**Sustained-Release Cysteamine Nanowafer Drug Delivery for Corneal Cystinosis**

Semi-Annual Progress Report

Cystinosis Research Foundation

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**Introduction**

The **goal** of this research project is to develop effective sustained release delivery of cysteamine for the treatment of corneal cystinosis. Nanopatterened wafer (*nanowafer*) technology will be utilized to overcome several drug delivery challenges that are unique to the eye and which have limited the effectiveness of topical therapy. The physiology of the ocular surface enables highly efficient removal of topically delivered eye drops in the first 15-30 seconds, leading to reduced drug efficacy. Because of this rapid clearance, as well as poor cornealpenetration and instability, cysteamine eye drops must be administered several times in a day resulting in a high-and-low drug profile. Frequent dosing also is inconvenient and uncomfortable, both of which affect patient compliance. Alternative drug delivery strategies have mainly focused on topical formulations, with modest to negative impact on effectiveness. To overcome these issues, we propose to develop a nanofabricated drug delivery system that can deliver the drug in a controlled release fashion for prolonged periods, thereby enhancing the drug efficacy and patient compliance.

Current nanofabrication technologies can be integrated with controlled drug delivery technology to develop a programmable *nanowafer* for sustained-release delivery of cysteamine, using materials (polyvinyl alcohol (PVA), cysteamine, PLGA, and collagen) that are already in clinical use. The *nanowafer* contains a patterned array of nano reservoirs fabricated using a hydrogel template strategy and then loaded with cysteamine for sustained-release delivery. The drug molecules slowly diffuse out from the nano reservoirs to the surrounding tissue, thus enabling the drug availability for a longer duration of time. In addition, the desired drug release profiles can be obtained by fabricating *nanowafers* containing drug reservoirs of different dimensions. The availability of an animal model that demonstrates a quantitative response to topical cysteamine therapy facilitates the testing of such *nanowafers*.

Based on the above, we hypothesize that hydrogel nanowafers loaded with PLGA coated cysteamine may be effectively tested for therapeutic delivery of topical cysteamine to reduce intracorneal cystine crystals in Cystinosin (Ctns-/-) knockout mice. To test this hypothesis we propose the following three specific aims:

**Specific Aim 1: Fabrication of cysteamine nanowafer *via* hydrogel template strategy**

Hydrogel wafers containing arrays of nanoreservoirs that can be readily filled with cysteamine-PLGA matrix will be fabricated using the hydrogel template strategy. The hydrogel nanowafers will be fabricated with 200 nm, 500 nm, and 1µm, and 1.5 µm features. The nanoreservoirs in the hydrogel wafer will be filled with cysteamine-PLGA by microinjection. The nanowafer integrity will be characterized by optical and SEM imaging.

**Specific Aim 2: Evaluation of *in vitro* and *in vivo* pharmacokinetics in mouse model**

Nanowafers will be analyzed for total cysteamine content and the *in vitro* cysteamine release kinetics in PBS-Tween 20 release medium by HPLC. *In vivo* cysteamine pharmacokinetics will be evaluated by instilling cysteamine nanowafer in a healthy mouse eye and collecting tear washings at predefined time intervals. The tear washings will be analyzed for cysteamine content by spectrophotometry.

**Specific Aim 3: Evaluation ofefficacy of cysteamine nanowafer**

Cysteamine nanowafer efficacy will be assessed via quantitative measurement of corneal cystine crystal volume (CVI) using *in-vivo* confocal microscopy in twenty 8-month-old Ctns-/- mice treated over a three-month period.

**Progress made during the first year of the grant period (10/1/2012 to 9/30/2013**

We have made significant progress and the project is moving as per the schedule. We have recently acquired a Shimadzu HPLC system to conduct the in vitro and in vivo pharmacokinetics and this will further accelerate the progress of this project. To date, we have focused on all the three specific aims.

**Specific Aim 1:**

Recently, we have developed the hydrogel template strategy for the fabrication of nanopatterned hydrogel wafers that can function as controlled release drug delivery systems. The first step is the nanofabrication of arrays of vertical posts on a silicon wafer by e-beam lithography. The silicon wafers thus fabricated were used for the imprinting of hydrogel nanowafers. On top of the silicon wafer, a hydrogel forming polymer solution was poured and heated for 30min at 70 °C. Once the polymer layer is solidified, it is peeled off from the silicon wafer. The nanoreservoirs will be filled with drug-PLGA matrix using an ultrasonic atomizing nozzle or by microinjection. PVA is an FDA approved material and has been used as a lubricant in eye drops.

As a first step nanowafers having 500nm diameter wells has been fabricated (**Figure 1**). These nanowafers were filled with cysteamine hydrochloride by microinjection. The cysteamine nanowafers containing cysteamine concentrations of 500ng, 250ng, 200ng, 175ng, 150ng, 100ng, 50ng and 0ng were fabricated. The cysteamine molecules slowly diffuse out from the nanowafer thus enabling a constant availability of the drug in the eye.



