WAFER-SCALE HIGH-RESOLUTION PATTERNING OF BIOSTRUCTURES USING SILK LIGHT CHAIN PROTEIN PHOTOLITHOGRAPHY

Wanpeng Liu[1], Shaoqing Zhang[1], Woonsoo Lee[1], and Tiger H. Tao[1,2]
[1]Department of Mechanical Engineering, The University of Texas at Austin, Texas, USA
[2]The State Key Laboratory of Transducer Technology, Shanghai Institute of Microsystem and Information Technology, Chinese Academy of Sciences, China

ABSTRACT

We report on wafer-scale high resolution patterning of bio-microstructures using silk fibroin light chain (L-fibroin) as the photoresist material. The L-fibroin fragments with the well-defined molecular weight have been successfully isolated from the integral silk fibroins consisting both heavy and light chains. Under facile biochemical modification, the L-fibroin photoresist can be synthesized via conjugating commercial photocrosslinkers as crosslinking sites in the presence of ultraviolet light. The enhanced patterning resolution, the improved etching selectivity and the inherent biocompatibility of such protein-based photoresist provide opportunities in large scale biocompatible functional microstructures manufacturing.

INTRODUCTION

Precise spatial patterns and micro and nanostructures of peptides and proteins have widespread applications in tissue engineering, bioelectronics, photonics, and therapeutics. High resolution fabrication platforms using materials from biological precursors have been proposed as feasible routes not only for sustainable bionanomanufacturing, but also to form organic devices to interface and interact with biological systems [1, 2]. In this context, natural silk biopolymers provide green alternatives to synthetic materials with advantages of mechanical strength, optical properties, biocompatibility and controllable degradation.

Silk exists in a self-assembled fibrous configuration, in which a mechanically robust protein - fibroin (70%) comprises the core, surrounded by a glue protein - sericin (30%) [3]. Recently, exciting opportunities for silk have opened up a wide range of applications in photonics, implantable bioelectronics and nanostructured scaffolds, revealing the need for innovative approaches to multi-scale fabrication with manufacturing scalability.

To date, micro-architectures of silk protein have been formed using techniques such as imprinting, molding via soft-lithography, electrospinning, embossing, inkjet printing and photolithography. Among them, photolithography provides an option to rapidly and directly fabricate complex features with high fidelity and without the need for high temperature and pressure or molding masters [4]. Two strategies have been reported with indirect and direct methods to pattern proteins using photolithography [5]. The application of optical lithography to proteins has been relatively uncommon and primarily based on indirect methods in which proteins are attached to functionalized surfaces. Light-induced activation, deposition or passivation using different types of chemistry are used to form patterns, followed by covalent protein immobilization. Another method to directly pattern proteins inherently requires that the materials used be photoreactive. Examples include photoactivable derivatives of biotin, and elastin-like proteins with photoreactive, non-canonical amino acids incorporated via site-specific and residue-specific techniques. In particular, patterning of silk microstructures using UV-photolithography has been reported using chemically modified silk protein as the photoresist [6].

However, the silk proteins patterns, either silk fibroin or sericin, suffered the issues of low resolution and high line width roughness due to the inevitable wide molecular weight distribution (ranging from a few tens to a few hundreds of kDa) [7]. Such resolution and roughness limits hindered their practical uses in the semiconductor industry where reliability and repeatability are paramount. The silk fibroin is composed of two components, namely heavy chain (85%) and light chain (15%), which are linked by a disulfide bridge [8]. The light chain indicates comparatively more hydrophilic properties. It has well-defined molecular weight of 25 kDa and is relatively elastic with little or no crystallinity. In addition, light chain has a more undifferentiated and hydrophilic amino acid composition resulting in facile chemical modification [9]. Without losing the advantages of integral silk protein, the light chain can provide higher resolution, more mechanical stability, hydrophilic character, degradation, and cell adhesion than either the mixed system or the heavy chain alone.

EXPERIMENTAL METHODS

Fabrication of pure L-Fibroin: Silk fiber was weighed and dispersed in 98 − 100% formic acid at a range of concentrations (0.01 − 8% w/v) for 30 min and then centrifuged at 4,000 rpm for half an hour to sediment the undissolved material. The supernatant was filtered using glass fiber filters to remove any remaining suspended particles/ fibers. Then, the soluble fractions were left under a flow of air at room temperature to evaporate to constant weight.

