Award Number: W81XWH-11-2-0064

TITLE: Preventing Vision Loss from Blast Injuries with Regenerative Biomaterial

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REPORT DATE: December 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-12-2011	Annual	30 Nov 2010 - 29 Nov 2011

4. TITLE AND SUBTITLE
Preventing Vision Loss from Blast Injuries with Regenerative Biomaterial

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-2-0064

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr. Denise Barbut

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Sarentis Ophthalmics Inc.
Eagan, MN 55123

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Ocular trauma from blast injuries is a common and serious condition. We have identified thin silk film biomaterials which may be fabricated to allow for stabilization and healing of these injuries in the acute setting. We have identified critical parameters, including optimal curvature and dissolution rate, for the successful use of these silk bandages using animal models. Moreover, we have developed novel methods to impart these characteristics on the silk film bandages.

15. SUBJECT TERMS
Cornea, Blast Injury, Silk, Biomaterial

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	12	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION:

Sarentis Ophthalmics in collaboration with Cornell University is developing the first biodegradable "green" corneal bandage that accelerates corneal healing. The bandage resembles a contact lens. When this bandage is placed on a wounded eye it reduces inflammation and stimulates the healing process. It is made of a novel biomaterial, which can be programmed to "dissolve" within days providing patients with a tailored product.

Over 2 million Americans sustain traumatic injuries to the cornea each year, and over 4 million Americans undergo surgery that leaves the cornea wounded annually. Such corneal wounds cause intense pain and may lead to blindness depending on the severity. Sarentis' corneal bandage accelerates corneal healing and adheres to the surface of the eye to aid in alleviating pain. This regenerative bandage is inexpensive and currently produced in the lab for less than a few pennies. This will result in a less expensive procedure for treating cornea trauma resulting in millions in savings to the American health care system, while allowing for the expansion of the point of care environments (i.e. clinic, home use, hostile environments).

BODY

Feasibility of the project has been successfully demonstrated in that the silk film bandage was shown to enhance corneal healing upon a rabbit animal model. During the course of the study the physical design of the silk bandage was successfully optimized, which included the production of a curved body shape to enable better application and comfort to the cornea. Reproducible rabbit models were developed to assess corneal injury, which were then utilized to demonstrate the silk film's capability to enhance wound healing. The funding provided by the DoD has allowed a conceptual device to be converted into a physical product capable of moving to the next steps of GMP production and human clinical studies. Silk films are a novel form of ocular bandage, and the use of these films on the ocular surface provided unique challenges and opportunities in developing devices with the appropriate material properties . Significantly more material work was supported by the award than anticipated in the original proposal. However, through these efforts a number of new silk material processing modalities have been developed. Furthermore, greater control and understanding of silk film material properties has been realized, which will ultimately lead to a more capable platform technology with the potential adaption to a number of applications both within and outside the field of ophthalmology. Of significant focus during the project period was a series of rabbit cornea studies utilized to assess the impact of the silk biomaterial properties while applied to the corneal surface. These experiments were performed in an effort to validate prior *in vivo* mouse animal findings, which suggested silk films could aid in expediting the wound healing response post corneal injury.

Task 1. Irritation Response

Silk biomaterials have a low toxicity and irritative response in a host of tissue engineering settings. We have demonstrated that this observation holds true for silks films applied to the ocular surface.

We have applied silk films to the rabbit eye and looked for clinical and histopathologic evidence of irritation. This analysis has been performed in both inured and non-injured eyes. Eyes were examined via slit lamp biomicroscopy for evidence of ocular surface injection, ulceration, or cellular infiltrate, as well as for any evidence of intraocular inflammation. By all metrics, eyes receiving silk films were consistently found to have inflammation equal to or less than that of control eyes not receiving silk films.

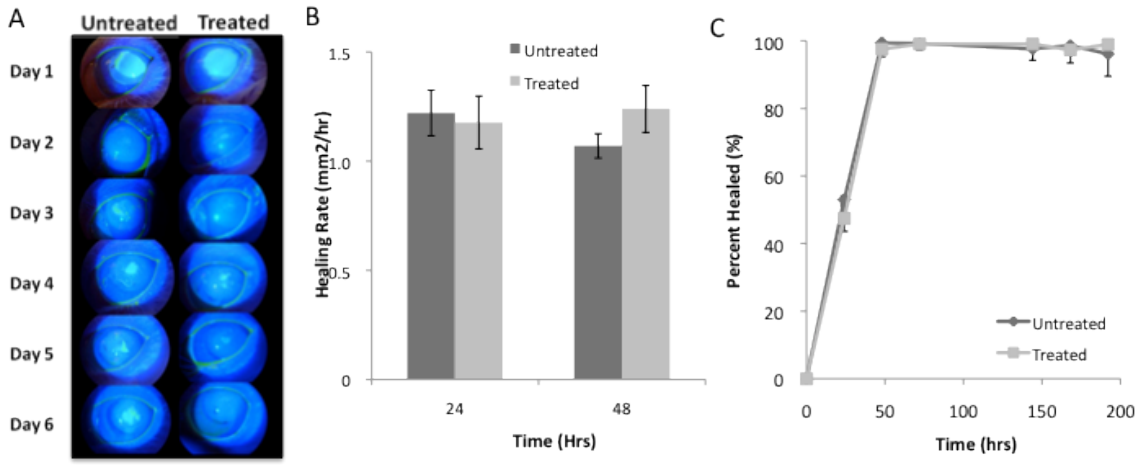
Eyes from animals receiving silk films and those receiving sham application of films were harvested from rabbits and evaluated histopathologically. Masked evaluation of samples demonstrated no evidence of untoward effects from the application of silk films. Application of silk films did not lead to corneal stromal loss, corneal melt, delay in epithelialization or increased numbers of inflammatory cells.

