REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
June 10, 2010	Final Report	02/01/2007 - 01/31/2010

4. TITLE AND SUBTITLE

Functionalized Silk Materials

5a. CONTRACT NUMBER

5b. GRANT NUMBER

FA9550-07-1-0079

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

David L. Kaplan

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Tufts University
4 Colby Street
Medford, MA 02155

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

AFOSR
875 N. Randolph St
Arlington, VA 22203

10. SPONSOR/MONITOR'S ACRONYM(S)

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

AFRL-SR-AR-TR-10-0259

12. DISTRIBUTION/AVAILABILITY STATEMENT

A = Approved for public release; distribution is unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The significance of the project is that a new family of functional materials and coatings derived from the silk systems will be generated, with the silk component serving as the organizing moiety and the functional domains added to the silk providing enhanced properties for the materials – thus, light weight materials, electronic properties and related features are anticipated through the precise control of functional domains within and on the silk well-defined material templates. As examples, light weight strong porous matrices formed from silks and silk composites, by exploitation of silk gelation features will be pursued. A next generation set of robust materials for a range of functional applications of potential relevance to Air Force needs is envisioned with the technology under study.

15. SUBJECT TERMS

silk, materials, gelation, self-assembly, processing

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			David L. Kaplan
					19b. TELEPHONE NUMBER *(Include area code)* (617) 627 3251

AFOSR: Functionalized Silk Materials [FA9550-07-1-0079]

Abstract

The goal is to exploit the novel structural, physical and biological features of silk proteins towards functionalization of materials systems generated from this unique family of protein. A new generation of functional silk systems is sought to provide novel materials with precise control of material features. Three main directions are planned, building off of continuing insight into the self-assembly and structural control of silk-based materials: (a) protein chimeras to form organic (silk) – inorganic nanocomposites by extending our recent studies with silk-silica designs but to new variants such as additional components, (b) formation of electronic materials using a similar design strategy but based on enzymatic coupling reactions to form conducting polymers, and (c) continuing to understand and exploit novel processing approaches with these proteins towards new functional materials systems. The significance of the project is that a new family of functional materials and coatings derived from the silk systems will be generated, with the silk component serving as the organizing moiety and the functional domains added to the silk providing enhanced properties for the materials – thus, light weight materials, electronic properties and related features are anticipated through the precise control of functional domains within and on the silk well-defined material templates. As examples, light weight strong porous matrices formed from silks and silk composites, by exploitation of silk gelation features will be pursued. A next generation set of robust materials for a range of functional applications of potential relevance to Air Force needs is envisioned with the technology under study.

Objectives

(a) silk protein chimeras for new functions
Year 1 - design new silk chimeras to combine silk protein domains with silica forming domains or new metal binding domains
Year 2 - clone and express these new silk variants, purify and characterize the recombinant proteins
Year 3 - characterize functional features of the new systems

(b) formation of electronic materials with silk
Year 1 - determine flexible electronic displays on silk
Year 2 - determine impact of electric fields on silk assembly
Year 3 - explore tyrosine coupling reactions to generate conductive silk systems

(c) exploit novel processing approaches for silk
Year 1 – PEGylation of silks to alter surface wetting
Year 2 – sonication induced silk assembly
Year 3 - determine control of surface patterning on silk for optical materials

Findings

(a) Silk protein chimeras for new functions:

Year 1 - design new chimeras to combine silk domains with other silica forming domains and new metal binding domains

New variations in the functional silk designs were prepared by bioengineering, with variants void of purification tags to allow better interpretation of structure-assembly relationships related to materials function, and new insight into self-assembly. By incorporating peptide sequences identified by phage display into silk, new materials which incorporate mineral binding functional of the peptide while retaining the useful functional properties of the silk are attained. A family of fusion proteins with silk and metal binding peptides was prepared via genetic engineering (Figure 1). The various structures were

