Micro-Patterned Drug Delivery Device for Light-Activated Drug Release

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INTRODUCTION

The method by which a general drug is delivered can have a significant effect on the drug’s therapeutic efficacy [1,2]. Conventional drug delivery systems such as tablets [3–5], pumps [6–8], implants [9,10], injectable microspheres (such as Luprom Depot [11]), and patches [12] often produces a sharp initial increase in concentration to a peak above the therapeutic range, followed by a fast decrease in concentration to a level below the therapeutic range.

Many polymeric implants achieve pulsatile release of a chemical via timed triggering by specific stimuli including (1) changes in pH [13–15], temperature [16–18] or electrical [19–23] or magnetic [24,25] fields; (2) exposures to ultrasound [24,26], enzyme [27] or photochemical [28] reagents or (3) molecules such as antigens [29] or water [29] present in the tissues. The conventional treatment for ocular disease is particularly difficult due to several factors including physiological barriers, space limitations within and surrounding the eye, and trauma to eye from invasive therapies. For example, treatment methods for wet age-related macular degeneration (ARMD) require coagulation/ablation by Argon laser, intra-vitreal drug delivery, or periodic injection of antiangiogenic drugs such as Lucentis (ranibizumab) through the sclera. Photocoagulation with Argon laser may worsen the vision in treated patients with disciform ARMD. There is about a 20% chance of vision loss within 3 months in the treated group versus 11% of controls [30]. Other side-effects occur from intra-vitreal drug injection includes pain, multiple risks of endophthalmitis (0.16% over total treatment period or 0.05% per injection), lens trauma (0.7%), retinal detachment (0.6%) [31]. Systemic VEGF-A inhibition predisposes patients to...
excess thrombo-embolic complications in the first year of follow-up [32].

Also, therapeutic efficacy of a drug delivery via these conventional treatment methods are not always optimum due to the fact that efficacy depends not only on how the drug is delivered but also the therapeutic half-life of the drug in the target tissue. The interval of optimum concentration range for therapeutic effect can be very short for conventional drug delivery such as intra-vitreal injection or oral delivery. In addition, non-invasive drug delivery to the anterior and vitreous chambers of the eye is severely impeded by physiological barriers. For example, most pharmacologic management of ocular disease uses topical application of eye drops (such as topical timolol and inulin) [33] and oral medications must permeate through the modified mucosal membrane of the cornea or the blood–retina barrier, respectively. A study by Geroski and Edelhauser [34] reported that only 5% of the dispensed eye drops may reach the anterior intraocular tissues through the cornea. Furthermore, drug is diluted by lacrimation, tear drainage, and turnover limit the drug contact time with the cornea. On the other hand, according to Fraunfelder oral medications require larger doses to reach therapeutic levels within the eye due to presence of the blood–retina barrier. This may result in serious systemic side effects [35–39]. Therefore, there is a need for an improved method of drug delivery to treat ocular diseases.

The primary goal of this study was to design and fabricate a novel drug delivery device that contained membrane covered reservoirs for storage of aqueous solutions of ophthalmic drugs. The device was implanted between the supra-scleral and sub-tenons space through an incision in the conjunctiva. Important aspects of the study included investigation of the long-term stability of the device implanted in in vivo rabbit eye and comparisons between two methods of dye (10% Na fluorescein) release by membrane rupture produced by (i) mechanical puncture and (ii) photodisruption with an ophthalmic Q-switched Nd:YAG laser irradiation. The comparisons were made from determinations of the fluorescence half-life of the released dye into the eye tissues over time. The fluorescence half-life was defined as the period of time required for the concentration or amount of deposited dye in the tissues to be reduced by one-half of the original released amount. We had considered the half-life of the 10% Na fluorescence dye in relation to the amount of the dye present in the vitreous humor of the eye.

Another important findings of this study was the difference in threshold energy of the pulsed Nd:YAG laser to release the content from the reservoir by ablating the membrane while the drug delivery device was implanted in the eye and when it is not. Reproducible, controlled, and effective photorupture of the reservoir membranes in the implanted device requires optical clearing of the opaque white conjunctival scar tissue and sub-tenons space (not sclera) using hyperosmotic agents such as 100% anhydrous glycerol to visualize the reservoir [40].

MATERIALS AND METHODS

Animal Preparation

Female SPF Dutch Belted (n = 2) rabbits weighing 4–5 lbs were used for the implant surgery. All experimental procedures were conducted according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin (AUP-2009-00061). Prior to the experiment, the rabbits were anesthetized with 35 mg/kg Ketamine, 5 mg/kg Xylazine, and 0.75 mg/kg Acepromazine. One hour prior to termination of the surgery, Buprenorphine 0.01–0.05 mg/kg was administered IM for any pain or discomfort. During the surgery, 0.5% Proparacaine Hydrochloride Ophthalmic sterile solution USP (Falcon Pharmaceuticals, Ltd., Fort Worth, TX) was used to anesthetized the eye.

Rabbits were positioned in the ventral recumbent position. Oxygen saturation, pulse, and respiration rate were monitored every 15 minutes throughout the study using a vet/ox G2 Digital pulse oxymeter (Heska Corp., Loveland, CO). This monitor was connected via a shaved area of the feet or the ear depending on the best contact for recording. Animals were kept warm with warm water blankets. The anesthetic and monitoring procedures were performed by a skilled technician from the Animal Resources Center (ARC) of the University of Texas at Austin. Rabbits used in this study were treated humanely during and after the experiments.

Two different methods of dye release. After complete recovery (2–3 weeks) from the implant surgery, the conjunctiva was optically cleared with topical application of hyper-osmotic agent (100% anhydrous glycerol). The glycerol was poured from the original container into smaller airtight screw cap bottle for each experiment to prevent the glycerol from absorbing any moisture from the air due to its hygroscopic nature. The rest of the glycerol after each experiment was discarded to prevent contamination. Initial optical clearing process started at 3 minutes after application. However, to clear the conjunctiva and part of sclera completely took about 8 minutes. After 8 minutes the tissue started to become opaque again. Thus, multiple topical applications of glycerol were needed to get best results. Once the eye tissue became optically clear, the drug delivery device became visible and the content of the drug delivery device was released in two different methods. During the first method the dye from the reservoir of the rabbit 1 was released by mechanical puncture using a 28 G ½ needle as it was not possible to rupture the membrane just by depression. In the second experiment, an ophthalmic Q-switched Nd:YAG laser (ELLEX, LaserEX, SuperQ) was used to disrupt the membrane of the implanted device in rabbit.

