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TITLE: Antimicrobial Silk Ocular Drug Delivery Implant for Chronic Posterior Segment Diseases

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14. ABSTRACT

We proposed to develop a novel, safe, and long-term drug delivery system for the eye and adnexa for treatment of chronic posterior segment diseases. Anti-vascular endothelial growth factor (VEGF) drugs and corticosteroids are in widespread clinical use for age-related macular degeneration (AMD), diabetic retinopathy and retinal vascular disease. New drugs for treating chronic eye diseases are being rapidly developed. These drugs are delivered via repeated intraocular injections that not only require frequent (monthly) physician visits but also carry the risk of infections (endophthalmitis), intraocular hemorrhage, retinal detachment, and increased intraocular pressure. Development of a safe, refillable, sustained-release and long-term implantable intraocular device has the benefit of delivering constant therapeutic levels of drug directly to the site of action without the side effects of systemic or intravitreal injections. The silk based device has been successfully optimized to achieve a sustained release of anti-VEGF for more than 7 days upon application of HMPEI antibacterial treatment to prevent bacterial infection. The animal rabbit model showed excellent biointegration of the device into the ocular structure, no signs of infection and inflammation were observed over the 8-month period. Furthermore, no signs of retinal toxicity were observed upon anti-VEGF release in vivo.

15. SUBJECT TERMS

Intraocular device, sustain release,

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INTRODUCTION

We proposed to develop a novel, safe, and long-term drug delivery system for the eye and adnexa for treatment of chronic posterior segment diseases. Anti-vascular endothelial growth factor (VEGF) drugs and corticosteroids are in widespread clinical use for age-related macular degeneration (AMD), diabetic retinopathy and retinal vascular disease. New drugs for treating chronic eye diseases are being rapidly developed. These drugs are delivered via repeated intraocular injections that not only require frequent (monthly) physician visits but also carry the risk of infections (endophthalmitis), intraocular hemorrhage, retinal detachment, and increased intraocular pressure. Development of a safe, refillable, sustained-release and long-term implantable intraocular device has the benefit of delivering constant therapeutic levels of drug directly to the site of action without the side effects of systemic or intravitreal injections.

1. KEYWORDS:

Natural polymer, delivery system, anti-VEGF

2. ACCOMPLISHMENTS:

- **What were the major goals of the project?**

  As listed in the approved SOW, the major goals of the project were the design and *in vitro* pharmacokinetics studies of the silk-based anti-VEGF drug delivery device, the development of an antimicrobial, *N,N*-hexyl, methyl-polyethylenimine (HMPEI) derivatized intraocular silk drug delivery implant, the assessment of the anti-VEGF HMPEI-silk drug delivery implant system in normal pigmented rabbit models and in a diabetic retinopathy rabbit models *in vivo*.

- **What was accomplished under these goals?**

  Task 1 a) Silk Drug Delivery Implant Design:

- The final material format and design device is described in Figure 1A. The device was made from a gel-spun tube of silk. Briefly, the silk was spun at a concentrated 15 % (w/v) solution over a steel wire (diameter = 500 μm), then lyophilized to yield a silk tube with consistent inner diameter. Then the silk tubes (still wrapped around the steel wire) were submerged in methanol for 1 h to induce β-sheet formation and stabilize the protein structure. After separation of the tube, the gel-spun tube was then cut into smaller pieces (5 mm in length). Although the inner diameter of the device is fixed by the outer diameter of the steel wire, the outer shape of the device is determined by the deposition pattern of silk solution. Upon completion of the device fabrication, the protein structure was chemically functionalized by covalently attaching the cationic surfactant N,N-hexyl,methyl-polyethylenimine (HMPEI), which has shown antibacterial properties (Figure 1B). The polymer breaches the cell wall of

bacteria, leading to cell lysis and death. The antibacterial functionalization would decrease the occurrence of complications such as endophthalmitis after insertion of the device through *pars plana* into the eye socket the rabbit. First the silk formats were converted to azo-functionalized silk formats. An azo group with a terminal carboxylate group was attached to the tyrosine residues in silk, resulting in a bright orange color. Next the HMPEI groups were attached to those free carboxylate groups, leaving the orange color unchanged. Instead of a uniform outer surface, we chose to prepare a peanut-shaped device to lock the device in place and for ease of placing into the eye (Figure 1 C and D). The device is based on a single format and is thus has no junctions that may affect the mechanical integrity and structural continuity. The device has a consistent and well-defined delivery channel for storing the drug-releasing material.