Task 2. Burn Wound Assessment.

A primary goal of the proposed studies has been to examine the role of silk films following ocular injury. Application of an alkali burn to the rabbit eye with concomitant application of silk films is to be evaluated extensively.

As mentioned above, we have made marked improvements in the material properties of the silk bandage (described in detail below) which requires extensive evaluation in animal models. We have elected to first focus on the abrasive model (described below under Task 3) as it is the most reproducible model for optimizing the material properties of the bandage. We have performed initial evaluation of silk films in the alkali models and found that the bandages were adherent and well tolerated on the acutely burned ocular surface.

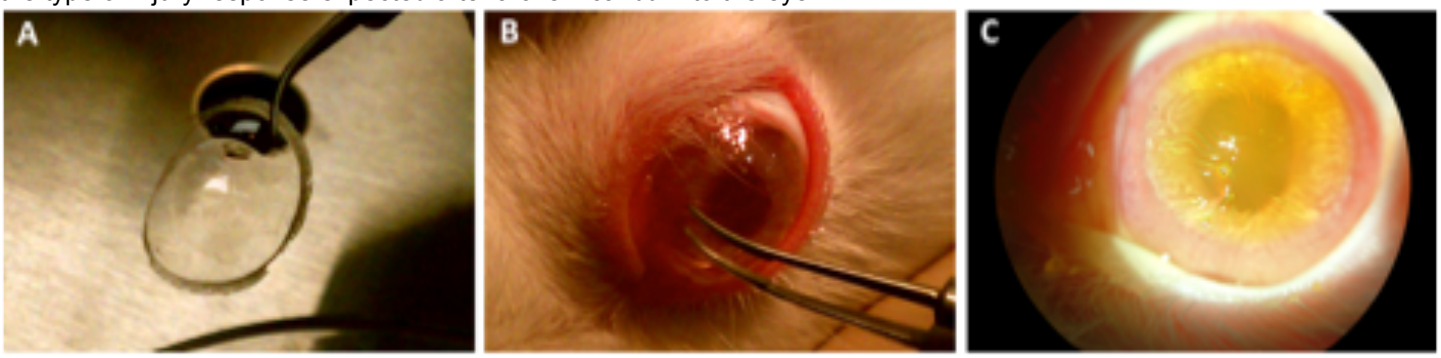
A rabbit alkali injury model was developed to assess the rabbit cornea's initial response to the application of the silk film bandage material. Bandages were produced as described above. The silk film bandage was heat sterilized at a minimum temperature of 150°C for 2 hours, which was shown to not affect dissolution rate. The corneal alkali injury was performed to create an 8.5 mm injury diameter, and then silk films were applied to the injury bed of the "treated" group of animals (n = 3). The corneal wounds were then followed using fluorescein, which indicates a de-epithelialized surface as denoted by green fluorescence under blue light. Animals were monitored until complete epithelial healing was reached by 72 hours as indicated by lack of fluorescein staining. These silk bandages dissolved on the ocular surface within 1 hour after initial application, and demonstrated no detrimental effect on healing when compared to the controls. From this result, it was decided that additional processing would be needed to optimize the dissolution time upon the cornea for possible improvement in corneal recover from the alkali injury.



(A) Fluorescein staining images of rabbit cornea healing progression for treated and untreated rabbits over a 6 day period. (B) Healing rate and (C) percent healed data comparing silk film bandage treated and untreated rabbits for the alkali injury model (n = 4, error bars = standard deviation).

Task 3. Abrasive Wound Assessment.

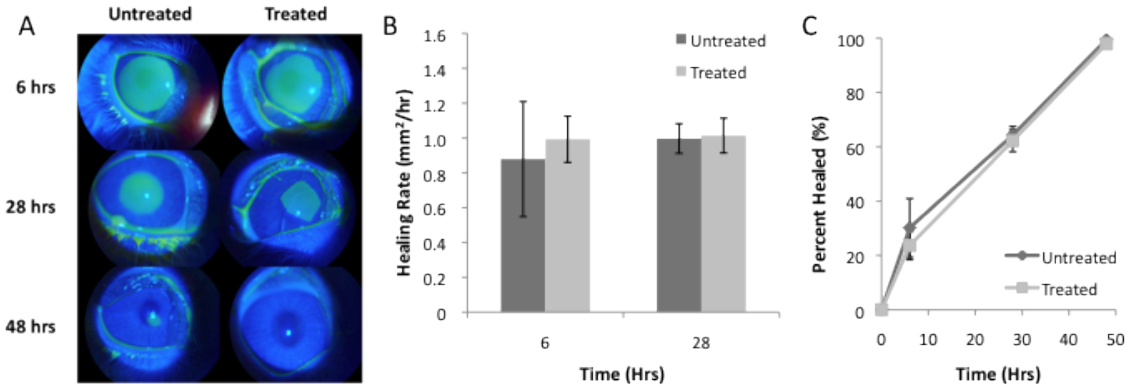
The larger corneas of the rabbit model offer a more physiologically relevant environment to humans than mice. Rabbit animal trials demonstrated that treatment of a denuded epithelial corneal surface with silk film biomaterial enhanced the observed 24-hour healing rates in rabbits. An epithelial debridement rabbit animal model was developed to simulate the surgical injury performed during a photorefractive keratectomy (PRK) procedure, which can be used to monitor epithelial healing rates. It was shown that the curved silk film bandage could be readily applied to the rabbit's eye and then monitored using slit lamp photography. In addition, an alkali burn injury model was also developed to simulate more substantial injuries, such as a chemical burn. This procedure has a greater damaging effect to the cornea and simulates the type of injury response expected after a chemical burn to the eye.



