight can be debilitating. In later stages, it manifests into corneal opacification, erosion, keratitis, and eventual blindness.[13]

Presently, corneal cystinosis is treated with topical cysteamine hydrochloride (Cys) eye drops.[14−19] The oral formulation of Cys cannot reach the cornea because of its lack of vasculature, and has negligible therapeutic effect on dissolving the corneal crystals.[20] Cys is a highly water-soluble small molecular drug. It cleaves the disulfide bond in cystine to form a lysine-like cysteine−cysteamine mixed disulfide, which will be cleared from the tissue.[21−23] Topical Cystaran

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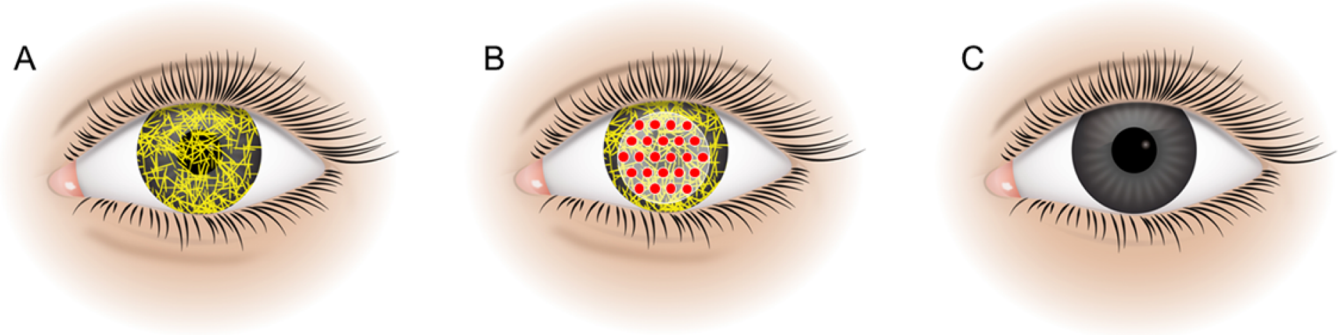

Figure 1. Cys delivery nanowafer as an efficacious treatment modality for corneal cystinosis. A schematic diagram depicting (A) a corneal cystinosis eye, (B) placement of a Cys-NW on the eye, and (C) clearance of corneal crystals after nanowafer treatment.

ophthalmic solution is the only medication approved by FDA for the treatment of corneal cystinosis.[24] Eye drops are rapidly cleared from the ocular surface due to reflex tearing, constant blinking, and nasolacrimal drainage, and have only a short contact time on the eye. Typically, less than 5% of the drug can penetrate through the ocular epithelium.[25,26] Hence, it is very difficult to maintain a therapeutically effective concentration of Cys in the eye for sufficient time to dissolve the cystine crystals. As a consequence, Cys eye drops need to be administered every hour while awake or at least 6−12 times in a day to be therapeutically effective, which is both inconvenient and impractical for school-going children and working adults.[27,28] Because of the continuous and prolonged usage of Cys eye drops, patients develop inflammation and ulcers in the eyes. The pungent smell of cysteamine also contributes to non-compliance and severely compromised treatment outcomes. Another major issue with topical Cys eye drops is its rapid oxidation in aqueous solutions to form a therapeutically inactive disulfide cystamine.[29,30] Hence, eye drop bottles need to be stored frozen. Once a bottle is opened, the drug is effective for only 1 week, after which it must be discarded.[31] This results in a high cost of treatment. To surmount these issues, we have developed a cysteamine nanowafer (Cys-NW) for the efficacious treatment of corneal cystinosis (Figure 1).

EXPERIMENTAL SECTION

Materials for Nanowafer Fabrication and Drug Release Studies. Poly(vinyl alcohol) (MW 146,000, 87−89% hydrolyzed), cysteamine hydrochloride, HPLC grade methanol, and red fluorescent quantum dots (6 nm) were obtained from Sigma-Aldrich, St. Louis, MO. Slide-A-Lyzer/mini dialysis units (MWCO 2000) were obtained from Pierce Biotechnology (Rockford, IL).

In Vivo Studies. All the experimental protocols involving mice were approved by the Institutional Animal Care and Use Committee prior to the initiation of this study. Healthy female C57BL/6 (8−10 weeks old) mice were used for tear collection and quantum dot (QD) diffusion experiments. Both genders of cystinosin knockout mice with C57BL/6 background ($Ctns^{-/-}$) were used for drug delivery experiments. Mice were randomly assigned to each experimental group.

Nanowafer Fabrication. Cys-NW was fabricated via hydrogel template strategy.[32−35] Briefly, a silicon wafer containing arrays of square wells (500 nm × 500 nm and 500 nm deep) was fabricated by e-beam lithography and was used to prepare poly(dimethylsiloxane) (PDMS) imprints containing vertical posts that were 500 nm × 500 nm and 500 nm in height. Poly(vinyl alcohol) (5 g in 100 mL water, 5% w/v) was poured onto the PDMS imprint and placed in an oven at 70 °C for 30 min to form a PVA wafer containing arrays of wells. These wells were filled with a thick solution of Cys (Sigma-Aldrich, St. Louis, MO) and PVA. Cys (10 mg) was dissolved in 1 mL of PVA solution (5%) and used for filling the nanowafers. The Cys-PVA solution (200 μL) was transferred with a pipet onto a dry PVA wafer containing nanoreservoirs facing up (previously peeled from the PDMS template). Then, the thick solution was swiped swiftly across the wafer using a razor blade to fill the nanoreservoirs and remove the excess solution. The filled Cys-NWs were vacuum-dried at room temperature for the solvent to evaporate. Thus, formed Cys-NW was punched into 2 mm circular disks with a paper punch. These 2 mm diameter Cys-NWs were used for the in vitro drug release, drug stability experiments, and for the *in vivo* studies in mice. Red fluorescent quantum dots (6 nm) were loaded into nanowafers to fabricate the QD-NW. These nanowafers were used for the study of quantum dot diffusion in the mouse cornea experiments.