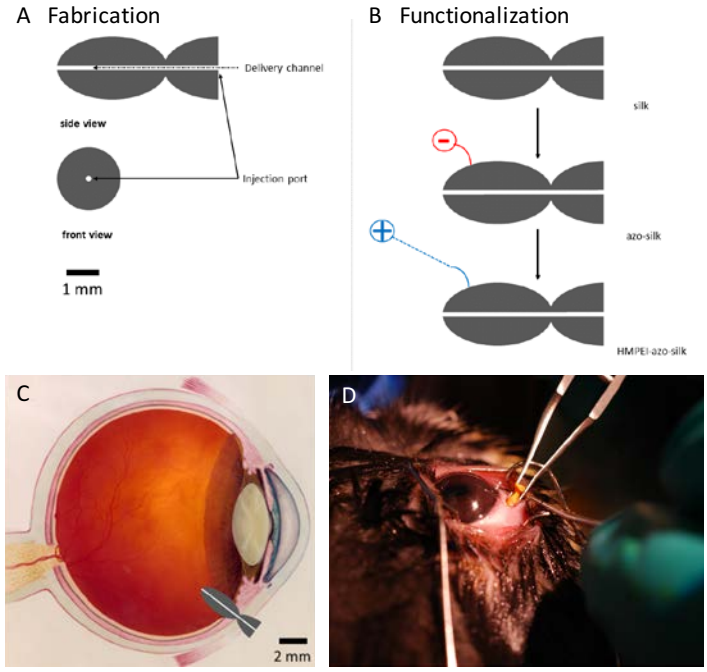

Figure 1: Silk device design. Schematics of the fabrication (A) and functionalization (B) of the silk-based device. (C) A graphic representation of the implant (The graphic of the eye has been adapted from an image by National Eye Institute: flickr.com/photos/nationaleyeinstitute/7544656020/in/album-72157646829197286/, Creative Commons 2.0 license). (D) HMPEI-functionalized silk device inserted into the *pars plana* of a rabbit eye *in vivo*.

Task 1b) In vitro Silk-based anti-VEGF Drug Delivery and Pharmacokinetic Studies:

Bevacizumab release from silk lyogel was initially assessed in vitro (Figure 2). Silk molecular weight was varied by altering the boiling time during the silk extraction process. Specifically, 30 minute of boiling time (30 BT) leads to a smaller molecular weight distribution of silk in comparison to protein fibers boiled for only 15 minute (15 BT). The cumulative drug release was affected by the molecular weight as well. 15 BT stabilized a much greater quantity of bevacizumab in comparison to 30 BT, which instead released significantly more over a 7 day period. Furthermore, 30 BT silk was able to provide a stable release of 4 ng/ml after 4 days in comparison to 15 BT. 30 BT silk was then selected to perform the animal studies.