Synthesis of photosensitive L-Fibroin (UV-LC): The L-fibroin photoresist was synthesized via chemical conjugation between L-fibroin and photocrosslinkers (2-Isocyanatoethyl methacrylate, IEM) in an anhydrous solvent of 1M LiCl in DMSO. L-fibroin was suspended at 1% (w/v) in a solution of 1M LiCl/DMSO and stirred at 65 ^{o}C in a dry N_2 atmosphere for 40 minutes. Immediately after, the IEM was added at a stoichiometric equivalent to reactive hydroxyl-containing amino acids and reacted for 5 hours at 65 ^{o}C. The product was precipitated out, centrifuged, washed and freeze-dried. For comparison, silk fibroins under 10, 30, 60, 90 minutes degumming

time, as well as, silk HTP (high temperature and pressure) have been prepared and synthesized to be photoreactive following the similar procedure.

s-SNOM: We utilized a commercially available scattering-type near-field microscope (s-SNOM, Neaspec GmbH, Germany) with a QCL IR laser (MIRCat, Daylight solutions Inc., USA) that is tunable between 1,495 and 1,790 cm^{-1}. During instrument operation, the laser was attenuated to ~10 mW such that the detector yields a nominal signal of 1.5 V. The AFM was operated in tapping mode with 65 nm tapping. Gold-coated AFM tips with about 250 kHz resonance (Tap300G-B-G, budgetsensors.com) were used to enhance the IR signal. The IR signal was detected simultaneously with AFM signals. The IR signal used for analysis in this work was measured by a lock-in amplifier at the second and third harmonics of the tapping frequency and the pseudo heterodyne technique, which provides both reflection and absorption that are (mostly) free of background. The image was scanned at 3.3 ms per pixel for a 500 × 500 pixel sized image.

RESULTS AND DISCUSSION

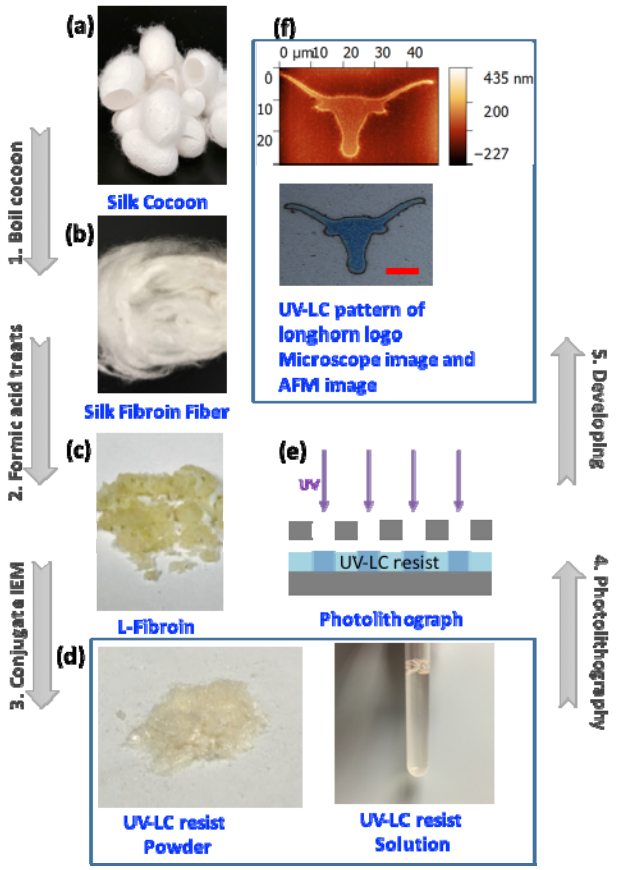

Figure 1: Synthesis of the UV sensitive silk fibroin light chain protein (i.e., UV-LC), subsequent photolithography process and patterning results (UV-silk30: UV sensitive silk fibroin with 30 minutes degumming time, consisting of both fibroin heavy and light chains. (a)-(c) Images of silk cocoon, silk fibroin fiber with sericin removed and silk L-Fibroin; (d) Images of UV-LC powder and solution after photocrosslinker conjugation; (e) Schematic of Silk UV-LC photolithography; (f) Microscope and AFM images.

In this study, silk fibroin was extracted from *Bombyx mori* silkworm cocoons. Then L-fibroin and H-fibroin fractions were separated based on formic acid solubility without protein degradation and at a scale and cost that makes the process amenable to scale up. After degumming, the silk fibroin was dissolved partially by repeat treatment with formic acid washes. The soluble fractions were separated and air-dried (Figure 1). The L-fibroin was modified via the reagent 2-isocyanatoethyl methacrylate (IEM) to yield a photocrosslinkable L-fibroin photoresist (UV-LC). Synthesized UV-LC was purified to separate out the insoluble product from water-miscible dimethyl sulfoxide (DMSO) solvent and soluble IEM oligomers. Micropatterns of silk fibroin on silicon and glass substrates were fabricated via photolithography using the UV-LC as a negative photoresist that is crosslinked in the presence of UV light. The protein precursor is spin coated on a substrate and exposure through a patterned mask results in crosslinking. Removal of the unexposed and uncrosslinked protein photoresist (development step) results in clearly defined micro-architectures. Patterns with thickness ranging from 80 nm to several micrometers can be easily fabricated in this manner.