Analysis of in Vitro Cys Stability in the Nanowafers. Cys stability studies were performed by placing Cys-NW of 2 mm diameter in separate plastic Petri dishes and sealed with parafilm. These Petri dishes were stored at room temperature for 10, 12, 14, 16, and 32 weeks at room temperature. Samples were dissolved in a mixture of methanol/water (50:50), centrifuged, and analyzed by liquid chromatography tandem mass spectrometry (Agilent 6490 Triple Quadruple LC−MS/MS system), equipped with a XDB C-18 column (50 mm × 4.6 mm, Agilent). A mixture of methanol/water (50:50) with 0.1% formic acid was used as the mobile phase with flow rate of 0.3 mL/min. The system was operated in a positive mode with electrospray ionization. Data acquisition in MRM mode and data quantification were performed with the corresponding Agilent MassHunter software. The MRM transition for Cys (monomer) was 78/61 and 153/108 for cystamine (dimer). Standards for calibration curves were always freshly prepared. Data was expressed as mean ± SEM.

Analysis of in Vitro Cys Release Studies. Cys-NWs (2 mm of diameter) were added to dialysis tubes provided with sterile water and transferred into a 5 mL size Eppendorf tubes containing also sterile water. The vials were constantly shaken (140 shakes/min, Brinkmann OrbiMix 1010 coupled with Incubator 1000, BioSurplus) at 37 °C. At different time points (1, 2, 3, 6, and 9 h), aliquots of 100 μL were taken out, and fresh water was used to replace the extracted aliquot volume. Samples were centrifuged (1000 rpm) for 10 s and stored at

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−80 °C until analyzed by mass spectrometry. Cys was measured using liquid chromatogram-tandem mass spectrometry (LC−MS/MS, 6490 QQQ Agilent). The samples were reconstituted in 100 μL of water/methanol (v/v 50:50), followed by centrifugation (RCF 15,000) for 15 min. The resulting supernatants were transferred to sample vials, and 5 μL was injected for analysis. The separation was achieved using a 50 mm × 4.6 mm XDB C-18 column (Agilent, Santa Clara, CA). The flow rate of the mobile phase was 0.3 mL/min with 50:50 methanol/water containing 0.1% formic acid. LC−MS/MS was operated in a positive mode with electrospray ionization. The data were acquired using Agilent MassHunter data acquisition software (Agilent) in the multiple reaction monitoring (MRM) mode. The quantification was performed by Agilent Mass Hunter quantitative analysis software. The MRM transition for Cys was 78/61. Standards for calibration curves were always freshly prepared. Results were reported as the mean ± SEM of quadruplicates.

Cys-NW Instillation and Measurement of Cys Concentration in Tear Samples. Healthy female C57/BL6 mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection, followed by the placement of a Cys-NW on the cornea. After mice were awake, tear fluid was collected hourly for 4 h.[36] Briefly, 2 μL of sterile water was instilled on the ocular surface, and after a few seconds, the tear washings were collected from the eye with a 1 μL volume glass capillary tube (Drummond Scientific, Broomhall, PA). The tear washings from a group of 50 mice were pooled, centrifuged, and stored at −80 °C prior to drug evaluation. In this experiment, sterile water, rather than a sterile balanced salt solution (BSS), was used, due to the interference of salt ions during mass spectrometry detection. Cys concentration was measured using LC−MS/MS as described previously. The MRM transition for Cys was 78/61. Standards for calibration curves were always freshly prepared. Results were reported as the mean ± SEM of triplicates.

Confocal Laser Scanning Microscopy of QD Diffusion in the Cornea. Healthy female C57/BL6 mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection. A QD-NW was placed on the cornea. At 4, 12, 24, and 48 h after the QD-NW instillation, mice were sacrificed and eyes were excised. Eye tissues were embedded in optimal cutting temperature compound prior to flash freezing in liquid nitrogen. Frozen tissues were sectioned at 10 μm thickness with a cryostat (HM505E; Micron International GmbH, Waldorf, Germany). Sections were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 min at room temperature and rinsed with phosphate buffered saline. Then sections were mounted with Fluoromount G (Southern Biotech, Birmingham, AL, USA) containing DAPI (1:300) prior to fluorescence imaging. Fluorescence images were obtained using a confocal laser scanning microscope (Eclipse Ni-E; Nikon, Melville, NY) with a 100× oil immersion objective (numerical aperture, 1.45; working distance, 1.3 mm). Obtained images were processed by NIS Elements software (Nikon, Melville, NY).

In Vivo Confocal Microscopic Analysis of Corneal Cystine Crystal Volume. Corneal crystal volume in cystinosis mice was determined as previously reported.[37,38] Briefly, in vivo corneal cystine crystal content was imaged by using a tandem scanning confocal microscope (TSCM; Tandem Scanning Corporation, Reston, VA) with a 24× surface-contact objective (numerical aperture, 0.6; working distance, 1.5 mm), encoder mike controller (Oriel 18011; Oriel, Stratford, CT) for focal plane control, and a low light level camera (MTI VE-1000; Dage MTI, Michigan City, IN). One drop of preservative-free, Refresh Tears (Allergan, Irvine, CA) was placed on the tip of the objective as a coupling gel. All camera settings were kept constant throughout the experiment. For each eye, three to five through-focus data sets were obtained from select central and peripheral corneal locations, including epithelium, stroma, and endothelium. To quantify the cystine crystal content in the cornea, several sets of through-focus images (3D images, *z*-stacking) were analyzed by Metamorph Image Processing Software (Molecular Devices). Initially, the stromal regions were extracted from the through-focus data set and then thresholded using the threshold subroutine to include all high intensity pixels representing light scattering from the cystine crystals. Threshold regions were set to include pixels intensity from 100 to 255. Pixels within the threshold region were then counted using the Measure subroutine for all planes in the image stack to record the crystal volume. To calculate a percent of crystal volume index (CVI), the crystal volume was divided by the extracted stromal volume multiplied by 100.