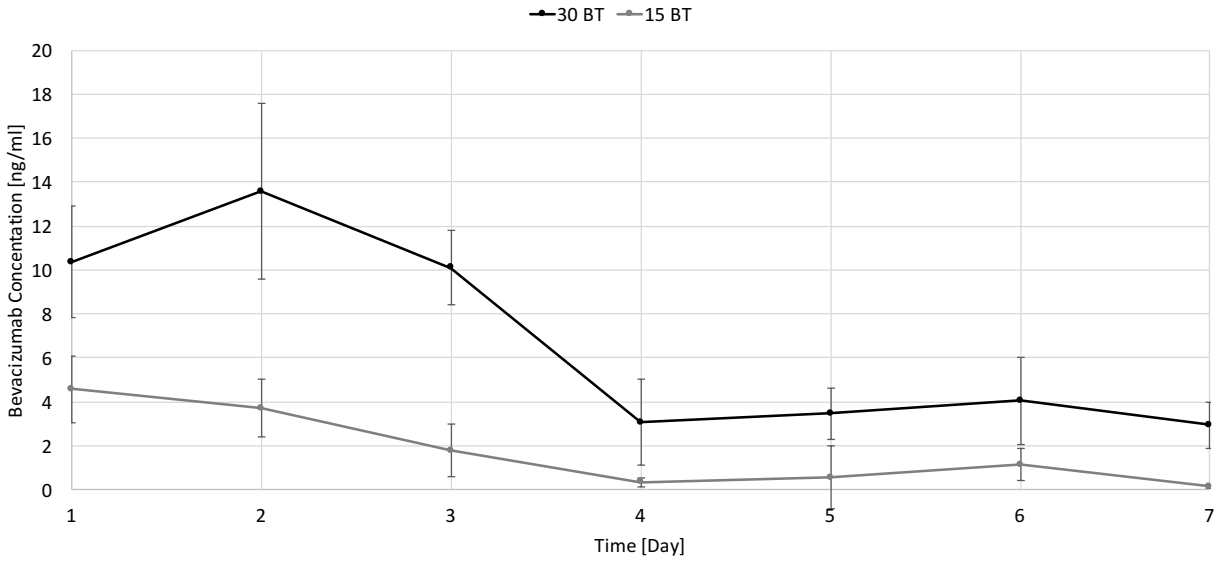

Figure 2: Silk lyogel loaded bevacizumab in vitro release profile over 7 days. Bevacizumab release profile was tuned by varying silk boiling time (BT). The release profile was stable after 4 days with silk boiled for 30 minutes (30 BT).

Task 2a: Maximization of HMPEI-bound silk surface area, host cell biocompatibility, and antimicrobial efficacy.

Diazonium coupling chemistry installs small functional groups onto the silk backbone. This enables silk to be further functionalized, increasing the opportunities to alter cell-specific responses and the development of novel multifunctional materials. We first attached the HMPEI to the carboxylic acid groups of the aspartic and glutamic acids of silk fibroin using modified

methods. This approach yielded 1.1% of the silk protein functionalized with HMPEI, theoretically sufficient coverage to confer antimicrobial activity. Nevertheless, we then targeted functionalized tyrosine amino acids that are homogenously distributed throughout the silk and to increase HMPEI functionalized silk to more than 5% coverage.

Cell viability staining and metabolic activity using human corneal stromal fibroblasts demonstrated the lack of cytotoxicity by HMPEI-derivatized silk (Figure 3). Of note, there was slightly less cellular proliferation with the attachment of HMPEI that may be due to the hydrophobic nature of HMPEI compared to the hydrophilicity of functionalized silk (RGD-silk, D-silk).

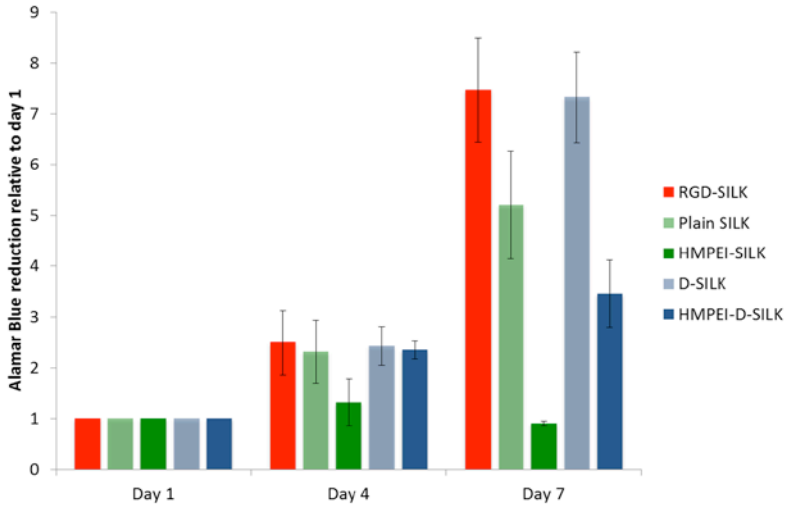

Figure 3. Comparison of Cell Metabolic Activity by Alamar Blue of corneal stromal fibroblasts co-cultured over 7 days with RGD-silk, Plain silk, HMPEI-Plain silk, D-Silk alone, and HMPEI-D-Silk.