In vivo eye model. In this study, we were primarily interested in the monitoring dye concentration in the anterior (aqueous humor) and vitreous (vitreous humor) chambers of the in vivo eye. The anterior chamber is the space in the eye that is behind the cornea and in front of
the iris. The anterior chamber is filled with a watery fluid known as the aqueous humor which is produced by the ciliary body. The vitreous chamber is the space in the eye behind the crystalline lens and is filled with vitreous humor. Vitreous humor is a transparent, colorless, gelatinous mass that consists of 99% water and remainder of the mixture is collagen such as vitrosin and a network of collagen type II fibers with hyaluronic acid, proteins, salts, and sugars. Although, the vitreous humor is mostly made of water, it has a jellylike consistency, and this viscosity helps the eye hold its shape. The vitreous humor has no vascularization, so once a drug gets into this part of the eye it will diffuse out slowly.

We were also interested in the three layers of the in vivo eye: the (i) bulbar conjunctiva, (ii) sclera, and (iii) choroid. The bulbar conjunctiva is a thin (54.7 ± 1.9 μm) clear outermost epithelium layer that covers the sclera [41–43]. The naturally white sclera is just beneath the conjunctiva and, which is opaque due to its normal level of hydration. Collagen (type I collagen) accounts for 90% of the dry weight of all mammalian sclera. In addition, some fine blood vessel arcades exist on both the surface of conjunctiva and sclera; however, the sclera is mostly avascular and is anatomically similar to dura mater of the brain. The thickness of the sclera varies from 1,000 μm at the vitreous pole to 300 μm just beneath the rectus muscle insertions. It is continuous with the dura mater and the cornea, and provides an attachment for the extra ocular muscles. The choroid lies between the sclera and retinal pigment epithelium (RPE) and consists of four layers (i) Haller’s layer (outermost layer), (ii) Sattler’s layer, (iii) choriocapillaris, and (iv) Bruch’s membrane (innermost layer). Haller’s and Sattler’s layers consist of large and medium diameter blood vessels, respectively; the choriocapillaris layer has capillaries [43]. Melanin, a darkly colored chromaphore synthesized by melanocytes, occurs throughout the choroidal layer and helps limit uncontrolled reflection within the eye [44]. Figure 1 depicts the anatomy of the cross-sectioned eye model (adapted from the National Eye Institute).

Micro-Patterned Drug Delivery Device Fabrication

We developed two different generations (first and second) of drug delivery devices. General description of the fabrication process is described in the following paragraphs.

A drug delivery device with 3 × 6 × 1.5 mm³ dimensions (width × length × depth) was fabricated (Zhang Research Group) from ultraviolet (UV) cured biocompatible polyurethane, a form of poly-methyl methacrylate (PMMA), under sterile condition. The device had a 82.0 μm thick clear cap or device membrane made from a transparent bio-compatible polymer (3% poly-1,1-dichloroethylene) [45,46]. The device consisted of two (2.6 mm diameter each) reservoirs containing 10% Na fluorescein USP sterile dye. Once the reservoirs were filled with the dye, the cap was bonded to the device with an optical adhesive using UV light.

The initial step of the device fabrication started with making polydimethylsiloxane (PDMS) mold (2.6 mm diameter reservoirs and 1.5 mm depth) on SU8 master (step 1 of Fig. 2a). The SU8 photore sist master was patterned on silicon wafers using a conventional photolithographic process (Microchem Corporation, Newton, MA; http://www.microchem.com/products/su_eight.htm) [47,48]. The actual drug delivery device was created from a UV curable polymer precursor called polyurethane.

The PDMS with curing agent (1:10 mixture ratio) was poured on the master mold and cured at 70°C for 30 minutes. The cured PDMS was then peeled off from the mold to create the template for the drug delivery devices. The polyurethane was then poured onto the PDMS template and cured under a UV lamp to create the reservoirs for the devices (step 2 of Fig. 2a). The devices were then peeled carefully from the PDMS template (step 3 of Fig. 2a) and 5.0 μl of the 10% Na fluorescein dye was pipetted into each reservoir. The devices were then bonded to a thin layer of plastic film (82 μm) using UV curing Norland Optic Adhesive (Thorlabs, Inc., Newton, NJ) under a UV lamp for 10 minutes. Once the devices were cured, they were washed with water to remove any unwanted debris and checked for leaks. The devices were then placed under the UV lamp for additional 20 minutes for sterilization.

The major difference between the first and the second generation device is that the second generation device had smoother edges and with rounded bottom for easier insertion (Fig. 2b). However, the curvature of the device did not match the curvature of sclera. Also, the bonding between the membrane and the device was much stronger for the second generation device due to a different optical adhesive called NOA68 unlike NOA65. Both adhesives were biocompatible (tested on culture of Hela cells) [49] and none of the ingredients were carcinogens in any state or known to cause reproductive toxicity [50]. Photographs and schematic cross-sections of the first generation device (not drawn to scale) are depicted in Figure 2c.

Device Stability Testing

The long-term stability of the drug delivery device was tested by submerging both generation devices (n = 48 for
each generation) under water and observing for any leaks of 10% Na fluorescein dye. These airtight containers holding the devices were agitated every day vigorously to generate maximum wear and tear to identify the breaking point of the drug delivery device by visual observation.

**Fluorescence Spectrophotometer (FS) System**

The fluorescence spectrophotometer (FS) system consisted of four main components: (i) a tungsten halogen lamp to shine white light (LS-1, Ocean Optics, Dunedin, FL), (ii) a low pass filter with transmission between 390 and 480 nm with cutoff at 505 ± 15 nm wavelength (FD1B, an additive dichroic color filter, blue, Thorlabs, Inc.), (iii) a custom-designed fiber-optic probe (core diameter = 200 μm; NA = 0.22; FiberTech Optica), and (iv) a spectrometer (USB4000, Ocean Optics). The flat-surfaced fiber-optic probe consists of two individual fibers with core to core separation of 370 μm that were terminated with SMA connectors. The source and detector fibers were connected to the lamp and spectrometer, respectively. The low pass-filter was placed between the light source and the fiber-optic probe. A built-in 74-VIS collimated lens was used in the LS-1 light source before termination with the fiber-optic probe. Figure 3 is the schematic illustration of the FS system.