Drug Escalation Study and in Vivo Efficacy of Cys-NW by Crystal Volume Measurements. A drug escalation study was conducted in 7 month old $Ctns^{-/-}$ mice, divided into 5 groups, with 5 animals per group. For this experiment, Cys-NWs containing a series of concentrations of Cys for this study were fabricated, i.e., Cys40-NW, Cys15-NW, Cys10-NW, and Cys5-NW (containing 40, 15, 10, and 5 μg of Cys, respectively). Mice were treated daily with the corresponding Cys-NW for 1 week. In addition, a blank PVA-NW was used as a control. The nanowafer was placed on only the right cornea. All mice were subjected to slit-lamp examination for corneal opacities. Because doses that showed questionable opacity levels were not used for the in vivo confocal microscopy experiments, the doses selected for crystal analysis were Cys5-NW and Cys10-NW.

$Ctns^{-/-}$ mice were divided into two groups of five animals each. One group was treated on the right eye with Cys5-NW, and the other group with one Cys10-NW per day for 27 days. Baseline corneal cystine crystal volume was quantified using in vivo confocal microscopy prior to the application of the nanowafers. At the end of the treatment period, the corneal crystal volume was remeasured under exactly the same image capture parameters. Cys10-NW were selected for the in vivo efficacy evaluation experiments.

Again, $Ctns^{-/-}$ mice were divided into two groups of five animals each. The first group was treated with 5 μL of 0.44% Cys solution twice a day, while the second group was treated with one Cys10-NW per day for 30 days.

Measurement of Cystine Concentration in Corneas by Mass Spectrometry. $Ctns^{-/-}$ mice (7 months old) were treated with Cys eye drops, PVA-NW, and Cys-NW for 30 days. Eye drop treatment (5 μL of 0.44% cysteamine solution) was performed twice a day. PVA-NW and Cys-NW treatments were applied once a day. An untreated set of animals was used as control. After the treatment period, mice were euthanized by cervical dislocation under anesthesia, and the eyes were enucleated and preserved in dextran solution (1%) at 4 °C. Corneas were then extracted, cleaned, and perforated with 2 mm biopsy punch, obtaining the central area of the cornea. Three corneas per sample (n = 7) were used. They were ultrasonicated in 100 μL of 0.1 N HCl, and the cystine was extracted using 400 μL of acetonitrile. Samples were

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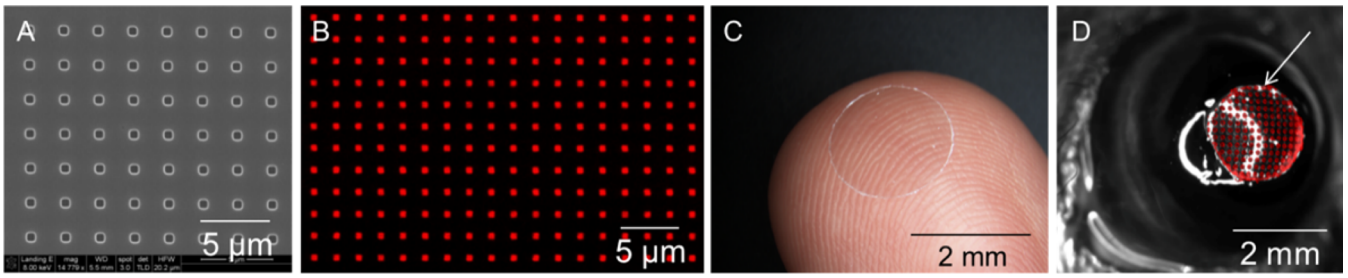

Figure 2. Ocular drug delivery nanowafer. (A) A scanning electron micrograph (SEM) presenting an unfilled nanowafer. (B) Fluorescence image of a red quantum dot-filled nanowafer. (C) Cys-NW on a fingertip demonstrating its transparency. (D) Nanowafer applied on a mouse eye.

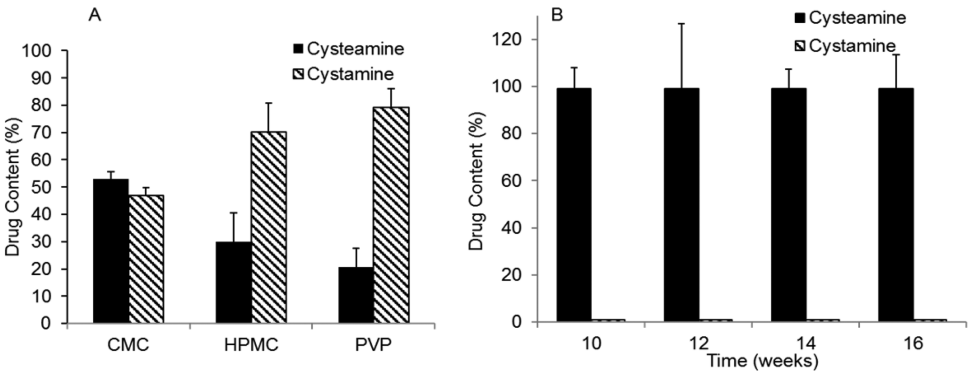

Figure 3. Cys-NW prevents the formation of therapeutically inactive cystamine and improves the drug stability. (A) A plot depicting the rapid deterioration of Cys (monomer) in CMC, HPMC, and PVP nanowafers within 2 weeks. (B) Cys is stable for up to 4 months in PVA nanowafer. Data is expressed as mean ± SEM.

concentrated using a pump dry system, reconstituted in methanol/water (50:50), and analyzed by LC−MS/MS. The multiple reaction monitoring (MRM) transition for cystine was 241/152. Data was expressed as mean ± SEM. Statistical analyses were performed using repeated measures one-way ANOVA with Tukey's multiple comparison post-tests (GraphPad Software, Inc.).

