

Controlled Delivery of the Anti-VEGF Aptamer EYE001 with Poly(lactic-co-glycolic)Acid Microspheres

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PURPOSE. To develop a controlled-drug delivery system for the long-term inhibition of vascular endothelial growth factor (VEGF) and its mediated responses.

METHODS. Poly(lactic-co-glycolic)acid (PLGA) microspheres containing anti-VEGF RNA aptamer (EYE001) formulations in the solid-state were developed by an oil-in-oil solvent evaporation process. In vitro experiments were performed to characterize the release profiles. Stability and bioactivity of the released drug were assayed by monitoring the RNA aptamer's ability to inhibit VEGF-induced cell proliferation in human umbilical vein endothelial cells (HUVECs). Cell proliferation experiments were conducted with aptamer aliquots collected after short-, mid-, and long-term release time points. To investigate the feasibility of this polymer device as a potential transscleral delivery device, an in vitro apparatus was developed to assess polymer hydration and degradation through rabbit sclera and subsequent delivery through it.

RESULTS. PLGA microspheres were able to deliver EYE001 in a sustained manner, with an average rate of 2 $\mu\text{g}/\text{d}$ over a period of 20 days. Solid-state stabilization of the aptamer with disaccharide trehalose before lyophilization and encapsulation in PLGA rendered the drug more stable after release. Cell proliferation experiments demonstrated that the bioactivity of the aptamer was preserved after release, as indicated by inhibition of endothelial cell proliferation after incubation with VEGF. Microspheres packed into a sealed chamber and placed onto the "orbital" part of a rabbit sclera for a period of 6 days became hydrated and started to degrade, as shown by scanning electron microscopy (SEM). As a result, the aptamer was delivered from the microspheres through the sclera, as determined spectrophotometrically.

CONCLUSIONS. The loading of aptamer-containing microspheres into a device and placing it on the orbital surface of the sclera was assessed and shown to be feasible. RNA aptamer EYE001 encapsulated in PLGA was delivered over a period of 20 days with retained activity. This method represents a promising approach for the transscleral delivery of drugs and the treat-

ment of choroidal and retinal diseases. (*Invest Ophthalmol Vis Sci.* 2003;44:290–299) DOI:10.1167/iovs.01-1156

Vascular endothelial growth factor (VEGF) has been identified as a key positive regulator of angiogenesis.¹ It acts as an endothelial cell mitogen and chemoattractant in vitro^{2,3} and induces vascular permeability and angiogenesis in vivo.^{2,3} Elevated VEGF expression is correlated with several forms of ocular neovascularization that often lead to severe vision loss, including diabetic retinopathy,⁴ retinopathy of prematurity,⁵ and macular degeneration.⁶ Thus, agents that specifically inhibit VEGF may have great utility in combating a variety of human diseases for which few effective treatments are presently available.⁷

Recently, a method used to isolate oligonucleotide ligands (aptamers) from libraries of RNA, DNA, or modified nucleic acids that bind with high affinity and specificity to various molecular targets, including proteins and peptides, has been described.⁷ In particular, an RNA-based aptamer has been developed with high affinity toward VEGF₁₆₅.⁸ After being isolated and determined to bind specifically to VEGF₁₆₅, RNA aptamer EYE001 (formerly referred to as NX1838) was further modified chemically to render it nuclease-resistant and thermally more stable, thus enhancing its potential for therapeutic utility.^{7,8} The promising results displayed by the aptamer's biological response both in vitro and in vivo against diseases associated with the growth of new blood vessels or angiogenesis, especially those threatening to vision, suggest that it has excellent potential as a therapeutic agent.^{7–9} Currently, EYE001 is undergoing clinical trials for the treatment of age-related macular degeneration (AMD). Because choroidal neovascularization is a severe complication of AMD and because patients with subretinal neovascularization, including those with AMD, show increased expression of VEGF, it is believed that antiangiogenic and/or antivascular permeability factors could delay or reverse the pathogenesis of AMD.^{10–12}

Widespread clinical use of EYE001 will necessitate a practical and effective method of delivery to the eye. Application in current clinical trials relies on intravitreal injections of the aptamer. Although this method allows for assessment of the potential use of the aptamer as a therapeutic drug, it is a less than optimal way to treat patients on a day-to-day basis, because of its invasive nature. The mode of delivery should provide exposure to the drug for the required period it must be minimally invasive and, preferably, localized.

Recent studies have highlighted the applicability of transscleral delivery for various macromolecules, including globular proteins.^{13,14} The potential for transport or diffusion through the sclera lies in the large and accessible surface area of this tissue, its high degree of hydration, hypocellularity, and permeability that does not decline significantly with age.^{15–17} Thus, this approach would circumvent the limitations and problems presented by other modes of delivery to treat posterior segment diseases—intravitreal, systemic, and eye drops—that include, among other drawbacks, retinal detachment, systemic side effects, and diffusional limitations, respectively.^{18–20}

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Because certain methods of transscleral delivery can be destructive (i.e., iontophoresis), causing, in some cases, retinal necrosis and gliosis,²¹ we have focused our attention on biodegradable polymer sustained-delivery devices. Sustained delivery of proteins or nucleic acids from polymer matrixes offers the advantage of targeting specific tissues and increasing the comfort and compliance of patients.^{22–24} Specifically, we have used poly(lactic-co-glycolic) acid (PLGA) as the encapsulation matrix of choice. PLGA, an FDA-approved material, has been extensively studied for its biocompatibility, toxicology, and degradation kinetics.^{25,26} It has been used clinically as a suture material since the 1970s,²⁷ and recently it has been used as scaffold in tissue engineering techniques.^{28,29} An important characteristic of PLGA carrier systems is their ability to be applied locally, which allows intrascleral concentrations of the drug to be sustained while systemic deleterious side effects are minimized, thus providing a pharmacological advantage at the treatment site.^{30,31} In vivo studies in which PLGA was used as a carrier system to the eye for various types of drugs used to treat various diseases have reported no sign of ocular toxicity or significant inflammatory responses for periods of up to 2 months.^{31,32} These studies, however, involved either the encapsulation of small synthetic drugs and molecules or the intravitreal injection of such polymer devices after a sclerotomy, which in itself is invasive.

