EXTRACTED NATURAL SILK FIBROIN AS A DUAL-TONE PROTEIN RESIST FOR ECO-FRIENDLY ELECTRON BEAM LITHOGRAPHY

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ABSTRACT
We report on the ability to reshape extracted natural silk fibroin with energetic electrons and on the application of advanced spectroscopic imaging for nanoscale structural analysis. Silk fibroin films under electron irradiation at various exposure dosages have been investigated using infrared scattering near-field optical microscopy (s-SNOM) for the first time to decipher the electron-regulated structural transitions of silk fibroin, which determines its solubility in water. Our work provides important guidelines for utilizing silk fibroin as a dual-tone protein resist for all-water-based eco-friendly electron beam lithography (EBL), with no hazardous chemicals used or generated in this process.

INTRODUCTION
Silk fibers, spun by silk worms, have been used in the textile industry across the globe for centuries mainly due to their extraordinary mechanical properties such as toughness and extensibility [1]. Recently, there has been increasing interest on silk-based biomaterials for a wide range of applications, such as optics, wearable electronics, implantable devices, and tissue engineering [1] owing to its excellent mechanical property [2] and inherent bio-compatibility [3]. In particular, the fibroin protein extracted from the natural silk fibers, which is known for providing the mechanical robustness of the fiber, has been intensively studied.

As a protein, the functionality and property of silk fibroin is fundamentally influenced by its secondary structure. By regulating the secondary structure, silk fibroin has been found to be micro/nanofabrication-compatible [2] and has been reported to be used as both a positive and a negative tone Electron Beam Lithography (EBL) resist under different pre-treatments [4]. For example, with a simple spin coating process, the resulting silk film (dominated by amorphous structures which are water-soluble) can be used as a negative tone EBL resist. In contrast, immersing the spin coated film in methanol for a short period of time for crosslinking (i.e., from amorphous to crystalline structures) will yield a positive tone EBL [4,5]. Despite initial experimental success, the fundamental mechanism has yet to be explored to tailor silk protein to specific applications. This is mainly due to the lack of deep understanding of material behaviors at its fundamental functional domain as the characterization tools currently used in analyzing the silk nano-structures, such as Scanning Electron Microscopy (SEM) and far-field Fourier Transform Infrared Spectroscopy (FTIR) [6][7], were either limited in chemical sensitivity (e.g., SEM) or spatial resolution (e.g., FTIR).

In this work, comprehensive nanoscale studies of silk fibroin films under electron irradiation at various dosages have been carried out using an Infrared Scattering Type Scanning Near-field Optical Microscopy (IR s-SNOM) with a spatial resolution of ~ 20 nm in our current setup. Taking advantage of nanoscopic structural information, important insight can be gained into electron-protein interactions at nanoscale. For the first time, we have visualized a complete "life cycle" of the silk fibroin protein under the irradiation of electrons, and found that the applied electron dosage is indeed the primary tuning parameter to engineer the crystallinity and therefore the water solubility of silk fibroin protein and plays a more important role than the crystallinity level of the starting materials, as required in [4]. The results provide important guidelines to use silk fibroin as a dual tone resist with only water used as the developing solution. No hazards are used or generated.

SETUP AND MATERIALS
Materials Preparation
The aqueous silk solution (Figure 1a) can be prepared by following the protocol described in [8]. B. mori cocoons were boiled for 30 min in aqueous 0.02 M Na_2CO_3 (Sigma-Aldrich, USA) to remove sericin and then rinsed for 30 min in distilled water. The degummed silk fibers were allowed to dry for more than 12h and then subsequently dissolved in 9.3 M LiBr (Sigma-Aldrich, USA) solution at 60 °C for 4 h. The solution was dialyzed for 2 days in distilled water using Slide-a-Lyzer dialysis cassettes (MWCO 3,500, Pierce, USA). The solution was centrifuged for 20 min at 18,000 r.p.m for purification. The concentration was determined by measuring a volume of solution and the final dried weight.