Evaluation of Safety Profile of Cys-NW on Human Corneal Explant Culture. Human corneoscleral tissues, which were not suitable for clinical use, from donors 23−64 years, were obtained from the Lions Eye Bank of Texas (Houston, TX, USA). Human tissues were handled according to the tenets of the Declaration of Helsinki.

Human corneal explant was cultured and maintained using a previously described method with a modification.[39] Briefly, corneoscleral tissues were rinsed with Hank's balanced solution containing gentamicin (50 μg/mL) and amphotericin B (1.25 μg/mL). Once excess sclera tissue was removed, the remaining tissue was cut into equal pieces (approximately 2 × 2 mm^2) using a surgical scalpel. Each explant tissue with epithelium side up was transferred into an 8-well chamber slide. The explants were cultured in Supplemental Hormonal Epithelial Medium (DMEM/F12, 1:1) containing EGF (5 ng/mL), insulin (5 μg/mL), transferrin (5 μg/mL), sodium selenite (5 ng/mL), hydrocortisone (0.5 μg/mL), cholera toxin A (30 ng/mL), 0.5% DMSO, gentamicin (50 μg/mL), and amphotericin B (1.25 μg/mL) and 5% FBS and were maintained at 37 °C with 5% CO_2 and 95% humidity. The culture media were exchanged every 3 days for 14 days.

After 14 days, explant tissues were removed and PVA-NW, Cys eye drop, and Cys-NW were placed in each well; then the culture was maintained at 37 °C with 5% CO_2 and 95% humidity for 24 h. Each well was gently rinsed with PBS and incubated with Hoechst 33342 (10 μg/mL) for 7 min at 37 °C and counterstained with propidium iodide (PI; 2.5 μg/mL). Fluorescence images were obtained using an inverted fluorescence microscope (Nikon Eclipse Ti), and images were analyzed using software IMARIS (Bitplane AG, Zurich, Switzerland) for quantification of stained cells. Cells stained with PI indicated dead cells, and total cell numbers equaled to cells stained with Hoechst 33342. The number of live cells was calculated by subtracting dead cell counts from the total number of cells.

RESULTS

In this study, we present the development of a Cys-NW, wherein the drug carrying polymer and the drug work synergistically to provide an augmented therapeutic effect compared to conventional eye drop therapy.

Cys-NW Fabrication. The Cys-NWs were fabricated via a slightly modified hydrogel template strategy (Figure S1). In this study, poly(vinyl alcohol) (PVA), polyvinylpyrrolidone (PVP), hypromellose (HPMC), and carboxymethyl cellulose (CMC) nanowafers loaded with Cys were fabricated. These polymers were selected for their water solubility, biocompatibility, transparency, and mucoadhesive properties so as to readily adhere to the wet mucosal surface and conform to the curvature of the eye.[40] Aqueous solutions of these polymers are currently in clinical use as artificial tears, and therefore nanowafers fabricated with these polymers can function both as a drug delivery system and also as lubricant.[41] The polymer wafer containing square wells (500 nm × 500 nm and 500 nm depth, with 2 μm spacing) were filled with a thick solution of drug/polymer mixture and punched with a 2 mm paper punch to obtain circular Cys-NW of 2 mm diameter and 80 μm thick circular discs. The nanowafer is a tiny transparent circular disc

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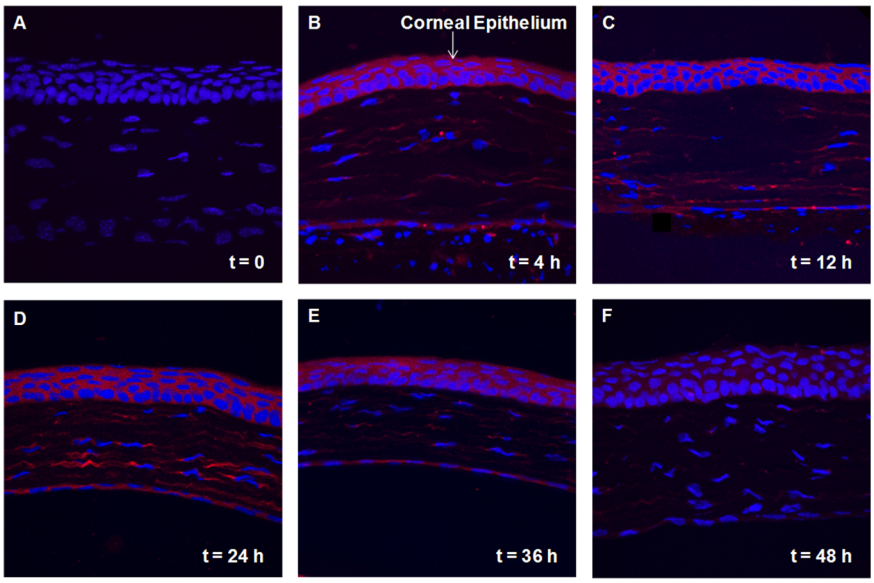

Figure 4. Nanowafer drug delivery enhances the drug diffusion into the cornea. Confocal laser scanning microscopic images of untreated cornea (A) and QD-NW treated corneal sections obtained at regular time intervals demonstrating the QD diffusion and retention in the corneas for up to 48 h (B–F).

that can be applied on the ocular surface with a fingertip and can withstand constant blinking without being displaced (Figure 2). It contains arrays of drug-loaded nanoreservoirs from which the drug will be released in a tightly controlled fashion for an extended period of time. The slow drug release from the nanowafer increases the drug residence time on the ocular surface and its subsequent absorption into the surrounding ocular tissue. At the end of the stipulated period of drug release, the nanowafer will dissolve and fade away.