In our present study, we considered the potential of transscleral delivery of drugs in a sustained and controlled manner in an in vitro setup. We were able to deliver the anti-VEGF aptamer EYE001 for a period of up to 20 days in a biologically active state, showing no destabilization due to the encapsulation procedure. We hypothesize that delivering drugs in a sustained manner through the sclera is a viable approach for the treatment of various vision-threatening diseases.

METHODS

Lyophilized RNA Aptamer EYE001

EYE001 was produced at Gilead Sciences, Inc. (Boulder, CO) by the systematic evolution of ligands by exponential enrichment (SELEX) process as described^{7,33} and supplied as a liquid formulation of 3 mg/200 μ L saline solution. EYE001 is a pegylated RNA aptamer of 50 kDa, with an A-type secondary structure, 40 mg/mL solubility, and a net negative charge of -28 . Samples were lyophilized in a (model SNL315SV; Savant Instruments, Farmingdale, NY) at a chamber pressure of 80 mbar and a shelf temperature of -45°C for 48 hours to obtain excipient-free aptamer. The lyophilized material was sealed in sterilized glass vials and stored at -20°C until use. Lyophilized samples containing trehalose (Sigma Chemical Co., St. Louis, MO) at a 1:3 weight ratio were prepared by adding an appropriate amount of concentrated excipient solution to the excipient-free aptamer solution before lyophilization.^{34,35} The ratio was selected based on the mass amounts of aptamer to trehalose needed to stabilize aptamer structure and function on lyophilization and thus prevent lyophilization-induced structural changes.

Circular Dichroism Experiments

Circular dichroism (CD) spectra were recorded on a CD spectrometer (model 202; Aviv Instruments, Lakewood, NJ). Data were collected at 25°C using a bandwidth of 0.5 nm and an average time of 0.1 second. The CD spectra were collected from 200 to 330 nm with a 0.5-cm quartz cells and corrected for the phosphate buffer signal contribution measured under identical conditions.

Microsphere Preparation

PLGA microspheres were prepared by a non-aqueous oil-in-oil methodology, as described previously.²⁴ Briefly, 25 to 30 mg of solid

aptamer was suspended in a solution of 200 mg/2 mL PLGA (Resomer 502 H, i.v. (inherent viscosity) 0.16–0.24 dL/g, 0.1% in chloroform, 25°C , molecular weight [M_w] 10 to 12 kDa, half-life for degradation approximately 1 to 1.5 months; Boehringer Ingelheim Pharma KG, Ingelheim, Germany) in methylene chloride with a homogenizer (Polytron, model PT 1200C; Brinkman, Westbury, NY) using a standard 12-mm diameter generator at approximately 20,000 rpm for 1 minute. After suspension of the aptamer, the coacervating agent poly(dimethylsiloxane) was added at a rate of 2 mL/min under constant homogenization, to ensure homogeneous dispersion of the coacervating agent, phase separation of PLGA dissolved in methylene chloride, and formation of microspheres. The coacervating mixture containing the microspheres was then poured into an Erlenmeyer flask containing 50 mL heptane under constant agitation and stirred for 3 hours at room temperature to allow for hardening of the microspheres. Microspheres were collected by filtration with the use of a 0.22- μm nylon filter, washed twice with heptane, and dried for 24 hours at a vacuum of 80 mbar.

Encapsulation Efficiency

Encapsulation efficiency was determined as described previously.²³ Ten milligrams of PLGA microspheres was placed in 2 mL methylene chloride and stirred for 30 minutes to dissolve the polymer. The solution was then centrifuged at 10,000 rpm for 10 minutes to precipitate the insoluble RNA aptamer, supernatant was removed, and the remaining methylene chloride was allowed to evaporate. To ensure evaporation of the methylene chloride, the sample was placed in a vacuum for 24 hours. The aptamer was then dissolved in Dulbecco's phosphate-buffered saline (DPBS; GibcoBRL, Grand Island, NY), and the concentration of entrapped aptamer in PLGA was determined spectrophotometrically. The percentage encapsulation efficiency was calculated by relating the experimental aptamer entrapment to the theoretical aptamer entrapment: (experimental/theoretical) $\times 100$.

In Vitro Release Profiles

Ten milligrams of solid microspheres was placed in 2 mL of DPBS, 1 \times (pH 7.3) and incubated at 37°C . Every 24 hours, the microspheres were gently centrifuged at 500 rpm for 1 minute, and the supernatant was removed for determination of aptamer concentration at 260 nm, $\epsilon_{\text{PBS}} = 25.08 \text{ cm}^{-1} (\text{mg/mL})^{-1}$ as described.^{24,36} Microspheres were then resuspended in 2 mL fresh DPBS to maintain sink conditions and control the pH.^{24,37} Ten milligrams of blank (empty) PLGA microspheres were subjected to the same conditions as PLGA-loaded microspheres, and the supernatant collected from these was used as a blank in the spectrophotometric analysis. Data are presented as the average of three independent experiments with standard deviations.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Cascade Biologics, Inc. (Portland, OR). Cells were maintained in growth-factor supplemented medium, including 2% vol/vol fetal bovine serum (FBS), 1 $\mu\text{g/mL}$ hydrocortisone, 10 ng/mL human epidermal growth factor, 3 ng/mL basic fibroblast growth factor, and 10 $\mu\text{g/mL}$ heparin under standard tissue culture conditions (5% CO_2 , 37°C , 100% relative humidity). Medium was changed every 48 to 72 hours, and cells were passaged by standard trypsinization and plated at a cell concentration of 2.5×10^3 cells/cm².

VEGF-Induced Endothelial Cell Proliferation

Proliferation assays were performed as described previously⁷ with few variations. Briefly, HUVECs were seeded into 6-well or 12-well plates (2.5×10^3 cells/cm²) as required in growth-factor-deficient medium (Medium 200; 5% FBS, 1 $\mu\text{g/mL}$ heparin; Cascade Biologics) for 24 hours before experimentation. Aptamers (10 nM) collected after release from PLGA microspheres at specific time points and then

VEGF₁₆₅ (10 ng/mL; R&D Systems, Minneapolis, MN) was added to cells and incubated for 4 days. Cells were trypsinized and counted with a cell counter (model Z1; Coulter, Bids, UK). Wells containing cells without addition of aptamer or VEGF₁₆₅ were trypsinized and counted for basal growth estimation (blanks).

