Dexamethasone nanowafer as an effective therapy for dry eye disease

Terry G. Coursey[†], Johanna Tukler Henriksson[†], Daniela C. Marcano, Crystal S. Shin, Lucas C. Isenhardt, Faheem Ahmad, Cintia S. De Paiva, Stephen C. Pflugfelder, Ghanashyam Acharya*

Ocular Surface Center, Department of Ophthalmology, Baylor College of Medicine, Houston, TX 77030

[†] These authors contributed equally

*Corresponding author

E-mail: gacharya@bcm.edu

Abstract

Dry eye disease is a major public health problem that affects millions of people worldwide. It is presently treated with artificial tear and anti-inflammatory eye drops that are generally administered several times a day and may have limited therapeutic efficacy. To improve convenience and efficacy, a dexamethasone (Dex) loaded nanowafer (Dex-NW) has been developed that can release the drug on the ocular surface for a longer duration of time than drops, during which it slowly dissolves. The Dex-NW was fabricated using carboxymethyl cellulose polymer and contains arrays of 500 nm square drug reservoirs filled with Dex. The *in vivo* efficacy of the Dex-NW was evaluated using an experimental mouse dry eye model. These studies demonstrated that once a day Dex-NW treatment on alternate days during a five-day treatment period was able to restore a healthy ocular surface and corneal barrier function with comparable efficacy to twice a day topically applied dexamethasone eye drop treatment. The Dex-NW was also very effective in down regulating expression of inflammatory cytokines (TNF- α , and IFN- γ), chemokines (CXCL-10 and CCL-5), and MMP-3, that are stimulated by dry eye. Despite less frequent dosing, the Dex-NW has comparable therapeutic efficacy to topically applied Dex eye drops in experimental mouse dry eye model, and these results provide a strong rationale for translation to human clinical trials for dry eye.

Key words

nanowafer; drug delivery; dry eye; cornea; dexamethasone; inflammation

1. Introduction

Dry eye is one of the most common eye diseases that affects approximately 30-40 million people in the United States alone and represents a major public health problem [1,2]. It is accompanied by eye irritation, blurred vision, light sensitivity, and ocular surface epithelial disease [3,4]. Also, it is one of the primary causes for clinical visits and approximately 30% of patients report symptoms of mild or chronic dry eye disease [5-7]. The causative factors include inflammation, hormonal imbalance, and aging. In addition, dry eye is a common complication of LASIK surgery [8,9]. A steady growth in computer screen-related visually demanding tasks and contact lens use can exacerbate dry eye [3,10]. Many of these causative factors adversely impact one or more components of the lacrimal functional unit including the ocular surface, lacrimal glands, meibomian glands, and the interconnecting neural network [11,12]. Mild dry eye is primarily treated with artificial tear drops that hydrate and lubricate the eye, do not have any pharmacologic activity, and provide only temporary relief [13,14]. Chronic dry eye is associated with inflammation that often responds to topically applied corticosteroids and cyclosporine-A eye drops [15-17]. Because of the rapid clearance, eye drops must be administered several times in a day resulting in a high-and-low drug concentration profile and potentially toxic side effects [18,19]. Also, frequent dosing is inconvenient and can cause discomfort that can compromise compliance. To address these issues, several new strategies have been developed [20]. Recently, nanoparticle formulations have been used in ocular drug delivery [21-24]. The rapid clearance of these formulations from the eye contributed to their limited drug efficacy. As another advancement, drug soaked contact lenses were developed [25-27]. These contact lenses released the drug very rapidly. Despite these methodological advances, controlled delivery of drugs in therapeutically effective concentration to tissues in the anterior segment of the eye, such as cornea and conjunctiva, is still very inefficient. These limitations have lead to a call for the development of a novel nanowafer therapeutic with extended release attributes and enhanced efficacy to treat dry eye disease.

