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TITLE:
Broadly Applicable Nanowafer Drug Delivery System for Treating Eye Injuries

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**ABSTRACT**

Eye injuries require immediate and effective treatment to prevent corneal opacification, neovascularization, irregularity and occasionally ulceration of the cornea, which can be potentially blinding. Eye injuries are generally treated with eye drops for 4-8 times per day, which may not be feasible in critically injured patients in intensive care. This research project aims to develop a nanowafer drug delivery system that can deliver the drug to the eye for longer periods of time to treat eye injuries and prevent potential loss of vision. During the third year of this project, dexamethasone loaded nanowafers have been fabricated and evaluated for the in vivo therapeutic efficacy in ocular burn induced mouse model. These studies revealed that the dexamethasone nanowafers are very effective in corneal wound healing and the suppression of corneal neovascularization as revealed by the laser scanning confocal microscopy. The efficacy of the dexamethasone was also evaluated by PCR analysis. This study also revealed that once a week Dexamethasone nanowafer treatment is as effective as twice a day Dexamethasone eye treatment. Presently, further studies are underway to optimize the therapeutic efficacy and dosing frequency of the nanowafer treatment.
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1. INTRODUCTION

Soldiers affected by eye injuries require immediate and effective treatment. The acute phase occurs at the time of the injury and results in corneal and conjunctival epithelial damage or necrotic death. These events lead to opacification, neovascularization, irregularity and occasionally ulceration of the cornea, which can be potentially blinding. Eye injuries are generally treated by simply introducing drug solution in the form of eye drops; however achieving sustained therapeutic levels on the ocular surface remains a challenge due to the continuous tear clearance through the lacrimal drainage system. Modulation of the ocular surface response to trauma requires multiple dosing (4-8 times per day) of the eye drops to achieve an effect, which may not be feasible in critically injured patients in intensive care. Hence, there is a strong need for the development of broadly applicable nanowafer drug delivery systems with high drug content and long term drug release attributes. This research project focuses on the development of a nanowafer drug delivery system that can deliver the drug to the eye in a controlled release fashion for longer periods of time to treat eye injuries and prevent potential loss of vision. In this project, by integrating the nanotechnologies and controlled release drug delivery technology, a nanowafer drug delivery system will be developed that can surmount the limitations of conventional eye drop formulations. The nanowafers will be fabricated via the hydrogel template strategy. The nanowafer contains an array of nanoreservoirs loaded with drug matrix. Upon instillation, because the nanowafer is very thin and comprised of mucoadhesive biomaterial, it readily adheres to the conjunctiva and can remain intact for several days without being displaced due to constant blinking. The nanowafer drug delivery system can release the drug in therapeutically effective concentration from a day to a week. The broadly applicable nanowafer drug delivery system upon development can be used for treating ocular surface injuries and also dry eye, corneal ulcers, glaucoma, and infections, and improve the performance efficiency and effectiveness of the soldiers in the warzone.
2. Keywords

nanowafer, nanofabrication, drug delivery, ocular burn, doxycycline, dexamethasone, cyclosporine-A, pharmacokinetics
3. ACCOMPLISHMENTS:

What were the major goals of the project?

This project focuses on accomplishing the following 5 defined tasks as proposed in the SOW:

**Task 1.** Regulatory approvals (IACUC/ACURO/HRPO). Duration: 3 months (months 1-3)

**Task 2.** Fabrication of nanowafer drug delivery systems. Duration 18 months (months 1-18)

**Task 3.** Evaluation of *in vitro* and *in vivo* pharmacokinetics. Duration 24 months (months 6-30)

**Task 4.** Study of the efficacy of doxycycline-nanowafers, dexamethasone-nanowafers, and cyclosporine-A-nanowafers in an ocular burn mouse model. Duration: 18 months (months 18-36)

**Task 5.** Data Analysis. Duration: 6 months (months 30-36)

What was accomplished under these goals?

This section summarizes the results obtained in our laboratories during this reporting period: October 1, 2015 to September 30, 2016. Specifically, we have accomplished: the following objectives defined under each task.

**Task 1.** Regulatory approvals (IACUC/ACURO/HRPO)
Duration: 3 months (months 1-3)

*This Task has been accomplished and reported in the Year-1 Report (09-2014)*

**Task 2.** Fabrication of nanowafer drug delivery systems
Duration 18 months (months 1-18)

Objective (i) Nanofabrication of silicon master templates with feature dimensions of 100, 200 nm, 500 nm, 1 μm, 1.5 μm, and 3 μm

*This objective has been accomplished and reported in the Year-1 Report (09-2014)*

Objective (ii) Fabrication of polyvinylpyrrolidone (PVP), dextran (DTR), carboxymethyl cellulose (CMC), and hydroxypropyl cellulose (HPMC) nanowafers

*This objective has been accomplished and reported in the Year-1 Report (09-2014)*

Objective (iii) Fabrication of drug-filled nanowafers: doxycycline-nanowafers; dexamethasone-nanowafers; and cyclosporine-A-nanowafers