Task 2b) HMPEI-silk implant mechanical stability and in vitro drug release studies:

The functionalization involves two steps, as described in Figure 1 A and B. The two steps produce azo-silk and HMPEI-azo-silk formats, respectively. By sputter coating the devices at each of the steps of functionalization and then performing SEM characterization, we investigated the changes in the morphology of the device in response to the covalent functionalization. Based on the results (Figures 4), we determined that the effect of functionalization was minimal and did not alter the morphological structure of the device. We did not observe differences in the drug release for the HMPEI-functionalized device in comparison to plain silk device, as the main key factor is the lyogel where the drug is stabilized.

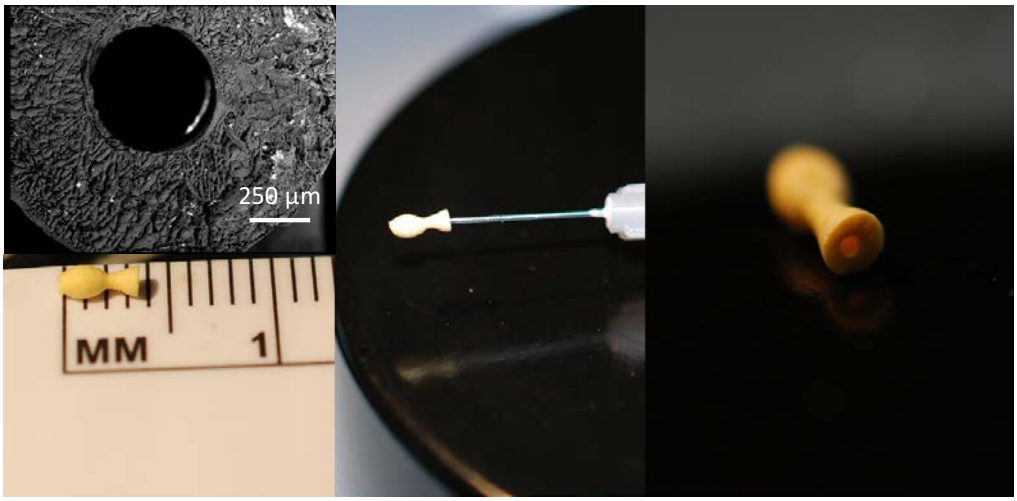

Figure 4: Silk device morphological characterization. Macro images of the HMPEI-functionalized silk device and scanning electron micrograph of the inner channel of the silk device, scale bar = 250 µm.

Task 3a): Assessment of the anti-VEGF HMPEI-silk drug delivery implant system in normal pigmented rabbit models in vivo.

All animals used in this study were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Tufts University Institutional Animal Care and Use Committee (Boston, MA). Only one eye per animal was used. The Dutch Belted rabbits (Covance, Lab, Amherst, MA) weighed between 1.8 and 2 kg were anesthetized with 4% isoflurane. Topical proparacaine HCl 0.5% was instilled into the conjunctival sac of the eye, and the operating area was sterilized with 5% povidine-iodine and repeated after 5 min. Topical ophthalmic antibiotics (Polytrim) were given immediately pre-operatively and one dose postoperatively. Animals were placed in a laterally recumbent position for surgery. Photography was performed using Nikon D90 digital single-lens reflex (SLR) camera and Zeiss Macrozoom lens attached to iPhone 6S. Immediately after euthanasia, animal eyes were enucleated and fixed in neutral buffered formalin, dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

There was minimal cellular reactivity and microvascularization at the surgical incision site, and no inflammatory response surrounding the HMPEI-implant, as showed in Figure 5. There is a time-dependent fibroblastic response with progressive ingrowth of epithelial and stromal cells into the porous HMPEI - silk fibroin implants over 3 months and 9 months, as demonstrated in

Figure 6. The porous HMPEI coated silk architectural structure is naturally yellow and remained yellow during H&E staining. The results of the in vivo biocompatibility evaluations conducted in this work are further strengthened by the fact that the rabbit eye is generally more sensitive than the human eye [1,2]. It also has a heightened tissue response to minor surgical procedures, foreign materials, drugs, or chemicals [1,2]. Another factor strengthening our findings is that topical antibiotics were only used once postoperatively and no steroids were needed.

