

# PRECISE CONTROL OF NATURAL AND SYNTHETIC SILK NANOSTRUCTURES USING ELECTRON BEAM LITHOGRAPHY

Nan Qin<sup>1</sup>, Shaoqing Zhang<sup>2</sup>, Jianjuan Jiang<sup>1</sup>, Zhitao Zhou<sup>1</sup>, Xiaoxia Xia<sup>3</sup>, Hu Tao<sup>1,2</sup>, and Keyin Liu<sup>1</sup>

<sup>1</sup>State Key Laboratory of Transducer Technology, Shanghai Institute of Microsystem and Information Technology, Chinese Academy of Sciences, Shanghai 200050, CHINA

<sup>2</sup>The University of Texas at Austin, Austin, TX 78712, USA

<sup>3</sup>Shanghai Jiao Tong University, Shanghai 200240, CHINA

## ABSTRACT

Silk protein fibers produced by silkworms and spiders are renowned for their unparalleled mechanical strength and extensibility as natural materials. We demonstrate the ability to finely control the structure of silk proteins (both naturally derived and genetically engineered) at the nanoscale. This fine control is obtained by careful water-based reverse engineering and synthesis of silk materials through self-assembly coupled with controlled, directed electron beam interaction with the protein's matrix. Genetically engineered recombinant spider silk proteins show considerably higher resolution thanks to the added control over the molecular weight of the proteins. This approach enables nanoscopic structures that push resolution approaching the molecular level, offering new rules to design protein-based architectures of unprecedented resolution.

## INTRODUCTION

Proteins, the elementary building blocks of biological materials, possess many unique properties that are of fundamental importance to modern technology. Silk has been heavily investigated because of its superior native mechanical properties (strength and toughness), biocompatibility and biodegradability, controllable water-solubility and degradation rate [1,2]. Precise spatial micro- and nano-structures of proteins have a wide range of applications in tissue engineering, bioelectronics, photonics, and therapeutics [3,4]. However, the ability to form proteins into complex nanostructures at high resolution has been challenging. Both top-down and bottom-up approaches have been proposed, such as nanoimprinting, soft lithography and inkjet printing. However, they are usually constrained in the formation of intricate architectures or require mold fabrication and multiple transfer steps.

Electron beam lithography (EBL) has been reported to precisely pattern silk fibroin proteins extracted from natural silkworm fibers with both negative and positive tones [5,6]. This builds on the ability to reshape silk with energetic electrons. In this work, multiple samples were prepared to elucidate the fundamental structural variations of silk by systematically exposing silk samples under different e-beam radiation dosages. We find that the nanolithographic performances of silk protein resists (including both the exposure sensitivity and contrast) highly depend on the material properties, such as the average molecular weight and the molecular weight distribution. In general, less energy is needed to crosslink long polymer chains with high molecular weights to form negative nanostructures, and in contrast, higher energy is

needed to "break" the bonds formed in long chains to form positive nanostructures.

## DESIGN AND FABRICATION

Silkworm silk proteins and recombinant spider silk proteins were prepared using the established purification and gene engineering protocols reported in [7] and [8], respectively. In brief, *Bombyx mori* cocoons were boiled for 30 min, 60 min and 120 min in an aqueous solution of 0.02 M sodium carbonate (Sigma-Aldrich, USA) respectively and then rinsed thoroughly with distilled water to remove the sericin proteins. The HTP silk was prepared at 121 °C and 15 psi for 3 h (HTP: high temperature and pressure). The extracted silk fibroin was dissolved in 9.3 M lithium bromide (Sigma-Aldrich, USA) solution at 60 °C for 4 h. The solution was dialyzed against distilled water using Slide-a-Lyzer dialysis cassettes (molecular weight cutoff (MWCO) 3500, Pierce) at room temperature for 48 h to remove the lithium bromide. The dialysate was centrifuged three times, each at 4 °C for 20 min, to remove impurities. Finally, the 6-8% (w/v) silk fibroin proteins solution was collected.