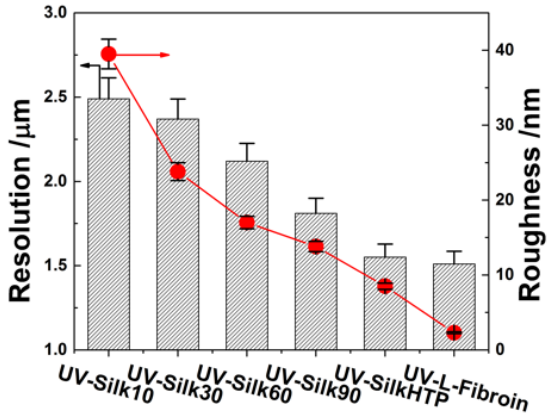

Figure 2: Patterning resolution and surface roughness of various silk protein photoresists.

The resolution and surface roughness of the patterns were observed directly and quantified via microscope and AFM. Figure 2 shows that under the same developing duration (30 min), the resolution of the as-prepared patterns increases with the increase of degumming time for the integrated silk fibroin protein, since the molecular weight decreased with increasing the degumming time. The resolution of UV-SilkHTP and UV-LC were the similar and the highest which reached the minimum feature size of the mask (1.5 μm). The surface roughness of UV-LC was less than that of UV-SilkHTP which was attributed to the well-defined molecular weight of L-Fibroin.

The conjugation of the multifunctional acrylate moiety on the purified product was verified via both FTIR and s-SNOM (Figure 3). In addition to peaks of amide I–III, preserved from native to photosensitive protein, the latter showed peaks at 1,720 cm^{-1} (C=O stretch), 1,635 cm^{-1} (terminal C=C stretch which confers photoreactivity) and 1,160 cm^{-1} (C–O stretch). After exposing under UV light, the peak intensity at 1,635 cm^{-1} will decrease with the exposure time due to photocrosslinking from terminal C=C group sites.

Figure 3(b) shows that before exposing 90s, the absorbance intensity of UV-Silk30 decreased quickly and linearly. After that, the rate is almost constant because the photocrosslnking degree of the IEM increase linearly with the exposure time before 90s. And after that, since the limitation of the active group site for the photocrosslinking, the rate keep constant with more time of exposure. The PP with decrease of molecular weight reached the constant intensity earlier comparing to the higher molecular weight, since the longer protein chain contains more IEM active group sites which provides the photocrosslinking function. Comparing with the integrated silk protein, the UV-LC possesses the most sensitivity to the UV light as shown in Figure 3(b).

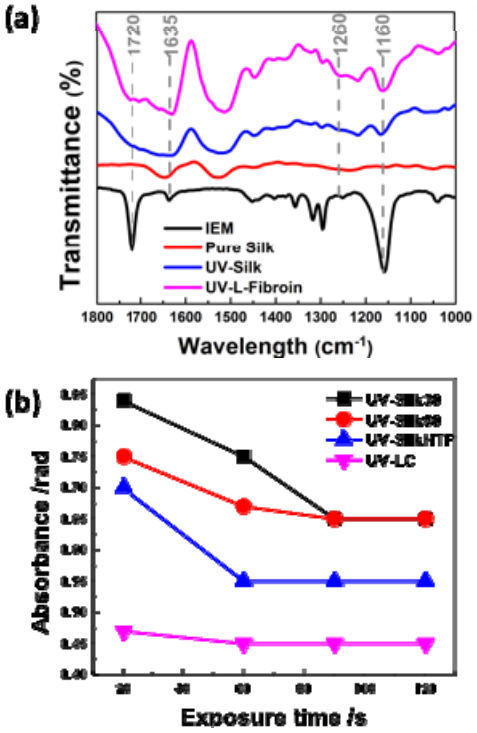

Figure 3: Characterization of the patterns Resolution and surface roughness of the patterns. (a) FTIR spectrum of IEM, pure silk, UV-silk and UV-LC; (b) Absorbance spectrum of UV-Silk30, UV-Silk90, UV-SilkHTP and UV-LC with various exposure time.

Figure 4 shows etching rate measurements of the patterns and the mechanism. The etching rate with different exposure time of UV-LC decreased faster than that of UV-Silk30 which match the results of s-SNOM, since the UV-LC is more sensitive to UV light (Figure 4(a)). The eventual etching rate of UV-LC is higher because of the less amount of active group on the chains. Three factors influence the etching rate of patterns: the secondary structure, the molecular weight, and the crosslinking degree. But the influence of secondary structure is weak comparing to others (Figure 4(b)). For the crosslinking degree, according to the comparison of blue and red line, with longer exposing time, the crosslinking degree is higher, resulting in lower etching rate.