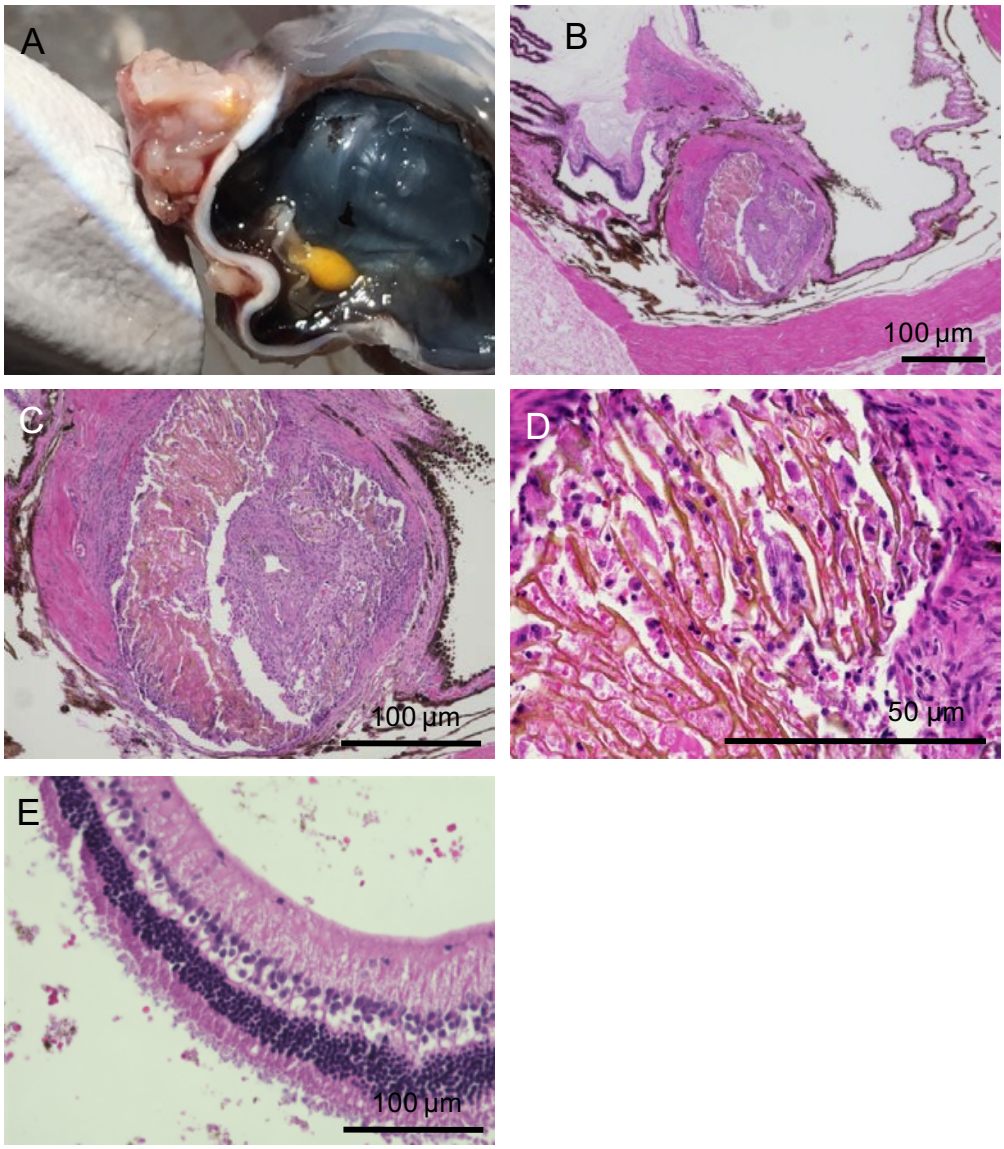

Figure 5. Unloaded HMPEI-Silk Implant at 3 months. A. Horizontal Cross-Sectional View of the surgical wound site had ecchymosis and implant slipped into the eye over time but remained attached. B-C-D. Hematosilin eosin stained histological sections show fibroblastic growth within the porous silk scaffold and channel. E. Representative normal retina was also observed.