We collected the 10% Na fluorescein emission spectrum using the FD1B from the eye tissue over a wavelength range of 450–700 nm by filtering the excitation spectra at

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**Fig. 2.** A schematic illustration of the implantable drug delivery (a) fabrication processes; (b) middle row left: 1st generation, right: 2nd generation; (c) last row left: photograph from the top, right: schematic side-view cross-section (not drawn to scale).
495 nm. The fiber-optic probe was placed at 90° angle in contact with the surface of the cornea, and the average was taken from five measurements of blue light spectra at each time point. Each fluorescence spectra had integration time of 500 milliseconds. Fluorescence spectra from the cornea were collected at the same location during baseline (before implant), immediately after implant, and once every week until the membrane of the device was ablated either with mechanical puncture or ophthalmic Nd:YAG laser. After the release of the dye, fluorescence emission spectrum was collected twice a week until the intensity returned to its baseline measurement. Prior to spectral analysis, recorded signals were corrected for system response by subtracting the detector dark current and adjusting the spectral intensity with respect to the fluorescence intensity of Rhodamin B fluorophores. Due to the corrosive nature of the Rhodamine B dye, the fiber-optic probe of the FS system was placed at the outside wall of the cuvette containing the fluorophore molecules at each calibration of the LS-1 before starting any animal measurements.

**Sampling Depth Measurement**

The sampling depth of optical fibers was a function of the source–detector separation distance and the optical properties of the tissue. Determination of the sampling depth assumes importance due to the location of the dye in the eye. It is important to know if the probe can sample fluorescence emission spectrum from the anterior chamber or vitreous humor. Therefore, knowledge of sampling depth is necessary to know the location of the dye in the eye.

Estimates of the changes in sampling depth were obtained using a container (base painted with non-reflective black paint), which was filled with 3.0 ml 10% Na fluorescein dye in 6.0 ml water (dye/water with 5:1,000 ratio) solution to mimic the fluorescence after the maximum release from the drug delivery device, and measured through the rabbit’s dilated pupil. A LabVIEW interface (San Diego Instruments, San Diego, CA, MC2000 controller) controlled the position of the fiber optic probe of the FS system. The fiber-optic probe was initially placed at the base of the phantom. The fluorescence intensity measured was close to zero due to the non-reflective black paint. Subsequently, the probe was moved in the z-direction and the fluorescence intensity was recorded at each step (50 μm). The experiment was continued until the fluorescence intensity reached a constant value. The slope of the curve provided a measure of this particular probe’s sampling depth. Based on our experiments, the average sampling depth was identified to be 17.6 mm for the case of dilated pupil (Fig. 4).
For the sampling depth measurement study the phantom could not accurately mimic the rabbit eye as we could not implement the optical properties of cornea and lens. Thus, we have performed a Monte Carlo model to simulate the sampling depth in the various layer of the rabbit eye tissue.

Monte Carlo Modeling

A Monte Carlo method is a stochastic model that has been used to simulate problems that can be readily characterized by radiative transport theory such as light propagation in biological media. The method consists of a detailed bookkeeping of the scattering and absorption experiences of each individual photon as they propagate through a medium.

Monte Carlo algorithm. The Monte Carlo program continuously scatters photons as they propagate through a medium until they are absorbed by a chromophore, reflected from, or transmitted through, the medium. The tissue is assumed to be infinitely wide and is characterized by its (a) thickness $D$ (mm), (b) refractive index $n$, (c) absorption coefficient $\mu_a$ (cm$^{-1}$), (d) scattering coefficient $\mu_s$ (cm$^{-1}$), and (e) anisotropy factor $g$. Photons are launched normal to the air–tissue interface and distributed along a radial line of width equal to the beam radius 200 $\mu$m. The physical quantities of interest that are estimated with the Monte Carlo simulation is fluence rate $\Phi(\lambda_{ex}, r, z, \theta) \text{ (W/cm}^2\text{)}$, and number of fluorescent emitted photon at various emission wavelength.

The path of each photon in the medium is characterized by steps of randomly varying lengths and an angle of deflection from the previous scattering site [51]. The step sizes and the angles are sampled from their respective probability distributions. Upon scattering, the directional cosines for the deflection angle $\cos\theta$ are determined by the Henyey–Greenstein scattering function, which provides a good analytical representation of single scattering in tissue [52].

The Monte Carlo simulation algorithm used in this study incorporates a variance reduction technique whereby a packet of photons, representing many photons, is propagated simultaneously through a particular path within the medium [53]. Each photon packet starts with an assigned weight $W$ that is set equal to 1. Photons can
either be terminated by reflection or transmission out of the tissue or absorbed within the medium to create fluorescence. To ensure conservation of energy, and to avoid a bias in the distribution of photon deposition, a random termination technique known as the roulette is used. When the weight of a photon packet is reduced below a threshold value $W_{\text{th}}$ (e.g., $W_{\text{th}} = 0.0001$), the roulette gives the photon bundle one chance in $m$ (e.g., $m = 10$) of survival, with a weight of $mW$. Once the photon packet is terminated, its weight is set to zero, and this interaction marks the end of the photon's propagation within the medium. Given a pseudorandom number $\xi$, the roulette criterion for survival can be summarized as

$$W = \begin{cases} mW & \text{if } \xi \leq 1/m \\ 0 & \text{if } \xi > 1/m \end{cases}$$  

When the photon packet is fully absorbed (terminated), a new photon packet is launched into the tissue and the same recordkeeping procedure is followed.

**Optical modeling of multi-layer rabbit eye tissue.** Healthy tissues of the anterior rabbit eye chamber such as cornea and lens are highly transparent for visible light because of their ordered structure and the absence of strongly absorbing chromophores. Scattering is an important feature of light propagation in eye tissues. To apply the Monte Carlo simulation to the particular tissue geometry involved in fluorescence emission from anterior and vitreous chamber from 10% Na fluorescein dye, we developed a four-layered model of the tissues from cornea to vitreous humor. These layers, as illustrated schematically in Figure 5, are (i) cornea, (ii) aqueous humor (anterior chamber), (iii) crystalline lens, and (iv) vitreous humor (vitreous chamber). The anterior interface is with air ($n = 1.00$) and the posterior interface is with the vitreous humor of the eye ($n = 1.336$) with infinite thickness.