Scanning Electron Microscopy

Samples were affixed with double-sided carbon tape to an aluminum stub and sputtered with approximately 100 nm gold (Sputter Coating System; SPI, West Chester, PA). SEM images were then obtained (model S360; Cambridge Instruments, Monsey, NJ).

Isolation and Preparation of Rabbit Sclera

The experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and guidelines developed by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary. Dutch belted rabbits (Myrtle's Rabbitry, Inc., Thompson Station, TN), each weighing 2 to 3 kg, were anesthetized and killed with an intramuscular combination of 40 mg/kg ketamine (Abbott Laboratories, North Chicago, IL) and 10 mg/kg xylazine (Bayer, Shawnee Mission, KS) as described.¹⁴ The eyes were enucleated immediately before the rabbits were killed and were immersed in DPBS. The adherent muscles were excised, and scleral tissue was removed. Areas free of nerve and vessel emissaries were used to obtain 12 × 20-mm slices of sclera under microscope caliper guidance. Each piece of sclera was immersed on PBS and used on the day of isolation.

In Vitro Transscleral Delivery Apparatus

The in vitro apparatus used for these experiments was modified from one previously described (Fig. 1).¹⁴ Briefly, a 10 × 18-mm window was created on one face of a polystyrene cuvette (Sigma, St. Louis, MO) with use of a vertical milling machine (Bridgeport Machines; Bridgeport, CT), and a piece of sclera was blotted dry and placed over this window without stretching, avoiding asymmetrical stress. The tissue was sealed to the cuvette with a small amount of cyanoacrylate tissue adhesive (Ellman International, Hewlett, NY) applied continuously around its rim. PLGA-loaded solid microspheres (5 mg) were packed into a device 9 mm in diameter and 4 mm in depth made from a polypropylene cap of a 26.5-gauge needle (BD Biosciences, Lincoln Park, NJ). Cyanoacrylate tissue adhesive was placed around the border of the device and sealed against the orbital surface. A second identical

cuvette was aligned with the first cuvette and glued in place along the margins of the tissue. Both sides of the cuvette were then filled with DPBS (2.5 mL), and the apparatus was placed in an incubator at 37°C without agitation. One side was considered the "uveal" chamber where diffusion of the aptamer would occur if the delivery were successful. The other side facing the "orbital" surface of the sclera would comprise any part of the sclera not covered by the device containing the microspheres and would serve as a control to assess any leakage from the device. A protease inhibitor cocktail (Complete Mini; Roche Diagnostics, Indianapolis, IN), at concentrations recommended by the manufacturer, was added to avoid proteolytic degradation of the tissue. In addition, 0.1 mM sodium azide was added to inhibit growth of bacteria in the medium.¹⁷ After 24 hours the "uveal" chamber was sampled for aptamer concentration at 260 nm with a spectrophotometer (UV-Vis LambdaBio 40; Perkin Elmer, Wellesley, MA), and the "orbital" chamber was sampled as a control. Each side was replenished with fresh DPBS. To assess microsphere hydration and degradation, scleral tissue was analyzed by SEM after incubation for a determined period.

Transmission Electron Microscopy

Tissue was placed in modified Karnovsky fixative consisting of 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer with 8 mM CaCl₂ and fixed for 12 to 24 hours at 4°C. The specimens were subsequently changed to 0.1 M cacodylate buffer for storage at 4°C. The tissue was trimmed to block size and postfixed in 2% aqueous OsO₄ for 2 hours at room temperature. After the tissue was rinsed in buffer, it was dehydrated in ascending concentrations of ethanol, transitioned through propylene oxide, and infiltrated with mixtures of propylene oxide and Epon (EMBed 812; Electron Microscopy Sciences, Fort Washington, PA), embedded in pure Epon, and polymerized at 60°C for 18 to 24 hours. One-micrometer sections and thin sections were cut on an ultramicrotome (Ultracut E; Leica, Deerfield, IL). The 1-μm sections were stained with 0.5% toluidine blue and the thin sections with saturated aqueous uranyl acetate and Sato lead stain, and then examined with a transmission electron microscope (model CM-10 Philips, Eindhoven, The Netherlands).

Statistics

The paired Student's two-tailed *t*-test was used to compare cell counts after each incubation condition. An α level of 0.05 was used as the criterion to reject the null hypothesis of equality of means.

RESULTS

Characteristics of PLGA Microspheres

SEM images of the microspheres after preparation indicated the formation of nonporous spheres with an average diameter of 14 ± 4 (Fig. 2A) and 16 ± 4 μm (Fig. 2B) after hydration. The encapsulation efficiencies of aptamer into PLGA varied with the original amount of drug used as starting material. Encapsulation efficiency for microspheres containing aptamer colyophilized with trehalose was $80\% \pm 5\%$ when 32.1 mg was used as the starting material, whereas for excipient-free aptamer-containing microspheres, the encapsulation efficiency was $71\% \pm 2\%$ when 6.6 mg was used. Analysis of the microspheres after 10 days of release showed degradation of the polymer matrix and the formation of pores through which the aptamer was slowly released (Fig. 2B).

RNA Aptamer EYE001 Release from PLGA Microspheres

In vitro release profiles (Fig. 3A) for both excipient-free aptamer and aptamer colyophilized with trehalose at a 1:3 weight ratio of aptamer to trehalose (herein referred to as

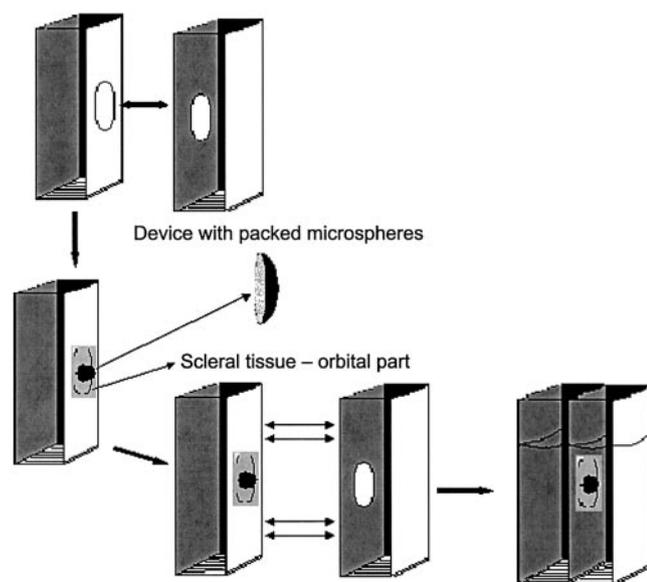


FIGURE 1. Schematic of the in vitro transscleral delivery apparatus.