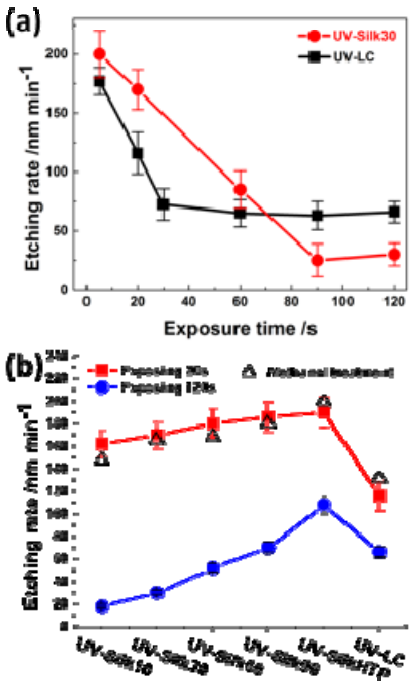

Figure 4: Etching rate measurements of the patterns and the mechanism. (a) Etching rate of the UV-silk30 and UV-LC patterns with increase of exposure time; (b) Etching rate of various silk protein photoresists.

CONCLUSION

We report on wafer-scale high resolution patterning of bio-microstructures using silk fibroin light chain (L-fibroin) as the photoresist material. The L-fibroin fragments with the well-defined molecular weight have been successfully isolated from the integral silk fibroins. Under facile biochemical modification, the L-fibroin photoresist was synthesized via conjugating commercial photocrosslinkers as crosslinking sites in the presence of UV light. The enhanced patterning resolution, the improved etching selectivity and the inherent biocompatibility of such protein-based photoresist provides opportunities in fabricating large scale biocompatible functional microstructures.

ACKNOWLEDGEMENTS

The work has been partially supported by the National Natural Science Foundation of China (Grant No. 61574156, No. 61527818), MOST of China (Grant No. 2016YFA0200800) and the Science and Technology Commission of Shanghai Municipality for the support under the International Collaboration Project (Grant No. 14520720400). We thank the NSF for support under the CMMI Division (Grant No. 1563422 & 1562915).

REFERENCES

[1] H. Tao, D. L. Kaplan, F. G. Omenetto, "Silk Materials – A Road to Sustainable High Technology" *Advanced Materials*, Vol. 24, pp. 2824-2837, 2012.

[2] F. G. Omenetto, D. L. Kaplan, "New Opportunities for an Ancient Material" *Science,* Vol. 329, pp. 528-531, 2010.

[3] B. B. Mandal, S. C. Kundu, "Cell Proliferation and

Migration in Silk Fibroin 3D Scaffolds" *Biomaterials,* Vol. 30, pp. 2956-2965, 2009.
[4] B. D. Gates, Q. B. Xu, M. Stewart, D. Ryan, C. G. Willson, G. M. Whitesides, "New Approaches to Nanofabrication: Molding, Printing, and Other Techniques" *Chemical Reviews,* Vol. 105, pp. 1171-1196, 2005.
[5] N. E. Kurland, T. Dey, C. Z. Wang, S. C. Kundu, V. K. Yadavalli, "Silk Protein Lithography as a Route to Fabricate Sericin Microarchitectures" *Advanced Materials,* Vol. 26, pp. 4431-4437, 2014.
[6] N. E. Kurland, T. Dey, S. C. Kundu, V. K. Yadavalli, "Precise Patterning of Silk Microstructures Using Photolithography" *Advanced Materials,* Vol. 25, pp. 6207-6212, 2013.
[7] M. S. Zafar, D. J. Belton, B. Hanby, D. L. Kaplan, C. C. Perry, "Functional Material Features of Bombyx mori Silk Light versus Heavy Chain Proteins" *Biomacromolecules,* Vol. 16, pp. 606-614, 2015.
[8] K. Tanaka, S. Inoue, S. Mizuno, "Hydrophobic interaction of P25, containing Asn-linked oligosaccharide chains, with the H-L complex of silk fibroin produced by *Bombyx mori*" *Insect Biochemistry and Molecular Biology,* Vol. 29, pp. 269-276, 1999.
[9] K. Tanakaa, N. Kajiyamaa, K. Ishikuraa, S. Wagaa, A. Kikuchia, K. Ohtomoa, T. Takagib, S. Mizuno, "Determination of the site of disulfide linkage between heavy and light chains of silk fibroin produced by *Bombyx mori*" *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology,* Vol. 1432, pp. 92-103, 1999.

CONTACT

*Tiger H. Tao, tel: +1-512-4756571;
tiger.tao@austin.utexas.edu