Silk bandage application and observation procedure. (A) The silk film bandage is administered using forceps, (B) applied directly to the rabbit cornea, and (C) then observed using slit lamp photography.

Effect of expedited silk dissolution time on epithelial regeneration

The PRK injury model was utilized to assess the rabbit cornea's initial response to the application of the silk bandage material. A 80 um thick silk film bandage was produced aseptically by casting 100 uL of 8% silk solution onto a 14 mm diameter silicone rubber substrate, air-dried overnight, and air lifted from the mold using forceps (Lawrence, Omenetto, Chui, & Kaplan, 2008). The corneal epithelial debridement surgeries were performed to create an 8.5 mm injury diameter, and then silk films were applied to the injury bed of the "treated" group of animals (n = 3). The corneal wounds were then followed using fluorescein dye, which indicates a de-epithelialized surface as denoted by green fluorescence under blue light. Animals were monitored until complete epithelial healing was reached (typically 48-72 hours) as indicated by lack of fluorescein staining. Silk film bandages dissolved on the ocular surface within five minutes after application, and demonstrated no effect on healing when compared to the controls. From this result, it was demonstrated that the material caused no adverse effect on healing. It was hypothesized that a longer residence time upon the cornea may promote healing to greater extent. Therefore, additional processing was utilized to extend the dissolution time of the silk film bandage upon the cornea.



(A) Fluorescein staining images of rabbit cornea healing progression for treated and untreated rabbits over a 48 hour period. (B) Healing rate and (C) percent healed data comparing silk film bandage treated and untreated rabbits for the epithelial debridement injury model.

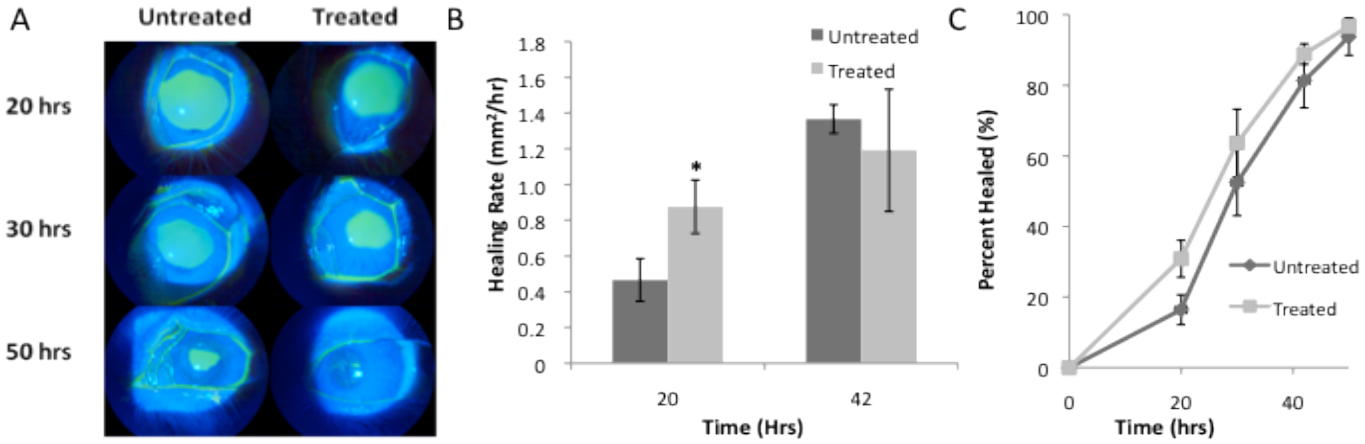
Effect of extended residence time of silk film bandage on epithelial regeneration

Silk film bandages were produced using the process described above, and in addition these bandages were water-annealed for 30 minutes. Water-anneal processing is a common silk film treatment method that extends the dissolution time of the material by increasing hydrogen bonding within the material through beta-sheet secondary structure formation (Jin, et al., 2005). This additional processing resulted in a near 10-fold increase in silk bandage dissolution time as compared to trial 1 within water. The PRK debridement procedure was performed and tracked over time with fluorescein staining. The films resided on the ocular surface for up to ten hours post-procedure. In addition, the silk bandage treated group demonstrated a significant increase (30%, n =3) in healing rate over the first 24 hour period while the film was still present on the wound bed when compared to untreated controls . Once the film had dissolved from the corneal surface it was shown that the healing rate was similar to untreated controls.