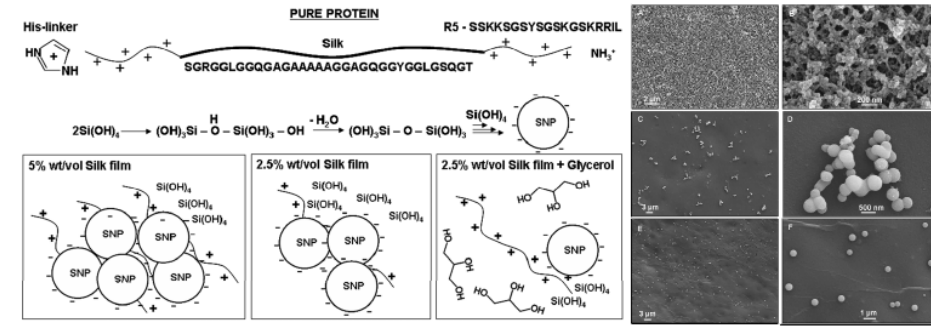

Figure 1. Silica nucleation and particle formation. Left side – mechanisms with and without glycerol present. Right side – SEM images of the differences in morphologies generated. Manuscript in review.

studied in solution and on surfaces with respect to metallization. In addition, ambient conditions were used for the control of silica morphology and distribution on the surface of silk films utilizing genetically engineered chimeric proteins. A genetic combination of spider dragline silk sequence (*Nephila clavipes*) and the silaffin derived R5 peptide of the diatom (*Cylindrotheca fusiformis*) led to the bioinspired synthesis of 3D porous silica networks, clustered silica nanoparticles (SNPs), or single/isolated SNPs. We anticipate that these novel silica-based biomaterials will have widespread applications due to the ability to regulate the location and features of silica. The silk component serves as an organic scaffold that controls material stability and allows multiple modes of processing. Si-derived nanostructures with strong morphological and spatial control are attractive in electronics, biosensors, microfluidic devices, and DNA microarray technology. The novelty in material design also allows for applications in biodopants and protein-silica nanocomposites. Different mineral phase loading and morphologies can be tailored synthetically, thus altering the biomaterial properties.

Year 2 - clone and express these new silk variants, purify and characterize the recombinant proteins

The design, construction, and characterization of a novel family of spider silk-like block copolymers was described. The design was based on the assembly of individual spider silk modules, in particular poly-alanine (A) and glycine-rich (B) blocks, that display different phase behavior in aqueous solution. Spider silk was chosen as a model for these block copolymer studies based on its extraordinary material properties, such as toughness, biocompatibility and biodegrability. Trends in spider silk-like block copolymer secondary structure and assembly behavior into specific material morphologies were determined as a function of the number of hydrophobic blocks, the presence of a hydrophilic purification tag and solvent effects. Structures and morphologies were assessed by Fourier Transform Infrared spectroscopy and Scanning Electron Microscopy. In terms of structure, β-sheet content increased with an increase in the number of poly-alanine blocks and the purification tag had significant impact on the secondary structure. In terms of morphology, spheres, rod-like structures, bowl-shaped micelles, and giant compound micelles were observed and the morphologies were linked with the size of the hydrophobic block, the presence of the purification tag and solvent environment. This study provides a basis for future designs of smart biomaterials based on spider silk chemistries, with controlled structure-architecture-function relationships.

Year 3 - characterize functional features of the new systems

Genetically Engineered Chimeric Silk/Metal Binding Proteins **–** Similar to the above systems, fusion proteins with metal binding domains and silks were prepared and studied. As an example, fusion proteins for titanium interactions were studied for function in controlling interactions with the metal phase. Various silk chemistries were compared for outcomes as shown in Figure 2, with some conditions demonstrating improved precipitation of titanium for functional outcomes. These would be the lead chemistries to pursue for scale up. The reaction conditions included TiO_2 from Ti[BALDH] in water, 1 mg/ml protein, with the peptides Ti1: QPYLFATDSLIK and Ti2: GHTHYHAVRTQT. The peptides