*This objective has been accomplished and reported in the Year-1 Report (09-2014)*
Objective (iv) Optimization of in vivo compliance after the instillation of nanowafers on cornea and conjunctiva and evaluate their adherence and dissolution by bright field and fluorescence microscopy

*This objective has been accomplished and reported in the Year-2 Report (09-2015)*

Task 3. Evaluation of *in vitro* and *in vivo* pharmacokinetics
Duration 24 months (months 6-30)

Objective (i) Study of *in vitro* drug release and drug concentration in doxycycline-nanowafers, dexamethasone-nanowafers, and cyclosporine-A-nanowafers by fluorescence spectrophotometry and high performance liquid chromatography (HPLC) method

*This objective has been accomplished and reported in the Year-1 Report (09-2014)*

Objective (ii) Study of pharmacokinetics of doxycycline-nanowafers, dexamethasone-nanowafers, and cyclosporine-A-nanowafers in the tear washings of mice

*This objective has been accomplished and reported in the Year-2 Report (09-2015)*

Objective (iii) Study of *in vivo* pharmacokinetics after the instillation of drug-nanowafers on the cornea by real-time drug molecular transport, distribution, and residence times in the cornea and conjunctiva by laser scanning confocal fluorescence image analysis in mice for 1-10 days

*A part of this objective has been accomplished and reported in the Year-2 Report (09-2015)*

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, red quantum dots loaded nanowafer (QD-NW) was fabricated and tested on a healthy mouse cornea. Because Dexamethasone drug 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 (Figure 1A). The fluorescence emission wavelength of the QD-NW is \( \lambda_{em} \) 580 nm. Although, this is not an exact replication of drug diffusion into the cornea, this study provides evidence for the drug diffusion into the cornea.

The mouse cornea is \( \sim 3.2 \) mm in diameter, and for the in vivo experiments in mice, nanowafers of 2 mm diameter and 80 \( \mu \)m thick were fabricated, so as to exactly fit within the cornea. Since, the nanowafers are fabricated with a mucoadhesive polymer, they readily adhere to the corneal surface. The nanowafer is very soft and stretchable in dry state. The nanowafer, upon application, readily adheres and conforms to the curvature of the cornea. Prior to the application of the nanowafer, the mouse was anesthetized by ketamine (100 mg/kg) and xylazine (10 mg/kg) injection. A nanowafer was applied on the mouse cornea with forceps while observing under a stereomicroscope followed by the instillation of 5 \( \mu \)L of balanced salt solution (BSS). The BSS was added to wet the cornea and further improve the...
nanowafer adhesion (Figure 1B-C). During the application of the nanowafer, no pressure or bending is required.

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 2). At this point, the fluorescence intensity 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.

Figure 1. Ocular drug delivery nanowafer. (A) Fluorescence image of a red quantum dot-filled nanowafer. (B&C) Nanowafer applied on a mouse eye

Figure 2. Nanowafer drug delivery enhances the drug diffusion into the cornea. Confocal laser scanning microscopic images of (A) Untreated cornea (control). (B-F) QD-NW treated corneal sections obtained at regular time intervals demonstrating the QD diffusion and retention in the corneas for up to 48 h.
Nanowafer can release the drug for up to 14 days

To demonstrate the ability of the nanowafer to release the drug for an extended period of time, increase drug residence time and its diffusion into the cornea, real-time *in vivo* corneal imaging experiments were performed in an ocular burn induced (OB) mouse model. For this study, Dextran nanowafers filled with fluorescein coupled dextran (FITC-Dextran) were fabricated. After the instillation of this nanowafer on a mouse eye, the cornea was subjected to real-time fluorescence imaging to monitor the presence of FITC-Dextran (*Figure 3*). This study revealed that the green fluorescent FITC-Dextran molecules were present in the cornea for up to 14 days (*Figure 3*). This study also confirmed that a longer drug residence time on the cornea will allow the drug molecules to effectively diffuse into the cornea and improve the bioavailability of the drug molecules. By optimizing the drug retention time in the cornea, a therapeutic concentration of the drug can be maintained in the cornea thus enhancing the drug efficacy.

*Figure 3. Nanowafer drug delivery enhances the drug residence time on the cornea for up to 2 weeks.* (A) Bright field micrograph demonstrating the application of a nanowafer on an ocular burn induced mouse cornea. (B-L) Fluorescence micrographs demonstrating FITC-Dextran release from the nanowafer for 2 weeks.

In comparison, ocular burn induced eyes treated with FITC-Dextran eye drops became non fluorescent within 4h, indicating its rapid clearance from the ocular surface (*Figure 4*). Also, after instillation of the FITCDextran drops on the eye, most of it is concentrated on the eye lids in addition to the cornea, indicating its clearance from the ocular surface (*Figure 4B*), compared to the nanowafer drug release, wherein little fluorescence intensity was observed around the eyelids and most of it in the eye (*Figure 4*). Because of the very short drug residence time on the cornea, eye drops need to be administered several times in a day for an observable therapeutic efficacy. This study has qualitatively demonstrated the ability of nanowafer to release the drug for an extended period of time and improve the drug diffusion into the corneal tissue for up to 14 days.