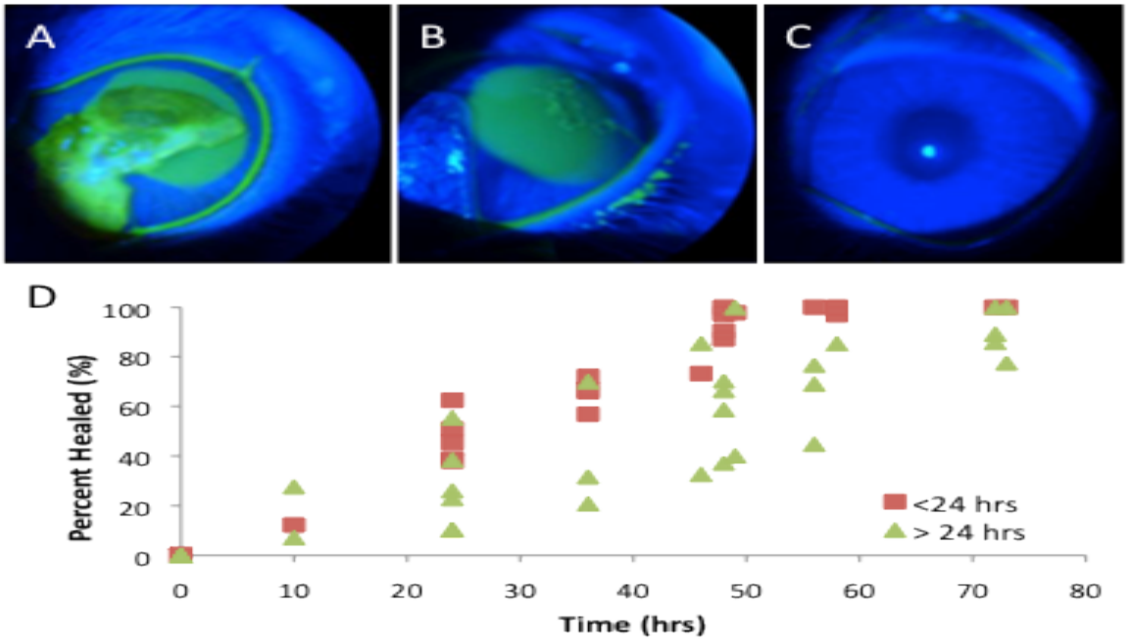
. (A)Time course images of fluorescein stained epithelial debridement area for untreated and silk film bandage treated rabbit groups. (B) A statistically significant increase in healing rate was demonstrated over the first 20 hours post-procedure for treated animals when compared to untreated controls. (n=3, error bars indicate standard deviation, * indicates $p < 0.05$ compared to untreated controls) (C) Wound healing profile demonstrated enhanced healing profile on average for treated animals over 40 hour period post-procedure.

Effects of silk film bandage non-dissolution upon healing rate

PRK studies were undertaken to assess the affect that non-dissolving vs. dissolving silk film bandages have on corneal epithelium healing.



Non-dissolving silk film bandages were compared to films had extended residence time or rapid dissolution time collectively. Non-dissolved films were found to negatively impact healing rate when compared to dissolvable films, especially at time points at 24-hours and longer. As a result it was concluded that non-dissolving silk bandages caused adverse effects on corneal healing, most likely due to a mechanical disruption of the healing epithelium. These conclusions indicated that less than 24 hour silk film bandage dissolution is an important material property for stimulating the healing process.