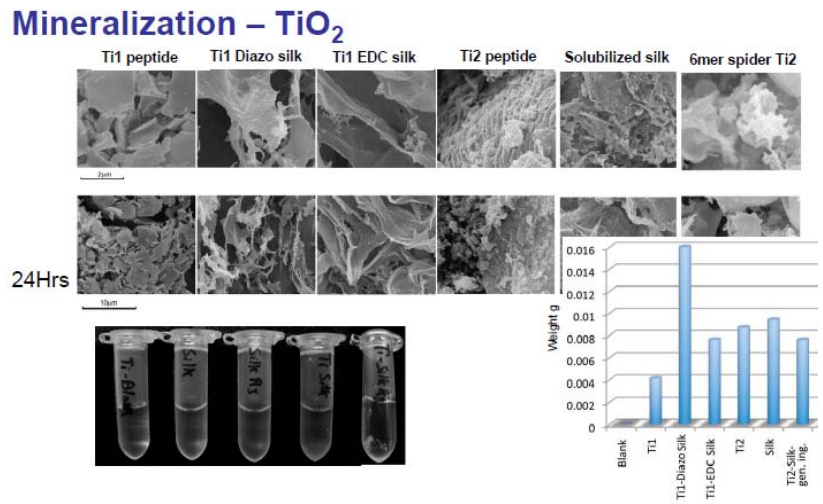

Figure 2. TiO2 formation from silk-peptide chimeras designed from phage display titanium binding sequences. Various reactions are shown based on modifications in silk chemistry along with the genetic variants. Precipitation of metal can be seen in the sample on the right in the bottom panel of Eppendorf tubes with quantitation on the right figure.

were determined by the Air Force Labs through phage display, and were fused to the silk at the genetic level, followed by expression, purification and then functional studies as shown in the Figure 2.

Silk Degradation – the studies on the synthesis of new silk proteins also led to detailed characterization of the degradation mechanisms for these proteins, to fully understand the materials lifecycle (Figure 3).

(b) <u>formation of electronic materials with silk</u>

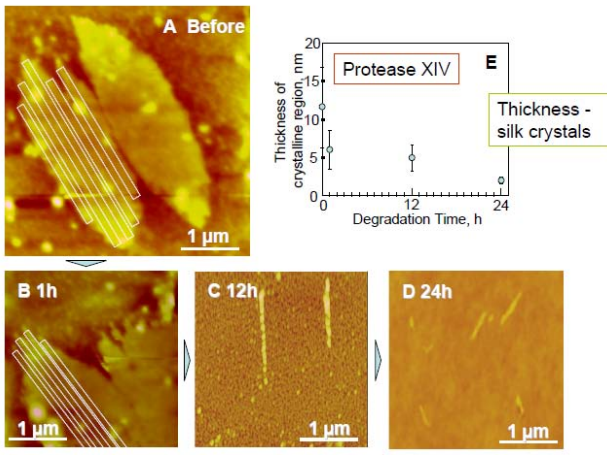

Figure 3. Enzymatic degradation of silk beta sheet crystals using protease XIV. Slow erosion of the crystals is evident by AFM images with time, as well as by measuring thickness in of the crstals as shown in the insert.

<u>Year 1 - determine flexible electronic displays on silk</u>

We developed unconventional material processing and device fabrication procedures by combining silk and with silicon nanomaterials, to generate new flexible electronic devices (Figure 4). A technology of this type could open various possible applications for insertion of high performance flexible electronics into implantable devices. For the fabrication process, single crystalline nanomembranes of silicon were used to construct transistors on ultrathin sheets of polyimide. Briefly, the doped silicon nanomembranes were transfer printed onto a film of polyimide and then cast onto a thin sacrificial layer of polymethylmethacrylate on a silicon wafer wafer for processing. After printing, a spin coating a layer of polyimide was used to encapsulate the active devices. Dry etching the polymer layers completed the fabrication of an array of isolated devices on PMMA, which was then dissolved with acetone to release the devices from the carrier wafer. These devices were lifted onto the surface of a transfer stamp of polydimethylsiloxane and then transfer printed to a spun cast film of silk on a silicon substrate or a freestanding silk membrane. A layer of silk served as an adhesive for transfer, which involves first establishing conformal contact with the silk substrate on a hot plate and then slowly retrieving the stamp.

