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Sustained Ocular Delivery of Bevacizumab Using Densomeres in Rabbits: Effects on Molecular Integrity and Bioactivity

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Purpose: To demonstrate that a single administration of an anti-angiogenic monoclonal antibody, when integrated into a novel biodegradable Densomere composed only of the active pharmaceutical ingredient and polymer, maintains molecular integrity, sustained release, and prolonged bioactivity in vitro and in vivo for up to 12 months.

Methods: Bevacizumab, a high-molecular-weight antibody (140,000–150,000 Da) was incorporated at 5% loading into Densomere microparticle carriers (DMCs) for injection to observe in vitro release over time from an aqueous suspension. The molecular integrity of the released bevacizumab was assessed by enzyme-linked immunosorbent assay (ELISA) and size-exclusion chromatography–high-performance liquid chromatography (SEC-HPLC). Anti-angiogenic bioactivity in vivo was assessed using the rabbit corneal suture model for suppression of neovascular encroachment from the limbus following a single subconjunctival administration.

Results: Continuous release of bevacizumab in vitro was observed in serial samples over a period of 12 months. ELISA and SEC-HPLC yielded profiles from aqueous supernatant samples indistinguishable from the reference bevacizumab. A single subconjunctival administration in rabbit eyes significantly suppressed corneal neovascularization in vivo compared to control eyes for 12 months.

Conclusions: The Densomere carrier platform maintained the molecular integrity of bevacizumab with a prolonged release profile in vitro and demonstrated sustained in vivo drug delivery with continuous bioactivity in the rabbit cornea eye model for 12 months.

Translational Relevance: The Densomere platform provides a significant opportunity for prolonged delivery of biologics in ocular and other tissues.

Introduction

Biotherapeutic antibodies and other biologics with high biospecificity have become widespread effective therapies for reducing the impact of ocular conditions affecting vision such as wet age-related macular degeneration (AMD) and diabetic retinopathy. Maintaining effective, therapeutic levels of these potent drugs in ocular tissues has meant repeated intravitreal injections, often monthly.[1] Although efforts continue to lengthen the intervals between ocular treatments with new products and regimens, extending retreatment intervals beyond 3 months has not yet been reliably achieved.[2,3] We developed a technology platform for extended release of an active pharmaceutical ingredient (API) utilizing poly(D,L-lactide-co-glycolide) (PLGA), a biodegradable polymer carrier utilized in several approved drug and device products, including an intravitreal implant.[4–6] When PLGA is processed using our technology with an API—without adding other excipients or coatings—it can be formulated into microparticle Densomeres (Sustained Nano Systems, Stony Brook, NY). By varying the production process, natural hydrolysis of the PLGA becomes a controllable parameter resulting in API release profiles lasting

from weeks to many months. Densomere microparticle carriers (DMCs) can be formulated as suspensions of micro- or nanoparticles in a range of desired sizes for injection or inhalation, or they may be shaped into implantable solid forms (e.g., rods, disks, blocks) for local delivery in various tissues. This set of experiments tested Densomeres designed to release the bioactive monoclonal antibody (mAb) bevacizumab over an extended period of at least 12 months.

Methods

A commercial bevacizumab solution (Avastin; Genentech, South San Francisco, CA) was frozen and lyophilized as the API. Under aseptic conditions, the dried material was combined with a PLGA solution (Resomer, RG 755 S; Evonik, Parsippany, NJ) in proportions to make 5% bevacizumab in the PLGA and dissolved in methylene chloride. The mixture was homogenized to make a solid–oil–water dispersion in multiple steps. Bevacizumab–PLGA microparticles were recovered by centrifugation, washed, and freeze dried. Following a controlled proprietary technique (Sustained Nano Systems), the bevacizumab–PLGA microparticles were processed into active DMCs. Aliquots of dry, active DMCs were placed in 2-mL, snap-top Eppendorf centrifuge tubes for storage. In a similar manner, control DMCs were prepared without an API.