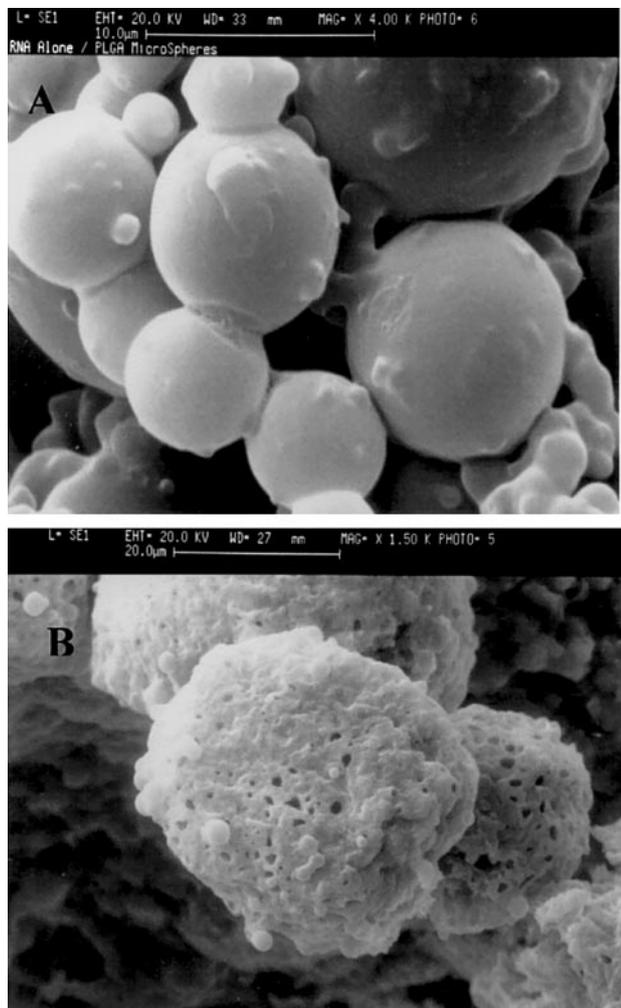


FIGURE 2. (A) PLGA microspheres loaded with anti-VEGF aptamer EYE001 before incubation with release medium and (B) after 10 days of exposure to aqueous release medium. The average diameter of the microspheres was $14 \pm 6 \mu\text{m}$. Determination of average diameter was a result of diameter estimations of approximately 30 individual microspheres.

EYE001-Tre) exhibited a controlled release of the drug in a period of more than 20 days. Release kinetics were characterized by a very low-burst release during the first 24-hour period, followed by a continuous release with no evidence of a lag phase. Both formulations were completely released, indicating no adsorption of the aptamer to the polymer core. The average amount of drug released was $2 \mu\text{g/d}$, regardless of the amount of drug originally encapsulated. The encapsulation efficiency for both formulations in PLGA was 70% to 85%, with a theoretical loading of 3.95% and actual loading of $2.76\% \pm 0.80\%$, indicating that the presence of trehalose had no effect in the encapsulation efficiency of the polymeric system.

Secondary Structural Determination of EYE001 Formulations upon Lyophilization

To assess any structural changes due to the nature of the formulation of EYE001 upon lyophilization, EYE001 formulations lyophilized as described herein were reconstituted in PBS and its CD spectra determined and compared with an aqueous EYE001 standard. Given that EYE001 has an A-type RNA structure (duplex formation, right-handed helix),⁷ the CD spectra

exhibit a maximum of approximately 260 nm and a minimum of approximately 210 nm.^{38,39} A decrease in molar ellipticity in either maxima or minima is a reflection of a secondary structural change.^{38,39} The CD spectrum of EYE001 in the absence of any excipient on lyophilization and further reconstitution exhibited a slight decrease in intensity at both wavelengths. It was observed that when increasing the mass ratio of the disaccharide stabilizer trehalose^{22-24,34,35} to EYE001 before lyophilization, there was an improvement in the retention of structure, as evidenced by molar ellipticities at both wavelengths comparable with those of the aqueous EYE001 standard (Fig. 4).