The nanowafer is a tiny disc-like or rectangular membrane that contains drug loaded nano-reservoirs (Fig. 1). The nanowafer can be readily applied on the eye by the patient's finger tip without any clinical procedure (Fig. 1). Although, instillation of drug solution or nanoparticle suspension as topical eye drop formulation is the simplest mode of ocular drug delivery, application of a nanowafer on the eye is as easy as applying a contact lens. The nanowafer, after its instillation on the ocular surface, can release the drug for a longer duration of time than eye drops, thus improving its therapeutic efficacy. During the course of the drug release, the nanowafer slowly dissolves. In this study, hydrogel-forming carboxymethyl cellulose (CMC) polymer was chosen for the nanowafer fabrication because of its water solubility, mucoadhesiveness, and use as an active ingredient in artificial tear eye drops [28,29]. The mucoadhesive property of CMC facilitates quick adhesion and retention of the nanowafer on the ocular surface to withstand reflex tearing and blinking without being displaced. Furthermore, CMC stimulates re-epithelialization of the cornea through its binding to the matrix proteins [30]. The nanowafer contains arrays of nano-reservoirs that are filled with a glucocorticosteroid dexamethasone (Dex). Dex was chosen to fill the nanowafers because of its potent anti-inflammatory properties and has well documented efficacy for treating ocular inflammation [31]. It inhibits the production of inflammatory cytokines, chemokines, and decreases the synthesis of matrix metalloproteinase MMP-3, in addition to other factors [32]. Dex has been successfully used to treat dry eye related corneal epithelial diseases [33].

Fig. 1. Schematic of the nanowafer drug delivery to treat dry eye disease. (A) dry eye; (B) instillation of a nanowafer on the conjunctiva; (C) restoration of a healthy cornea.

2. Materials and Methods

2.1. Materials

Sodium carboxymethyl cellulose (CMC, MW 90,000) and HPLC solvents (acetonitrile) were obtained from Sigma Aldrich. USP grade dexamethasone sodium phosphate (Dex) was obtained from Spectrum Chemicals. PCR reagents and Oregon Green Dextran (72 KDa) were purchased from Life Technologies.

2.2. Animals

All animals were treated in accordance with the Association of Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Female C57/BL6 mice (6-8 weeks old) were purchased from the Jackson Laboratory.

2.3. Nanowafer fabrication

The nanowafers were fabricated by a previously published procedure with slight modifications [34-36]. A clear CMC solution (4% w/v, 12 mL) was transferred with a pipette onto a polydimethylsiloxane (PDMS) imprint (3" diameter) containing square posts (of 500 nm x 500 nm, 500 nm high) placed on a flat glass plate and left to dry at room temperature. Thus formed CMC nanowafer was carefully peeled away from the PDMS imprint. The concentration of the polymer solution can be varied to obtain the required thickness of the nanowafer. The CMC nanowafer obtained was ~3" diameter, ~75 μm thick, and has arrays of wells (500 nm x 500 nm, 500 nm deep).

The CMC wafers were filled with Dex by transferring a thick solution of dexamethasone-CMC with micropipet on to the nanowafer followed by gently swiping with a Teflon swiper. A similar procedure was used for filling the CMC wafers with fluorescein (a green fluorescent dye). The drug or dye filled wells are open on one side of the nanowafer. The open face of the nanowafer will be placed in direct contact with the ocular surface, so that the drug molecules can directly diffuse into the ocular tissue. The drug/dye filled nanowafers were punched into 2 mm diameter discs and used in the *in vitro* and *in vivo* experiments. Each 2 mm diameter nanowafer contained 5.027×10^5 wells.

2.4. *In vitro* drug release study

Accurately weighed Dex-NWs were placed inside dialysis tubes (MWCO 2,000). Each loaded dialysis tube was placed inside a 5 mL Eppendorf tube containing phosphate buffered saline solution (PBS, pH 7.4) and constantly shaken at 37°C. Aliquots were obtained at different time points and analyzed using a Shimadzu Prominence UV-HPLC system with a Kinetex 5uXB-C18 100A (150 mm x 4.6 mm) column from Phenomenex. Fresh PBS was added to replace the aliquot extracted. Each sample was filtered through a 0.2 µm syringe filter, and drug concentration was calculated by comparing the peak area of standards and sample detected at 240 nm. The UV-HPLC system was equipped with autosampler, in line degasser, and column oven set at room temperature. The mobile phase for Dex analysis was a mixture of 0.1 M monosodium phosphate (90%) at pH 4.6 and acetonitrile (10%). Injection volume was 5 µL, the flow rate was 0.8 mL/min, and the pressure was lower than 2500 psi. The total drug content in the nanowafer was determined by dissolving an accurately weighed nanowafer in 1 mL PBS solution and 2 mL of ethanol to precipitate the polymer. The suspension was centrifuged to remove the polymer. The clear solution was filtered through a 0.2 µm syringe filter followed by UV-HPLC analysis. The total drug content in the nanowafer was quantified by comparing with the standard curve. This experiment was performed in triplicates.