*Figure 4. Rapid clearance of topically applied FITC-Dextran eye drops from the ocular surface in ocular burn mouse eye.*

Duration: 18 months (months 18-36)

Objective (i) Quantification of the drug-nanowafer efficacy by time to epithelial healing
Using confocal fluorescence imaging

To evaluate the therapeutic efficacy of the nanowafer on wound healing, ocular burn (OB) induced mice were treated with dexamethasone nanowafers (Dex-NW) and compared with topical Dex eye drop treatment. One group of OB induced mice were treated with Dex-NW once on the first day and waited for a week. Another group of mice were treated with Dex eye drops twice a day for a week. On the eighth day both groups of mice were sacrificed and the corneas were collected and subjected to hematoxylin & eosin staining, followed by microscopic imaging. In a healthy cornea, epithelium and the collagen fibers in the stroma are tightly packed (Figure 5A). In OB induced corneas, greater stromal swelling (edema), fibrosis, and invasion of inflammatory cells (non-bacterial keratitis) was observed (Figure 5B). Once a week Dex-NW treatment was very effective in wound healing with minimal stromal edema and fibrosis, and the stromal collagen fibers were tightly packed (Figure 5D). In comparison, twice a day topical Dex eye drop (0.1%) treatment for a week was able to minimize fibrosis, however the stromal collagen was very loosely packed with several empty spaces (Figure 5C). These studies have clearly demonstrated an enhanced efficacy of Dex-NW compared to topical Dex eye drop treatment on corneal wound healing.

We are presently optimizing the drug content and dosing frequency of the nanowafer treatment.

Figure 5. Nanowafer drug delivery is more effective in corneal wound healing and preventing fibrosis. (A) healthy cornea, (B) ocular burn (OB) induced cornea, (C) OB induced cornea treated twice a day with dexamethasone eye drops. (D) OB induced cornea treated with Dex-NW once a week. The images were obtained after one week of treatment.

Efficacy of Dexamethasone Nanowafer on Suppressing Corneal Neovascularization

Because of the physiological barriers of the ocular surface, topical eye drops must be applied several times a day for a therapeutic effect, thus increasing the potential for toxic side
effects. Excessive use of Dex eye drops are known to cause side effects such as, cataract formation and increased intraocular pressure leading to glaucoma.

In this study, the efficacy of once a week dexamethasone nanowafer (Dex-NW) treatment was compared with twice a day topical dexamethasone eye drop (Dex-eye drops, 0.1%) treatment on inhibiting corneal neovascularization (CNV) in an ocular burn (OB) induced mouse model. A circular Dex-NW (2mm diameter) was applied on the injured cornea under general anesthesia, once in a week. Another group of OB-induced mice were subjected to topical Dex eye drop treatment twice a day for a week. On the 8th day, the mice were sacrificed, eyes were enucleated for corneal whole mount staining. Corneas including limbal area were dissected from freshly enucleated eyes, and surrounding conjunctiva, Tenon capsule, uvea, and lens were carefully removed, followed by making four slits with a scalpel blade at 90°, 180°, 270°, and 360° to flatten the corneas, then fixed in 4% (wt/vol) paraformaldehyde solution at room temperature for 1 h. Tissues were blocked with 10% goat serum and 0.5% Triton X-100 prepared in PBS for 1 h. Rat anti-mouse CD31 antibody (1:300) supplemented with 5% goat serum and 0.1% Triton X-100 was added to the tissues and allowed to incubate at 4°C for 3 days. After a series of washing with PBS, the tissues were incubated with Alexa-Fluor 594-conjugated goat anti-rat secondary antibody (Jackson Immuno Research, West Grove, PA, USA) in a dark chamber for 1 h at room temperature. The tissues were then mounted on slides using Fluoromount G (Southern Biotech, Birmingham, AL, USA) containing DAPI (1:300) (Life Technologies, Grand Island, NY, USA).

Thus prepared whole-mounted corneas were subjected laser scanning confocal fluorescence imaging on Nikon Eclipse Ni A1R confocal microscope provided with a 20X objective (Plan APO20X-0.75/OFN25-DIC-N2 by Nikon) and a 561 nm laser (blood vessel detection, red). Each Z-stack was captured using nonresonant galvano scanners, 512 × 512 pixel size, unidirectional scan, 0.5 scan speed, 2.2 pixel dwell, 0.9 μm Z-space, and 19.2 μm pinhole size. The Z stack Images were stitched by NIS Elements software, and deconvolved in the NIS Deconvolution module in order to improve the signal intensity.