The Cys-NWs are highly transparent. The refractive index of a Cys-NW is very close to that of a soft contact lens. Also, when a Cys-NW is placed on a fingertip, the fingerprint lines are very clearly visible through the nanowafer (Figure 2C). Hence, nanowafer application on the cornea will not affect the normal vision.

Analysis of Cys-NW Stability. Cys is chemically unstable in eye drop formulation and rapidly oxidizes to its therapeutically inactive dimer cystamine. Hence, the stability of Cys was evaluated in the nanowafer at room temperature. In this study, Cys-NWs fabricated with CMC, HPMC, PVP, and PVA were evaluated. After fabrication, all the nanowafers were stored at room temperature (25 °C). The Cys-NWs were analyzed for pure Cys and its dimer cystamine concentrations by mass spectrometry. Experiment was run in triplicates. This study revealed that, within 2 weeks Cys in CMC, HPMC, and PVP, nanowafers began to dimerize to therapeutically inactive cystamine. As can be seen in Figure 3A, ∼45% of cystamine was formed in CMC nanowafers, while up to 80% of cystamine was formed in HPMC and PVP nanowafers. In the case of PVA nanowafers, Cys is stable and in therapeutically effective form for up to four months when stored at room temperature (Figure 3B), compared to the eye drop formulation in which Cys is stable only for 1 week even under refrigerated conditions.[31] This is possibly because of the ion pairing of Cys with the polymers. In the case of PVA nanowafer, because of the neutral PVA, the Cys molecules are protected in the polymer matrix from dimerization to therapeutically inactive cystamine, thus enhancing its stability. However, Cys slowly started to oxidize after 6 months to its dimer (Figure S2).

Nanowafer Improves Cys Retention Time in the Eye. The mouse cornea is ∼3.2 mm in diameter, and for the in vivo experiments in mice, nanowafers of 2 mm diameter and 80 μm thick were fabricated, so as to exactly fit within the cornea.[42] Since, the nanowafers are fabricated with a mucoadhesive polymer, they readily adhere to the corneal surface. Cys-NW is very soft and stretchable in dry state. The nanowafer, upon application, readily adheres and conforms to the curvature of the cornea. Also, after application of a Cys-NW on the mouse cornea, 5 μL of balanced salt solution (BSS) was added to wet the cornea and further improve the nanowafer adhesion. During the application of the cysteamine nanowafer, no pressure or bending is required. Furthermore, PVA nanowafer is known to elicit negligible inflammatory responses when applied on the eye.[32] The nanowafer was applied on the mouse cornea with forceps followed by the instillation of 5 μL of BSS to wet the surface (Figure 2D).

The in vitro Cys release from the Cys-NW was monitored by mass spectrometry. During the first hour ∼35% Cys was released, and it continued for 6 h under sink conditions (Figure S3). To measure the Cys concentration in the tear washings, Cys-NW were applied on the corneas of healthy mice, and the tear samples were collected at hourly time intervals. Tear samples from Cys-NW treated eyes contained measurable levels of Cys for up to 2 h and was not detectable in the third hour tear samples (Figure S3). However, in the case of topically applied Cys eye drops, Cys was not detected even in the first hour tear samples, indicating its rapid clearance from the ocular surface. PVA is a water-soluble nondegradable polymer. During the course of the drug release the Cys-NW completely dissolves. The drug release from the Cys-NW is a combination of both diffusion and dissolution. No degradation process is required to release the drug. This study reaffirms that the drug molecules do not get sufficient time to diffuse into the ocular tissue because of the rapid clearance of topically applied eye drops due to reflex tearing, blinking, and nasolacrimal drainage. This limits the bioavailability of the drug and results in a compromised therapeutic effect. The nanowafer, upon placement on the eye, slowly releases Cys, thus increasing the drug

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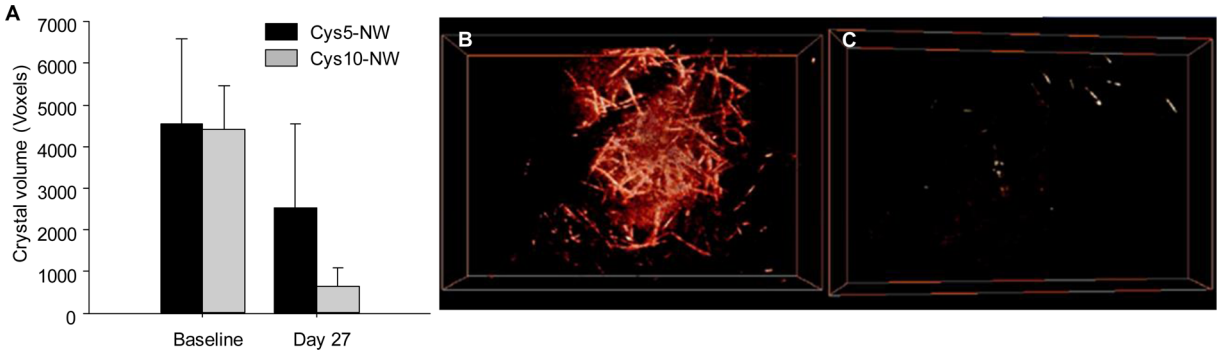

Figure 5. Determination of therapeutically most effective Cys concentration in the nanowafer. (A) A plot depicting the efficacies of Cys5-NW and Cys10-NW on dissolving corneal cystine crystal volume. (B,C) Confocal images of cystine crystal volume change in Cys10-NW treated corneas before and 27 days after Cys-NW therapy.