In our prior work studying HMPEI covalently attached to an artificial cornea composed of PMMA and titanium, we found as others that the rabbit eye readily extrudes foreign material, limiting long-term studies beyond a few months [3]. This is the first time implant has remained localized (and without any sutures or retaining procedures) without antibiotics or immunosuppressants for greater than 6 months. We did note as silk went through its programmed degradation, it did become slightly smaller and slipped into the eye as our newer fishtail design enabled.

This is the first known ocular implant that is residing on the ocular surface demonstrating no signs of infection, long-term biocompatibility without any signs of inflammation or implant reactivity. This is the first known ocular implant for the posterior eye residing on the surface that is safe, infection free, and without reactivity.

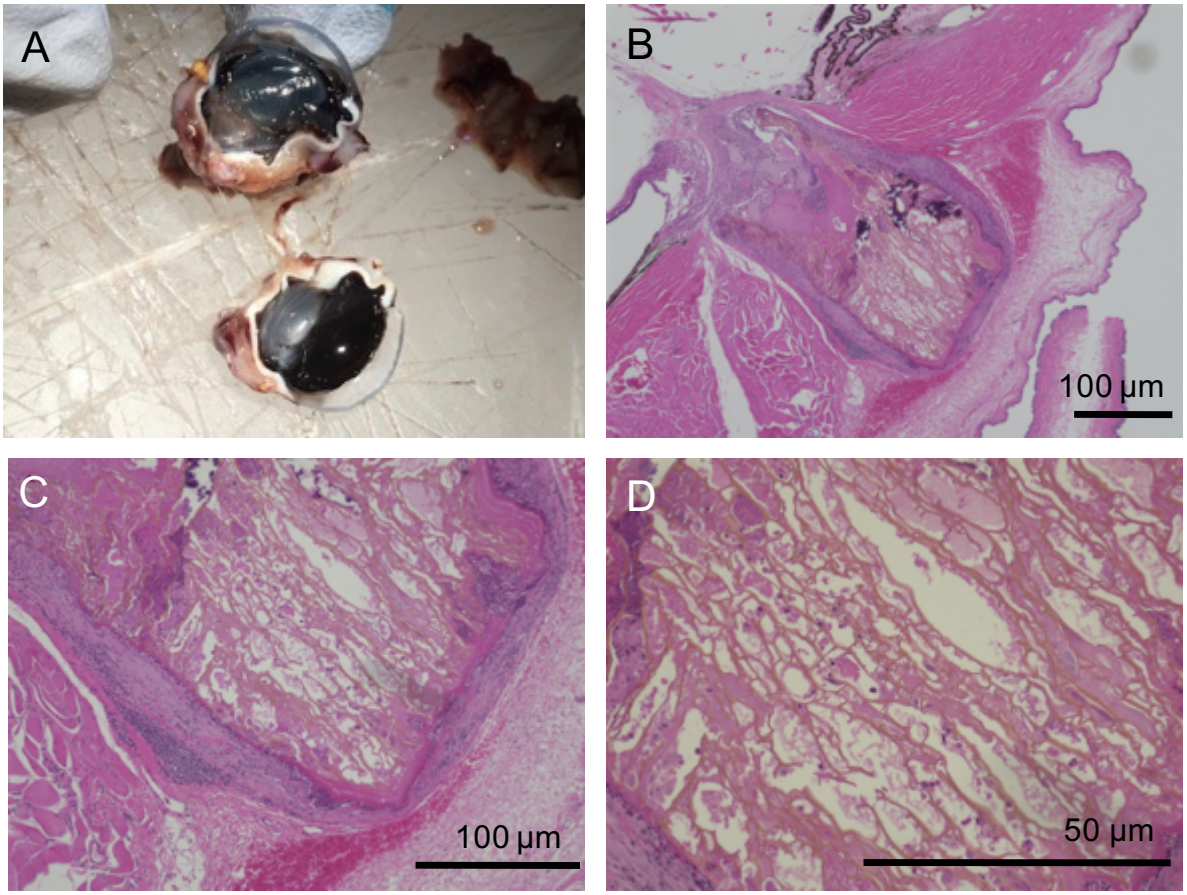

Figure 6. Bevacizumab Preloaded HMPEI-Silk Implant at 8 months. A. Longitudinal Cross-Sectional View of the Surgical wound site that showed more bleeding associated with the bevacizumab preloaded implant. B-C-D. H&E stained histological sections showed more fibroblastic response and inflammatory response associated with this bevacizumab preloaded HMPEI-Silk implant. Fibroblastic growth within the porous silk scaffold and channel can be seen.