The images of whole mount corneas clearly demonstrated a strong therapeutic effect of the Dex-NW treatment compared to the untreated OB control group (Figure 6D). The Dex-NW treatment has restricted the proliferation of blood vessels to the limbal area and very closely resembled the healthy uninjured cornea. However, the OB control, PVA-NW, and Dex eye drop treated corneas exhibited an extensive neovascularization (Figure 6). The new blood vessels were highly branched and extended from the limbal area toward the center of the cornea. In the case of Dex-NW treatment, the amount of drug delivered to the cornea was 10 μg per week, and for Dex eye drop treatment it was 10 μg per day, i.e., 70 μg per week). Although, eye drop treated mice received a lot more drug than the Dex-NW treated group, still the nanowafer was more effective than the topical eye drop treatment (Figure 6). These results also confirmed that the controlled drug release from Dex-NW is more effective in inhibiting CNV compared to the eye drop treatment even at a substantially lower dosing frequency.
Figure 6. Efficacy of Dexamethasone nanowafer on inhibiting corneal neovascularization. Representative 3D reconstructed laser scanning confocal fluorescence images of the cornea demonstrating the enhanced therapeutic efficacy of Dex-NW compared to the Dex eye drop treatment. (A) Healthy cornea. (B) OB-induced cornea. (C) PVA-NW. (D) Dex-NW. (E) Twice a day Dex-eye drop (0.1%) treatment.

Presently we are performing the image analysis using IMARIS software (Bitplane AG, Zurich, Switzerland) to quantify the blood vessel volumes. Completion of this study will provide the quantitative data to compare the therapeutic efficacy of Dex-NW and topical eye drop treatment on inhibiting the corneal neovascularization after ocular burn injury.

Objective (ii) Measurement of expression of relevant inflammatory mediator genes by real-time PCR

Therapeutic efficacy of the of nanowafer drug delivery was quantified by the measurement of the expression levels of proinflammatory cytokines and proangiogenic factors by reverse transcription polymerase chain reaction (RT-PCR) analysis in ocular burn (OB) induced mouse model. In this study, the therapeutic efficacy of once a week dexamethasone nanowafer (Dex-NW) treatment was compared with twice a day topical dexamethasone eye drop (Dex-eye drops) treatment by PCR analysis. The nanowafer (tiny circular discs of 2 mm diameter and 100 μm thickness) were applied once a week on the corneas of OB-induced mice. Another group of OB-induced mice were subjected to topical Dex eye drop treatment twice a day for a week. After one week, the mice were sacrificed. The corneas were collected and processed for evaluating proinflammatory and proangiogenic genes by RT-PCR analysis.

During the wound-healing process, the expression levels of proinflammatory cytokines, IL-1β, and IL-6, proangiogenic IL-8, VEGF-A, and MMP-12, tumor necrosis factor TNF, and transforming growth factor TGF-β1 will be upregulated. Quantification of the expression levels of these factors gives insights into the efficacy of Dex-NW on the regulation of inflammation and angiogenesis. Once a week Dex-NW was as effective as twice a day Dex-eye drop treatment for a week in suppressing the expression levels of proinflammatory cytokine IL-6, proangiogenic IL-8 and MMP-12, tumor necrosis factor TNF, and transforming growth factor TGF-β1 (Figure 7). Most importantly, the amount of Dex delivered by the Dex-NW was 10 μg per week, compared to 70 μg of Dex delivered as a topical eye drops twice a day for a week. These results reaffirmed the enhanced efficacy of Dex-NW once a week compared to the twice a day Dex eye drop treatment for the same period of time.
Figure 7. Enhanced therapeutic effect of Dexamethasone nanowafer. RT-PCR analysis revealing the suppression of the expression levels of proinflammatory and proangiogenic factors by Dex-NW. \( n = 3 \) (5 animals per group). *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \). All error bars represent standard deviation from the mean.

Presently, further studies are in progress to evaluate the efficacy of Dexamethasone-Nanowafers in controlling inflammation and wound healing in ocular burn induced mouse model.
What opportunities for training and professional development has the project provided?

1. **Dr. Daniela Marcano, Ph.D.** A postdoctoral research associate working on this project was provided necessary training and professional development opportunities. Specifically, Dr. Marcano was trained: (i) in the fabrication of nanowafer drug delivery systems, (ii) use of Nikon laser confocal fluorescence microscope and image analysis, (iii) pharmacokinetic analysis by HPLC, and (iv) preparation of animal protocols. As part of professional development, Dr. Marcano actively participated in the lab meetings, attended BCM seminars, and ARVO meeting held in Denver, CO. In addition, during one-on-one meetings, we have systematically reviewed and analyzed Dr. Marcano’s experimental protocols and results. All these activities have helped Dr. Marcano accomplish the defined objectives of the project and develop into a trained scientist.

2. **Dr. Crystal S. Shin, Ph.D.** A postdoctoral research associate working on this project was provided necessary training and professional development opportunities. Specifically, Dr. Shin was trained: (i) in the fabrication and optimization of nanowafer drug delivery systems, (ii) use of Nikon laser confocal fluorescence microscope and image analysis, (iii) in vitro pharmacokinetic analysis by HPLC. For professional development, Dr. Shin actively participated in the lab meetings, attended seminars and symposiums hosted by BCM, Rice University, and Texas Medical Center, and attended ARVO meeting held in Denver, CO. During one-on-one meetings, we have reviewed and analyzed Dr. Shin’s experimental progress and results. Dr. Shin was awarded the NIH-NEI travel grant to attend ARVO 2016 conference at Seattle, WA, and present this research work. With all these activities Dr. Shin was able to achieve the defined objectives of the project and develop into an independent scientist.

