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Broadly Applicable Nanowafer Drug Delivery System for Treating Eye Injuries

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14. 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 first year of this project, dexamethasone and doxycycline loaded nanowafers have been fabricated. The in vitro drug release kinetics from the nanowafers has been studied by HPLC analysis. The in vivo drug release in the mouse cornea by laser confocal fluorescence imaging study revealed that the nanowafers upon instillation on mouse eye were able to release the drug for up to 24 hours. We are presently optimizing the drug release for up to 5 days.					
15. SUBJECT TERMS Nanowafer, fabrication, drug delivery, doxycycline, dexamethasone, cyclosporine-A, pharmacokinetics, ocular burn					
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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, fabrication, 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: 1 Dec 2013 to 30 Sep 2014. Specifically, we have accomplished: the following objectives defined under each task.

Task 1. Regulatory approvals (IACUC/ACURO/HRPO)

Duration: 3 months (months 1-3)

We have obtained IACUC/ACURO protocol approval from Baylor College of Medicine and DOD.

Protocol Title: Broadly applicable nanowafer drug delivery system for treating ocular burn injury
Protocol Number: AN-6366.

Task 2. Fabrication of nanowafer drug delivery systems

Duration 18 months (months 1-18)

This task involves the completion of three objectives: **Objective (i)** Nanofabrication of silicon wafer master templates and PDMS imprints with feature dimensions of 100nm, 200nm, 500nm, 1µm, 1.5µm, and 3µm; **Objective (ii)** Fabrication of polyvinylpyrrolidone (PVP), dextran (DTR), carboxymethyl cellulose (CMC), and hydroxypropyl methyl cellulose (HPMC) nanowafers with feature dimensions of 100nm, 200nm, 500nm, 1µm, 1.5µm, and 3µm; and **Objective (iii)** Fabrication of drug-filled nanowafers: doxycycline-nanowafers; dexamethasone-nanowafers; and cyclosporine-A-nanowafers with feature dimensions of 100nm, 200nm, 500nm, 1µm, 1.5µm, and 3µm. Completion of each objective and the outcomes are described below.

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

Duration: 12 months (months 1-12)

Under this objective, silicon wafer master templates having a series of well diameters were fabricated. The feature patterns to be written on the silicon wafers were designed using AutoCAD program and transferred to the e-beam lithography system. The silicon wafer templates with arrays of wells of 100nm, 200nm, 500nm, and 1µm diameters were fabricated by electron beam lithography, and wells of 1.5µm, and 3µm diameters were fabricated by photolithography. The silicon wafer master templates were examined by scanning electron

microscopy (SEM) for the feature integrity and uniformity. The SEM images revealed the presence of regular arrays of square and circular wells of predefined dimensions (**Figure 1**).

Representative experimental procedures for the fabrication of a silicon wafers containing 200nm and 3μm features is described below.

(a) Fabrication of a silicon wafer containing 200nm features by E-beam lithography

Making surface patterns of submicron size features requires e-beam lithography. Circular patterns of 200nm squares were designed using the Auto CAD2007 program. A 6-in. diameter silicon wafer covered with 1 μm thick SiO₂ layer (University Wafer, South Boston, MA) was spin coated with poly(methyl methacrylate) (PMMA, Microchem, Newton, MA) photoresist of 300nm thick layer was spin coated at 3500rpm for 30s (SCS P6708 spin coating system, Indianapolis, IN). The coated PMMA photoresist layer was exposed to e-beam in a pre-programmed pattern using a Leica VB6 High Resolution Ultrawide field e-beam lithography Instrument (Bannockburn, IL) operating at 100KV, transmission rate 25MHz current 5nA. After e-beam lithography, the silicon wafer was developed in 3:1 isopropanol:methyl isobutyl ketone solution to remove exposed regions of the photoresist. A 5nm thick chromium layer and a 20nm thick gold layer were successively deposited on to this pattern followed by liftoff of the residual PMMA film in refluxing acetone. The pattern was transferred to the underlying silicon oxide by deep reactive ion etching with SF₆/O₂ plasma. The silicon master templates having features sizes of 100nm, 200nm, 500nm, 1μm were fabricated by this method.

(b) Fabrication of a silicon wafer containing 3μm wells by UV photolithography

A silicon wafer was spin coated with SU8 2005 photoresist (Microchem, MA) at 3500rpm for 30s followed by baking at 95 °C for 3min. The photoresist coated silicon wafer was exposed to UV radiation through a mask containing a 3 μ m diameter circular pattern for 12s. After exposure, the silicon wafer was post baked at 95 °C for 3 min followed by development in SU- 8 developer for 2 min. The silicon wafer was rinsed with isopropanol and dried with nitrogen gas. The wafer thus fabricated contained 3 μ m diameter wells. Similarly, a silicon wafer template having 1.5 μ m diameter wells was fabricated following this procedure with minor modifications.