The contact fiber-optic probe has a diameter of 200 $\text{mm}$, and because the flat-surfaced fiber was in contact with the eye tissue at 90° angle, a flat beam profile was chosen for the simulation. The 200 $\text{mm}$ diameter of the fiber is considered the spot size of the incident beam on the corneal surface. The simulated response of the tissue to a flat-top beam of 200 $\mu\text{m}$ radius, whose total energy was fixed at 65 mJ or 130 mW for 500 millisecond integration time, was obtained with a total of 2,000,000 photons.

To model the fluorescence escape process, the transport parameters ($\mu_{\text{at}}, \mu_{\text{s}}, g$) for the eye at different wavelengths from 450 to 650 nm in 10 nm intervals from using the data from the literature by Hughes [54]. A slab geometry, as required by the Monte Carlo simulation, is used for all layers, and the tissues are assumed to be of infinite lateral extent, which is a justified assumption for the wavelengths under study (495–530 nm). The optical properties for 495 nm excitation wavelength are listed in Table 1 for the above eye layers.

### Energy Measurement

It was important to know how much energy was required to ablate the membrane of the drug delivery device before we could implant the device in the *in vivo* rabbit eye. Also, this value must be within the physiological range (0–12 mJ) that could be used in humans safely. Thus, before any implant surgeries, the device was placed vertically in a beaker filled with water where the membrane was facing towards the laser beam. An ophthalmic Nd:YAG laser was used starting from zero until the threshold energy was reached at which the membrane was ablated. The threshold energy was recorded since that value would be the starting point for the membrane ablation of the implanted drug delivery device in the *in vivo* rabbit eye.

### Surgical Implant Procedures

A wire eyelid speculum was placed in the rabbit eye to hold the eyelid open. The position of the eye was stabilized.

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**Fig. 5. Multi-layered eye tissue structure used in generating fluorescence photons in the presence of the 10% Na fluorescein dye in the various layers. Visible blue was used as a light source to simulate the Monte Carlo model.**

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**TABLE 1. Four-Layer Eye Optical Model for Rabbit (Transport Parameters $\mu_{\text{at}}, \mu_{\text{s}}, g$ Are for 495 nm Only)**

<table>
<thead>
<tr>
<th>Layer</th>
<th>$D$ (mm)</th>
<th>$n$</th>
<th>$\mu_{\text{at}}$ (cm$^{-1}$)</th>
<th>$\mu_{\text{s}}$ (cm$^{-1}$)</th>
<th>$g$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>—</td>
<td>1.0000</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cornea</td>
<td>0.4000</td>
<td>1.3760</td>
<td>2.2000</td>
<td>19.400</td>
<td>0.9</td>
</tr>
<tr>
<td>Aqueous humor</td>
<td>2.9000</td>
<td>1.3370</td>
<td>0.0462</td>
<td>17.070</td>
<td>0.9</td>
</tr>
<tr>
<td>Crystalline lens</td>
<td>7.9000</td>
<td>1.4200</td>
<td>0.2370</td>
<td>17.070</td>
<td>0.9</td>
</tr>
<tr>
<td>Vitreous humor</td>
<td>6.7000</td>
<td>1.3360</td>
<td>0.0537</td>
<td>17.070</td>
<td>0.9</td>
</tr>
</tbody>
</table>
with a traction suture placed near the limbus (6-0 prolene). The suture helped to position the eye to gain access to the superior temporal sclera. The device was implanted by making a 6.5 mm incision in the bulbar conjunctiva so that the device could be aligned with the cornea horizontally. However, the implant surgery in the two rabbits was slightly varied to identify the best method for delivering the content of the device. For one of the rabbits we made a 4.0 mm scleral flap with 300 μm depth adjacent to the place of the implant. The original intent of the surgery was to place the drug delivery device under the scleral flap to create a direct access for the content of the device to reach the blood vessels of the highly vascularized choroidal layer. However, the choroidal layer started to herniate and we decided to abandon the implantation under the scleral flap. Based on the observation from the implant in rabbit one no scleral flap was made for the second rabbit. For simplicity, the rabbits with scleral flap and without scleral flap are called rabbit 1 and rabbit 2, respectively. For both cases the drug delivery device was placed between the sub-tenons and supra-scleral space of rabbit’s eye that was 0.5 mm away from the corneal limbus. Although, the implant was placed at the same location for the both rabbits, the incisions were toward and away from the cornea, respectively. The membrane of the device was facing toward the exterior of the eye, or the tenons layer. Surgical sutures, 6-0 fast absorbing plain gut (Ethicon, Inc., Cornelia, GA), were used to close the conjunctival incision for rabbit 1 and rabbit 2, respectively. At the end of the implant surgery Bacitracin Zinc and Polymyxin B Sulfate Ophthalmic Ointment USP (Bausch & Lomb, Rochester, NY) was used to prevent any post-operative infection. Figure 6 illustrated the surgical procedure and the implant location. At the limbus, the implanted location, the scleral thickness was 300 μm.

Once the drug delivery devices were implanted into the eye, the rabbits were placed under observation in the ARC facility for 2 weeks until the eyes completely recovered from the surgery. At this time, any leaks of 10% Na fluorescein dye from the device was checked by dilating the pupil of the implanted eye using three drops of 1% Tropicamide every 1 and 1/2 minutes for 5 minutes. Once the pupil dilated, a fiber optic probe with a non-invasive FS system (Device Stability Testing Section) was used to measure the intensity of 10% Na fluorescence from the eye.

Photography of Eye After Implant

Two different methods were applied to photograph the implanted device. For the rabbit 1 an EOS digital SLR Canon camera (Digital Rebel XT, Tokyo, Japan) attached to a slit lamp (Topcon SL-6E) was used to capture the condition of the implant. The camera shutter was triggered by digital single-lens reflex (DSLR) software using a computer. For the rabbit 2 a CCD camera (HV-C20 Hitachi Denshi, Ltd., Woodbury, NY) attached to a surgical microscope (Topcon OMS75, NJ) was used to capture the image as the photographs with Canon camera were not desirable. A photograph was taken of the in vivo conjunctiva immediately after implant and every time the fluorescence intensity was measured from the implanted eye.