How were the results disseminated to communities of interest?
"Nothing to Report"

What do you plan to do during the next reporting period to accomplish the goals?

For the next reporting period (1 Oct 2015 to 30 Sep 2016), we plan to work on the following Tasks defined in the SOW: **Task 3**: Evaluation of *in vitro* and *in vivo* pharmacokinetics; and **Task 4**: Study of the efficacy of doxycycline-nanowafers, dexamethasone-nanowafers, and cyclosporine-A-nanowafers in an ocular burn mouse model.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
"Nothing to Report"

What was the impact on other disciplines?
"Nothing to Report"

What was the impact on technology transfer?
"Nothing to Report"

What was the impact on society beyond science and technology?
"Nothing to Report"
5. **CHANGES/PROBLEMS:**
The progress of the project was delayed due to unexpected delays in procuring mice and PCR reagents. Because of this a no cost extension of the project approval has been obtained from DOD.

6. **PRODUCTS:**

**Journal publications.**


**Conference papers**


**Presentations**


**Books or other non-periodical, one-time publications.**

"Nothing to Report"

**Website(s) or other Internet site(s)**

"Nothing to Report"

**Technologies or techniques**

"Nothing to Report"

**Inventions, patent applications, and/or licenses**

"Nothing to Report"

**Other Products**

"Nothing to Report"
### 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

**What individuals have worked on the project?**

<table>
<thead>
<tr>
<th>Name</th>
<th>Contribution to the Project</th>
<th>Funding Support</th>
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<tbody>
<tr>
<td><strong>Stephen C. Pflugfelder, M.D.</strong></td>
<td>Directed and oversaw the project performance of all experiments defined under Tasks 1, 2, and 3. Reviewed and analyzed the experimental results. Reviewed the animal protocol for IACUC/ACURO approval.</td>
<td>(Complete only if the funding support is provided from other than this award).</td>
</tr>
<tr>
<td><strong>Ghanashyam Acharya, Ph.D.</strong></td>
<td>Directed Tasks 2 &amp; 3. Fabricated the silicon wafer master templates, by e-beam lithography and photolithography. Fabricated PDMS imprints. Fabricated doxycycline and dexamethasone nanowafers. Developed HPLC methods for in vitro and in vivo drug release study of doxycycline and dexamethasone from the nanowafers. Designed the experiments, reviewed and analyzed the experimental results.</td>
<td>Cystinosis Research Foundation, DOD</td>
</tr>
<tr>
<td><strong>Daniela Marcano, Ph.D.</strong></td>
<td>Prepared the animal protocol for IACUC/ACURO submission. Fabricated the PDMS imprints. Fabricated doxycycline and dexamethasone loaded nanowafers. Performed in vitro drug release study of the nanowafers. Performed the fluorescence confocal imaging to monitor the in vivo doxycycline release in mouse eye. Performed the animal studies to evaluate the therapeutic efficacy of nanowaffer drug delivery and compared it with topical eye drop treatment in ocular burn induced mice.</td>
<td></td>
</tr>
<tr>
<td><strong>Crystal S. Shin, Ph.D.</strong></td>
<td>Performed and optimized the nanowaffer compliance experiments on mouse eyes. Performed in vivo drug release experiments. Performed laser confocal imaging experiments and quantified the</td>
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epithelial recovery and corneal neovascularization using IMARIS data analysis software.

**Funding Support**

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
"Nothing to Report"

What other organizations were involved as partners?
"Nothing to Report"

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:
"Not Applicable"

9. APPENDICES

**Appendix 1:** Journal publication

**Appendix 2:** Conference paper

**Appendix 3:** Conference abstract
Marcano et al. Extended release cysteamine nanowafer as an efficacious treatment modality for corneal cystinosis. Controlled Release Society Meeting 2016, July 17-20, Seattle, WA.

**Appendix 4:** Conference abstract
Appendix 1: Journal publication
Synergistic Cysteamine Delivery Nanowafer as an Efficacious Treatment Modality for Corneal Cystinosis

Daniela C. Marcano,† Crystal S. Shin,‡ Briana Lee,§ Lucas C. Isenhart,† Xing Liu,§ Feng Li,§ James V. Jester,‡ Stephen C. Pflugfelder,† Jennifer Simpson,*‡ and Ghanashyam Acharya,*†

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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 matrices 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 vascularature, 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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ophthalmic solution is the only medication approved by FDA for the treatment of corneal cystinosis.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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).

\section*{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\textsuperscript{−/−}) were used for drug delivery experiments. Mice were randomly assigned to each experimental group.