2.5. *Dex- NW instillation and tear collection*

Female C57/BL6 mice were anesthetized with an intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg). The mice were treated with either one Dex-NW containing 10 µg of Dex placed on inferior bulbar conjunctiva or 2 µL eye drops (containing 10 µg of Dex in 2 µL of 0.1% CMC solution). Tear fluid was collected at regular intervals. 2 µL of balanced salt solution (BSS) was instilled on the ocular surface, after a few seconds, the tears, mixed with the isotonic solution, were collected from the conjunctival sac close to the lateral canthus with a 1 µL volume glass capillary tube (Drummond Scientific). The tear washings from a group of 15 mice were pooled, centrifuged, and stored at -80 °C prior to drug quantification. UV-HPLC analysis was performed following the same parameters established for the *in vitro* experiments except, using 10 µL as the injection volume.

2.6. *Experimental mouse dry eye model*

Desiccating stress (DS) was used to induce experimental dry eye in female C57/BL6 mice, six to eight weeks of age, by subcutaneous injection of 0.5 mg/0.2 mL scopolamine hydrobromide (Sigma Aldrich) into alternating hindquarters administered four times a day (8:30 a.m., 11 a.m., 1 p.m. and 4:30 p.m.) to inhibit tear secretion, exposure to an air draft, and <30% ambient humidity. Mice were euthanized after five days of desiccating stress (DS) treatment [37]. A group of age- and gender-matched mice that were housed in normal environmental conditions were used as non-stressed (NS) controls.

2.7. *Evaluation of Corneal smoothness*

Reflected images of a white ring from the fiber-optic ring illuminator of the stereoscopic zoom microscope (SMZ 1500; Nikon) were obtained immediately after euthanasia. This ring light is firmly attached and surrounds the bottom of the microscope objective. Because the illumination path is nearly coincident with the optical axis of the microscope, the viewing area is evenly illuminated and nearly shadowless. The projected ring light will reflect off a wet surface and the regularity of the reflected ring depends on the smoothness of the ocular surface. [38-40].

2.8. Corneal barrier function measurement

On the morning of the fifth day of DS, corneal staining was measured by Oregon Green Dextran (OGD). 0.5 μ L of OGD was instilled on the cornea of both eyes and the mouse was left in the dark for 1 minute before euthanization. The eyes were then washed with 2 mL of BSS. Excess liquid was blotted off the eye with tissue paper and digital images were captured and the mean fluorescence intensity within a 2 mm central corneal ring was measured with NIS Elements (Nikon). The data are presented as mean $\pm$ SEM of fluorescence gray levels from three independent experiments using 5 mice per group per experiment [38].

2.9. RNA isolation and real time RT-PCR

The corneal epithelium was scraped off with a scalpel and the tissue was collected for PCR analysis. Total RNA from corneal epithelium was isolated using a QIAGEN RNeasy Plus Micro RNA isolation kit (Qiagen) following the manufacturer's protocol. After isolation, the concentration of RNA was measured and cDNA was synthesized using the Ready-To-GoTM You-Prime First-Strand kit (GE Healthcare). Real time PCR was performed using specific Taqman probes for MMP-3 ((*Mmp3*) (Mm00440295_m1)), CCL-5 ((*Ccl5*) (Mm00445235-m1)), CXCL-10 ((*Cxcl10*) (Mm01302427_m1)), TNF- α ((*Tnfa*) (OriGene MP217748)), and IFN- γ ((*Ifng*) (Mm00801778_m1)) genes (Taqman Universal PCR Master Mix AmpErase UNG) in a commercial thermocycling system (StepOnePlusTM Real-Time PCR System, Applied Biosystems) according to the manufacturer's recommendations. The results were analyzed by the comparative threshold cycle method and normalized by beta 2 microglobulin (B2M) as the control.

2.10. Statistical analysis

Prism 6.0 software (GraphPad Software Inc.) was used for statistical analysis. One-way analysis of variance (ANOVA) was used to determine overall differences among groups, followed by a post-hoc test (Tukey's post hoc). An unpaired t-test is used to evaluate statistical differences between 2 experimental groups. The statistical significance was considered to be $P \leq 0.05$ and data are presented as mean $\pm$ SEM.