Then the silk fibroin solution was spin-coated on oxygen plasma treated silicon wafers to form a uniform film (Figure 1a). The thickness of the film was controlled to be ~ 150 nm by using a 5% silk fibroin solution (w/w) spun at a maximum speed of 4000 r.p.m. for 40s. The crystallization of the film (if necessary) can be done by immersing the film into methanol for 5 min.

Experimental Details
Using a commercial EBL tool (with 25 KeV acceleration voltage), precise delivery of energy (in terms of both spatial precision and the amount of energy) is realized to enable well-controlled nano-patterning on extracted natural silk fibroin thin films (Figure 1b). The fabricated silk fibroin can then be developed using DI water for 2min for both positive and negative patterns.

To characterize the structural transition of silk under electron irradiation, we subsequently customized a commercially available s-SNOM (Fig. 1c), with a near infrared quantum cascade laser (QCL) in continuous wave

(CW) mode. The output wave of the QCL ranges from 1,500 – 1,750 cm^{-1}. The modified s-SNOM can achieve a spatial resolution down to ~20 nm, determined by the apex size of metal-coated AFM tip. The scattered light combined with an interfering reference light will be collected by an HgCdTe detector. The SNOM technique is operated based on the near-field interaction between a sharp conductive tip and the sample surface within a few tens of nanometer [9]. Due to the non-propagating nature of the near-field interaction, the s-SNOM can break the diffraction limit of conventional far-field FTIR and enabling understanding of proteins approaching their molecular limits [10-12]. Combining these two systems (i.e. EBL for nano-patterning and s-SNOM for nano-characterization), the electron induced nanoscale structural transition of silk protein has been investigated with unprecedented details.

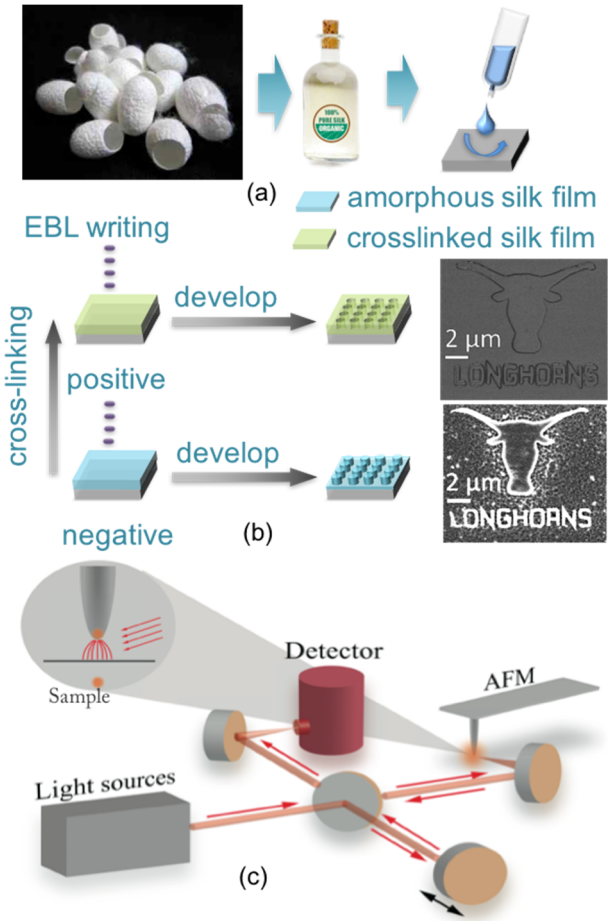

Figure 1: (a) Bombyx mori silk cocoons and extracted natural fibroin protein solution spin coated into thin films (b) Silk fibroin can be used as both positive and negative tone EBL resists. (c) The schematic of s-SNOM. A beam of IR laser is focused on the AFM tip and induces near-field interaction between the tip and the sample.