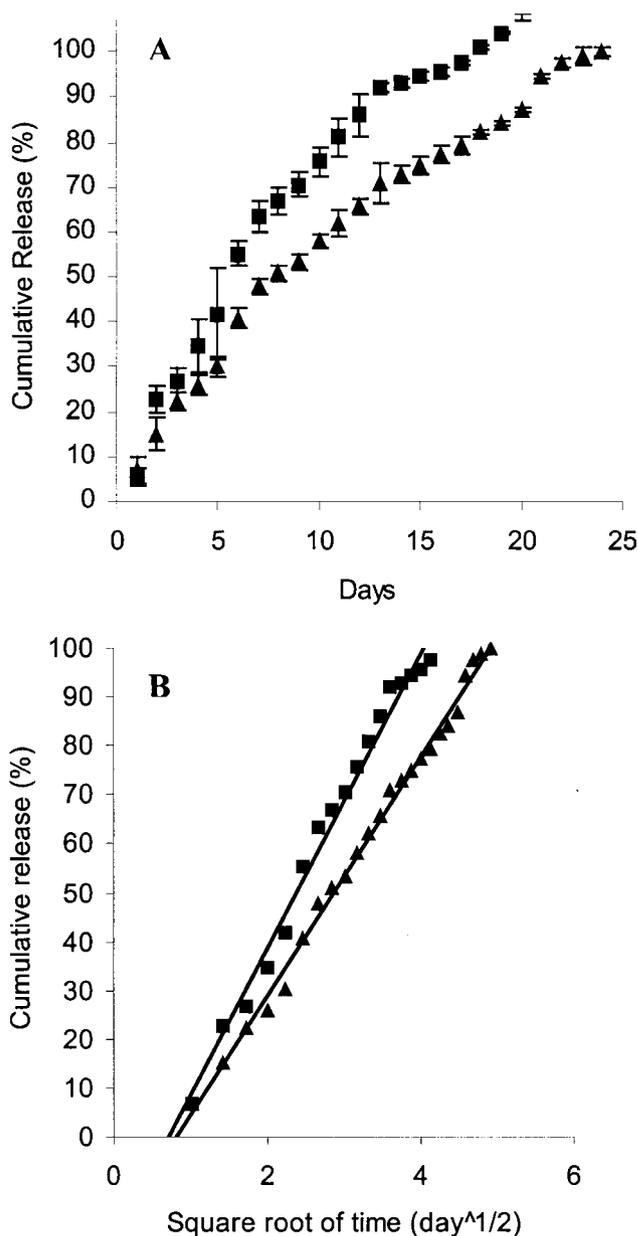


FIGURE 3. (A) Cumulative release profile of (■) excipient-free EYE001 and (▲) EYE001-Tre from PLGA microspheres. (B) Correlation between the amount of EYE001 released and the square root of time for (■) excipient-free EYE001 and (▲) EYE001-Tre. The correlation coefficients of the released formulations were 0.9852 and 0.9946, respectively.

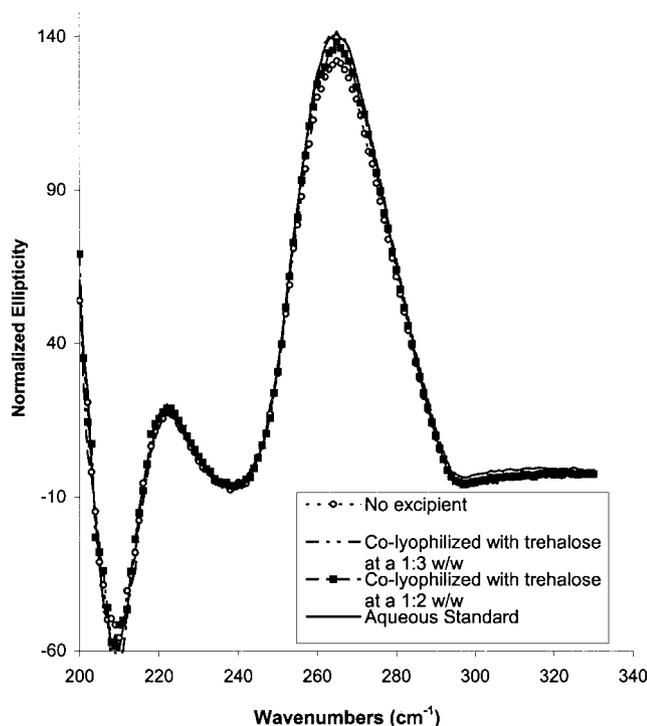


FIGURE 4. CD spectra of reconstituted EYE001 formulations under different lyophilization conditions. The spectra were recorded after reconstitution of samples in PBS.

Anti-VEGF Aptamer Activity after Release from PLGA

EYE001 activity after encapsulation and further release from PLGA microspheres was assayed by monitoring its ability to inhibit VEGF-induced proliferation of HUVECs. To determine the feasibility of polymer microspheres as a viable delivery device, proliferation assays were conducted at various stages during the release period. The representative time points chosen were at early, intermediate, and late stages of release. We were interested not only in the stability of the aptamer over time but also in the stability of the formulation state in which the aptamer was encapsulated in PLGA.

The VEGF-induced proliferation of HUVECs after a period of 4 days showed a threefold average increase in cell counts compared with those found in blanks. On HUVEC incubation with VEGF in the presence of the different aptamer formulations after release, it was evident that regardless of the formulation state, the aptamer was capable of at least partially inhibiting VEGF-induced cell proliferation (Fig. 5). However, it is worth noting that the inhibition showed by the aptamer was, in general, enhanced when EYE001 was co-lyophilized in the presence of trehalose and then encapsulated in PLGA. EYE001 preserved its bioactivity after encapsulation in PLGA and during its release over a period of 20 days (Fig. 5). Incubation of HUVECs with PLGA-loaded microspheres containing EYE001 formulations and degraded PLGA supernatant had no effect on HUVEC proliferation (data not shown).

Transscleral Delivery of EYE001 Released from PLGA Microspheres

After active EYE001 was delivered from PLGA microspheres in a controlled manner for an extended period in vitro, we sought to determine whether the hydration of the sclera would be sufficient to degrade the microspheres and result in aptamer

release and diffusion through the sclera. To do this, PLGA-loaded microspheres were loaded into a device and placed it on the sclera of Dutch belted rabbits as described in Figure 1 (see the Materials and Methods section). The degree of polymer degradation was monitored qualitatively by analyzing the morphology of the microspheres. SEM pictures show the morphologic state of the microparticles after exposure to scleral hydration after a period of 18 hours and after 6 days (Fig. 6). During the first 18 hours, the polymer microspheres seemed to adhere to the tissue, but no significant degradation was observed (Fig. 6B), as expected, because of the short incubation time. However, after 6 days, PLGA microspheres showed significant degradation and formation of pores along its surface (Fig. 6C). The visible signs of degradation indicated that scleral hydration was sufficient to degrade the PLGA-loaded microspheres, indicating feasibility of the delivery method for EYE001 through the sclera.

To determine whether diffusion of EYE001 through the sclera was indeed possible after delivery from PLGA microspheres, aptamer concentration was monitored in the uveal chamber (sampling the chamber with the uveal side of the sclera exposed), and, as a control, the aptamer concentration in the orbital chamber was monitored as well (sampling chamber with the orbital side of sclera exposed and containing the device loaded with microspheres). Having determined the characteristics of the in vitro release profiles of EYE001 from the microspheres, aptamer diffusion through the sclera was monitored for 6 days. Table 1 presents the data showing the amount of aptamer diffused through the sclera. As can be observed, the amount of aptamer delivered from PLGA microspheres and diffused through the sclera is comparable with that released in vitro from isolated microspheres. An average of 2 $\mu\text{g}/\text{d}$ was sampled in the uveal chamber, indicating that EYE001 diffused readily through the sclera, as reported previously for molecules of similar molecular weight.¹⁴ An average of 0.5 $\mu\text{g}/\text{d}$ was sampled in the control chamber. SEM analysis of lyophilized powder obtained after freeze drying of the volume sampled in the uveal chamber revealed that there were no microspheres present, indicating that the drug permeated in its free, nonencapsulated form.