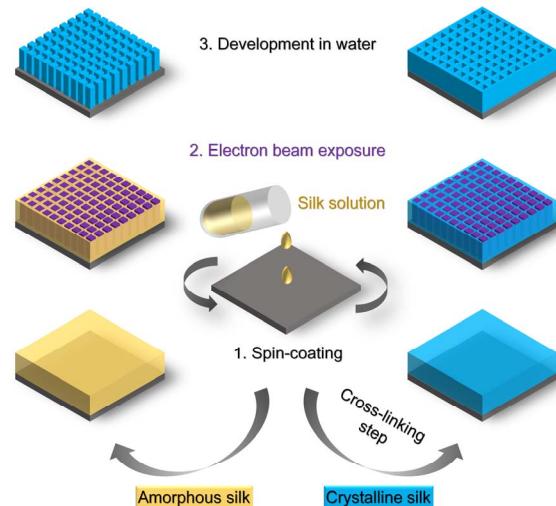


Figure 1: Illustration of precise control of silk nanostructures using EBL, redrawn from [5].

As shown in Figure 1, the silk solution was spin-coated on silicon substrates. Film thickness can be controlled by the spin speed and the concentration of the silk solution. In our case, 150-nm-thick silk films were prepared by spin-coating 5% silk solution at a maximum speed of 5,000 r.p.m. for 45 s. The samples were baked on a hotplate at 120 °C for 20 min to dry off the residual water. Crosslinking (i.e., crystallization) of the film was obtained by dipping it in methanol for 5 min or water-annealing in a water vapor filled chamber for 24 h. An EBL system

(Hitachi-4800) was used to expose the silk protein thin films with electron doses varied from 0 to 6,000  $\mu\text{C}/\text{cm}^2$  at 25 keV with a probe current of 10 pA. The solubility of as-exposed silk proteins in water depends on their conformational structures, regulated by the applied electron beam radiation. Amorphous silk proteins (i.e., water-soluble) will be folded to form the intermolecular crosslinks, which make the silk protein water-insoluble. In contrast, the inelastic collision of electrons with crystalline (water-insoluble) silk proteins results in the degradation of the protein chains to form the short polypeptides that are water-soluble and therefore the areas exposed to the electron beam will be washed away during the following water development process.

## RESULTS AND DISCUSSION

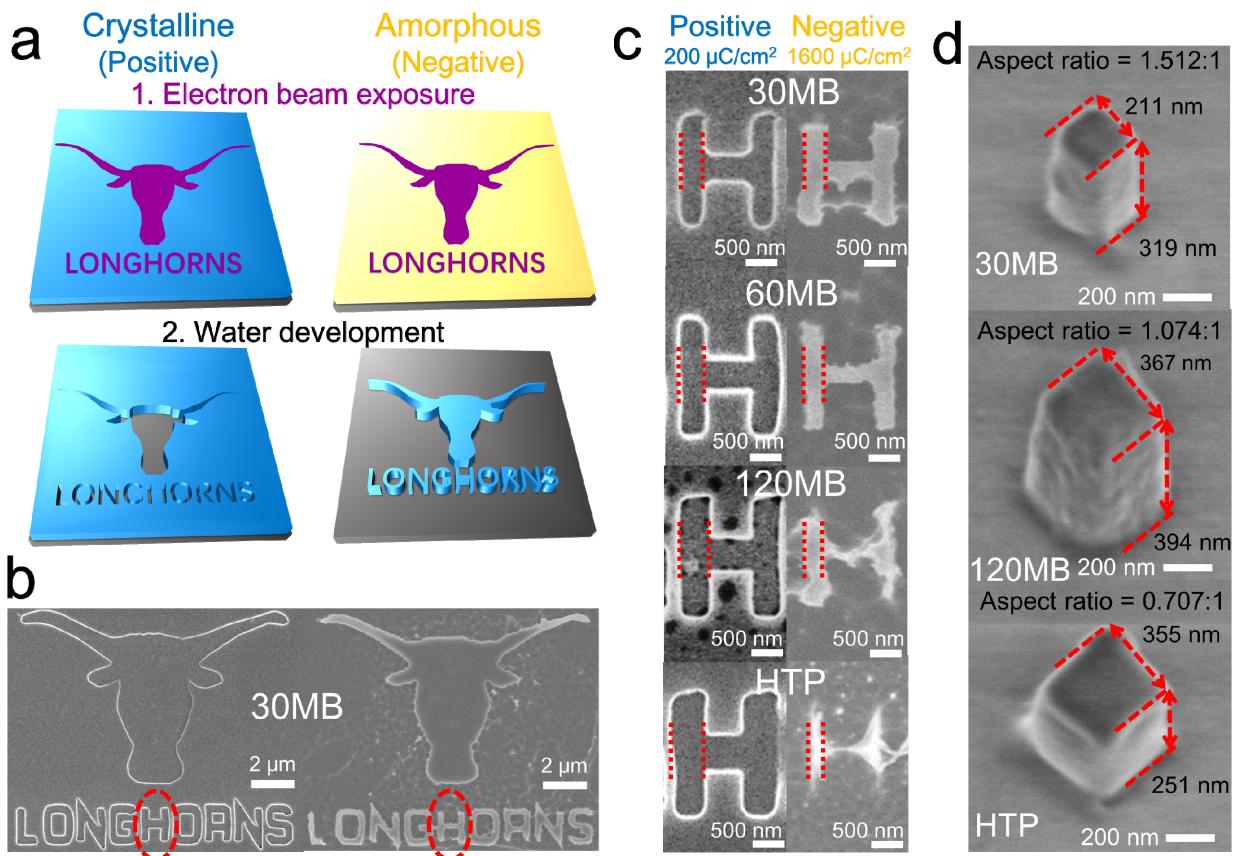
Figure 2 shows the designed and resulting patterns in silk after electron beam exposure and water development (MB: minutes-boiled). We found that the sensitivity of silk fibroin resists increased with the increased boiling time during the extraction process in the positive tone and decreased in the negative tone, as shown in Figure 2c.