3. Results and discussion

In this article, we describe the development of a nanowafer therapeutic and demonstrate its *in vivo* efficacy in an experimental mouse dry eye model (dry eye mice) [37,38]. The nanowafers were fabricated by hydrogel template strategy [34-36]. A schematic of the nanowafer fabrication procedure has been presented in Fig. 2A-F. Carboxymethyl cellulose (CMC) was chosen for the nanowafer fabrication because of its water solubility, mucoadhesiveness, and use as a constituent in artificial tear eye drops [28-30]. The nanowafer contains arrays of wells that were filled with a glucocorticosteroid dexamethasone (Dex, MW = 392 g/mol). For conducting *in vivo* studies in dry eye mice, 2 mm diameter nanowafers were fabricated to be placed on the conjunctiva.

The Dex loaded nanowafers (Dex-NW) were examined for their *in vitro* drug release kinetics by HPLC. The total Dex content in the Dex-NW was 10 μ g. The initial release after 5 h was ~40% and the drug release continued for 24 h (Fig. 2G). To assess the drug release from the Dex-NW after its instillation on the conjunctiva, tear samples were collected at hourly intervals and analyzed for Dex concentration by HPLC. This study revealed the presence of Dex in tear

samples for up to 2 h at significantly greater concentrations than eyes treated with Dex eye drops containing the same amount (10 μ g in 2 μ l) of the drug (Fig. 2H). Also, in the case of Dex release from the nanowafer, more drug was present in the first hour tear samples compared to the second hour samples. Because of the ocular surface barriers, such as reflex tearing and tight epithelial junctions, the drug diffusion into the cornea is very slow in the beginning, hence more drug was present in the first hour tear samples. However, with longer drug residence time provided by the nanowafer, more drug penetrates into the cornea, resulting in a decrease in drug concentration was observed in the tear samples collected at later time points.

Fig. 2. Nanowafer fabrication and the drug release from Dex nanowafer. Fabrication of nanowafer by hydrogel template strategy: (A) a PDMS template containing vertical posts; (B) preparation of a CMC imprint of the PDMS template; (C) a CMC nanowafer; (D) a CMC nanowafer filled with drug/dye; (E) bright field image of a CMC nanowafer; (F) fluorescence image of a CMC nanowafer filled with fluorescein. Dex release from a nanowafer: (G) *In vitro* drug release profile; (H) Dex concentration measured in tear samples collected from eyes treated with Dex-NW or Dex eye drops. *P < 0.05

To study the efficacy of the nanowafer in improving the drug molecular residence time on the ocular surface and its subsequent diffusion into it, fluorescein (a green fluorescent dye, MW = 332 g/mol) loaded nanowafers (Flo-NW) were fabricated. The Flo-NWs were instilled on the corneas of healthy mice and were examined by fluorescence imaging at every hour for 5 h.

The ocular surface was initially non fluorescent (Fig. 3A). After the placement of a nanowafer, the fluorescein molecules were slowly released on the cornea and it was green fluorescent. Since, the fluorescein released from the nanowafer is in direct contact with the cornea, most of the drug will be able to penetrate into it. After 4 h, the green fluorescence of the cornea started to fade because of the diffusion of fluorescein molecules into the anterior chamber, and its clearance by tear secretion (Fig. 3B-G). Once the fluorescein molecules pass through the cornea and reach the aqueous humor in the anterior chamber, they are cleared through the trabecular meshwork. In comparison, eyes treated with fluorescein eye drops were non-fluorescent after 5 min, indicating its rapid clearance from the ocular surface (Fig. 3H-L). Also, after instillation of the fluorescein drops on the eye, within a few minutes, most of it is concentrated on the eye lids, indicating its clearance from the ocular surface, compared to the nanowafer drug release, wherein only a small amount of fluorescein concentrated around the eyelids and most of it in the eye. This study has qualitatively demonstrated the ability of nanowafer to release the drug for a few hours thus improving the drug residence time on the cornea.