(c) Fabrication of PDMS imprints using the silicon wafer master templates

Using the silicon wafer master templates, polydimethylsiloxane (PDMS) imprints were fabricated. First, a prepolymer solution of Sylgard 184 elastomer (90g) mixed with 9g of the curing agent (10:1 by weight). The prepolymer solution was poured on to the silicon master template and cured at 80 °C for 10h in an oven (Isotemp 500 Series Economy Lab Ovens, Fisher Scientific). At the end of this period, the PDMS template was carefully peeled away from the silicon master template. Using this process, PDMS templates for each feature size (100nm, 200nm, 500nm, 1 μ m, 1.5 μ m, and 3 μ m) were fabricated (**Figure 2**).

Figure 2. SEM and bright field micrographs of PDMS templates containing vertical posts of: (A) 100 nm; (B) 200 nm; (C) 500 nm; (D) 1 μ m; (E) 1.5 μ m; and (F) 3 μ m. Scale bars: for the micrographs (B) to (E) is 10 μ m and for (F) 20 μ m.

Objective (ii) Fabrication of polyvinylpyrrolidone (PVP), dextran (DTR), carboxymethyl cellulose (CMC), and hydroxypropyl methyl cellulose (HPMC) nanowafers with feature dimensions of 100nm, 200nm, 500nm, 1 μ m, 1.5 μ m, and 3 μ m.
Duration: 15 months (months 3-18)

Under this objective, PVP, DTR, CMC, and HPMC nanowafers with well dimensions of 100nm, 200nm, 500nm, 1 μ m, 1.5 μ m, and 3 μ m have been fabricated. These nanowafers were characterized for integrity of the well dimensions and array patterns by atomic force microscopy (AFM). As representative examples, the AFM images of CMC nanowafers are presented in

Figure 3. The AFM study revealed that all the nanowafers have arrays of wells of predefined dimensions without any defects.

We have fabricated the polymer nanowafers and as a representative example, the fabrication of CMC nanowafers is described here.

(a) Fabrication of CMC nanowafers

A clear thick CMC solution (5% w/v) was prepared by dissolving CMC (5g) in 100 ml of ethanol:water mixture (6:4) on a stirring hot plate (RT Elite Stirring Hotplate, Fisher Scientific). Thus prepared polymer solution (10 ml) was transferred with a pipette onto a PDMS template containing vertical posts placed on a spin coating system. The spin speed was adjusted to 500rpm and spun for 45sec to obtain a 100μm thick nanowafers. The concentration of the polymer solution, speed and spinning times were optimized for each polymer to obtain the required thickness of the nanowafers. After spin coating, the wafers were baked in an oven (Isotemp 500 Series Economy Lab Ovens, Fisher Scientific) at 60 °C for 15min. This procedure resulted in the fabrication of nanowafers having nanoreservoirs of predefined dimensions (e.g.

1µm diameter and 1µm deep). The nanowafer were characterized by AFM (**Figure 3**). By this method, nanowafers of PVP, DTR, CMC, and HPMC for each feature size (100nm, 200nm, 500nm, 1µm, 1.5µm, and 3µm) were fabricated.

(b) Atomic force microscopy (AFM)

A Catalyst Bioscope Atomic Force Microscope (Bruker) combined with a fluorescence microscope (Olympus) was used for testing and imaging the nanowafers. The AFM probe is a Scanasyst-Air (Bruker Nano) with a tip diameter of 2-5nm, cantilever dimensions: 115µm (l), 25 µm (w), and 250nm (thick). The cone shaped probe length is 10 µm with 18° cone. Cantilever spring constant is 0.4Nm. The AFM experiment was conducted under ambient conditions. The scan rate is 1Hz.

Objective (iii) Fabrication of drug-filled nanowafers: doxycycline-nanowafers; dexamethasone-nanowafers; and cyclosporine-A-nanowafers with feature dimensions of 100 nm, 200 nm, 500 nm, 1 µm, 1.5 µm, and 3 µm.
Duration: 12 months (months 6-18)

Under this task, nanowafers filled with doxycycline and dexamethasone were fabricated by microinjection. The following drug filled nanowafer have been fabricated for further study: Dexamethasone-PVP, Doxy-PVP, Dexamethasone-DTR, Doxy-DTR, Dexamethasone-CMC, Doxy-CMC, Dexamethasone-HPMC, Doxy-HPMC, with drug reservoir dimensions of 100nm, 200nm, 500nm, 1µm, 1.5µm, and 3µm. As representative examples, fluorescence micrographs of Doxy-CMC nanowafers are presented in **Figure 4**. Fluorescence imaging study was performed because of the intrinsic fluorescence property of doxycycline. This study revealed the presence of arrays of doxycycline filled drug reservoirs in the Doxy-CMC nanowafers.