Given that we monitored diffusion for 6 days in an in vitro setup, an important consideration was the integrity and viability of the sclera during the transport study. To this end, we examined cultured scleral tissue immersed in PBS and incubated at 37°C for 6 days by transmission electron microscopy (TEM). As a control, we analyzed a fresh scleral tissue, fixed the same day it was detached. As can be observed in Figure 7, there were signs of swelling of the collagen fibrils in the cultured sclera when compared with fresh rabbit sclera, as evidenced by the thickness of the collagen fibers, but the general ultrastructure of the tissue was preserved, as determined by TEM. This is consistent with the observations in other investigations in which similar in vitro experiments were performed to determine diffusion of solutes through the sclera, with the results indicating that normal scleral physiology can be maintained over the course of short- and long-term perfusion periods.⁴⁰

DISCUSSION

The goal of the present study was to develop a drug delivery modality that could release the anti-VEGF aptamer EYE001 in a sustained and controlled manner over a significant period and could be applied locally to the outer part of the sclera. The retina and choroid are the target tissues, because this aptamer is intended to block the contribution of VEGF to choroidal neovascularization and diabetic macular edema, respectively. Transscleral administration, no more frequently than every 6

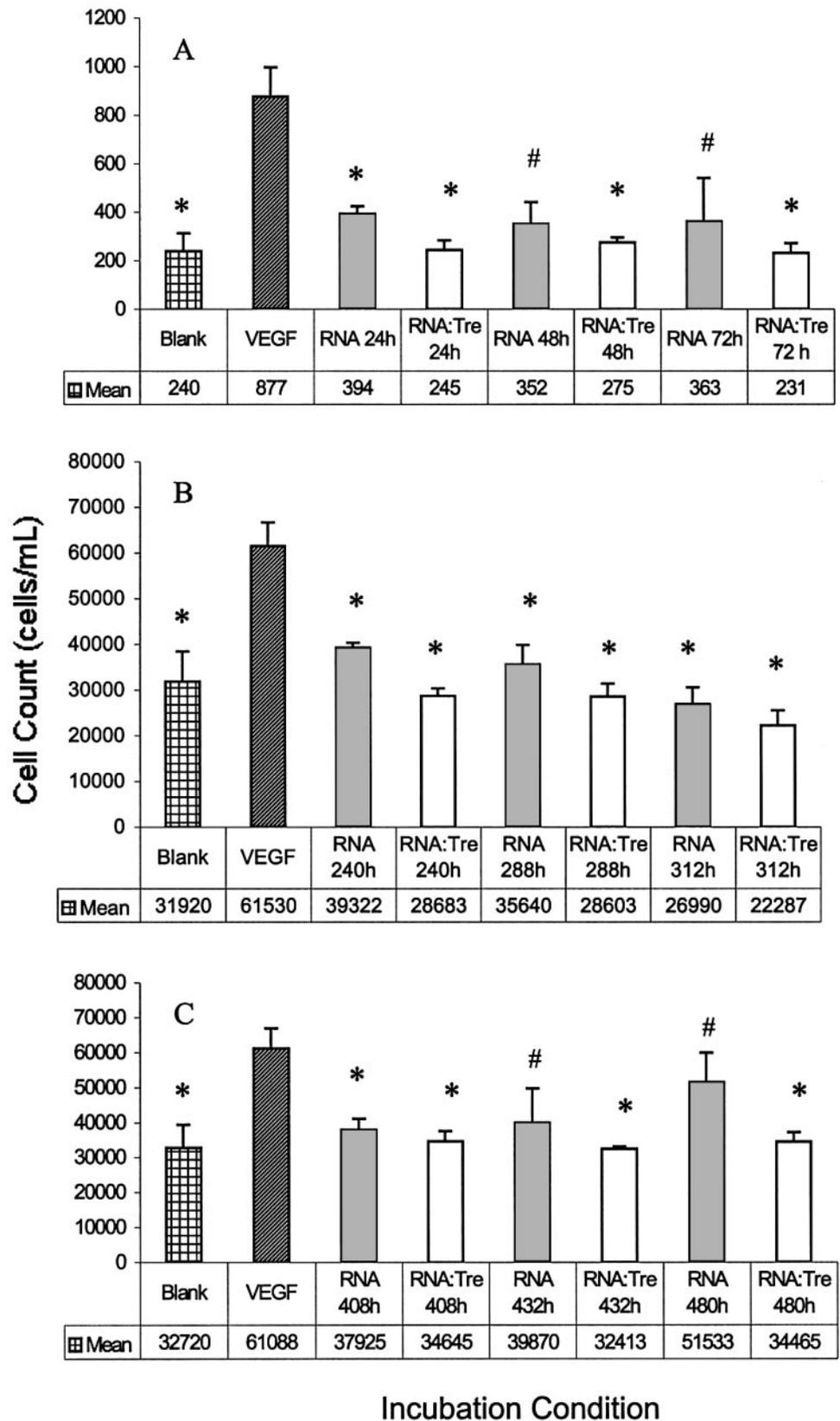


FIGURE 5. Cell proliferation assays of HUVECs incubated with EYE001 formulations after release from PLGA microspheres. Each graph indicates the incubation condition and the time-point at which EYE001 was collected after release from PLGA. The mean values represent the average cell count results for each condition in three independent experiments. (A) Short-, (B) mid-, and (C) long-term release time points. **P* < 0.05; #*P* > 0.05 versus VEGF-induced cell proliferation. *n* = 3 for all time points.

weeks, would prove an attractive substitute to intravitreal injections of the aptamer, currently occurring at a similar frequency in two separate clinical trials.