Fig. 3. Nanowafer improves the drug residence time on the cornea. (A) Fluorescence micrograph of the eye prior to fluorescein nanowafer (Flo-NW) instillation; (B-G) fluorescence micrographs depicting the presence of fluorescein dye at the specified time points in the corneal tissue after Flo-NW instillation; (H) fluorescence micrograph of the eye prior to fluorescein eye drops instillation; (I-L) rapid clearance of fluorescein eye drops within 5 min. (M) A plot depicting the fluorescence intensities (n=3). *P<0.05, ***P<0.001. All error bars represent standard error of the mean. Scale bar: 1 mm.

1 The *in vivo* efficacy of Dex-NW was evaluated in dry eye mice. A Dex-NW was placed
2 on the inferior bulbar conjunctiva of a mouse (on days 1 and 3) that was subjected to desiccating
3 stress for five days with no topical treatment. The efficacy of the Dex-NW was evaluated by
4 corneal smoothness, corneal barrier function, and expression of pro-inflammatory cytokines,
5 chemokines, and MMPs and compared with control CMC-NW (without Dex) treated and
6 untreated dry eye mouse groups.

7
8 Chronic dry eye disease can desiccate the ocular surface and create corneal epithelial
9 erosions that alter corneal smoothness and permeability [38-40]. Accordingly, corneal
10 smoothness was monitored as a study parameter for the evaluation of nanowafer efficacy. The
11 regularity of a white-light ring reflecting off the mouse cornea was used to evaluate corneal
12 smoothness. A regular circular ring is reflected off the smooth normal corneal surface. In dry eye
13 mice treated with control CMC nanowafers, the reflected rings were very irregular, while regular
14 and uniform ring reflections were observed from eyes treated with Dex-NW, indicating
15 maintenance of a smooth normal corneal surface (Fig. 4). Improved corneal smoothness over
16 control affirms the efficacy of Dex-NW. These results indicate that the drug release from the
17 nanowafer is effective in restoring or maintaining a healthy corneal surface that is subjected to
18 desiccation.

19
20 **Fig. 4.** Maintenance of a smooth corneal surface by Dex-NW treatment observed by the
21 reflection of a ring of light in mice. (A) Healthy eye; (B) Desiccating stress induced dry eye; (C)
22 CMC-eye drops; (D) Dex-eye drops; (E) CMC-NW; (F) Dex-NW. Scale bar: 1 mm.

23
24 To study the therapeutic effect of Dex-NW on maintaining a healthy corneal surface in
25 mice exposed to DS, corneal epithelial barrier function was measured by staining the cornea with
26 green fluorescent Oregon Green Dextran (OGD, 70 kDa). Corneal epithelial disruptions created
27 due to the damage or loss of epithelial cells was visualized with the dye, where fluorescence
28 intensity positively correlates with the degree of disruption in corneal barrier function [38]. For
29 the corneal epithelial barrier function measurement, OGD was delivered as topical eye drops.
30 Because the mice are exposed to DS, the corneas develop epithelial disruptions due to the
31 damage or loss of epithelial cells. The corneal surface is no longer smooth with tight epithelial
32 junctions. Upon instillation of OGD eye drops on these corneas, the OGD molecules will rapidly
33 (<1 min) penetrate into the corneal tissue through the epithelial disruptions compared to healthy
34 and drug treated corneas. Because of the short duration of the eye drops on the cornea (1 minute)
35 and the low blink rate of mouse, most of the eye drop stays on the ocular surface and won't get
36 washed away.

Fig. 5. Measurement of corneal epithelial barrier function. (A) Healthy eye; (B) subjected to five days of desiccating stress (5D); (C) CMC drops; (D) Dex drops; (E) CMC-NW; (F) Dex-NW; (G) a plot depicting the fluorescence intensities. ** $P < 0.05$, **** $P < 0.0001$. All error bars represent standard error of the mean.