Figure 4. Drug filled nanowafers. Fluorescence micrographs of Doxy-CMC nanowafers: (A) 500nm; (B) 1.5µm; and (C) 3µm

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).
Duration: 15 months (months 6-21)

The drug-loaded nanowafers were investigated for their drug release kinetics by HPLC analysis and are being optimized for extended drug release. As representative examples, the *in vitro* drug release kinetics of Doxy-CMC and Dexamethasone-CMC nanowafers is described here. The

Doxy-CMC and Dexa-CMC nanowafers were evaluated for their total drug content and in vitro drug release kinetics by HPLC. In this study, CMC nanowafers having an array of 1 μ m diameter reservoirs filled with doxycycline and dexamethasone were used.

Analysis of *in vitro* drug release from the CMC nanowafers

The total drug content in each nanowafers was determined by dissolving accurately weighed nanowafers in 1ml PBS, followed by addition of ethanol (9ml). The precipitated CMC was removed by centrifugation. The clear solution was rotary evaporated and the solid formed was redissolved in the mobile phase (10ml). An aliquot of this solution was filtered through a 0.2 μ m syringe filter, analyzed by HPLC, and was compared with the standard curve to quantify the content. From this study, (1) the doxycycline content in a 6mm diameter CMC nanowafers was 298 μ g and is 14% of the total mass of the nanowafers, and (2) the dexamethasone content in a 6mm diameter CMC nanowafers was 433 μ g and is 16% of the total mass of the nanowafers.

The drug release from the CMC nanowafers was studied by HPLC analysis. Accurately weighed Doxy-CMC nanowafers were placed in 5 separate 1ml dialysis tubes filled with PBS buffer as release medium. Each dialysis tube was placed in 5ml conical tube filled with 5ml of the release medium. The conical tubes were kept in an orbital shaker maintained at 37 °C with constant agitation. At predefined time intervals 1ml of the release medium was withdrawn from each tube and replaced with the same amount of the fresh medium. Thus collected samples were transferred into glass vials and stored in the refrigerator. Sampling of the release medium was continued for 24h. The samples thus collected were subjected to HPLC analysis. Exactly the same procedure was followed for the dexamethasone release study from Dexa-CMC nanowafers.

The HPLC analysis of the drug release study revealed that the Doxy-CMC nanowafers released ~18% (150 μ g) of doxycycline after 6h and it was ~39% (340 μ g) after 24h (**Figure 5**). In the case of Dexa-CMC nanowafers, the dexamethasone released was ~17% (215 μ g) after 6h and ~39% (490 μ g) after 24h (**Figure 6**). Taken together, the in vitro drug release study confirmed that, both doxycycline and dexamethasone release from the CMC nanowafers was slow and there was no initial burst release, and followed a zero order release kinetics. Presently, work is in progress to optimize the fabrication, well diameters, and drug loading parameters for the nanowafers to accomplish drug release for up to 5 days.

Figure 5. *In vitro* doxycycline release from the CMC-Doxy nanowafer analyzed by HPLC: (A) cumulative amount of drug released and (B) cumulative percentage drug released in a 24 hour time period.

Figure 6. *In vitro* dexamethasone release from the CMC-Dexa nanowafer analyzed by HPLC: (A) cumulative amount of drug released and (B) cumulative percentage drug released in a 24 hour time period.

The experimental procedures for the HPLC analysis are presented below.

(a) Determination of total drug content in a nanowafer

The total amount of a drug loaded in the nanowafer was determined by dissolving an accurately weighed nanowafer in 5 ml PBS solution, followed by addition of ethanol (5 ml). The precipitated polymer was removed by centrifugation. The clear solution was rotary evaporated to separate the solid drug. The solid drug was re-dissolved in 10 ml of the mobile phase (90% methanol and 10% ammonium acetate, pH 7). An aliquot of this solution was filtered through a 0.2 μ m syringe filter, analyzed by HPLC, and compared with the standard curve to quantify the total drug content in the nanowafer. This experiment was performed in triplicate.