For this purpose, the biodegradable, biocompatible, and FDA-approved polymeric material PLGA was selected.²³ The

release profiles of EYE001 from these microspheres were characterized by a low initial burst, followed by continuous release in the absence of a lag phase. Typical release profiles from PLGA microspheres are triphasic, characterized by an initial burst as drug entrapped near the surface releases, followed by

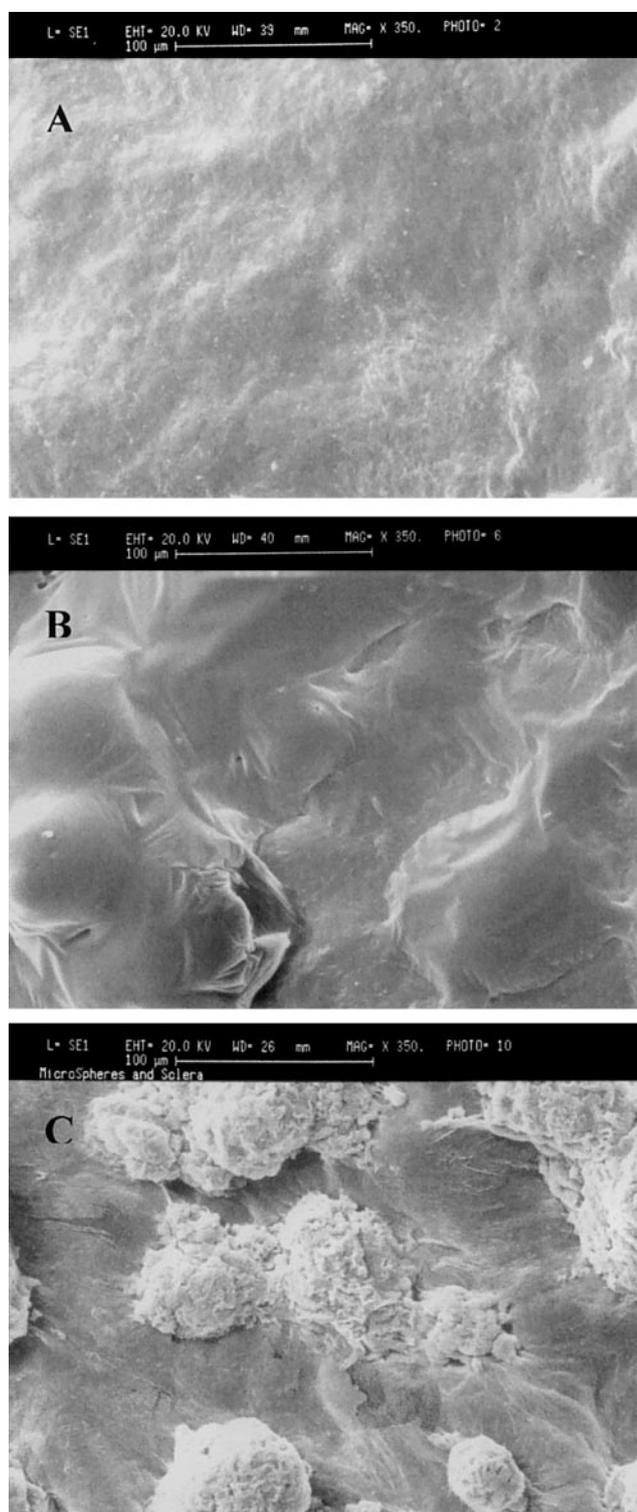


FIGURE 6. SEM images of (A) bare rabbit sclera and (B) rabbit sclera with PLGA microspheres after 18 hours and (C) 6 days of exposure to the orbital surface.

a lag phase controlled by polymer degradation and final release of the drug as it diffuses from the polymer core as erosion takes place.²⁶ In the scenario observed, it is probable that EYE001 formulations encapsulated in PLGA were homogeneously distributed throughout the polymeric matrix. As described by Higuchi^{41,42} and reported elsewhere,^{43,44} when a drug releases from a homogeneous matrix-type delivery system, the process

is diffusion-controlled and is evidenced by a proportionality between the amount of drug being released and the square root of time. The process is described by the following equation:

$$Q = \sqrt{2WDC_s t}$$

where Q is the rate of released drug, D is the diffusion coefficient of the drug in the matrix, W is the total amount of the drug per unit volume of matrix, C_s is the solubility of the drug in the matrix, and t is the drug release time.

The release of both excipient-free aptamer and EYE001-Tre from PLGA as a function of the square root of time ($t^{1/2}$) show a linear relationship with correlation coefficients of 0.98 and 0.99, respectively (Fig. 3B). These data support the hypothesis that both aptamer formulations were released through a diffusion-controlled process.

An important consideration in our development of a long-term delivery device for a nucleic acid such as EYE001 was its stability before, during, and after the encapsulation process in PLGA. Nucleic acids are known to suffer depurination and become susceptible to free radical oxidation in aqueous solutions.^{45,46} Such a phenomenon was recently reported by a group evaluating the potential development of pharmaceutical formulations of plasmid DNA with long-term storage stability.⁴⁷ To this end, we colyophilized EYE001 with the known potent stabilizer trehalose,^{34,48} and used a completely nonaqueous oil-in-oil methodology^{48,49} for the creation of polymer microspheres that has been effective in the delivery of biologically active proteins with native secondary structures.^{24,48-51}

The cell proliferation assays conducted to monitor aptamer bioactivity after release from PLGA microspheres reveal that the conditions chosen to create the polymer microspheres were satisfactory. As shown in Figure 5, EYE001 preserved its ability to inhibit VEGF-induced cell proliferation during all the representative time points along its release from PLGA. Although bioactivity was retained regardless of its formulation state, an improved level of bioactivity was observed in general when EYE001 was colyophilized with trehalose before encapsulation.

Incubation of PLGA microspheres directly with HUVECs revealed the same trend as that of the aptamer collected after it was released from isolated microspheres in vitro. No evident signs of toxicity or cell death were observed when blank PLGA microspheres were incubated with HUVECs from microscopic observations and cell counts (data not shown). These results are in agreement with reports by other groups that conducted cell proliferation assays with polylactides of various molecular weights with rat epithelial cells, human fibroblasts, and osteosarcoma cells under culture conditions.⁵² Overall, it was determined that satisfactory biocompatibility was exhibited.^{52,53}

TABLE 1. Amount of Aptamer Diffused through the Sclera after Release from PLGA Microspheres

| Day | EYE001 _{sc} * (μg) | EYE001 _{cc} † (μg) |
|-----|-----------------------------|-----------------------------|
| 1 | 3.4 ± 0.8 | 0.7 ± 0.3 |
| 2 | 2.3 ± 0.5 | 0.5 ± 0.3 |
| 3 | 2.1 ± 0.6 | 0.5 ± 0.2 |
| 4 | 1.8 ± 0.3 | 0.6 ± 0.4 |
| 5 | 2.4 ± 0.1 | 0.5 ± 0.2 |
| 6 | 2.6 ± 0.2 | 0.4 ± 0.3 |

Data are presented as the average results of three experiments ± SD.