For in vitro tests, approximately 100 mg of dried DMCs were suspended in 2 mL of phosphate-buffered saline (PBS). At predetermined time points, the tubes were centrifuged and the supernatant removed to assay for protein content representing the released amount of the bevacizumab API. The removed diluent was then replaced with an equal volume of PBS, washed, and refilled again with PBS, and the tube was stored for the next time period. This sampling was repeated at intervals for up to 12 months from the initial bevacizumab–DMC preparation. Protein content (as bevacizumab) was determined using a bicinchronic acid assay (QuantiPro BCA assay; MilliporeSigma, St. Louis, MO) and read at 562 nm with a spectrophotometer. The entire in vitro study was performed in triplicate.

Random samples of the supernatant were selected from the same PBS suspensions at nine time periods to assess in vitro bevacizumab content as determined by enzyme-linked immunosorbent assay (ELISA) (Bevacizumab ELISA Assay Kit; Eagle Biosciences, Nashua, NH). Samples were read at 450 nm with a spectrophotometer and compared to a previously prepared standard plot that used a fresh, authentic bevacizumab solution at known concentrations.

Purity and molecular integrity were assessed on additional samples using size-exclusion chromatography–high-performance liquid chromatography (SEC-HPLC). Supernatant samples were extracted in PBS as described above and placed on an Agilent 1100 HPLC device (Agilent Scientific Instruments, Santa Clara, CA) using a TSKgel G3000SWXL (7.8 × 300 mm, 5 µm) silica column (Tosoh, Tokyo, Japan). Readings were made at 280 nm and compared to the authentic reference bevacizumab sample.

An in vivo assessment of anti-angiogenic bioactivity over time was conducted using the rabbit corneal model for suppression of neovascular encroachment from the limbus in response to corneal suture injury.[7,8] All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Stony Brook University and met the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Twelve adult male New Zealand white rabbits (Charles River Laboratories, Wilmington, MA) were randomly assigned to one of two groups: six active and six control. One pair, consisting of one active and one control, was followed for 3 months and then euthanized. Their eyes were enucleated, fixed in 10% neutral buffered formalin, processed, paraffin embedded, and sectioned, and the specimens were stained with hematoxylin and eosin for histological evaluation. Four pairs were studied for 5.5 months to observe and photograph the neovascularization response, and one pair was followed for 12 months. Under general anesthesia, a single, radial 9-0 silk suture (Ethicon, Somerville, NJ) was placed in clear cornea (mid-stromal) 2 to 3 mm from the limbus to stimulate corneal neovascularization in the study eye. The fellow eye was not used. Reconstituted in approximately 0.5 mL of sterile normal saline, the control or bevacizumab–active DMC suspension was administered as a single injection of 30 mg subconjunctivally using a 27-gauge needle (targeting the sub-Tenon space) in the same meridian as the suture near the limbus. To ensure continuous stimulation of the angiogenic response, the corneal silk sutures were replaced when necessary without additional DMC administration. Photography of the conjunctiva, cornea, and sclera was performed at set intervals with a standardized digital camera (Nikon D3300 with a 105-mm Micro-NIKKOR f/2.8G lens; Nikon, Tokyo, Japan). Images were subsequently matched for color balance, exposure, apparent magnification, and size (DxO PhotoLab 4.1.2, Boulogne-Billancourt, France) with the goal to focus on the

limbal area at the same meridian as the suture and injection. Sets of images were presented at the end of the study in a randomized, masked manner (in terms of both sequence and treatment group) to a group of eight clinical ophthalmologists as graders, to assess corneal neovascularization and assign a standardized, semiquantitative grade based on the intensity of neovascularization for each image. The in vivo data analysis was performed using hierarchical linear models to account for the clustered nature of the data (i.e., each animal had more than one score over time); each animal could be scored differently at different time points. The fixed effects were treatment group and time (in days). Random effects were animals, graders, and time. To test for differences between treatment groups, this variable was entered in the model to predict the scores given by all graders over time. To test for the trajectories (slopes) of scores over time, we tested the interaction term treatment group $\times$ time. A positive slope reaching statistical significance means that the scores increased significantly between the two time points, for example. A non-significant slope (negative or positive) means that the scores did not change significantly. Statistical significance was defined as $P < 0.05$ (type 1 error). Computerized statistical analyses were performed with Stata 14.2 (StataCorp, College Station, TX).