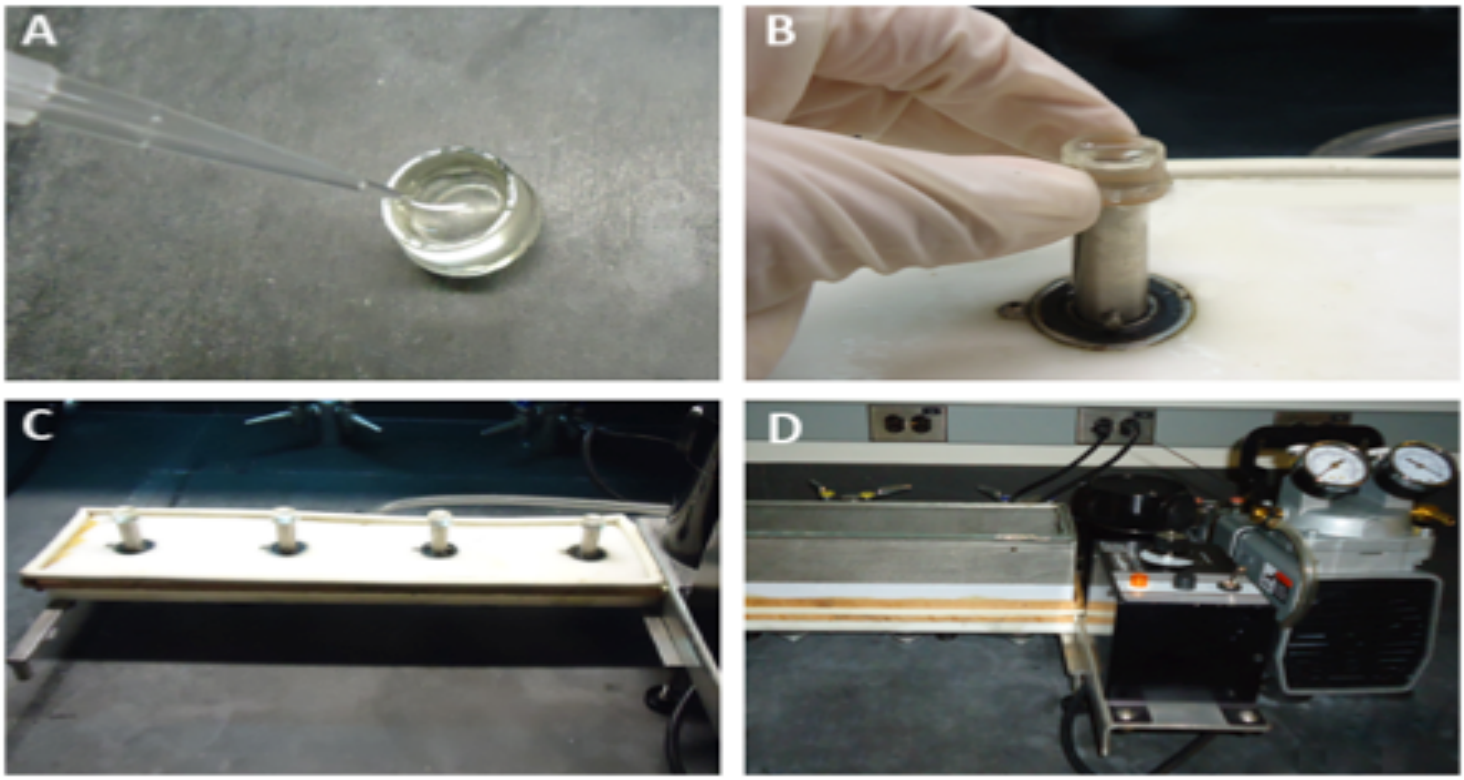


Fluorescein staining of debrided rabbit corneal epithelium for eyes with silk film bandages that are (A) non-dissolving (24-hours post procedure), (B) enhanced residence time (10 hours post procedure), and (C) completely dissolved (48 hours). (D) Collective scatter plot comparing percent healing for non-dissolving (green) and dissolving (red) silk film bandages.

As indicated above, a substantial amount or work has been performed to optimize the material properties of the bandage including developing new methods to create a curved bandage that approximated the curvature of the eye, new methods to control the dissolution of the film, and new methods to sterilize the bandages.

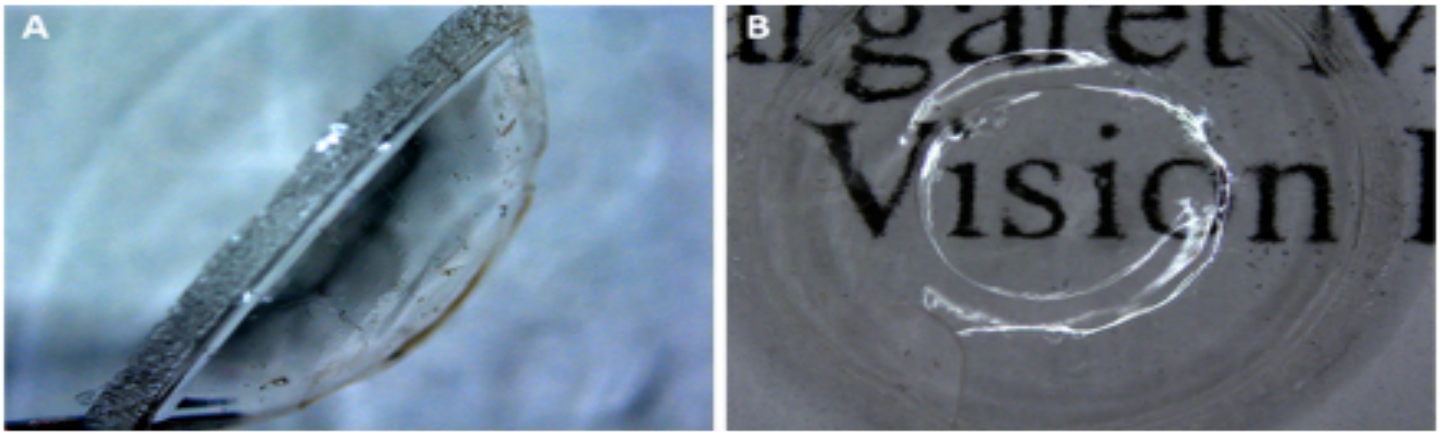
Producing a shaped silk film bandage

Prior to beginning the studies, we had preliminary *in vivo* mouse data to suggest that silk films may serve as regenerative bandages to promote corneal wound healing. During the course of the studies, these films would be evaluated in more relevant wounding models in the larger rabbit eye. The rabbit model has been the ideal test bed for a number of successful ocular devices and procedures that are now used clinically in humans. The original silk biomaterials used to treat ocular wounds to the mouse were flat, and when moved to the rabbit model this design proved problematic. The flat film did not conform to the ocular surface, making it difficult to get the film to readily adhere to the corneal surface uniformly. This could cause potential discomfort to the animal and also affect the outcomes of the study due to non-uniformity in film coverage over the cornea area. In an attempt to produce a curved silk film bandage a spin casting process was developed within the lab. The spin casting process has been used to create standard contact lenses in the past and was a good candidate technique for shaping silk films. A prototype spin casting device was built in which a curved silicone rubber mold could be mounted onto 1 of 4 spindles which were connected to a variable speed motor through a power transfer belt system. 70 uL of silk solution was pipetted into the curved molds and then the spun at a fixed rate for a 1.5 hour period until the solution dried. In addition, we have expedited the drying time of the curved films by controlling the spindle environment.