Procedure of Histology

After extraction of the device from the rabbit eye 1 after euthanasia, a portion of the eye including the scar tissue and adjacent sclera and retina, conjunctiva and iris was removed from the globe and submitted for plastic embedding and sectioning. Step sections (5 μm thick, n = 16) were collected at different levels beginning at one end of the scar and proceeding into the cavity in the scar tissue with the embedded device. The sections were mounted on glass microscopic slides, stained with hemotoxylin and eosin reagents and covered with glass cover slips.

The microscopic slides were examined with an Olympus BX51 microscope fitted with diffuse white light (LM) and transmission polarizing (TPM) optics and 2×, 4×, 10×, 20×, and 40× objectives. Selected fields were imaged using Zeiss Axioscam MRc5 digital camera mounted on the microscope and Zeiss AxioVision SE64 Rel 4.8 software supported by a Dell Optiplex 980 Desktop Computer. Selected images were further modified using Adobe Photoshop Elements 9 software.

Scaling Factor

We conducted additional experiments to identify the correlation between the emission spectra intensity of the 10% Na fluorescein dye in vitreous humor and the volume of the dye. Five enucleated pig eyes were provided by the Air Force Research Laboratory at Brooks City Base. The eyes were harvested and stored in antibiotic and buffer solution (0.5 L 1x Hank’s Balanced Salt Solution (HBSS) with Ca and Mg, 10 mM HEPES buffer,
100 IU/ml penicillin, 100 IU/ml streptomycin, and 0.05 mg/ml gentamicin) immediately after enucleation. Storage containers holding the eyes were placed in ice to minimize sample degradation during transport. Eyes were placed in a 4°C refrigerator for one night. The time between enucleation of the eyes and experimental studies was 16 hours. The next day eyes were removed from the solution and held until temperature reached between 24 and 25°C (room temperature). Once the eye reached this specific temperature, 3.3 ml of vitreous humor was extracted from the vitreous chamber of each enucleated pig eye using a 18 G needle and fluorescence intensity was measured each time 1.0 μl of 10% Na fluorescein dye was added to the liquid. This process was repeated five times for each addition of 1.0 μl dye to find the correlation between the fluorescence intensity of the dye associated with a specific volume of the dye. However, volume of rabbit vitreous humor (1.67 ml) is about one and half times smaller than the porcine. This information was used to correct the scaling factor for the in vivo rabbit fluorescence measurements to calculate the amount of dye present in the rabbit eye after the release from the drug delivery device.

Emission Spectra of Rabbit Vitreous Humor

Female SPF Dutch Belted (n = 5) rabbits weighing 4–5 lbs were used for this experiment. Five microliters 10% Na fluorescein dye was added with 25.05 lbs were used for this experiment. Five microliters 10% stock chambers of the same volume of 30.0 ml of 0.9% blood bank saline (Fisher Scientific, Pittsburg, PA) to make a 24 and 25 solution and held until temperature reached between enucleation of the eyes and experimental studies was 16 hours. The next day eyes were removed from the 8°C refrigerator for one night. The time between enucleation of the eyes and experimental studies was 16 hours. The next day eyes were removed from the solution and held until temperature reached between 24 and 25°C (room temperature). Once the eye reached this specific temperature, 3.3 ml of vitreous humor was extracted from the vitreous chamber of each enucleated pig eye using a 18 G needle and fluorescence intensity was measured each time 1.0 μl of 10% Na fluorescein dye was added to the liquid. This process was repeated five times for each addition of 1.0 μl dye to find the correlation between the fluorescence intensity of the dye associated with a specific volume of the dye. However, volume of rabbit vitreous humor (1.67 ml) is about one and half times smaller than the porcine. This information was used to correct the scaling factor for the in vivo rabbit fluorescence measurements to calculate the amount of dye present in the rabbit eye after the release from the drug delivery device.

RESULTS

In Vitro Study

The yield of the drug delivery device was found to be 46% for the first generation drug delivery device. In another words, 46% of the devices did not leak 10% Na fluorescein dye during the stability testing. However, the second generation device had a 100% yield. This increase yield in the second generation drug delivery device was due to the higher adhesion strength of the optical glue. Both generation devices were submerged under water and stayed intact for 365 days or a year for the entire duration of the observation. The membrane stayed intact and no leaks of 10% Na fluorescein dye were found from any of the submerged devices.

The threshold energy of the ophthalmic Q-switched Nd:YAG laser to ablate the membrane of the ex vivo drug delivery device submerged in water was measured at 6.5 mJ to create a 100.0 μm diameter hole, unlike the implanted device which was measured as 10.0 mJ. To measure the diameter of the hole a photograph (Fig. 7a) was captured using a fluorescence microscope (Olympus BX51, Leeds Instruments, Inc., Irving, TX) and the same device was imaged (Fig. 7b) using the Spectral Domain Polarization Sensitive Optical Coherent Tomography (SD-PSOCT) to measure the exact thickness of the membrane after the ablation.

Figure 8 illustrated the scaling factor of the fluorescence intensity versus volume of 10% Na fluorescein dye in the vitreous humor that was extracted from vitreous chamber of the enucleated pig eye. There was a linear relationship with the volume of the dye and its associated fluorescence intensity.

In Vivo Study

Only one of the two reservoirs was ablated mechanically for rabbit 1 to find out the stability of the membrane of the intact reservoir. For rabbit 2, membrane of only one of the reservoirs was ablated using ophthalmic Nd:YAG laser. Photographs were taken up to the end of the experiment when the fluorescence intensity returned to baseline measurement. Figures 9 and 10 verified that the device did not leak the dye from the intact reservoir of both rabbits during the entire duration of the experiments. Although, the fluorescein staining surrounding the photo-disrupted membrane was more visible in the rabbit 2 after ablating the membrane until 18 days, no staining was observed in rabbit 1 after a few days. At the end of the study, the implanted device was extracted from the rabbit 1. However, for rabbit 2, the implanted device extruded from the surrounding tissue 1 day after the last measurement (day 20 since the dye release). Thus, only the surrounding tissue with the device from the rabbit 1 was collected for histology (Fig. 11a) unlike the rabbit 2 where only device was analyzed for the laser ablation pattern on the membrane (Fig. 11b). Figure 11c,d was the enlarge
figures of the holes shown in Figure 11b using a BX51 fluorescence microscope without fluorescent filter exposure.