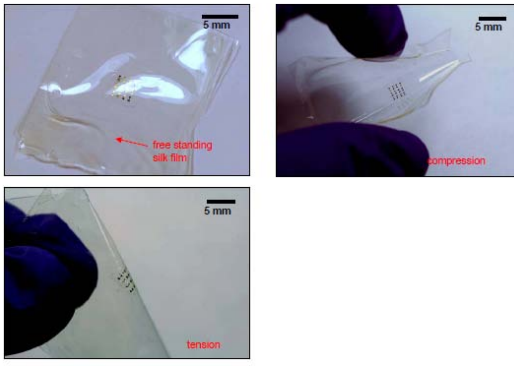

Figure 4. Flexible clear silk film with printed electrodes. Flexing the film does not dislodge the metal, thus the interface is strong.

Year 2 - determine impact of electric fields on silk assembly

We reported novel electrochemical behavior of silk proteins to generate adhesives. This biomimetic system demonstrates reversible materials properties and novel behavior on both hydrated and dry surfaces, suggesting a broad range of applications. The mechanisms and functional outcomes demonstrated with this silk electrogelation or egel process are not observed with other commonly studied polymers. We anticipate that this difference is due to the unique block copolymer structure of silk and dominant hydrophobocity, combined with the polymorphic behavior of the protein. The system functions when silk solutions are placed in a low voltage direct current system, where the gel forms at the positive electrode. The process appears to be due to a combination of local changes in pH due to electrolysis, combined with conformational changes in the protein with an increase in helical content. There is no beta sheet formed under these conditions, thus the reversibility of the process. Upon reversing the poles, the gel dissipates from the original positive electrode and now forms at the new positive electrode. If the voltages is increased, then the gel can be induced to form beta sheets and lock in the structure.

Year 3 - explore tyrosine coupling reactions to generate conductive silk systems

We conducted preliminary reactions between silk films and tryrosines as a mode to functionalize the silk surface for conducting polymer features. The reactions were conducted with peroxidase enzymes to carry out a free radical coupling reaction. However, the consistency in film properties was poor and we need to improve the control of the surface chemistry. One approach we have begun to pursue is to embed the enzyme within the film, to be able to control the surface reactions better. In addition we have conducted a series of studies related to the impact of electric fields on silk properties. First, we have pursued questions of breakdown voltage silk thin films, where preliminary data suggested about 1 GV/m. These efforts are continuing in collaboration with the Air Force Materials lab to refine the measurements and determine more accurate numbers.

(c) exploit novel processing approaches with silk

Year 1 – PEGylation of silks to alter surface wetting

Silk fibroin film surfaces were PEGylated by reaction with cyanuric chloride-activated poly (ethylene glycol) (PEG). Reactions with different concentrations of activated PEG generated films with PEG graft densities from 0.02 to 0.4 mg per square cm of silk fibroin. Increased PEGylation resulted in increased hydrophilicity as analyzed by contact angle, and a smoother morphology based on scanning electron microscopy (SEM). Increased PEGylation decreased protein adsorption and decreased the attachment and proliferation of human fibroblasts over two weeks. Increased concentration of PEG on the silk fibroin surfaces also decreased the proliferation of cells. Surface PEGylated silk fibroin films could be useful anti-adhesion materials for many applications when considered along with the unique mechanical and tailorable degradation profiles of silk fibroin.

Year 2 – Sonication induced silk assembly

Purified native silk fibroin forms b-sheet-rich, physically cross-linked, hydrogels from aqueous solution, in a process influenced by environmental parameters. Previously we reported gelation times of days to weeks for aqueous native silk protein solutions, with high ionic strength and temperature and low pH responsible for increasing gelation kinetics. We reported a novel method to accelerate the process and control silk fibroin gelation through ultrasonication (Figure 5). Depending on the sonication parameters, including power output and time, along with silk fibroin concentration, gelation could be controlled from minutes to hours, allowing the post-sonication addition of cells prior to final gel setting. Mechanistically, ultrasonication initiated the formation of b-sheets by alteration in hydrophobic hydration, thus accelerating the formation of physical cross-links responsible for gel stabilization. K+ at physiological concentrations and low pH promoted gelation, which was not observed in the presence of Ca2+. The hydrogels were assessed for mechanical properties and proteolytic degradation; reported values matched or exceeded other cell-encapsulating gel material systems. Cells were successfully incorporated into