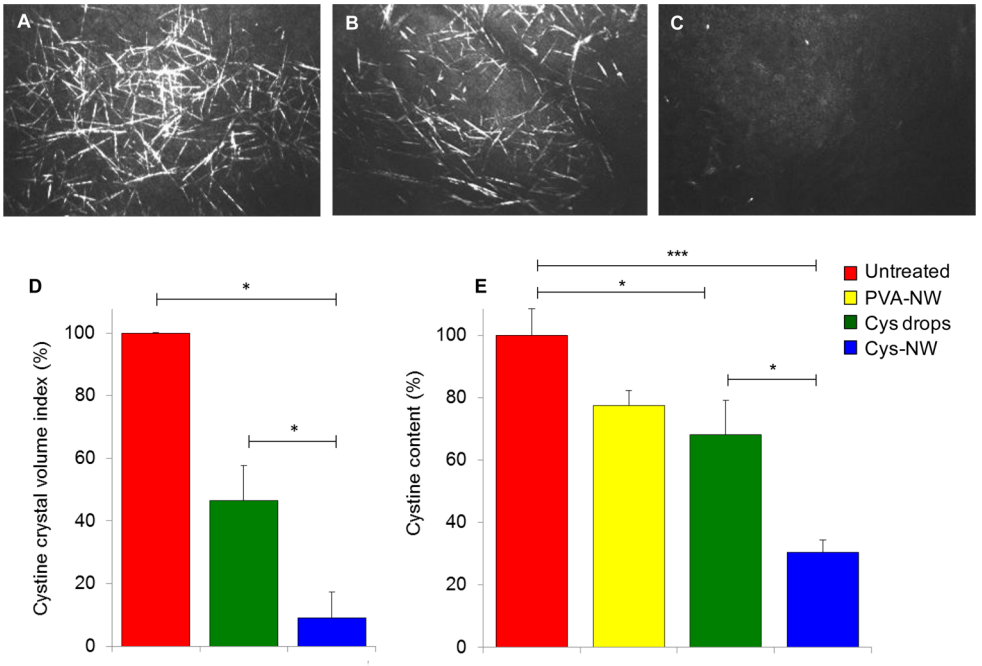

Figure 6. Cys-NW is more efficacious than topical Cys eye drop treatment. Representative laser confocal images of (A) untreated cystinosis cornea, (B) twice a day topical Cys eye drops treated cornea, and (C) once a day Cys-NW treated cornea. (D) A plot depicting the total cystine crystal content in the corneas quantified by laser confocal image analysis (mean ± SD). (E) A plot depicting the total cystine mass content in the corneas quantified by mass spectrometry (mean ± SEM).

residence time on the ocular surface and its subsequent diffusion into the ocular tissue. This increased drug diffusion improves the bioavailability of Cys and enhances its therapeutic efficacy in dissolving the corneal cystine crystals. Furthermore, the desired drug content in Cys-NW and drug release profiles can be modulated by fabricating nanowafers containing drug reservoirs of requisite dimensions. During the course of the drug release, the nanowafer slowly dissolves and eventually disappears. To demonstrate the dissolution and disappearance of the nanowafer with time after its instillation on the ocular surface, a fluorescein loaded nanowafer was placed on the mouse cornea. Bright field microscopy revealed that the nanowafer began to disappear after 1 h of its instillation on the cornea. However, fluorescence microscopy revealed the presence of fluorescein loaded nanowafer on the cornea for up to 4 h. The nanowafer was completely dissolved in 4−5 h and cleared from the ocular surface due to constant blinking (Figure S4). Furthermore, because the nanowafer was fabricated using PVA, which is also in use as artificial tear eye drops, the nanowafer provides lubrication and relief during its dissolution.

To evaluate the ability of a nanowafer to increase the drug molecular residence time on the cornea and its subsequent diffusion into the corneal tissue, a red QD-NW was fabricated and tested on a healthy mouse cornea. Because Cys is nonfluorescent, it cannot be monitored by fluorescence microscopy. Hence, a QD-NW was fabricated to monitor QD diffusion and residence times in the cornea by fluorescence microscopy. The QD-NW were fabricated using hydrophobic CdSe/ZnS core−shell-type quantum dots stabilized with octadecylamine ligands. The fluorescence emission wavelength of the QD-NW is λ_{em} 580 nm. Although, this is not an exact replication of Cys diffusion into the cornea, this study provides evidence for the drug diffusion into the cornea. Upon placement of the QD-NW on the mouse cornea, the QDs began to diffuse into the corneal tissue, and it was observed for up to 48 h (Figure 4). At this point, the fluorescence intensity