Figure 12a,b shows the half-life of the 10% Na fluorescein dye in the eye of the rabbit 1 and rabbit 2, respectively. In order to identify whether the fluorescence signal was acquired from the anterior or the vitreous humor, additional experiments were performed and the results were described in the Signal Variation of Emission Spectra of Rabbit’s Eye Chambers Section. The variability in signal emission peak was considered as an identifier of the location. Also, experimental result of the sampling depth measurement of the probe geometry was also verified with the multi-layers rabbit eye tissue simulating Monte Carlo model (Monte Carlo Modeling Section). The normalized fluorescence intensity showed no fluorescence activity between the implant and before the content was released by ablating the membrane. At 21 days since the first implant, the membrane was ablated mechanically for the rabbit 1. However, 2 weeks were found to be adequate time for recovery from the implant surgery based on the observation of the rabbit 1. Thus, at 14 days the membrane was ablated for the implanted device in the rabbit 2 using an opthalmic Nd:YAG laser with a threshold energy of 10 mJ. About 17 minutes after the release of the dye, the fluorescence intensity peaked corresponding to a volume of $3.82 \pm 0.7$ and $3.4 \pm 0.4 \mu l$ (standard deviations were shown in Fig. 12a,b) for rabbit 1 and rabbit 2, respectively. The concentration of the 10% Na fluorescein dye in the vitreous humor of rabbit 1 and rabbit 2 was 60.84 and 54.15 mmol/L, respectively, at about 17 minutes post-release.

Fig. 7. a: Fluorescence microscope (Olympus BX51) image of a 100 µm diameter hole at the reservoir membrane of the drug delivery device by the Nd:YAG laser ablation after the device was placed in a water filled glass jar, (b) same drug delivery device was photographed using the spectral domain polarization-sensitive optical coherent tomography (SD-PsoCT) to measure the thickness of the membrane.

Fig. 8. Scaling factor calculation using 10% Na fluorescein dye in the vitreous humor from the vitreous chamber of five enucleated pig eyes and measured five times for each additional 1.0 µl of dye (a) intensity versus volume, (b) intensity versus concentration. These information were used to calculate the volume of 10% Na fluorescein dye inside the eye.
The measured half-life of 10% Na fluorescein dye from the eye vitreous chamber for both rabbits was found to be 13 days based on the peak emission spectra at 520 nm wavelength unlike the anterior chamber at 534 nm (Signal Variation of Emission Spectra of Rabbit's Eye Chambers Section). During this time the fluorescence intensity reduced to half of the original intensity. At 19 days from the dye release, the fluorescence intensity reached its baseline measurement for both rabbits. Thus, at 21 days since the release, the rabbits were euthanized before the extraction of the implanted device with or without the surrounding eye tissue. The second generation device in the rabbit 2 extruded at 19 days after the dye release due to the boat shape curvature of the device that did not match the curvature of sclera and the semi-circle shape of the limbus.

Pathology of Histology

The results of the histology study on rabbit 1 are shown in Figure 13. The irregular space that contained the device is outlined by scar tissue. The cavity surface adjacent to the sclera is associated with dense inflammatory infiltrates and focal accumulations of blood vessels (granulation tissue) characteristic of early wound healing. This early wound tissue is continuous to the more mature fibrous scar of the sclera flap incision. Farther away from the scleral flap wound tissues, the inflammation is less prominent, and the cavity surfaces, particularly the surfaces of the sides and the cavity opposite to sclera, are lined by a thin layer of inflammatory cells supported by loose fibrous connective tissue. Fragments of birefringent material consistent with formed foreign material that could represent retained suture or pieces of the membrane of the device are found at the edges of the cavity particularly close to the scleral flap. A few foreign body giant cells are scattered among the material fragments.

The scar tissue of the sclera flap incision extends through the sclera to the choroid producing local fibrous scar tissue containing melanin-containing phagocytes in the sclera and choroid. The adjacent retina showed disruption of the sensory cells (rods and cones) and the adjacent neural cells with increased glial cells some of which contained melanin. However, the retina away from the scleral flap incision scar shows normal cellular features and anatomical arrangements.

Monte Carlo: Rabbit Eye Model

Figure 14 shows the excitation light distribution as a function of tissue depth $z$ (mm) and radial distance $r$ (mm) for incident power density or fluence rate in W/mm$^2$. Fluence is very high in cornea, anterior chamber, and...
part of crystalline lens. Although, fluence rate is reduced in the vitreous chamber, the incident power is still high enough to excite fluorophores in the vitreous chamber a total depth of 17.6 mm. This fluence rate was further verified with the Monte Carlo for Multi-Layered Media (MCML) free software developed by Lihong Wang and Steven Jacques.

**Signal Variation of Emission Spectra of Rabbit’s Eye Chambers**

For *in vivo* rabbit eye, fluorescence emission spectra varied between the anterior and vitreous chambers. For the *in vivo* rabbit eye, the signal from the anterior chamber emission spectra peaked at 524 nm at 0 minute or immediate after injecting the dye. As time elapsed, the peak of the emission spectra started to shift to the longer wavelength and at 30 minutes the emission spectra peaked at 534 nm. On the other hand, immediately after the injection of the fluorescein dye into the vitreous chamber, the fluorescence emission spectra peaked at 505 nm wavelength. However, the spectral peak started to shift to a longer wavelength and was 520 nm at 30 minutes (Fig. 15).

**DISCUSSION**

One of the unique advantages of this drug delivery system is that it allows separation of the components (reservoirs) that control device performance from those that affect drug stability. The formulation that controls the drug release (the reservoir membranes) is to a first approximation independent of any formulation that may...
Fig. 11. a: Photograph (EOS digital SLR Canon camera attached to a slit lamp) of the implanted device extracted with surrounding eye tissue from the rabbit 1 for histology at 39 days after the implant. The intact reservoir with dye is highlighted with a circle and empty reservoir (no trace of dye) shown at the bottom; (b) photograph (CCD camera attached to a surgical microscope) of the implanted device that was extracted from the rabbit 2 at end of the experiment (fluorescence intensity reached at baseline) to observe the effect of laser ablation on the membrane of the reservoir. Two different holes are identified on the membrane of the device highlighted with arrow. c,d: Fluorescence microscope (Olympus BX51) images were taken without fluorescence filter exposure of the ablations shown in (b). The size of the hole is about 500 μm (represented with left arrow in b) and a dark ring presents surrounding the hole due to disintegration of the membrane due to laser. There is also a small penetration of about 100 μm (represented with right arrow in b). The round depression on the top (b) is an air bubble.