Task 3b): Assessment of the anti-VEGF HMPEI-silk drug delivery implant system in a diabetic retinopathy rabbit models in vivo

No retinal toxicity was reported upon VEGF injection and anti-VEGF HMPEI-silk drug delivery implant insertion, as from OCT and histopathology analyses. Results are still under investigation from the histopathological examiners to correlate the functionality of the device in the diseased animal model.

References:

[1] Wilhelmus KR. The Draize eye test. Surv Ophthalmol 2001; 45:493e515.

[2] Espana EM, Acosta AC, Stoiber J, Fernandez V, Lamar PD, Villain FL, et al. Long-term follow-up of a supradescemetic keratoprosthesis in rabbits: an immunofluorescence study. Graefes Arch Clin Exp Ophthalmol 2011; 249:253e60.

[3] Behlau I, Mukherjee K, Todani A, Tisdale AS, Cade F, Wang L, Leonard EM, Zakka FR, Gilmore MS, Jakobiec FA, Dohlman CH, Klibanov AM, Biocompatibility and biofilm inhibition of N,N-hexyl,methyl-polyethylenimine bonded to Boston Keratoprosthesis materials, Biomaterials. 2011 Dec;32(34):8783-96.

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

  Nothing to Report.

- **How were the results disseminated to communities of interest?**

  Nothing to Report.

- **What do you plan to do during the next reporting period to accomplish the goals?**

  Nothing to Report.

3. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**

This is the first known ocular implant that is residing on the ocular surface demonstrating no signs of infection, long-term biocompatibility without any signs of inflammation or implant reactivity. This is the first known ocular implant for the posterior eye residing on the surface that is safe, infection free, and without reactivity, opening the door to other sustained release ocular treatments that are currently suffering from ocular infections.

- **What was the impact on other disciplines?**

Nothing to Report.

- **What was the impact on technology transfer?**

  Nothing to Report.

- **What was the impact on society beyond science and technology?**

  Nothing to Report.

4. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

  Nothing to Report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

  Nothing to Report.

- **Changes that had a significant impact on expenditures**

  Not Applicable.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

  Not Applicable.

5. PRODUCTS:

Nothing to Report.

- **Publications, conference papers, and presentations**

  We are currently working on a journal publication that we are aiming to submit to a top journal in the ophthalmology field.

- **Journal publications.** Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes).

- **Other publications, conference papers, and presentations.**

  Nothing to Report.

- **Inventions, patent applications, and/or licenses**

  Nothing to Report at this stage.

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

- **What individuals have worked on the project (for period since last annual report, September 2016 – July 31, 2017)?**

Name:	*David Kaplan*
Project Role:	PI

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Project overview and supervision*
Funding Support:	Tufts University
Name:	*Irmgard Behlau*
Project Role:	*Research Assistant Professor*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Behlau has worked closely with Dr. Kaplan to complete the aims of the project, particularly focusing on the in vivo aims.*
Funding Support:	*This grant*
Name:	*Chiara Ghezzi*
Project Role:	*Research Assistant Professor*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Ghezzi has contributed to the design and development of the silk device and drug delivery system, as well as supervising all the aspects of this program.*
Funding Support:	*This grant*
Name:	*Biplab Sarkar*
Project Role:	*Postdoctoral Scholar*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to	*Dr. Sarkar contributed towards the design and chemical*

Project:	*functionalization of the device, optimization of the drug-delivery medium, as well as the determination of the drug release profile.*
Funding Support:	*This grant*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Nothing to Report.

- **What other organizations were involved as partners?**

  Nothing to Report.

7. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** Not applicable.
- **QUAD CHARTS:** Not applicable

8. APPENDICES:

Nothing to Report.