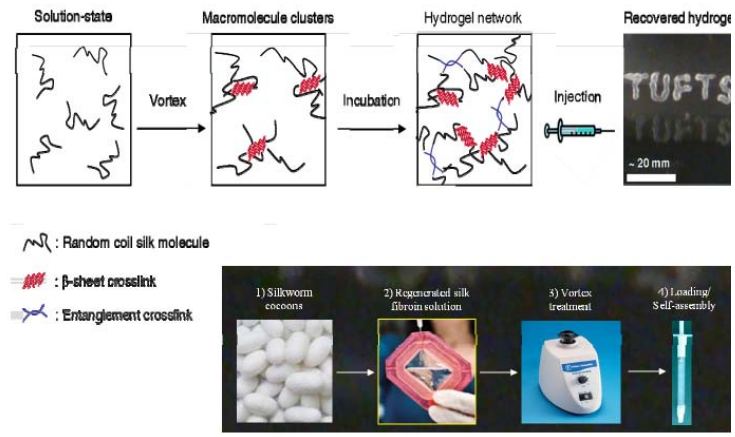

Figure 5. Sonication-induced assembly of silk into gels.

these silk fibroin hydrogels after sonication, followed by rapid gelation and sustained cell function. Sonicated silk fibroin solutions at 4%, 8%, and 12% (w/v), followed by mixing in cells, gelled within 0.5–2 h. The cells grew and proliferated in the 4% gels over 21 days, while survival was lower in the gels with higher protein content. Thus, sonication provides a useful new tool with which to initiate rapid sol–gel transitions, such as for cell encapsulation.

Year 3 - control of surface patterning on silk for new optical materials

We optimized the all aqueous processing of silk fibroin into novel surface nanopatterned protein materials. We exploited control of this nanomorphology to optimize the optical features of these silk protein systems. We demonstrated control of surface morphology down to 125 nanometers with fidelity over large length scales. This surface nanopatterning allows the silk protein to be formed into diffractive optics such as diffraction gratings, pattern generators and lenses, due to novel aqueous processing into optically clear materials via control of beta sheet crystallinity. Further, we incorporate biological components, such as hemoglobin and the enzyme peroxidase, during the process of forming the silk diffraction gratings. The ambient processing of the silk protein in water, in combination with these bioactive components, allows these entrained molecules to retain activity and provide added functions and selectivity to the optically active silk films. Thus, combinations of biochemical and optical readout is feasible and provides in a single, disposable/all degradable element with both spectral discrimination and biological function. These new surface nanopatterned, bioactive silk protein-based material systems offer a unique combination of features potentially useful for a range of biosensor needs, particularly when considered in concert with the remarkable mechanical properties of these proteins, their biocompatibility and controllable biodegradation. An example of nanoimprinting of silk films is shown in Figure 6.

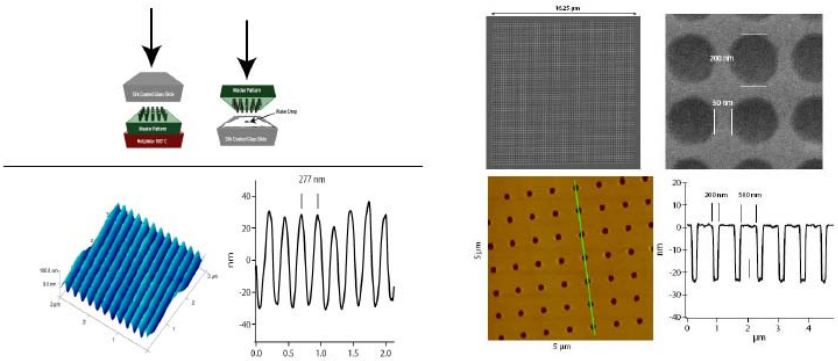

Figure 6. Nanoimprinting of silk films. Left side- heating or room temperature nanoimprinting with images shown at the bottom. Right side – images of nanoimprints and AFM measures to show high fidelity and pattern