Silk spin casting device. (A) Silk solution is cast into a curved silicone rubber mold and (B) then mounted onto a spindle and rotated at a controlled rate. (C) Lab prototype containing 4 spin casting spindles. (D) The spin casting area was covered, the spindles were attached to a variable voltage motor, and a compressor was used to provide a controlled pressurized source of convective air-flow through the chamber.

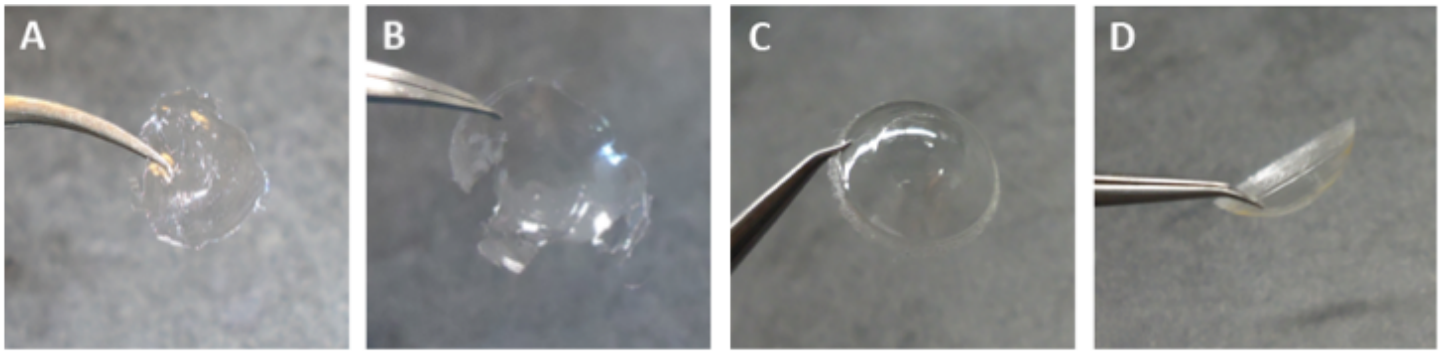
The dried curved film can then be easily removed by bending the silicone rubber mold and air-lifting the curved film from the casting surface with forceps (Lawrence, Cronin-Golomb, Georgakoudi, Kaplan, & Omenetto, 2008). The films that emerge are both curved in shape and are highly transparent. The process was found to be highly reproducible and silk film dimensions were easily controlled by optimizing the spin cast process parameters (i.e. air flow, RPM, and silk concentration).



Spin cast silk film showing the (A) curved cross-section, and (B) transparency from the en face viewpoint over black print lettering.

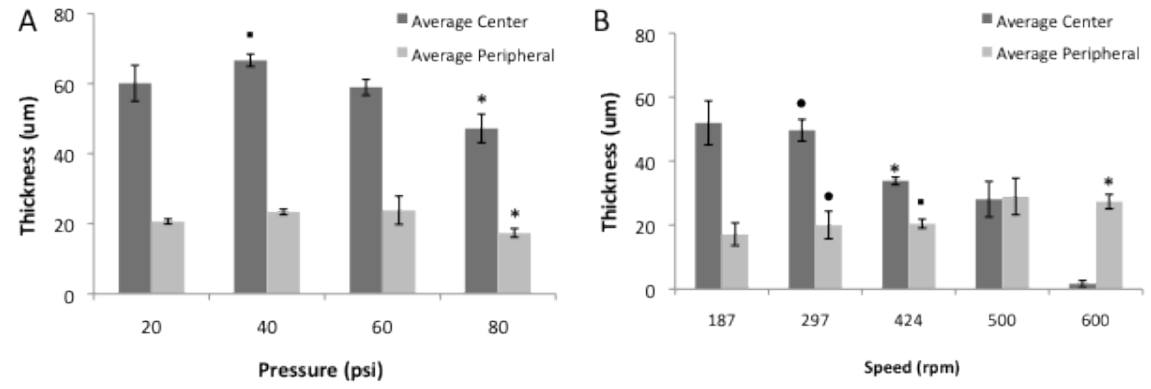
Factors affecting shaped silk film bandage design

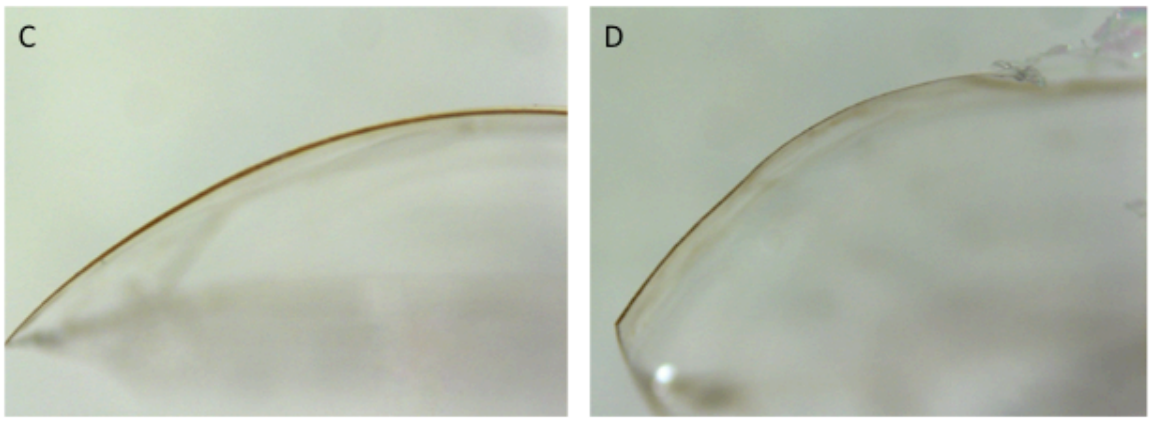
At present, manufacturing parameters are being studied to better understand how silk film shape and uniformity may be controlled for a desired material design. The concentration of the silk solution was determined to be an important factor in producing a uniform shaped silk film. It was found that solutions containing 4% silk had significant problems in wetting the silicone rubber surface during the spin cast process when compared to 8% silk solution, which produced a uniform and well shaped curved silk film bandage. This discrepancy in wetting is extremely evident in the final products, as the 4% silk solutions create irregularly shaped body in comparison to those made from 8% silk solution.