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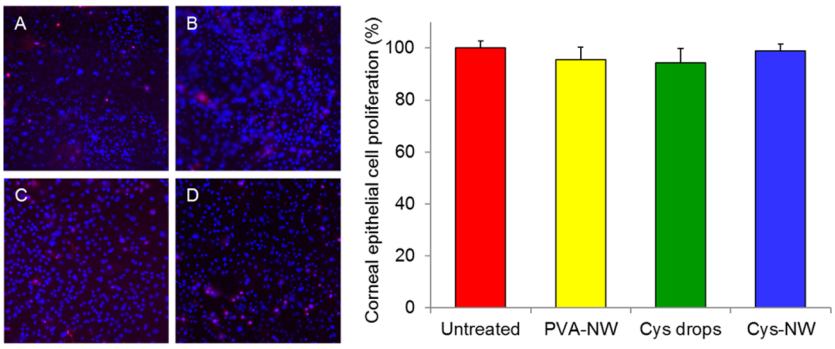

Figure 7. Safety profile of Cys-NW on human corneal explant epithelial cell cultures. Fluorescence micrographs of human corneal epithelial cells after live−dead cell assay: (A) untreated, (B) PVA-NW treated, (C) Cys-eye drop treated, and (D) Cys-NW treated human corneal epithelial cells. (E) A plot depicting the percentage of live human corneal epithelial cells after the treatments.

of the QDs in the corneal tissue began decreasing as the QDs diffuse through the cornea and reach the aqueous humor in the anterior chamber and cleared through the trabecular meshwork.

Determination of Therapeutically Most Effective Dose. Drug escalation studies were conducted to determine the maximum tolerated dose and to minimize the drug related acute toxicity. Administration of maximum tolerated dose is usually associated with maximum clinical benefit. Maximum tolerated drug dose was determined by monitoring corneal opacity in mice. Cys-NWs containing a series of concentrations of Cys for this study were fabricated, i.e., Cys40-NW, Cys15-NW, Cys10-NW, and Cys5-NW (containing 40, 15, 10, and 5 μg of Cys, respectively), and a blank PVA-NW was used as control. Maximum tolerated dose was evaluated in cystinosin knockout mice ($Ctns^{-/-}$ mice). In these mice, the cystine crystals begin to appear at 3 months of age in the corneal endothelium, and the crystal content progressively increases up to 7 months, followed by corneal scarring, thus mimicking the clinical disease progression seen in humans.[43] The $Ctns^{-/-}$ mice (7 months old) were divided into 5 groups, treated with the corresponding Cys-NW for 1 week, and examined using slit lamps imaging at the end of the treatment period. Slit lamp examination revealed no visible corneal opacities in mouse groups treated with PVA-NW, Cys5-NW, and Cys10-NW. In the case of Cys15-NW and Cys40-NW treated mouse groups, the eyes began to develop slight corneal opacification, and the experiments were discontinued. Based on these results, Cys10-NW and Cys5-NW were selected for the determination of therapeutically most effective dose.

The therapeutically most effective concentration of Cys-NW was evaluated in $Ctns^{-/-}$ mouse model by measuring the reduction in the corneal cystine crystal volume. In this study, $Ctns^{-/-}$ mice were divided into two groups. Baseline corneal cystine crystal volume was quantified using in vivo confocal microscopy in the right eye of each animal prior to the application of Cys-NW.[37,38] A Cys-NW was placed on the right eye every day for 27 days. At the end of the treatment period, the corneal crystal volume was remeasured under exactly the same image capture parameters. Cys10-NW was more effective in dissolving the corneal cystine crystals compared to Cys5-NW (Figure 5).

Cys-NW Is More Efficacious than Topical Cys Eye Drop Treatment. To determine the in vivo efficacy of Cys10-NW in comparison to topical Cys eye drop formulation (0.44%), two groups of $Ctns^{-/-}$ mice (three per group) were treated separately with Cys-NW (10 μg of Cys, once a day) and Cys eye drops (5 μL, 22 μg) twice a day for 30 days. At the end of this period, the cystine crystal content in the corneas was estimated by laser confocal image analysis.[33,34] These studies revealed that, compared to the baseline corneal cystine crystal volume, twice a day Cys eye drop treatment reduced the crystal volume by 55%, while the once a day Cys-NW treatment reduced the crystal volume by 90%, confirming that the Cys-NW treatment is significantly more efficacious than Cys eye drop treatment (Figure 6A-D).

At this point, although the Cys-NW is very effective in dissolving cystine crystals, the confocal microscopic analysis was able to quantify only up to the submicron sized cystine crystals and not the molecular cystine content present in the corneal epithelium. Therefore, the efficacy of the nanowafer was also evaluated by measuring the amount of cystine present in cystinosis mice corneas using mass spectrometry.

This study revealed that once a day Cys10-NW treatment for 30 days reduced the corneal cystine mass by 65%, while twice a day administration of Cys eye drops during the same treatment period resulted in a decrease in corneal cystine content by 34%, compared to the untreated control group (Figure 6E). Furthermore, the amount of Cys delivered per day by the nanowafer was 10 μg, while the twice a day Cys eye drop treatment delivered 44 μg. These results reaffirmed the enhanced efficacy of Cys10-NW with half the drug dosage compared to the topical Cys eye drop treatment, as observed in confocal microscopic analysis. The blank PVA wafer (control) was able to dissolve a small amount of the corneal cystine crystals. A possible reason could be, since the PVA wafer is in close contact with the cornea, some cystine crystals must have been dissolved by PVA due to hydrotropic effect.[44]

The enhanced efficacy of Cys-NW in dissolving cystine crystals is mainly because of the increased drug residence time on the cornea, which enabled the Cys molecules to diffuse into the corneal epithelium. Once the Cys molecules penetrate into the cornea, they react with cystine molecules to form cysteine-cysteamine mixed disulfide, which will be cleared from the cornea. Topically applied eye drops will be rapidly cleared from the ocular surface due to blinking and have a very short contact time on the ocular surface. As a consequence, the drug absorption is severely limited by the ocular surface barriers resulting in extremely low bioavailability. For this reason, topical Cys eye drops must be administered several times in a day to achieve a measurable therapeutic effect.