Fig. 12. Fluorescence intensity associated with the volume of the 10% Na fluorescein dye in the vitreous humor of the posterior chamber of in vivo rabbit. Half-life of the dye in both rabbits (rabbit 1 and rabbit 2) is found to be 13 days despite the release methods of the dye were different. Membrane of the device was ablated (a) mechanically using a 28 G ½ needle for rabbit 1 (b) light activated using an ophthalmic Nd:YAG laser for rabbit 2.
Fig. 13. **Top left:** The cavity (*) in scleral scar marks the placement of the drug delivery device. Dense infiltrates of inflammatory cells and numerous blood vessels are components of early scar tissue (short thick arrow) has formed between the device and the sclera. The cavity walls (short thin arrows) away from the sclera show less inflammation (original magnification 20×). **Top right:** Higher magnification of wound tissue (original magnification 100×). **Middle left:** Cavity located 1,000 μm from the previous tissue sample. The cavity lumen (*) is closer to the sclera and the inflammation (arrow) in the wall is much less (original magnification 20×). **Middle right:** Higher magnification of eye wall indicated by the arrow in the previous picture. A light infiltrate of inflammatory cells extend from the cavity wall surface into the outer layers of the sclera. The neural layers of the retina, the pigmented retinal epithelium (arrow), and the blood vessels of the choroid are in regular array (original magnification 200×). **Bottom left and right:** Representative of the cavity (*) wall away from the sclera. The smooth surface of the cavity wall has been due to presence of the device membrane revealed by transmission polarizing microscopy (right). Infiltrates of inflammatory cells are present in the fibrous tissue forming the cavity wall (original magnification 200×).
be included in the reservoir to control the drug stability. This is vastly different from other drug delivery systems that have been developed [55,56] such as a passive MEMS drug delivery device with mechanically controlled pump by Lo et al. [56].

Our drug delivery device was fabricated using PMMA which is not only biocompatible but also can be implanted in the eye for long time. A study by Lo et al. [55,56] showed that a form of PMMA which is a type of PDMS was used to build their refillable implant device for eye. Due to the refillable nature the device, it can be implanted for 2–3 years. However, they showed only in in vitro study on enucleated porcine eye. Lo study also reported that cyclosporine implant fabricated from PMMA can be implanted for 3–5 years [55]. Therefore, the PDMS is very stable and reliable for these types of implant method in drug delivery. Also, PMMA is used for ophthalmic intraocular lens in the eye when the original lens has been removed in the treatment of cataract [57]. In orthopedics, PMMA bone cement is used to affix implants and to remodel lost bone [58]. In cosmetic surgery, tiny PMMA microspheres suspended in some biological fluid are injected under the skin to reduce wrinkles or scars permanently [57]. Therefore, PMMA is very stable and safe to use for long-term use for treating any ocular diseases [57,58].

The required energy level (6–10 mJ) to ablate the membrane of the drug delivery device was within the safe range of 0–12 mJ for eye surgery. The implanted device extruded from the rabbit 2 showed multiple ablations (diameters between 50 and 500 μm) on the membrane without destroying the other reservoir. Thus, the content of the drug delivery device could safely be released from one chamber without inadvertently rupturing the second chamber to provide controlled dosing.

In general, the scar tissue surrounding the device was very thin and, in the loose connective tissue away from the sclera, the cavity surface was very flat probably reflecting the smooth surfaces of the device. Only a very thin layer of inflammatory cells separated the device from the surrounding soft fibrous tissues of the orbital cavity. The prominent accumulation of vascular and fibrous granulation tissue of early wound healing in the cavity wall adjacent to the sclera could have been associated with the surgical trauma of creation of the sclera flap and the insertion of the device. However, the mechanical

Fig. 14. Fluence rate of light source with specific probe geometry (power: 65 mJ and core diameter: 200 μm). The fluence rate was calculated by generating 2,000,000 photons per package for excitation wavelength of 495 nm. The roman numerical values represent (i) cornea, (ii) anterior chamber or aqueous humor, (iii) crystalline lens, (iv) posterior chamber or vitreous humor, (v) beyond posterior chamber of rabbit eye.

Fig. 15. Average fluorescence intensity for 12 time intervals from five in vivo rabbit eyes before and after injecting 10% Na fluorescein dye into (a) the anterior chamber, (b) vitreous chamber. The maximum fluorescence emission peaks for anterior and vitreous chambers varied about 14 nm at 30 minutes observation point.
disruption of the membrane covering the dye chamber may have also produced tissue damage and possibly fragmentation of the membrane leading to a stronger inflammatory and wound healing response. Since the device was removed, the location of the membrane rupture could not be correlated to the distribution of the inflammatory and wound healing responses in the tissues.

Although, our device flat form had anchoring holes, no anchor sutures were used. Thus, the first implanted drug delivery device in the rabbit 1 had shown about 2.0 mm shift from the original implanted location and settled in the cul-de-sac. Usually, sub-tenons implants shift unless the anchoring holes for suture and subsequent tissue in growth are attached. This shift was also thought to be due to the shape of the device as well as the horizontal incision (with respect to cornea) at the limbus. Thus, we changed the design of the second generation device to give a more stationary feature by creating a boat like bottom and the four corners of the device were angled to remove any sharp edges. Also, the horizontal incision was placed away from the limbus to create a physical barrier to prevent device movement. For the rabbit 2, a second generation device was implanted with a combination of a new incision method to hold the device in the original position. No shift was observed. This device stayed at the limbus area (sclera with thickness of 300 μm) and provided an additional advantage of fast tissue clearing effect from 100% anhydrous glycerol. For the rabbit 2, the tissue clearing took only 3 minutes to clear completely unlike the rabbit 1 that took about 8 minutes. The shape of implant 2 was not matched to the curvature of the sclera and that mismatch contributed to the extrusion. Ideally, the implant should be curved to match the curvature on the sclera and the device should be implanted with the long axis perpendicular to the limbus.