Personnel

Heather Currie, post doctoral fellow
Michael Curtis, PhD student
Sreevidhya Krishnaji, PhD student
Gary Leisk, research faculty, mechanical engineering
Fiorenzo Omenetto, faculty, biomedical engineering
David L. Kaplan, faculty, biomedical engineering

Collaborations

Carole Perry, Nottingham Trent University, chemistry
Vladimir Tsukuk, Geogia Tech, materials characterization
Rajesh Naik, Air Force Materials lab, materials preparation and characterization
James Grote, Air Force Materials lab, electronic properties of silk materials

Publications

2007

Bettinger, C.; Cyr, KM.; Matsumoto, A.; Langer, R.; Borenstein, JT.; Kaplan, DL. (2007). Silk fibroin microfluidic devices. Advanced Materials 19:2847-2850.

Georgakoudi, I.; Tsai, I.; Greiner, C.; Wong, C.; DeFelice, J.; Kaplan, DL. (2007). Intrinsic fluorescence changes associated with the conformational state of silk fibroin in biomaterial matrices. Optics Express 15(3):1043-1052.

Hu, X.; Kaplan, DL.; Cebe, P. (2007). Effect of water on the thermal properties of silk fibroin. Thermochimica Acta. 461(1-2): 137-144.

Jiang, C.; Wang, X.; Gunawidjaja, R.; Lin, Y-H.; Gupta, MK.; Kaplan, DL.; Naik, RR.; Tsukruk, VV. (2007). Mechanical properties of robust ultrathin silk fibroin films. Advanced Functional Materials 17:2229-2237.

Tilburey, GE.; Patwardham, SV.; Huang, J.; Kaplan, DL.; Perry, CC. (2007). Are hydroxyl-containing biomolecules important in biosilicification? A model study. J. Physical Chemistry 111:4630-4638.

Vepari, C.; and Kaplan, DL. (2007). Silk as a biomaterial. Progress in Polymer Science, 32(8-9):991-1007.

2008

Gupta, S.; Hunter, M.; Cebe, P.; Levitt, JM.; Kaplan, DL.; Georgakoudi, I. (2008). Non-invasive optical characterization of biomaterial mineralization. Biomaterials 29:2359-2369.

Lawrence, BD.; Cronin-Golomb, M.; Georgakoudi, I.; Kaplan, DL.; Omenetto, FG. (2008). Bioactive silk protein biomaterial systems for optical devices. Biomacromolecules 9:1214-1220.

Matsumoto, A.; Lindsay, A.; Abedian, B.; Kaplan, DL. (2008). Silk fibroin solution properties related to assembly and structure. Macromolecular Bioscience. 8:975-977.

Murphy, AR.;St. John, P.; Kaplan, DL. (2008). Modification of silk using diazonium coupling chemistry and the effects on hMSC proliferation and differentiation. Biomaterials 29:2829-2838.

Omenetto, FG.; and Kaplan, DL. (2008). A new route for silk. Nature Photonics 2:641-643.

Perry, H.; Gopinath, A.; Kaplan, DL.; Negro, LD.; Omenetto, FG. (2008). Nano- and micropatterning of optically transparent, mechanically robust, biocompatible silk fibroin films. Advanced Materials. 20:3070-3072.

Rice, WL.; Firdous, S.; Gupta, S.; Hunter, M.; Foo, CW.; Wang, Y.; Kim, HJ.; Kaplan, DL.; Georgakoudi, I. (2008). Non-invasive characterization of structure and morphology of silk fibroin biomaterials using nonlinear microscopy. Biomaterials 29: 2015-2024.