Results

In Vitro Protein Release

The net amount of bevacizumab released into the PBS supernatant per milligram of DMCs over time in vitro appeared at significantly higher rates during the first 5 weeks (day 1 to day 35) compared to the following weeks extending out to 12 months (Fig. 1). Rates ranged from 0.56 µg bevacizumab/mg Densomeres on day 4 to 0.01 µg/mg on day 151. Release rates remained consistently above the lower limit of detection for the bicinchronic acid protein assay method during the entire 12-month period. Figure 1 shows this effect as the net amount of bevacizumab (in micrograms) released per milligram of the DMCs for each measured time point. Figure 2 shows the cumulative bevacizumab content released (expressed as micrograms of bevacizumab released per milligram of microparticles). All points are the average of $n = 3$ independent tests $\pm$ SD.

In Vitro ELISA Bioactivity Results

Bioactive bevacizumab as detected by a quantitative ELISA assay was measured on nine selected

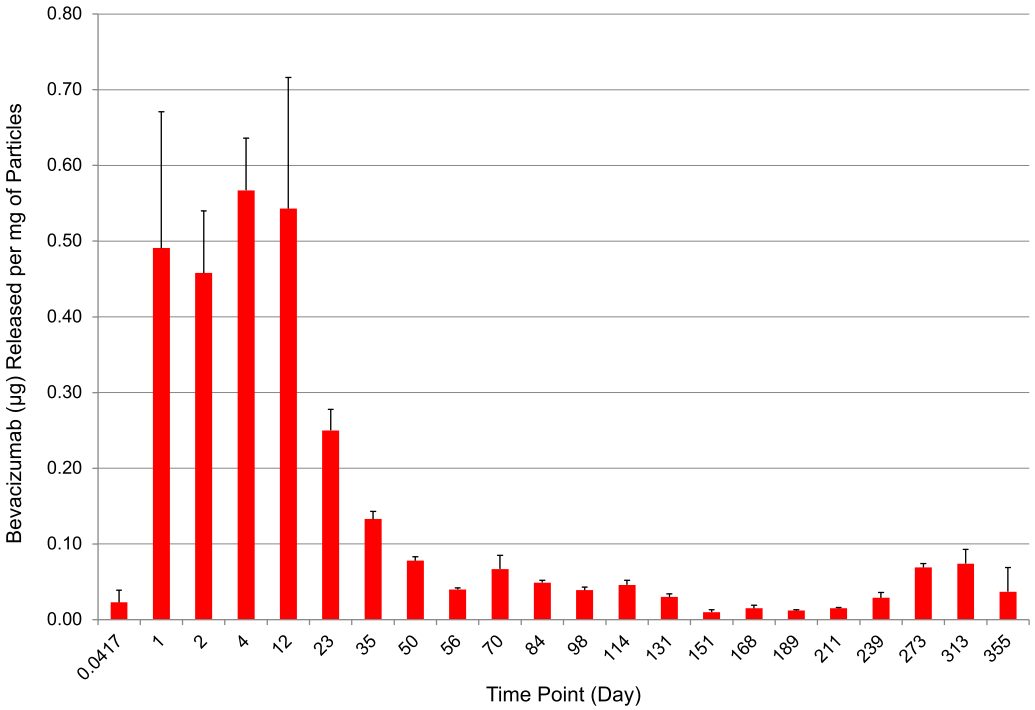

Figure 1. Micrograms of bevacizumab released into PBS supernatant in vitro at each measured day per milligram of biodegradable bevacizumab–DMCs from day 0 to day 355. *Red bars* represent the average of $n = 3$ observations $\pm$ SD bars.