In rabbit 2, the surrounding tissue of the ablated membrane was stained with the fluorescein dye. However, no such staining was observed in the rabbit 1. The difference could be due complex pharmacokinetic and the additional scleral flap created only in the rabbit 1. This scleral flap might have provided a direct access to the blood vessels of the choroid layer; thus, not enough time to be stained with the fluorescein dye.

Due to the large sampling depth of 17.6 mm both experimental result and simulated by the Monte Carlo model, the probe with our specific geometry could sample the rabbit anterior and vitreous chambers (Signal in vivo and Rhodamin B in these three layers of pigmented rabbit's eye (n = 3) after topical administration. In the three rabbits, the emission spectra of fluorescein dye (typical peaks at 520 nm) in the cornea and anterior chamber were shifted toward longer wavelength by 6–10 nm to longer wavelength. Similar red shift was also identified from the crystalline lens. This “red shift” is presumed to result from the fluorescein being bound by the protein in the cornea [62]. As the maximum fluorescence emission spectra collected from the two in vivo rabbits in this drug delivery study also matched with these supporting studies discussed above, we can conclude that the dye from the implanted device must have diffused in the vitreous chamber of the rabbit eye.

To determine if this type of implanted device can be used to treat ocular diseases such as disciform ARMD, the actual drug (Leucentis or Avastin) will need to be fluorescently tagged and half-life studies of drug concentration vs. time in the vitreous will need to be performed. The fiber optic probe discussed in this article shows promise for detecting fluorescently tagged drugs in the vitreous but will need to be verified with other techniques such as depth-resolved microscopy.

Although, the knowledge of half-life of a drug or substance is an important aspect for treating any ocular diseases, to our knowledge no other study has ever investigated the half-life of a fluorescent marker that is released from a sub-tenons implant into the eye. In our study, the measured half-life for both release techniques was found to be 13 days, but the volume or concentration of the dye present in the vitreous chamber differed. The rabbit 1 showed larger dye volume present in the vitreous chamber. This difference may due to direct diffusion through the scleral flap. Half-life is related with not only the rate of diffusion of the substance but also the inverse relationship to the molecular weight (MW) of that substance. Thus, one may assume that based on MW the 10% Na fluorescence dye (MW 332.3 Da) would have a much shorter half-life compared to other ocular drugs such as Ranibizumab (MW 48 kDa). However, the half-life for the dye was found to be much longer than that of Ranibizumab (delivered in a traditional method of intra-vitreal injection). Gaudreault et al. evaluated the pharmacokinetic (PK) and serum bioavailability of ranibizumab after a single intra-vitreal (ITV) or intra-venous (IV) dose in cynomolgus monkeys [60]. Ranibizumab cleared in parallel
from all ocular compartments, with a terminal half-life of 3 days. It distributed rapidly to the retina (6–24 hours), and concentrations were approximately one-third that in the vitreous humor. After IV injection, the drug will be released. Serum concentrations were very low, reflecting wider distribution and faster clearance when ranibizumab reached the serum. After IV administration, the terminal half-life was even lower (approximately 0.5 day). The longer half-life from the implanted drug delivery method compared to the traditional method could be due to the slow release of the dye over a couple of weeks following a very complex pharmacokinetics to the vitreous chamber unlike direct injection. With high concentration of drugs in a large number of reservoirs, the drug-delivery device presented here can supply on demand medication to patients over several months. Tissue clearing technique needs to be employed to access the implanted device in the eye whenever the drug needs to be released. This new technology can provide the option to the patient to have a one-time insertion of the carrier system containing the drug. Each time the patient requires treatment, the sclera will be cleared and a laser will be used to release the drug in measurable doses from the carrier system into the vitreous humor. The entire processes of tissue clearing and ablation with ophthalmic Nd:YAG laser takes only 10 minutes and can be performed in the office. Current treatment for wet type age-related macular degeneration (AMD) requires patients to take intra-vitreal injection through sclera multiple times based on the condition of the disease. The procedure is not only painful but also can cause multiple risks of endophthalmitis. An implanted drug delivery device will reduce the discomfort that the patients with wet AMD feel with repeated intra-vitreal injections and may reduce the risk of endophthalmitis.

We used 10% Na fluorescein dye as a tracer for this study. This molecule is hydrophilic and has much smaller molecular weight (MW 332.3 Da) than some ocular drugs such as Ranibizumab (MW 48 kDa). Since the device administers the content via a sub-tenons route, large molecules such as Ranibizumab may not pass in adequate amounts through human sclera to the retina due to thicker sclera compared to rabbit. Thus, in future studies, we may conduct experiments with ocular drugs such as steroids (small molecular weight) that are fluorescently tagged. The proposed drug delivery device may provide an alternative for recurrent administration of steroids by sub-tenons injection. In addition, more experiments testing the clinical safety and efficacy of an implantable device and the methods of drug release have to be done.

In this study, we attempted to implant the device under a scleral flap so that the device could be very close to the choroid; thereby, reducing the scleral impermeability to large molecules such as Leucenist. However, the sub-scleral implant did not work due to the large size of the device. Some of the data presented in this study were from a rabbit with an aborted flap (but thinned sclera). The rabbit with aborted flap and thin sclera in some ways mimicked the delivery system of a smaller device implanted under a scleral flap. We definitely recognize the problem for large molecules to penetrate thick sclera and the limitations of generalizing our results due to the thin sclera—in the rabbit models. Thus, if we could reduce the size of the devices and implant them in the sub-scleral space we would improve delivery of large molecules to the vitreous after the optical clearing of thick sclera using hyperosmotic agent. We have also designed a next generation drug delivery device that could release drug through a needle directly into the vitreous.

CONCLUSION

In this study, we have shown that the material for the drug delivery device is bio-compatible and can be implanted in the sub-tenons space. The device membrane can be ablated with an ophthalmic Nd:YAG laser after tissue clearing to create a controlled release of the content without disrupting the other chamber. The study also showed that small molecules such as 10% Na fluorescein dye can get into the vitreous chamber. The half-life or drug availability of 13 days is higher than other similar molecules in the eye most likely due to slow release from the implant once the membrane is ablated. Based on our observation, the device needs to be implanted with the long axis perpendicular to the limbus (not parallel). Thus, in the future the size of the device needs to be reduced by at least one-half of the current dimension with smooth and rounded edges. In addition, the device should be curved such that it fits the curvature of the sclera (radius, r = 12 mm).

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