Silk films spin casted using (A)(B) 4% silk solution and those produced using (C)(D) 8% silk solution. Silk film bandages produced with 8% silk solution showed uniform film formation and curvature when compared to the reduced silk concentration, which did not form complete curved body.

The casting chamber was also vented with pressurized air to reduce silk solution drying time. It was shown that the introduction of air-flow reduced drying time from 180 minutes down to 90 minutes (50% reduction). The addition of the pressurized air showed significant effects on silk film bandage thickness uniformity , however the effect on drying rate was negligible in that as long as vented air was flowing through the system drying time was decreased. It was shown that the higher drying rate produced thinner silk film bandage thickness profiles when compared to various other air pressure settings. In addition, 20, 40, and 60 PSI showed minimal differences in the center thickness while more significant differences where observed in the periphery regions of the film. As a result of these studies 40 PSI was chosen as a spin casting pressure, due to the ability to reduce drying time and successful production of a uniform product.
More profound was the effect of rotations per minute (RPM) settings on silk film thickness uniformity. It was found that 500 RPM produced the most uniform center to periphery thickness when compared to other selected RPM settings. It was demonstrated that lower RPM settings would produce films with a thicker center thickness and a thinning periphery thickness, while films produced at higher RPM would have relatively thick peripheries and extremely thin, or even non-existent, center thickness. The results form these studies indicated that 500 RPM was the best setting for producing a uniform silk film bandage thickness.





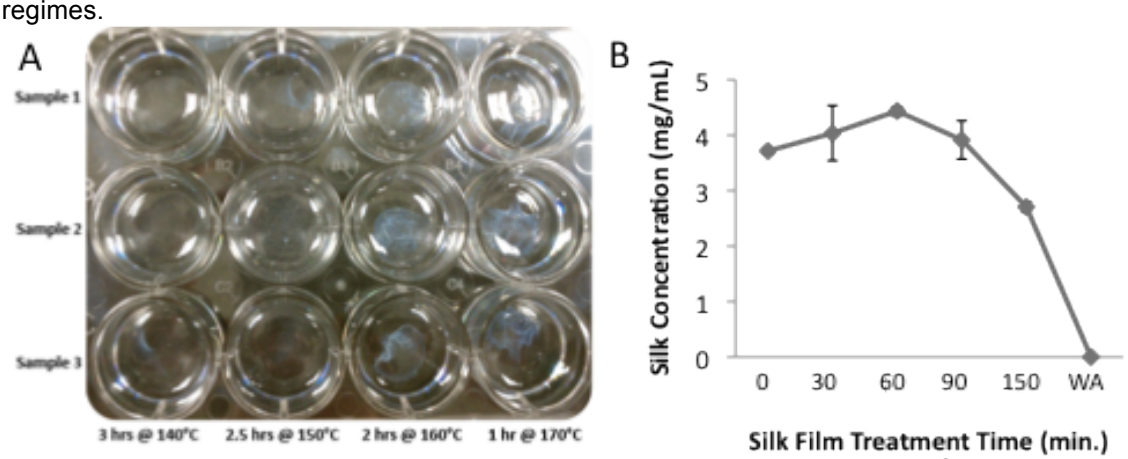
(A) Effects of chamber air pressure on spin casting of shaped silk film thickness for center and periphery areas (n = 4, error bars = standard deviation, * indicates $p < 0.05$ compared to 20, 40, & 60 psi. ▪ indicates $p < 0.05$ compared to 20 & 60 psi). (B) Effects of RPM speed of spin casting spindle on silk film bandage thickness for center and periphery areas (n = 4, error bars = standard deviation, * indicates $p < 0.05$ compared to all other speeds except 500 RPM in each group. ● indicates $p < 0.05$ when compared to all other speeds except 187 RPM in each group. ▪ indicates $p < 0.05$ compared to all

other speeds except 187 and 297 RPM in each group). Thickness profile images of silk film bandages produced at (C) 297 RPM and (D) 600 RPM demonstrating thickness uniformity differences between two spin rates.

Controlling silk dissolution rate utilizing heat-annealing

Silk casting produces a soluble film material form, which rapidly adheres and dissolves on the cornea surface post application (Kim, et al., 2010). Without further processing the silk film bandage dissolves on the order of minutes once applied to the cornea. This relatively quick dissolution time was found to have a limited effect on enhancing the regenerative capacity of the cornea. In order to lengthen silk film bandage residence time upon the cornea post application, silk film bandages were being treated using a water-annealing process, which involves placing the films within a vacuum in the presence of water vapor to allow protein secondary structures changes to take place and produce a more insoluble material (Jin, et al., 2005). However, once the material becomes overly processed the silk film will no long dissolve or adhere to the cornea surface. This is undesirable as non-dissolved films have a negative effect on cornea healing. Contrary to what had been predicted in the proposal, water-anneal processing was found to be highly uncontrollable when attempting to produce a uniform silk film bandage dissolution profile.