Nanowafer Fabrication. Cys-NW was fabricated via hydrogel template strategy.\textsuperscript{32–35} Briefly, a silicon wafer containing arrays of square wells (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 wiped 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 Quadrupole 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

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{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.}
\end{figure}
mice was determined as previously reported.37,38 Briefly, Cystine Crystal Volume. NIS Elements software (Nikon, Melville, NY). Obtained images were processed by counting 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 nanowafers were 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

−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 were always freshly prepared. Results were reported as the mean ± SEM of triplicates.

Confocal Laser Scanning Microscopy of OD Diffusion in the Cornea. Healthy female C57/B6L6 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.43 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.

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 24X 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.

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
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 mm²) 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) in 5% FBS and were maintained at 37 °C with 5% CO₂ 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% CO₂ 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 nanowafers are tiny transparent circular discs.
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 4 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. 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. 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. 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).

In 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.

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 cornea for up to 48 h (B–F).
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 $\lambda_{em}$ 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

**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).
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 S).

**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.35,34 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 nan wafer was also evaluated by measuring the amount of cystine present in cystinosin 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 nan wafer 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
proliferation of corneal epithelial cells. 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. 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. 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. 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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(31) Cystaran (cysteamine ophthalmic solution) 0.44% Sterile, Prescribing Information: http://www.cystaran.com/Cystaran_PI.pdf.


Appendix 2: Conference paper

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ARVO Annual Meeting Abstract | September 2016

Anti-Angiogenic Polymer Therapeutic for Corneal Neovascularization

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Footnotes

Commercial Relationships Crystal Shin, None; Xiaoyong Yuan, None; Daniela Marcano, None; Lucas Isenhart, None; Ken Simmons, None; Stephen Pflugfelder, None; Ghanashyam Acharya, None

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Abstract

http://iovs.arvojournals.org/article.aspx?articleid=2557616&resultClick=1
**Purpose**: Currently corneal neovascularization is treated with topical formulations of anti-inflammatory or anti-VEGF drugs which can cause adverse side effects. The purpose of this study was to develop a non-invasive biopolymer therapeutic nanowafer to treat corneal neovascularization. The *in vivo* efficacy of the nanowafer was evaluated in a murine ocular burn model.

**Methods**: A sulfated polysaccharide, dextran sulfate (DS) nanowafers and an antiangiogenic drug, axitinib, loaded DS nanowafers were fabricated by hydrogel template strategy. The chemical burn induced mouse corneas were treated daily with the DS nanowafers, axitinib loaded DS (Axi-DS) nanowafers, DS eye drops to compare their therapeutic efficacies and to evaluate synergistic effect of DS. After treatments, mouse corneas were subjected to immunofluorescence imaging and RT-PCR analyses.

**Results**: Overall, the nanowafer treatment minimized corneal neovascularization when compared to untreated ocular burn corneas in murine models. Immunofluorescence images presented CD31 positive vasculature endothelium in corneas and showed the DS nanowafer treatment was as effective as Axi-DS nanowafer treatment in suppressing neovascularization. The RT-PCR analysis revealed that after five days of DS nanowafer treatment, gene expressions of proinflammatory cytokines (IL-1α and IL-1β) and angiogenic factors (VEGFA, VEGFR1, and VEGFR2) were downregulated (Figure 1). This result showed that DS nanowafer had anti-angiogenic effect on inhibiting corneal neovascularization.

**Conclusions**: This study demonstrated the anti-angiogenic effect of a biopolymer, dextran sulfate, in a murine ocular burn model. In addition, an anti-angiogenic drug incorporated dextran sulfate nanowafer maximized their therapeutic efficacies.

This is an abstract that was submitted for the 2016 ARVO Annual Meeting.
Figure 1. RT-PCR analysis showing that downregulation of proinflammatory cytokines and angiogenic factors by nanowafer treatments.

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Appendix 3: Conference abstract

Marcano et al. Extended release cysteamine nanowafer as an efficacious treatment modality for corneal cystinosis. Controlled Release Society Meeting 2016, July 17-20, Seattle, WA.
2016 CRS Annual Meeting
Presentations
July 17-20, 2016
Seattle, Washington

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- Taking Stock of Progress and Challenges in Drug Delivery and Targeting
- Thinking Outside the Box Delivery Technologies: Nanocarriers from Nature
- Tissue Engineering
- Transdermal Delivery