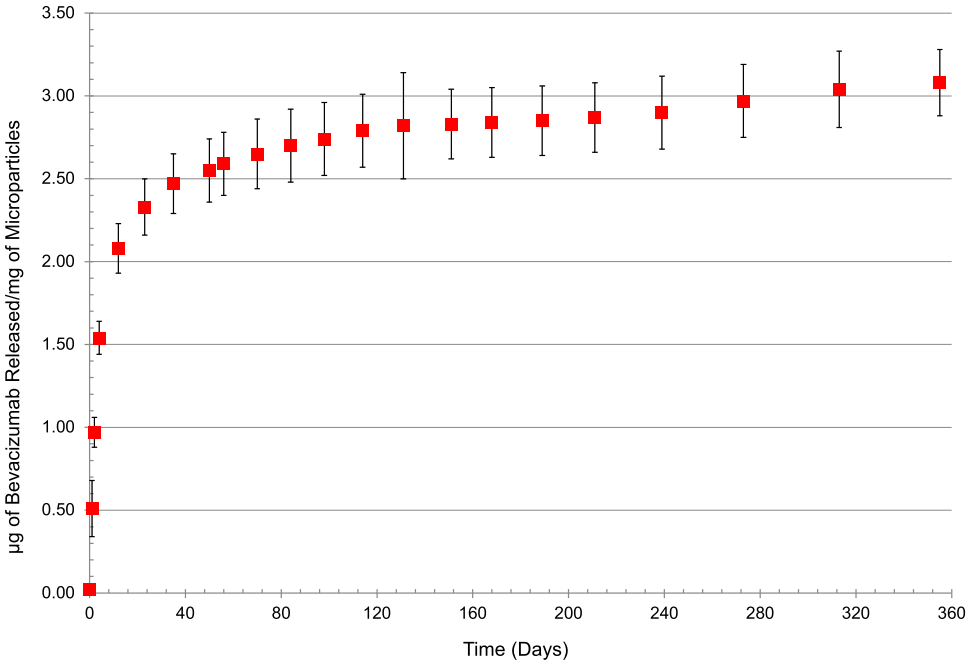

Figure 2. Cumulative amount of bevacizumab (micrograms) released into PBS supernatant in vitro at each measured time point per milligram of biodegradable bevacizumab–DMCs from day 0 to day 355. *Red squares* are the average of $n = 3$ assessments ± SD.

Table. Active Bevacizumab Released Into PBS Supernatant From Biodegradable Bevacizumab–Active DMCs Over Time by ELISA Assay

	Day of Sample								
	1	4	23	35	84	168	239	273	355
Bevacizumab concentration in sample (ng/mL)	6115	9231	5539	923	454	148	358	450	504
Total bevacizumab recovered (ng)	7950	12,000	7200	1200	590	192	465	585	655

days of aqueous exposure for the DMCs in PBS. The Table shows the range of recovered bioactive bevacizumab released from a peak of 12,000 ng on day 4 to an ebb observed with 192 ng on day 168. Maximum observed concentrations of bevacizumab in samples also occurred at day 4 (9231 ng/mL) and were lowest at day 168 (148 ng/mL).

In Vitro SEC-HPLC Results

Comparisons between samples from the authentic bevacizumab and the supernatant of DMCs showed matching peak retention times using SEC-HPLC to assess estimated molecular weight. Figure 3A shows the reference bevacizumab, and Figure 3B shows the bevacizumab recovered from the PBS supernatant of active DMCs. Both main peaks of the two samples contained over 91% of the areas under the curve, with peak retention times at 15.968 and 15.995 minutes, respectively.