* Sampling Chamber.

† Control Chamber.

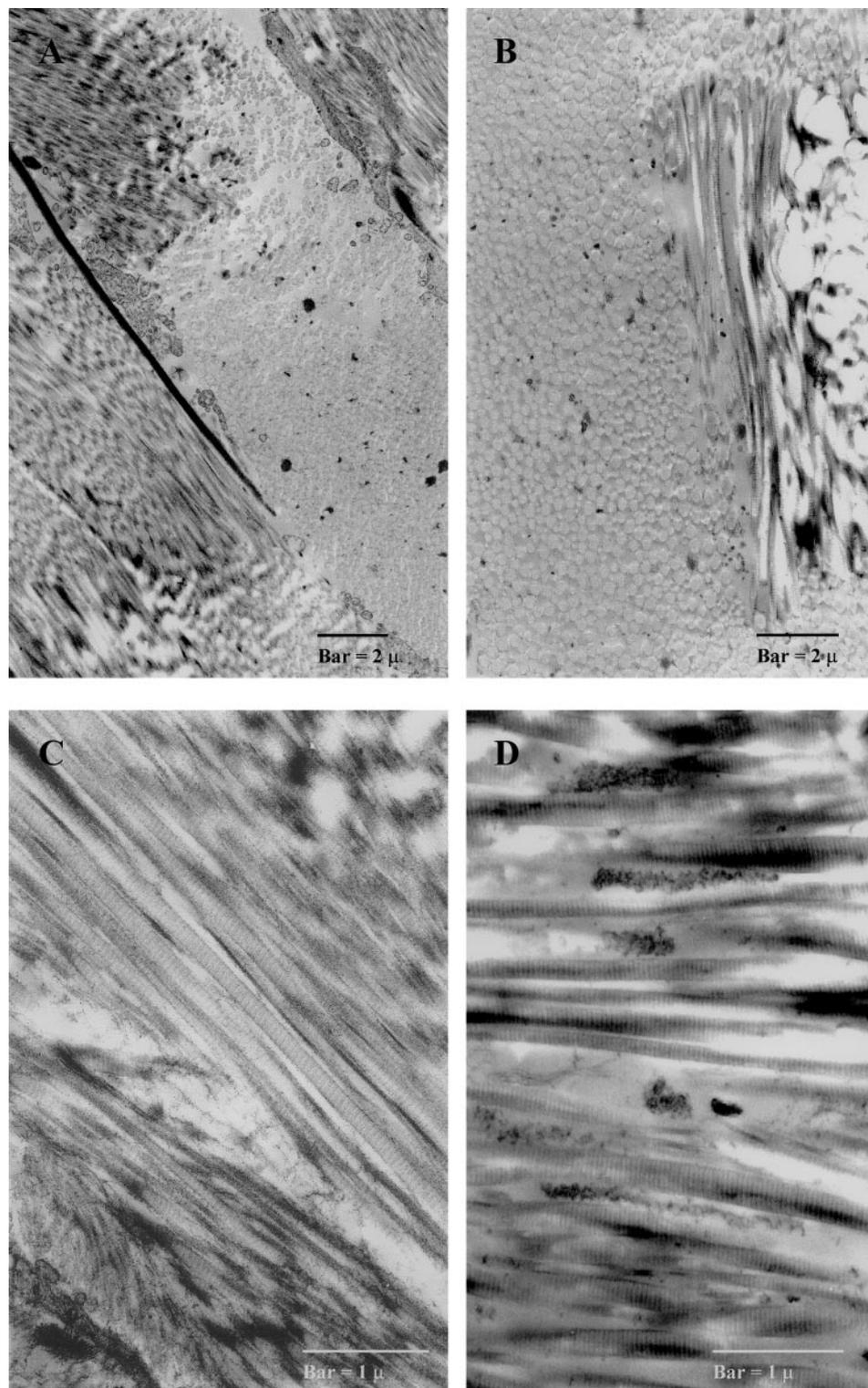


FIGURE 7. Cross-sections (A, B) and longitudinal sections (C, D) of rabbit sclera analyzed by TEM. Fresh rabbit sclera (A, C) was used as control to compare with sclera incubated in PBS at 37°C for 6 days (B, D).

These data add support for the conclusion that the method described in this report holds promise for the long-term inhibition of VEGF-mediated responses *in vivo*.

Rabbit sclera is 71% water¹⁷ and, as documented by electron microscopy (Fig. 6C), it served to hydrate and degrade the solid PLGA microspheres placed on the orbital side of the sclera, which were not in contact with any hydration medium other than the hydrated scleral surface itself. An important aspect of PLGA controlled-delivery devices is that they provide continuous release and avoid the repeated use of injections or

high concentrations of drug to achieve the desired pharmacological response. Even though controversy exists over how the flux over the sclera occurs and whether it achieves steady state,⁵⁴ our controlled-drug delivery device would increase drug-sclera contact, thus improving scleral absorption. The hypocellularity⁵⁵ and large surface area¹⁵ of the human sclera, as well as its remarkable tolerance of foreign bodies overlying its surface (e.g., scleral buckles⁵⁶) helps to facilitate diffusion through it and allow a long-term transscleral delivery device to be clinically feasible.

In this report, we present data showing the feasibility of delivering the anti-VEGF aptamer EYE001 in a sustained and controlled manner and in a biologically active form. The development of such an approach to drug delivery accompanies the advent of many potential antiangiogenic drugs for the treatment of various vision-threatening diseases that affect the posterior segment of the eye. Validation of this study would require testing the system in an in vivo model that would also address other important questions: how choroidal blood flow affects transscleral delivery and whether the concentrations of active drug delivered through the proposed system are sufficient to inhibit some or all the responses triggered by neovascularization in the posterior segment, among others. These studies are currently in progress in our laboratory.

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