Compared to a healthy eye, the dry eye mice absorbed more OGD. This study revealed that the corneal uptake of OGD significantly increased after five days in dry eye mice (Fig. 5). In contrast, treatment with Dex-NW or Dex drops maintained corneal epithelial barrier function. Treatment of dry eye mice with Dex eye drops (0.1%, 2 μ L) administered twice a day for five days or instillation of Dex-NW on the first and third days during five-days of experimental dry eye maintained OGD staining at baseline levels. No difference in efficacy was observed between the Dex-NW and Dex drops treated groups, despite the lower dosing frequency for the Dex-NW. These results confirm the ability of the nanowafer to deliver Dex in therapeutic concentrations to the ocular tissue. Interestingly, CMC eye drops appear to be slightly more effective, although not significant, compared to the CMC nanowafer in maintaining the corneal barrier function (Fig. 5G). This could be because of the ability of the CMC polymer molecules in eye drop formulation delivered twice daily to quickly bind to the corneal epithelium thus filling some of the corneal epithelial disruptions caused by the desiccating stress, compared to the CMC nanowafer which was applied only twice during the 5 day treatment period [30]. Also, when an eye drop is instilled on the ocular surface, it will have some wetting/moisturizing effect on the surface regardless of whether it has any effective ingredients (vehicle effect), the nanowafer on the other hand did not produce as much vehicle effect as it was instilled only twice during the treatment period (on days 1 and 3).

Dry eye is a multifactorial disease. It activates an innate immune response in the cornea and conjunctiva that ultimately affects the corneal epithelium by producing chemokines and cytokines. Chemokines CXCL-9, -10, -11 attract T helper cells (type 1) which produce IFN- γ , while other chemokines, such as CCL5 attract innate immune cells [41]. Important immune mediators in the conjunctiva also include cytokines IFN- γ and TNF- α [42,43]. In particular, IFN- γ is known to cause conjunctival goblet cell loss and apoptosis of the ocular surface epithelium [44-47]. The gene expression of these molecules was evaluated due to their importance in the pathogenesis of dry eye. Matrix metalloproteinase MMP-9 and its physiological activator MMP-3 have been found to increase in the ocular surface epithelium of both human and experimental dry eye [48-51]. The MMPs break down a variety of substrates, including components of the corneal epithelial basement membrane and tight junction proteins that maintain corneal epithelial barrier function. MMPs have also been detected in the tear fluid of both dry eye patients and dry eye mice [39].

Fig. 6. RT-PCR analysis revealing down regulation of drug target genes by Dex-NW in the corneal epithelium after 5 days of desiccating stress is comparable to that of twice a day topical Dex eye drop treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. All error bars represent standard error of the mean.

Inhibition of inflammatory cytokine and MMP expression confirms the efficacy of Dex-NW. Instillation of Dex-NW on the first and third days effectively inhibited expression of inflammatory cytokine and MMP production in the corneal epithelium compared to CMC-NW (control). The slight anti-inflammatory effect of CMC nanowafer is possibly due to the ability of CMC polymer molecules to bind to the corneal epithelium and protect it against the pro-inflammatory effects of the DS and modulate the healing process [30]. The efficacy of the Dex-NW (administered on first and the third days) was equal to twice a day topical administration of Dex eye drops for the five-day treatment period (Fig. 6). Both Dex-NW and Dex eye drop treatment inhibited the production of MMP-3, CCL-5, CXCL-10, TNF- α , and IFN- γ . Overall, the Dex-NW was effective in inhibiting inflammatory cytokine expression at the same level achieved with twice a day topically administered Dex eye drops.

4. Conclusions

Despite advances in the therapy of dry eye disease over the past two decades, challenges still remain in delivering therapeutic levels of anti-inflammatory drugs at a convenient dosing schedule and optimum tissue concentrations. This study evaluated the nanowafer drug delivery system to deliver Dex to the ocular surface. The Dex-NW was able to enhance diffusion of a corticosteroid into the cornea and maintain a smooth, healthy corneal surface with intact barrier function in mice with experimentally induced dry eye. In addition, the Dex-NW was effective in suppressing expression of inflammatory mediators associated with dryness of the cornea. The Dex-NW treatment was well tolerated on the mouse eye. Administration of Dex-NW only twice during the five-day treatment had equivalent efficacy to twice a day topical administration of Dex eye drops during same treatment period. The less frequent dosing schedule of the Dex-NW

will improve convenience and enhance treatment compliance among dry eye patients. Furthermore, release of dexamethasone from the nanowafer can be adjusted to the minimal effective concentration to minimize the drug related toxicity of corticosteroids that can cause glaucoma and cataract at higher concentration. These results confirm the therapeutic efficacy and translational potential of the nanowafer drug delivery system to treat dry eye disease. Upon further development, Dex-NW can provide a simple and effective treatment with a more convenient dosing schedule than eye drops for dry eye disease.

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