As an alternative to water-annealing, the use of heat-annealing was explored. Heat-annealing is the use of a dry heat environment (i.e. dry heat sterilizing oven) to induce protein secondary structure changes over time, which was shown to increase silk bandage dissolution time (Hu, Kaplan, & Cebe, 2007). Additionally, heat-annealing has the added benefit of sterilizing the material while simultaneously processing the silk bandage to increase dissolution time, thus simplifying the manufacturing process by combing two processing steps together. It was found that silk film dissolution time could be readily varied using a range of FDA recommended sterilization temperature and time ranges. For example, a silk film heated for 3 hours at 140°C would dissolve within a 10 second interval within water, while a silk film heated at 170°C for 1 hour would remain mostly non-dissolved after overnight incubation in water . In addition, silk film dissolution could be quantified using the bicinchoninic acid (BCA) protein content assay. The processed silk film bandages were placed in 1 mL of water, allowed to dissolve for 10 minutes, and then sampled for protein content. Results for the assay indicated that silk film bandage dissolution could be readily modified based on the length of heat-annealing time. Further investigation is underway to better understand how to control these profound material modifications to produce a variety of dissolution regimes.



Increasing heat-annealing time and temperature increases dissolution rate. (A) Qualitative results indicate that films pictured on the left have dissolved in 1 mL of water after 24 hours in solution, while those on the right have not dissolved to as great an extent that is represented by the remaining residual material. (B) Quantitative assessment of silk film bandage protein dissolution using the BCA assay indicate less silk protein dissolved in solution after greater heating time at a 165°C temperature.

Task 4. Puncture Wound Assessment

Work has continued in ex vivo porcine eyes to determine the most relevant models for this Task. We have identified factors that made the adherence of a flat film difficult and as described above have now constructed bandages with the appropriate curvature and with improved biomaterial properties.

Task 5. GMP Production of a Regenerative Lens

We have partnered with the GMP Facility at Weill Cornell Medical College to produce GMP grade lenses for further use and testing of the silk ocular bandages. Extensive interaction between Sarentis Ophthalmics and this Core Facility is leading to the development of SOPs for the creation of the requisite silk bandages. The process for extracting the silk proteins as well as the purification of the protein is well underway. Suitability od scale-up procedures is being evaluated. Final GMP production is pending final optimization of the biomaterials and design of the bandage.

Problem Areas

As described above, we have had some issues related to optimization of the bandage dissolution and shape. The above data demonstrates a systematic approach to better understanding the material properties of silk films and for creation of a

curved bandage. We have demonstrated the feasibility of creating a transparent curved bandage of uniform thickness and have more limited experiments remaining to optimize the dissolution properties of the films.

Work to be performed.
Task 1. This task has been essentially completed. GMP material will be analyzed a final time for safety.
Task 2. The optimization of the material properties of the lens is nearing completion. We anticipate significantly greater evaluation of the use of the films in the burn model one the design has been "frozen".

Task 3. Optimized curved bandages will be evaluated in the abrasion model and we would anticipate completion of abrasion model testing during the next period. We will have a more quantitative understanding of the factors which improve the regenerative properties of the bandage.

Task 4. We will utilize the optimized bandages on the models we have devised for ex vivo and in vitro testing. This will allow us to determine the resistance to bursting and regenerative role of these bandages following injury.

Task 5. We would anticipate that the design for the bandage will be completed during the next period and that this will allow us to develop the final SOPs for use by the GMP facility. This will allow for production to likely begin during the end of the next period.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of a novel silk-based film capable of supporting ocular regeneration after injury.
- Characterized the salient material properties of silk film bandages to promote corneal repair.
- Identified heat annealing as a new method to control silk film dissolution.
- Identified parameters for silk film dissolution which optimizes corneal healing.
- Identified film curvature as critical for proper bandage adherence and designed a novel method to produce curved silk films which have better apposition to the corneal surface.
- Have begun to create the requisite GMP processes to fabricate films suitable for human use.

REPORTABLE OUTCOMES

- Designed a novel method to produce curved silk films which have better apposition to the corneal surface.
- Identified heat annealing as a new method to control silk film dissolution.

CONCLUSION

Ocular trauma from blast injuries is a common and serious condition. There is a critical need to develop materials which could be used on the battlefield to treat these injuries. We have identified thin silk film biomaterials which may be fabricated to allow for stabilization and healing of these injuries in the acute setting. These films appear to be quite stable and suitable to use as a filed dressing for ocular injuries prior to definitive treatment in a more elaborate ophthalmic care setting.

Silk biomaterials have been developed as medical devices in a number of clinical settings. We have identified critical parameters, including optimal curvature and dissolution rate, for the successful use of these silk bandages using animal models of ocular injury. Moreover, we have developed novel methods to impart these characteristics on the silk film bandages. Novel heating strategies can both sterilize and anneal the films to control dissolution rate. We have adapted spin-casting techniques for use in silk film fabrication to create films with optimized curvature to adhere to the cornea in an appropriate manner. We are now developing the GMP protocols to allow for rapid and accurate fabrication of these optimized silk film bandages for possible use in human trials.

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APPENDICES

None

SUPPORTING DATA

Included in the body of the report.