In Vivo Assessment Results From Rabbit Cornea Model

Control rabbit eyes always scored significantly higher for corneal neovascularization by masked observers at all follow-up periods evaluated compared to eyes that received the active DMC injection. Over time, the average score difference on a scale of 0 to 5 was 1.86 ± 0.64 (mean ± SE) ($P < 0.004$). Resuturing was necessary to maintain the neovascularization stimulus

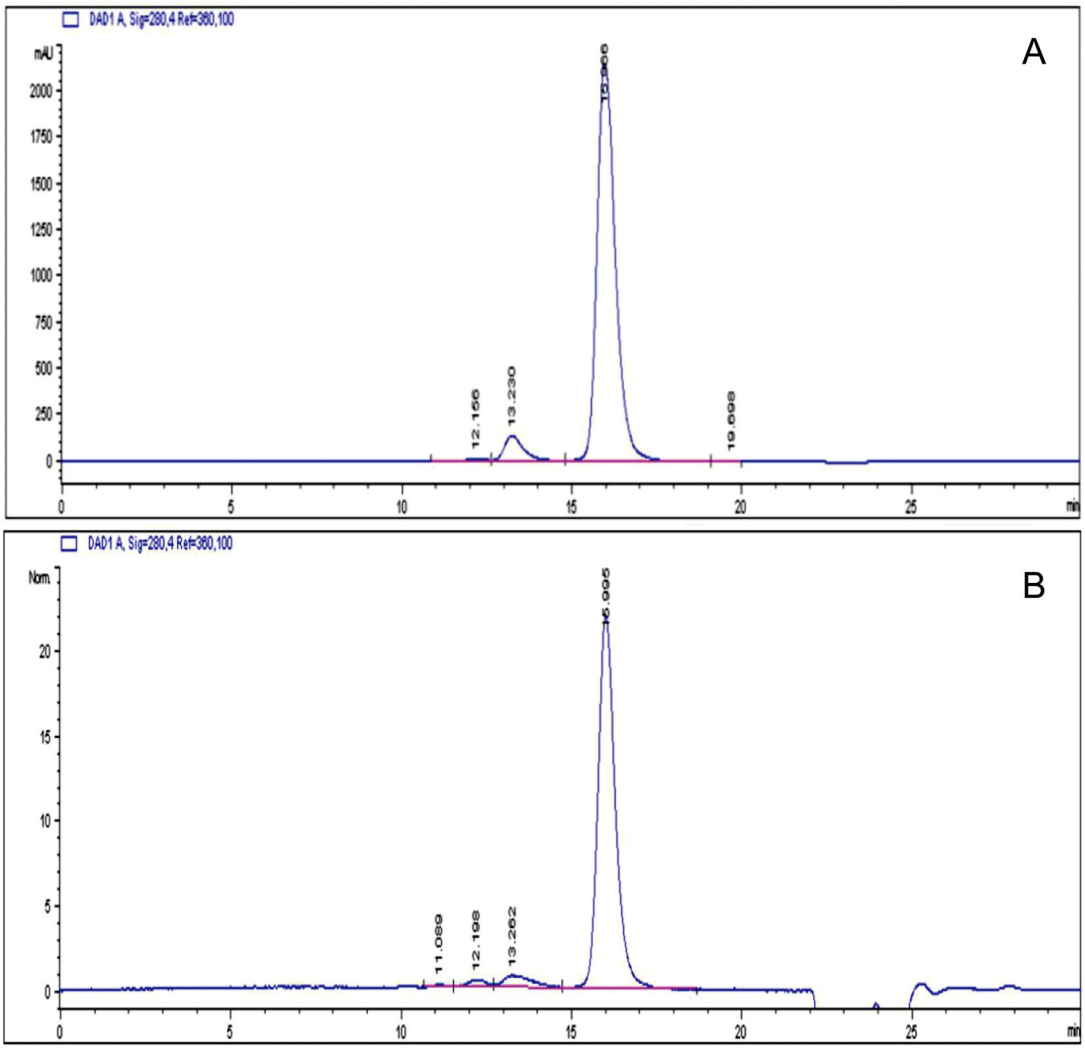

Figure 3. (A) SEC-HPLC for an authentic bevacizumab sample. (B) SEC-HPLC for released bevacizumab from the supernatant of bevacizumab–DMCs in PBS.

in all animals. The appearance as shown in Figure 4 remained similar over time since the first evaluations at day 14. For the remaining pair of rabbits followed for 12 months, the control eye scored a 5 (severe) on the scale of 0 to 5 (Fig. 4A), whereas the eye that received the single subconjunctival injection of active DMCs at day 0 was scored as 2 (mild) for the same period (Fig. 4B).