11 Silicon-Based Nanomaterials for Ocular Drug Delivery
Presenter: Sailor, Michael. Authors: M. SAILOR (1), J. Wang (1), T. Kumeria (1), L. Cheng (1), H. Hou (1), W. Freeman (1)
12 Bridging Product Design and Performance for Bioequivalence: A Journey through the Eye
Presenter: Xu, Xiaoming. Authors: X. XU (1)
(1) U.S. FDA, U.S.A.
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13 An Update on FDA's Research Program for Ophthalmic Generic Products
Presenter: Choi, Stephanie. Authors: S. CHOI (1)
(1) FDA, U.S.A.
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269 Smart Wireless Contact Lens for Ocular Theranosis
Presenter: Keum, Dohee. Authors: D. KEUM (1), S. Hahn (1)
(1) POSTECH, Korea
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270 Ultradeformable bilosomes for enhanced ocular delivery of terconazole: In vitro characterization and factorial analysis
Presenter: Abdelbary, Aly. Authors: A. ABDELBARY (1), A. Al-mahallawi (1), W. Abd-Elsalam (1)
(1) Faculty of Pharmacy-Cairo University, Egypt
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271 Ultrasound-responsive nanobubbles for enhanced posterior eye delivery of therapeutics
Presenter: Thakur, Sachin. Authors: S. THAKUR (1), H. Parekh (1), I. Rupenthal (2), E. Chen (2)
(1) School of Pharmacy, the University of Queensland, Australia; (2) Buchanan Ocular Therapeutics Unit, School of Medicine, the University of Auckland, New Zealand
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272 Extended Release Cysteamine Nanowafer as an Efficacious Treatment Modality for Corneal Cystinosis
Presenter: Marcano, Daniela. Authors: D. MARCANO (1), C. Shin (1), B. Lee (2), L. Isenhart (1), F. Li (1), J. Jester (2), S. Pflugfelder (1), J. Simpson (2), G. Acharya (1)
(1) Baylor College of Medicine, U.S.A.; (2) University of California, U.S.A.
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273 Convective loading and release of latanoprost via a silicone hydrogel contact lens in vitro
Presenter: Pitt, William. Authors: R. HORNE (1), W. Pitt (1), K. Judd (1)
(1) Brigham Young University, U.S.A.
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274 Retinylamine Modified Multifunctional Lipid DNA Delivery System for the Treatment of LCA2
Presenter: Sun, Da. Authors: D. SUN (1), B. Sahu (2), S. Gao (2), A. Maeda (2), K. Palczewski (2), Z. Lu (2)
(1) Case Western Reserve University, U.S.A.; (2) Case Western Reserve University, U.S.A.
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275 Potential Preclinical Approaches to Establish Bioequivalence of Ophthalmic Products
(1) Suven Life Sciences Limited, India
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276 Flexible Formulating for Problem Therapeutics
Presenter: Groves, Rhian. Authors: R. GROVES (1), P. Seaman (1)
(1) Midatech Pharma, United Kingdom
Extended Release Cysteamine Nanowafer as an Efficacious Treatment Modality for Corneal Cystinosis

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Purpose: To demonstrate the enhanced efficacy of stability of cysteamine nanowafer compared to cysteamine eye drops for the dissolution of corneal cystine crystals in a cistinosin knock-out mouse (CTNS−−) model.

Methods: Nanowafers of 2 mm in diameter and 80 µm thick were fabricated by a modified hydrogel template strategy (1,2). The nanowafer contains arrays of cysteamine filled nanoreservoirs (500 nm diameter). In treatment group CTNS−− mice (7 month old), cysteamine nanowafers were applied daily for 30 days. Another group CTNS−− of mice were treated once a day with 5 µL of the cysteamine eye drop solution (0.44%). The therapeutic effect of the cysteamine-nanowafer was evaluated both by measuring the reduction in the corneal cystine crystal volume (3) and by quantifying the amount of cystine by mass spectrometry. The stability of the cysteamine in the drug-loaded nanowafers was assessed by determining the concentration of cysteamine and cystamine (oxidation product) in nanowafers that were prepared and stored for 10, 12, 14, and 16 weeks at room temperature.

Results: Cysteamine-nanowafers were stable for up to 4 months, and no formation of cystamine was observed during this period. Cystine quantification in corneas by mass spectrometry showed that the cysteamine-nanowafer reduced the corneal cystine content by 65% and the eye drops by 34%. Remarkably, the amount of cysteamine delivered per day by the nanowafer was 10 µg, while the twice a day cysteamine eye drop treatment delivered 22 µg. Cystine quantification by cystine crystal content in the corneas, estimated by laser confocal image analysis, showed that in comparison to the baseline corneal cystine crystal volume, eye drop treatment reduced the crystal volume by 55%, while the cysteamine-nanowafer treatment reduced the crystal volume by 90%.

Figure 1. (A) Nanowafer applied on a mouse cornea. Laser confocal images of (B) cystinosis cornea, (C) eye drops treated cornea, (D) cysteamine-nanowafer treated cornea. (E) Quantification of corneal cystine crystals.

Conclusions: These studies revealed that cysteamine loaded in nanowafers was stable for up to 16 weeks when stored at room temperature, unlike the eye drop formulation, in which cysteamine is stable only for a week, even under refrigerated conditions. Reduction of the corneal crystals confirmed the efficacy of the loaded nanowafer.

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References:
Appendix 4: Conference abstract

### 2016 CRS Annual Meeting Presentations

**July 17-20, 2016**  
Seattle, Washington

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### 11 Silicon-Based Nanomaterials for Ocular Drug Delivery

Presenter: Sailor, Michael. Authors: M. SAILOR (1), J. Wang (1), T. Kumeria (1), L. Cheng (1), H. Hou (1), W. Freeman (1)
277 OpsiSporin - Development of Controlled Release Cyclosporin for the treatment of posterior uveitis
Presenter: Bamsey, Katherine. Authors: K. BAMSEY (1), P. Seaman (1), R. Groves (1)
(1) Midatech Pharma, United Kingdom

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278 Dextran Sulfate Wafer as an Anti-Angiogenic Polymer Therapeutic
Presenter: Shin, Crystal. Authors: C. SHIN (1), X. Yuan (1), D. Marcano (1), L. Isenhart (1), K. Simmons (1), S. Pfugfelder (1), G. Acharya (1)
(1) Baylor College of Medicine, U.S.A.