The designed feature size (i.e. the linewidth) of the letter "H" was 250 nm. When silk fibroin was used in the positive tone, the experimentally obtained size increased from 255 nm (for 30MB silk) to 435 nm (for HTP silk). When used in the negative tone, the feature size decreased from 250 nm (for 30MB silk) to 90 nm (for HTP silk).

Furthermore, a range of two dimensional square patterns were fabricated to explore the limits of aspect ratio of the nanostructures built with various silk fibroins (Figure 2d). The aspect ratio of the nano-pattern decreased by > 50% from about 1.5:1 (for 30MB silk) to 0.7:1 (for HTP silk), which is expected as shorter boiled time during the extraction process results in longer protein chains and thus better mechanical properties.

The exposure sensitivity and contrast - two major parameters of resist performances in this work - strongly depend on the starting material, mainly including the average molecular weight and the molecular weight distribution. Generally, the higher average molecular weight, the higher sensitivity of negative resists, as long chains with high molecular weights are easier to be cross-linked. On the contrary, higher energy is needed to break the bonds formed in long chains for positive resists. This has been verified by our experiment results where silk fibroin proteins with different boiling times and average molecular weights have been prepared and characterized.

Lower molecular weight material typically improves the contrast; however, this comes at the expense of the sensitivity, mechanical and etching performances (Table 1). In addition, narrower molecular weight distribution improves the contrast. The inevitable wide molecular weight distribution of silk fibroins is mainly due to the protein extraction from natural silk cocoons. It is difficult to control the reliability and repeatability of natural proteins as their synthetic counterparts.



*Figure 2: Performance evaluation of proteins as resists for nanolithography. Designed (a) and resulting patterns (b and c) in silk fibroin after electron beam exposure and water development. MB: minutes-boiled. (d) SEM images of nanopillars at different ultimate aspect ratios of silk proteins.*

Table 1: Performance evaluation of relevant silk proteins as resists. (Pos: crystalline silk; Neg: amorphous silk).

Source of materials	Young's modulus (GPa)	Etching selectivity (Si:fibroin)	Designed size 250 nm Obtained size (nm)
30MB Pos	3.5	11	255
30MB Neg	2.7	13	250
60MB Pos	2.6	9	302
60MB Neg	2.5	11	231
120MB Pos	2.5	6	359
120MB Neg	2.4	7	203
HTP Pos	2.4	2	435
HTP Neg	2.3	4	90
PMMA	1.8	3	N/A

Genetically engineered recombinant proteins with well-defined molecular structures may be the true key to controllable “green” nanolithography success. In Figure 3, it was observed that patterns fabricated using recombinant spider silk proteins showed considerably higher contrast (i.e., sharper edges) than patterns fabricated using natural silk fibroin proteins.