**Specific Aim 2:**

a. Analysis of *in vitro* and *in vivo* cysteamine release

We are presently studying the in vitro cysteamine release kinetics from the nanowafer by HPLC analysis. We have just purchased a Shimadzu HPLC system and this study will begin next month.

2. Demonstration of drug molecular distribution in the corneal epithelium

To demonstrate the diffusion of cysteamine molecules and their increased residence times in the cornea, we have fabricated: (i) red fluorescent nanospheres (60nm diameter) loaded and (ii) gold nanospheres (60nm diameter) loaded nanowafers. These nanowafers were instilled on the corneas of healthy mice. The mice were sacrificed after 2 and 5 days respectively and the eyes were microtomed to obtain 5 µm slices and were examined by confocal fluorescence imaging and transmission electron microscopy (TEM) to ascertain the diffusion and residence times of the nanospheres. As can be seen from the **Figures 3A-C**, the red fluorescent nanospheres diffused into the cornea and remained there even after 5 days. To further confirm the nanoparticle diffusion in the cornea, we placed gold nanosphere loaded nanowafer on the corneas of healthy mice and examined by TEM imaging. TEM analysis reaffirmed the results obtained by confocal fluorescence imaging. As can be seen from the **Figures 3D-F**, the gold nanoparticles (tiny black spots in the images) diffused into the corneal epithelium and stroma, and after 5 days the density of the gold nanoparticles was a little less. **Taken together, these results confirm that the enhanced efficacy of the cysteamine nanowafers in dissolving the corneal crystals is because of the enhanced residence time of the drug molecules in the cornea.**

**Specific Aim 3:**

(a) Drug escalation study

We conducted a preliminary study to determine potential corneal toxicity (corneal stromal opacity, corneal neovascularization or corneal epithelial changes) of the cysteamine nanowafer in mouse corneas. Five drug concentrations of cysteamine nanowafers were fabricated: 500, 200, 100, 50 and 0 ng/wafer ( 0= blank wafer). 25 CTNS+/- mice ages 3-5 months were divided into 5 groups with 5 animals per group. A cysteamine nanowafer was applied to the right cornea of each animal every third day for one week. **Table 1** shows the corneal changes that were noted. No visible corneal toxicity was noted in blank wafers, or at 50 or 100 ng/wafer doses. At day four, 50% of the 500ng/wafer corneas displayed corneal stromal opacity at day seven 100% displayed stromal opacity. For the 200 ng cysteamine/wafer group, one cornea displayed subtle questionable corneal opacity at Day 7.



Based on these results, we selected wafers with cysteamine concentrations of 150 and 175 ng/wafer to evaluate the effect on corneal crystal concentration. Seven month-old CTNS -/- mice were divided into two groups of 5. Baseline corneal cystine crystal volume was quantified using in-vivo confocal microscopy at time zero in the right eye of each animal. A cysteamine nanowafer of either 150 or 175 ng/wafer was placed on the right eye every third day for 27 days. At day 27 corneal crystal volume was re-measured using in-vivo confocal microscopy. **Figure 3** shows the results for this study, demonstrating a dose dependent reduction in corneal crystals.



Based on these preliminary results we repeated this study. We applied 150 and 175ng Cysteamine/wafer nanowafer once a week to two groups of ctns-/- mice (seven mice per group) 11-13 months of age for a 2-month duration. In vivo confocal corneal imaging was obtained at baseline and then every two weeks for the duration of the study. The percent change in crystal volume from baseline at 2 4, and 6 weeks is shown in the two groups combined (y-axis represents animal ID number in Figure 3b). At 2, 4, and 6 weeks, there is a trend toward a decreasing crystal volume over time in the treatment eye. It is noteworthy that by 6 weeks, 43% of treated corneas ( 6 out of 14) underwent scarring compared to 93% (13 out of 14) in the contralateral control eye.

***Comparison of Cysteamine Nanowafer Efficacy with Eye Drops***

To determine the efficacy of cysteamine nanowafer in comparison to its eye drop formulation, two groups of Ctns-/- mice were treated separately with cysteamine nanowafers (one nanowafer each time, thrice a week) and cysteamine eye drops daily 6 times for 30 days. At the end of this period, the cystine crystal content in the cornea was estimated by laser confocal imaging and by mass spectrometric analysis of the cystine extracts from the homogenized corneas. These studies suggest that application of one cysteamine nanowafer three times per week is more efficacious in reducing cystine crystals than the administration of eye drops six times a day (**Figure 4)**.



Figure 3b

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In summary, the preliminary experimental results demonstrate: (1) feasibility of the research idea; (2) nanowafer fabrication and controlled drug release attributes; (3) diffusion of nanoparticles from the nanowafer into the corneal epithelium and stroma; and (4) efficacy of the cysteamine nanowafer as compared to cysteamaine drops in rapidly dissolving the cystine crystals in the corneal by confocal image analysis.

**Future objectives**:

Future work will be directed at establishing the in vitro and in vivo pharmacokinetics in the mouse model as outlined in specific aim 2 and using the clinically effective dosage established in our preliminary studies.