2009

Amsden, J. J., H. Perry, S. V. Boriskina, A. Gopinahth, D. L. Kaplan, L. Dal Negro, F. G. Omenetto. (2009) Spectral analysis of induced color change on periodically nanopatterned silk films. Optics Express 17(23):21271-21279 [PMID 19997366]

Domachuk, P., H. Perry, J. J. Amsden, D. L. Kaplan, F. G. Omenetto. (2009) Bioactive self-sensing optical systems. Applied Physics Letters 95(25):253702 Epub 2009 Dec 22 [PMID 20087427]

Gupta, S.; Hunter, M.; Kaplan, D.; Georgakoudi, I. (2009). Optical characterization of the nano-scale organization of mineral deposits on silk films. Applied Optics, 48(10):D45-D51.

Lawrence, B.; Omenetto, FG.; Kaplan, DL. (2009). Processing methods to control silk fibroin film biomaterial features. Journal of Materials Science.
Lu, S., X. Wang, Q. Lu, X. Hu, N. Uppal, F. G. Omenetto, D. L. Kaplan (2009) Stabilization of enzymes in silk films. Biomacromolecules 10(5):1032-1042 [PMID 19323497]
Murphy, A., D. L. Kaplan. (2009). Biomedical applications of chemically modified silk fibroin. Journal of Materials Chemistry, 19(36):6443-6450 [PMID 20161439]
Rabotyagova, OS.; Cebe, P.; Kaplan, DL. (2009). Self-assembly of genetically engineered spider silk block copolymers. Biomacromolecules, 10(2):229-236 [PMID 19128057]
Vepari, C., D. Matheson, L. Drummy, R. Naik, D. L. Kaplan. (2009) Surface modification of silk fibroin with poly(ethylene glycol) for anti-adhesion and antithrombotic applications. J. Biomedical Materials Research. Jul 9 Epub ahead of print [PMID 19591236]
Yucel, T., N. Kojic, G. G. Leisk, T. J. Lo, D. L. Kaplan (2009) Non-equilibrium silk fibroin adhesives. Journal Structural Biology Dec 21 Epub ahead of print [PMID 20026216]

2010
Amsden, J. J., P. Domachuk, A. Gopinath, R. D. White, L. D. Negro, D. L. Kaplan, F. G. Omenetto. 2010. Rapid nanoimprinting of silk fibroin films for biophotonic applications. Advanced Materials 22(15):1746-1749 [PMID: 20496408]
Domachuk, P., K. Tsioris, F. G. Omenetto, D. L. Kaplan. 2010. Bio-microfluidics: biomaterials and biomimetic designs. Advanced Materials 22(2):249-260 [PMID: 20217636]
Lawrence, B., D., S. Wharram, J. A. Kluge, G. G. Leisk, F. G. Omenetto, M. I. Rosenblatt, D. L. Kaplan. 2010. Effect of hydration on silk film materials properties. Macromolecular Bioscience 10(4):393-403 [PMID: 20112237]
Leisk, G.G., T. Lo, T. Yucel, Q. Lu, D. L. Kaplan. 2010. Electrogelation for protein adhesives. Advanced Materials 22(6):711-7115 [PMID: 20217775]
Lu, Q., X. Wang, X. Hu, P. Cebe, F. Omenetto, D. L. Kaplan. 2010. Stabilization and release of enzymes from silk films Macromolecular Bioscience 10(4):359-368 [PMID: 20217856]
Rabotyagova, OS.; Cebe, P.; Kaplan, DL. (2010). Role of polyalanine domains in beta sheet formation in spider silk block copolymers. Macromolecular Bioscience 10(1):49-59 [PMID19890885]
Yucel, T., N. Kojic, G. G.l Leisk, T. J. Lo, D. L. Kaplan. 2010. Non-equilibrium silk fibroin adhesives. Journal Structural Biology 170(2):406-412 [PMID: 20026216]

Interactions/Transitions

Rajesh Naik - Air Force Materials Lab – joint group meetings and visits by researchers to promote synergy with the materials, functions and applications – has led to new directions in the effort towards optics and sensor systems with many of the applications continuing to be developed jointly

Holly Carpenter – clones and approaches for reflectin protein studies that evolved out of the program, hosting her student this summer in the lab to learn additional techniques

Reflectin Proteins – has spun off into a new AFOSR project