Histological Evaluations

A corneal section of the rabbit control eye at 3 months after subconjunctival injection of DMCs without an API is depicted in Figure 5A. Thickening of the epithelial layer stimulated by the bordering silk suture is evident; the suture remnant is surrounded by a fibrotic capsule. The silk suture apparently induced considerable inflammatory response in the surrounding corneal tissues, as indicated by the interspersed macrophages and the extensive development of vasculature (as revealed by the tubular structures lined with endothelial cells). Figure 5B depicts a corneal section of the rabbit from the active group at 3 months after subconjunctival injection of the active DMCs. Similar to Figure 5A, there is clear thickening of the epithelial layer stimulated by the presence of the silk suture. Underneath the thickened corneal epithelium is a zone occupied by fibrotic tissue (containing some interspersed macrophages) as part of the inflammatory response induced by the prolonged presence of

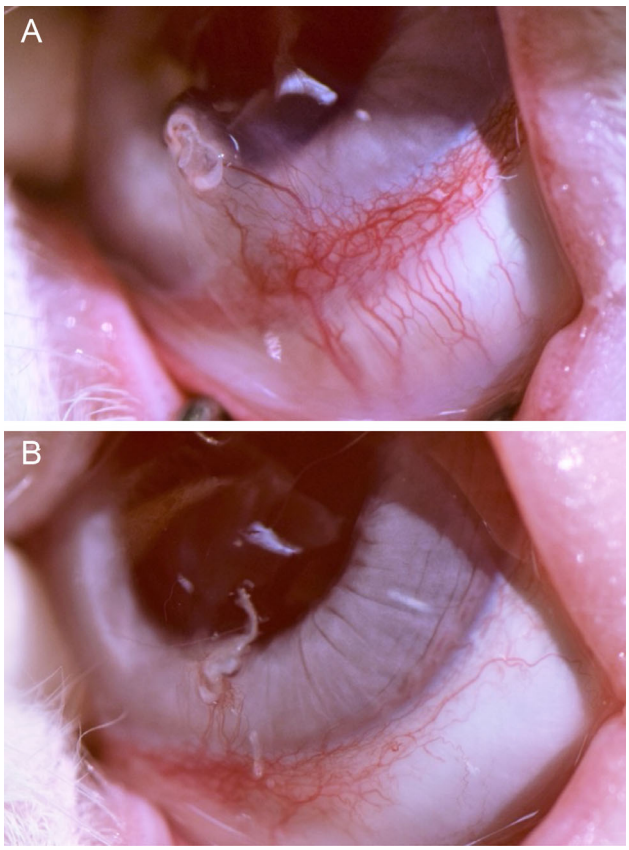

Figure 4. (A) Rabbit corneal neovascular response to continuous suture placement for 12 months after a single subconjunctival injection of biodegradable control DMCs. (B) Rabbit corneal neovascular response 12 months after a single subconjunctival dose of 5% extended-release biodegradable bevacizumab–active DMCs. Sutures and resulting scar tissue can be seen. Using a vascularization index ranging from 0 to 5, with 5 being severe neovascularization and 0 being absent, clinical ophthalmologists gave the control eye (A) an average score of 5 (severe neovascularization) and gave an average score of 2 (mild neovascularization) for the eye treated only once (12 months earlier) with the bevacizumab–loaded DMCs (B). Compared to the control eyes, intermediate assessments showed similar results with statistically significant suppression of neovascularization scores lasting throughout the entire 12-month period.