Safety Profile of Cys-NW on Human Corneal Explant Epithelial Cultures. The safety of Cys-NW was examined on human corneal explant epithelial cells. In this study, human corneal explants were cultured in a 12-well plate to promote the

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proliferation of corneal epithelial cells.[39] Monolayers of human corneal epithelial cells formed in the wells were separately treated with PVA-NW, Cys eye drops, and Cys-NW, respectively, followed by live−dead cell assay. In this study, no significant cell death was observed with Cys eye drops and Cys-NW treatments, when compared to the untreated human corneal cell monolayers (Figure 7). This study revealed that Cys-NW is as safe as Cys eye drops on human corneal explant epithelial cell cultures.

DISCUSSION

The ultimate goal of this work was to develop a nanowafer therapeutic that can efficiently deliver Cys for an extended period of time and enhance the therapeutic efficacy. A cystinosis patient has to instill the Cys eye drops on the eyes at least 6−12 times in a day for several years.[27] This is very inconvenient and also unlikely that it will be applied in a timely manner every day, resulting in patient noncompliance and compromised therapeutic efficacy. Furthermore, considering the fact that the cystinosis patients are generally school going children and young adults, noncompliance to Cys eye drop treatment and drug related side effects become more significant.

The results of this study confirm that Cys-NW is more efficacious than the topical eye drop formulations. This study also demonstrated that the enhanced efficacy of Cys-NW is due to the longer residence time of the drug molecules on the eye, which enabled their diffusion into the ocular surface epithelium, unlike eye drops, which will be cleared from the ocular surface within a few minutes due to reflex tearing and rapid blinking. The nanowafer readily adheres to the ocular surface and can withstand constant blinking without being displaced. The nanowafer will completely dissolve in 4−5 h and cleared from the ocular surface due to constant blinking. Hence, there will be no accumulation of the polymer on the eye. Drug release from the nanowafer can be controlled by choosing the right drug reservoir dimension and drug loading concentration. Thus, the drug release from the nanowafer can be programmed to have prolonged drug efficacy to suit patient's requirements. For the in vivo mouse study, 2 mm diameter nanowafers were fabricated; however for human applications, a larger nanowafer (of 8 mm diameter) will be fabricated that can be applied on the eye with a fingertip.

The nanowafer drug delivery system presents several advantages compared to the conventional ophthalmic solutions applied as topical eye drops and drug delivery contact lenses. Nanowafers are designed for controlled release ocular drug delivery applications. The nanowafers are easy to fabricate, more efficacious at a lower dosing frequency, provide a sustained drug availability in the eye, and protect the drug from deterioration. The nanowafer can release the drug in a therapeutically effective concentration and at the end of the stipulated period of drug release, the nanowafer will dissolve and fade away, i.e., "self-clearing" and does not need to be removed. Because the drug molecules slowly diffuse from the polymer matrix of the nanowafer, they are protected from degradation and rapid release. The nanowafers are fabricated with polymers that are currently in clinical use as artificial tear eye drops, and therefore, the nanowafers can function both as a drug delivery system and also as lubricant. The nanowafer therapeutic can provide mechanical barrier to protect the eye from desiccation. These attributes of the nanowafer will improve patient comfort and compliance to treatment. Since, the polymers and drugs used in the nanowafer fabrication are already in clinical use, the nanowafer can be rapidly translated to the clinic for human use.

The Cys-NW was fabricated using PVA, which is in clinical use as artificial tears. Also, PVA is noninflammatory and nontoxic on the ocular surface even after prolonged usage.[45,46] Therefore, as a nanowafer, PVA can function both as a drug delivery system and also as a lubricant on the ocular surface. Furthermore, PVA nanowafer is known to elicit negligible inflammatory responses when applied on the eye.[32] Because of its enhanced therapeutic efficacy, safety profile, and extended stability at room temperature, Cys-NW is a major advancement in the treatment and management of corneal cystinosis.

The enhanced therapeutic efficacy and translational potential of Cys-NW in comparison to topical Cys eye drop treatment has been demonstrated in treating corneal cystinosis in $Ctns^{-/-}$ mouse model. This study may not exactly represent the clinical situations, and whether Cys-NW can be effective in humans requires further investigation. Although, Cys and PVA are independently in clinical use as ophthalmic solutions, the efficacy of Cys and PVA as a nanowafer therapeutic needs to be thoroughly evaluated in human clinical trials. The nanowafer drug delivery system holds promise for future research not only in exploring its broad applicability in treating other ocular surface diseases but also in translational medicine.

CONCLUSIONS

This study has demonstrated the enhanced therapeutic efficacy and translational potential of Cys-NW in comparison to topical Cys eye drops in treating corneal cystinosis in $Ctns^{-/-}$ mouse model. For human use, the Cys-NW can be applied on the eye with a fingertip like a contact lens, and it will remain in the eye to deliver the drug for a longer duration of time, thus enhancing the drug residence time, bioavailability, therapeutic efficacy, and treatment compliance. The nanowafer is self-clearing, i.e., during the course of the drug release it will dissolve and eventually disappear. The human corneal explant epithelial culture studies have revealed negligible cytotoxic effects of Cys-NW. Furthermore, the nanowafer prevents oxidation of Cys to its therapeutically inactive disulfide form cystamine, thus enhancing its stability and shelf life at room temperature. The nanowafer drug delivery system is broadly applicable and holds promise for future research in treating other eye diseases, such as dry eye, ocular infections, eye injuries, and glaucoma.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.6b00488.

Schematic of the nanowafer fabrication, stability of Cys-NW, and the drug residence time on the ocular surface (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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