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279 Composite Nanotherapeutics for Long Term Ocular Delivery of Macromolecules
Presenter: Agrahari, Vibhuti. Authors: V. AGRAHARI (1), V. Agrahari (2), W. Hung (3), L. Christenson (3), A. Mitra (2)
(1) University of Missouri-Kansas City, U.S.A.; (2) University of Missouri Kansas City, U.S.A.; (3) University of Kansas Medical Center, U.S.A.

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280 Template Directed Chemical Polymerization of Electroactive Polypyrrole Particles Loaded With Dexamethasone
(1) The University of Auckland, New Zealand; (2) Monash University, Australia

View Abstract

281 Controlled Release of Avastin® from the Tethadur™ Biodegradable Matrix
(1) PsiMedica, United Kingdom

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Oligonucleotide Delivery: New Applications and Opportunities

14 Engineering Cyclodextrin Nanoparticles for the Delivery of siRNA
Presenter: Geall, Andrew. Authors: A. GEALL (1)
(1) Avidity NanoMedicines, U.S.A.

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15 Design of lipid nanoparticle delivery systems to enable therapeutic applications of siRNA and mRNA
Presenter: Cullis, Pieter. Authors: P. CULLIS (1)
(1) University of BC, Canada

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282 Exploring MicroRNA Expression Profiles Related to Cell Death Pathways in Mouse Embryonic Fibroblast Cells Treated with Polyethyleneimine
Presenter: Kuo, Jung-hua. Authors: J. KUO (1), M. Jan (2), C. Lin (2)
(1) Department of Pharmacy, Chia Nan University of Pharmacy and Science, Taiwan; (2) Institute of Microbiology and Immunology, Chung Shan Medical University, Taiwan

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283 Dual-sensitive mixed-micelles for co-delivery of miRNA 34a and doxorubicin into tumor cells
Presenter: Costa, Daniel. Authors: D. COSTA (1), G. Salzano (1), C. Sariosen (1), E. Luther (1), P. Dhargalkar (1), G. Mattheolabakis (1), V. Torchilin (1)
(1) Northeastern University, U.S.A.

View Abstract
Dextran Sulfate Wafer as an Anti-Angiogenic Polymer Therapeutic
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**Purpose:** Eye injuries, prolonged inflammation, infections, and chronic dry eye trigger corneal neovascularization (CNV) leading to impaired vision and blindness. Presently, eye injuries are treated with topical eye drop formulations of corticosteroid drugs: fluorometholone, prednisolone acetate, and dexamethasone which cause side effects, such as glaucoma, cataract formation, and temporary blurred vision, resulting in discomfort and poor patient compliance [1]. To surmount these issues, we have developed a dextran sulfate (DS) polymer wafer for the effective treatment of CNV with negligible side effects. In this study DS was chosen for wafer fabrication because of its demonstrated anti-angiogenic attributes [2].

**Methods:** The effect of dextran sulfate on cell proliferation was first evaluated in vitro. Dextran sulfate of different molecular weights, 500 kDa and 40 kDa were used to treat human mammary epithelial cell line (HMLE), human umbilical vein endothelial cell line (HUVEC), and murine endothelial cell line (2H11) and MTS assay was performed. DS (500 kDa) eye drop formulation was prepared and DS (500 kDa) wafers were fabricated by modified hydrogel template strategy [3]. The in vivo therapeutic efficacy of DS wafer was evaluated in ocular burn (OB) induced mouse model. The OB induced mice were treated daily with DS eye drops and DS wafer for 14 days. After treatments, mouse corneas were enucleated and incubated with a platelet endothelial cell adhesion molecule-1 marker, CD31, for immunofluorescence imaging.

**Results:** Our studies have demonstrated that cell proliferation in HMLE, HUVEC, and 2H11 cell lines was inhibited when cells were treated with DS; the higher concentration of 500 kDa DS was more effective in inhibiting proliferation in all cell lines (Fig 1). Therefore, high molecular weight DS was chosen for fabricating DS wafers for further animal studies. After 14 days of treatment, immunofluorescence imaging analysis showed that wafer treatments were effective in suppressing corneal neovascularization compared to untreated burned corneas and DS eye drop treated corneas (Fig 2). In untreated burned corneas, vascularization towards the center of the cornea was observed. The DS wafer treatment was more effective than the eye drop treatment in minimizing vascularization.

**Conclusions:** This study demonstrated that DS wafer is more efficacious as an anti-angiogenic polymer therapeutic compared to topical DS eye drop treatment for treating CNV in ocular burn induced mouse model.

**References:**