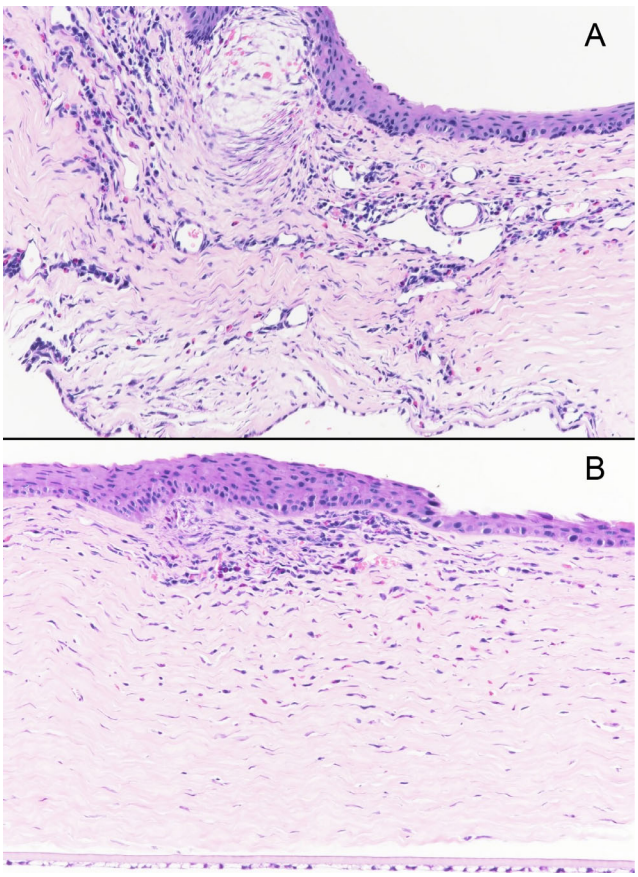

Figure 5. A (Top): (A) Corneal cross-section at the location of the silk suture in the control rabbit eye on day 91 after subconjunctival injection of biodegradable control DMCs without an API. Significant scarring, inflammation, and vascular infiltration are present. (B) Cross-section of rabbit cornea at the location of a silk suture on day 91 after subconjunctival injection of the biodegradable bevacizumab–active DMCs. Although an inflammatory response is clearly present, it appears significantly less intense than the control response seen in (A).

silk suture; the suture remnant is surrounded by an organized fibrous capsule. A much smaller amount of vasculature in the fibrotic tissue is visible as revealed by the tubular structure lined by endothelial cells in concert with the presence of some blood cells. Overall, the distinct contrast between Figures 5A and 5B suggests that the constant presence of bioactive bevacizumab inhibited uncontrolled angiogenesis, thereby indicating that the bevacizumab–DMC formulation for the sustained release of active bevacizumab is effective in reducing angiogenesis in this model.

Discussion

This study evaluated a Densomere drug delivery technology, a novel proprietary platform that integrates an API with biodegradable PLGA nano- and microcarriers. We demonstrated that DMCs can extend single-dose drug delivery for up to 12 months. Prolonged dosing options are especially important for chronic conditions that require frequent or near-constant maintenance therapy and where patient outcomes may suffer from poor compliance (e.g., self-administration of eye drops) or the burdens of costs and potentially serious risks associated with frequent in-office physician administration (e.g., intravitreal injections).[9]

The in vivo study was performed using subconjunctival DMC injections to observe if bevacizumab,

being slowly released from the DMC polymer, would have a continuing therapeutic impact on the neovascular response to corneal injury over time. The route of administration for the DMC injection is not likely to alter the release rate of an incorporated drug from the Densomeres themselves in a significant manner, as long as the DMC depot is located in an aqueous milieu where natural hydrolytic biodegradation of the polymer can occur.[4,5] Intravitreal injections would be expected to have different post-release distribution kinetics than observed in extraocular, vascularized tissues, but this in vivo study focused on prolonged drug release from the DMCs themselves and whether the released bevacizumab retained its functional antineovascular bioactivity in a nearby target tissue.