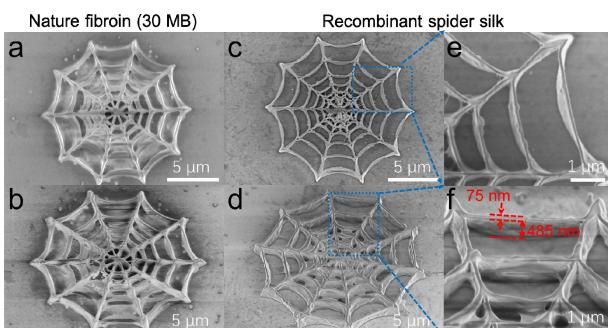


Figure 3: SEM images of (a, b) 30 MB nature fibroin and (c-f) recombinant spider silk nanostructures.

## CONCLUSION

In summary, we carried out a comparative investigation of nanofabrication processes using natural silk fibroin proteins and genetically engineered spider proteins as the EBL resists. We report - for the first time - nanopatterning of synthetic spider silk proteins using EBL, which offers significant improvements over their natural counterparts including the resolution, contrast and mechanical integrity due to their well-defined protein structures and high purity. The ultimate limits to controlled nanostructuring of this protein, including the maximum aspect-ratio and minimum feature size, depend on the starting material, as well as the side-chain bonds and protein conformation.

A deep understanding of the structure-property relations in protein-based biomaterials offers capabilities of building protein-based nanostructures and provides a

powerful route for building complex, mechanically robust 2D and 3D nanoarchitectures for cell culture, drug delivery, optoelectronic and tissue engineering applications.

## ACKNOWLEDGEMENTS

This work was partly supported by the National Natural Science Foundation of China (Grant No. 61574156, No. 61527818), MOST of China (Grant No.2016YFA0200800), the National Basic Research Program of China (973 Program, 2015CB755500), the International Science & Technology Cooperation Program of China (2014DFA31470) and the Science and Technology Commission of Shanghai Municipality for the support under the International Collaboration Project (Grant No. 14520720400).

## REFERENCES

- [1] F. G. Omenetto, D. L. Kaplan. “New Opportunities for an Ancient Material”, *Science* 329, vol. 5991, pp. 528-31, 2010.
- [2] Z. Z. Shao, F. Vollrath, “Materials: surprising strength of silkworm silk”, *Nature*, vol. 418, pp. 741–741, 2002.
- [3] N. E. Kurland, T. Dey, C. Wang, S. C. Kundu, V. K. Yadavalli, “Silk Protein Lithography as a Route to Fabricate Sericin Microarchitectures”, *Adv. Mater.*, vol. 26, pp. 4431-7, 2014.
- [4] R. A. Petros, J. M. DeSimone, “Strategies in the design of nanoparticles for therapeutic applications”, *Nat. Rev. Drug Discov.*, vol. 9, pp. 615, 2010.
- [5] S. Kim, B. Marelli, M. A. Brenckle, A. N. Mitropoulos, E. Gil, K. Tsioris, H. Tao, D. L. Kaplan, F.G. Omenetto, “All-water-based electron-beam lithography using silk as a resist”, *NAT. NANOTECHNOL.*, vol. 9, pp. 306-10, 2014.
- [6] N. Qin, S.Q. Zhang, J.J. Jiang, S.G. Corder, Z.G. Qian, Z.T. Zhou, W. Lee, K.Y. Liu, X.H. Wang, X.X. Li, Z.F. Shi, Y. Mao, H.A. Bechtel, M.C. Martin, X.X. Xia, B. Marelli, D.L. Kaplan, F.G. Omenetto, M.K. Liu, H. Tao, “Nanoscale Probing of Electron-regulated Structural Transitions in Silk Proteins by Near-field IR Imaging and Nano-spectroscopy”, *NAT. COMMUN.*, DOI: 10.1038/ncomms13079, 2016.
- [7] D. N. Rockwood, R. C. Preda, T. Yücel, X. Wang, M. L. Lovett, D. L. Kaplan, “Materials fabrication from *Bombyx mori* silk fibroin”, *NAT. PROTOC.*, vol.6, pp. 1612-31, 2011.
- [8] X. X. Xia, Z. G. Qian, C. S. Ki, Y. H. Park, D. L. Kaplan, S. Y. Lee, “Native-sized recombinant spider silk protein produced in metabolically engineered *Escherichia coli* results in a strong fiber”, *P Natl. Acad. Sci. USA*, vol. 107, pp. 14059-14063, 2010.

## CONTACT

\*K.Y. Liu, keyin.liu@mail.sim.ac.cn