(b) Study of drug release kinetics of the nanowafers by HPLC analysis

HPLC experiments were performed on a Shimadzu Prominence HPLC. The analytical column was Kinetex 5uXB-C18 100A (150 mm x 4.6 mm) from Phenomenex. The system was equipped with autosampler, in line degasser, and column oven set at room temperature. The mobile phase for doxycycline analysis was a mixture of 5% acetic acid (55%) and methanol (45%) ammonium acetate (10%, pH 7), and for dexamethasone it was mono phosphate buffer (NaHPO₄, 100mM, pH 4.6) and acetonitrile used in gradient mode (monophosphate buffer 90%-65% and acetonitrile 10%-35%). Injection volume was 65 μ l, the flow rate was 1.0 ml/min and the pressure was 1200 mm. Each sample was filtered through a 0.22 μ m syringe filter and

subjected to HPLC analysis. The UV detection wavelength for doxycycline was 274nm and for dexamethasone it was 240nm. The drug concentration was calculated by comparing the peak area of standards and sample. Exactly the same procedure was followed for the dexamethasone release study from Dexa-CMC nanowafers.

Objective (ii) Study of *in vivo* pharmacokinetics after the instillation of drug-nanowafers on cornea and conjunctiva, 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.
Duration: 21 months (months 9-30)

To demonstrate the controlled drug release from the nanowafer, diffusion of drug molecules and their increased residence times in the cornea, *in vivo* drug release studies were conducted in a healthy mouse model. In this study, doxycycline loaded nanowafers were fabricated. Doxycycline was chosen as a model drug because of its green fluorescence, which will be very useful in monitoring the drug diffusion and residence times in the cornea by real-time confocal fluorescence imaging. After the instillation of a doxycycline-nanowafer on a mouse cornea, the drug slowly diffused into the cornea and corresponding increase in fluorescence intensity was monitored for 5h (**Figure 7**).

The doxycycline-nanowafer treated mice were further subjected to intravital confocal

Figure 7. The *in vivo* drug release from the nanowafer in mouse eyes. The bright field (top row) and fluorescence images (bottom row) demonstrating the controlled release of doxycycline from the nanowafer into the cornea for up to 5 h. The nanowafer was instilled at t=0.

imaging to investigate the presence of doxycycline molecules in the cornea. This study revealed that the green fluorescent doxycycline molecules were present in the cornea for up to 48h (**Figure 7**).

At this point, to compare the drug diffusion into the cornea by eye drop administration with that of nanowafer delivery, healthy mice were treated with doxycycline eye drops. As can be seen from the **Figure 8**, mild green fluorescence was observed within a minute after the eye drops administration, however there was no observable fluorescence was detected after 15min, thus confirming that the doxycycline eye drops were completely wiped out from the ocular surface. Because of the very short drug residence times on the cornea, eye drops need to be administered several times in a day for an observable therapeutic efficacy.

Taken together, these results confirm that the nanowafer releases the drug in a tightly controlled fashion and the released drug remains in the cornea for a longer duration of time. The controlled drug release from the nanowafer will facilitate the drug molecules to effectively diffuse into the corneal and improve the bioavailability of the drug, consequently enhancing the drug efficacy. Presently, studies are underway to optimize the drug retention times and therapeutic concentrations in the cornea with nanowafer drug delivery for an enhanced efficacy. In conclusion, we have accomplished all the objectives defined in the SOW for the first year grant period.

What opportunities for training and professional development has the project provided?

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, Cullen Symposium held in Houston, and ARVO meeting held in Fort Lauderdale, FL. 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 well trained 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 2014 to 30Sep 2015), we plan to work on the following Tasks defined in the SOW: **Task 2:** Fabrication of nanowafer drug delivery systems will be completed; **Task 3:** Evaluation of *in vitro* and *in vivo* pharmacokinetics will be in progress; and **Task 4:** Study of the efficacy of doxycycline-nanowafers, dexamethasone-nanowafers, and cyclosporine-A-nanowafers in an ocular burn mouse model will be in progress.

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:

"Nothing to Report"

6. PRODUCTS:

"Nothing to Report"

Publications, conference papers, and presentations

"Nothing to Report"

Journal publications.

"Nothing to Report"

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

"Nothing to Report"

Other publications, conference papers, and presentations. Identify any other 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?

Name	Stephen C. Pflugfelder, M.D.
Project Role	Principal Investigator
Researcher Identifier	
Nearest person month worked	1

Contribution to the Project	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
Funding Support	No competing support
Name	Ghanashyam Acharya, Ph.D.
Project Role	Co-Principal Investigator
Researcher Identifier	
Nearest person month worked	4
Contribution to the Project	Directed Tasks 2 & 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.
Funding Support	No competing support
Name	Daniela Marcano, Ph.D.
Project Role	Postdoctoral Research Associate
Researcher Identifier	
Nearest person month worked	12
Contribution to the Project	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.
Funding Support	
Name	Xiaoyong Yuan, M.D., Ph.D.
Project Role	Postdoctoral Research Associate
Researcher Identifier	
Nearest person month worked	12
Contribution to the Project	Developed and optimized the ocular burn mouse model. Performed and optimized the nanowafers compliance experiments on mouse eyes.
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

"Nothing to Report"