With regard to the extensive and growing market related to chronic and degenerative ocular diseases, drug manufactures have had difficulty extending existing or newer anti-vascular endothelial growth factor (VEGF) treatment frequencies beyond 4 months.[2,3,10–12] As a result, there is a need to effectively extend the therapeutic bioactivity of both large mAbs and other potent APIs to reduce the frequency of repeated intravitreal injections for the major causes of blindness, including diabetic retinopathy, wet AMD, diabetic macular edema, and retinal vein occlusion. Although frequent intravitreal treatments have been associated with better long-term adherence and visual outcomes after the first 2 years of therapy for wet AMD, that experience has involved treatment intervals ranging from 4 to slightly more than 12 weeks and has not utilized drug products designed as long-acting therapies.[13] The safety and effectiveness of truly extended treatment intervals with products designed for continuous anti-VEGF bioactivity for 6 or more months have yet to be explored.

These in vitro and in vivo data demonstrate that Densomeres, bioengineered to control duration of drug delivery, can be prepared for injectable suspensions that include a mAb API for prolonged and controlled drug release kinetics lasting many months. Densomeres can be also formulated into nanoparticles suitable for inhalation, as well as solid rods, disks, or blocks, for implantation, and can incorporate small molecules as well as larger mAbs. Bevacizumab, an established mAb anti-VEGF therapy used in several anti-cancer and ocular indications,[14,15] was selected as the API in this study to represent a class of macromolecules whose bioactivity could be sensitive to manipulation and inactivation during formulation, as well as premature metabolic degradation when introduced into target tissues. Bevacizumab also has the advantage of prior ocular use in an established animal model, both topically and subconjunctivally, for corneal neovascularization.[7] Both clinical and laboratory use of subconjunctival bevacizumab was shown to have an immediate inhibitory effect on corneal neovascularization and inflammation, but, in contrast to our Densomere subconjunctival injection results, those effects were short lived.[16–18]

Unlike other PLGA-containing drug products,[4] Densomeres using only the API and PLGA polymer are processed to control release kinetics and appear to reduce tissue reactions. Although biodegradable PLGA has found significant commercial use in several approved medical products, most are used with added ingredients, coatings, and/or blends of polymer products to carry their APIs and help reduce foreign body tissue reactions to the polymer carrier.[4] The Densomere technology appears to protect the molecular integrity of the internalized API and reduce foreign body reactions without the need for additional ingredients, all while prolonging the API release profile, as the polymer hydrolysis occurs at a controllable, slower rate for up to 12 months. We verified this molecular integrity by observing the molecular weight and component chromatographic profiles of the bevacizumab appearing in the supernatant of Densomere suspensions compared to authentic bevacizumab. A Densomere composition and processing technology that results in a product with only the API and PLGA polymer obviates the need for using multiple particle layers, excipients, or solvents or the need for increased dosing, each of which could contribute to untoward responses. Densomere microparticles are considerably denser than water and tend to settle quickly rather than disperse, even in the rabbit vitreous. We tested central vitreous injections of Densomere microparticles in rabbits and observed that they settled and localized to the inferior periphery within 2 days, leaving the visual axis undisturbed, as determined by ophthalmoscopy.

In this study, Densomere microparticles were produced at a size allowing easy passage of the suspension through a 27-gauge needle, a diameter that would minimize injection trauma to ocular tissues. The Densomere production process can be tailored for smaller (nano) or larger particles based on the intended route of administration and clinical indication. This study demonstrated that Densomere technology can provide measurable in vitro release of an API without loss of molecular integrity, and, more importantly, it provided evidence of continued bioactivity in vivo extending up to 12 months after a single injection. This may give drug makers and patients new therapeutic options that utilize Densomere technology to reduce systemic toxicities and the burden of frequent

drug administration for both ocular and extraocular indications.

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