RESULTS AND DISCUSSION

s-SNOM spectrum mapping

Figure 2 shows the mechanism (Figure 2a) and the near-field characterization of silk fibroin protein used as positive (Figure 2b) and negative tone (Figure 2c) EBL resists by using different pre-treatment. The nanoscale conformational information of fabricated silk nanostructures was characterized using s-SNOM by scanning the same structure at different wavenumbers/wavelengths (Fig. 2b), therefore mapping the infrared absorption spectrum (Fig. 2c) covering both Amide I and Amide II band.

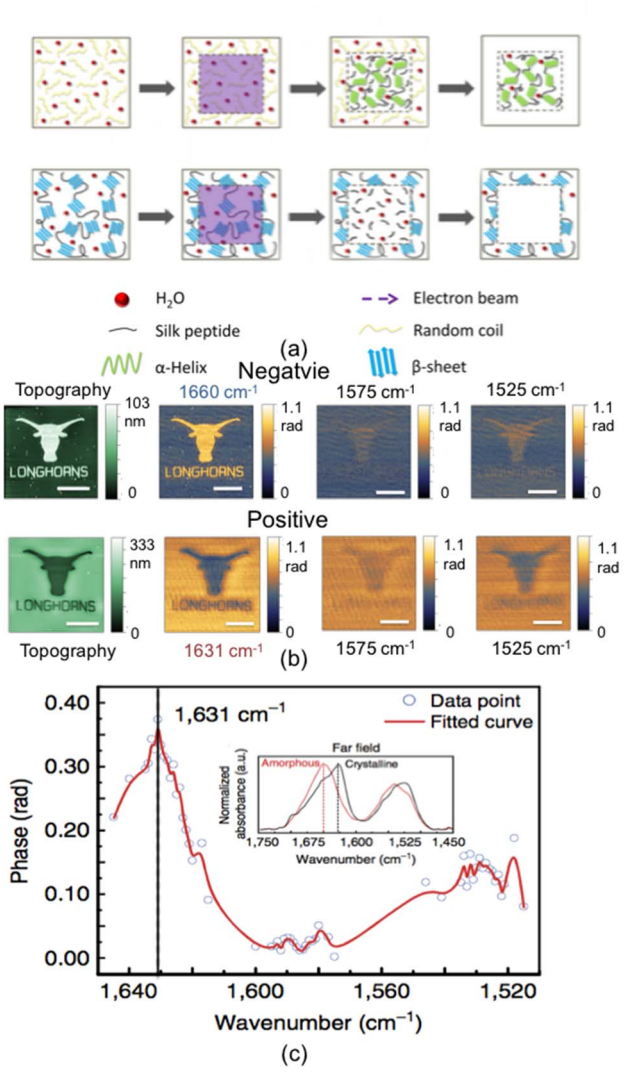

Figure 2: (a) The mechanism of using silk fibroin protein as positive and negative EBL resist with different starting secondary structure. (b) s-SNOM nanoscopic imaging of fabricated silk structure by scanning the same area with different wavenumber. The scale bar is 2 μm (c) full IR spectrum of silk protein used as positive and negative tone EBL resist, characterized by s-SNOM.

As shown in Figure 2b, at 1660 cm^{-1} and 1,631 cm^{-1} (for negative and positive tone silk fibroin respectively), the phase image exhibits a strong contrast between silk protein and silicon (silicon is used as the reference for IR imaging) owing to the amide I absorption corresponding to the secondary structure of α-helix [7] and β-sheets [13]. This phase contrast decreases when the illumination is tuned to 1,710 cm^{-1} where silk proteins have little absorption. Subsequently the normalized near-field phase contrast of the crystalline silk protein (predominantly β-sheet structure) in the range that covers both the Amide I and Amide II absorption of silk protein was acquired by tuning the wavenumber of the QCL laser during IR nano-scopic imaging. The absorption peak centered at 1,631 cm^{-}

[1] agrees well with the one obtained in far-field FTIR measurement, confirming the validity of this technology. The non-invasive nature of the s-SNOM measurement provides an ideal infrastructure of characterizing biologically active sample (silk fibroin protein in our case) because the measurement can be conducted at ambient conditions without sophisticated sample preparation or causing significant structural change during the measurement.

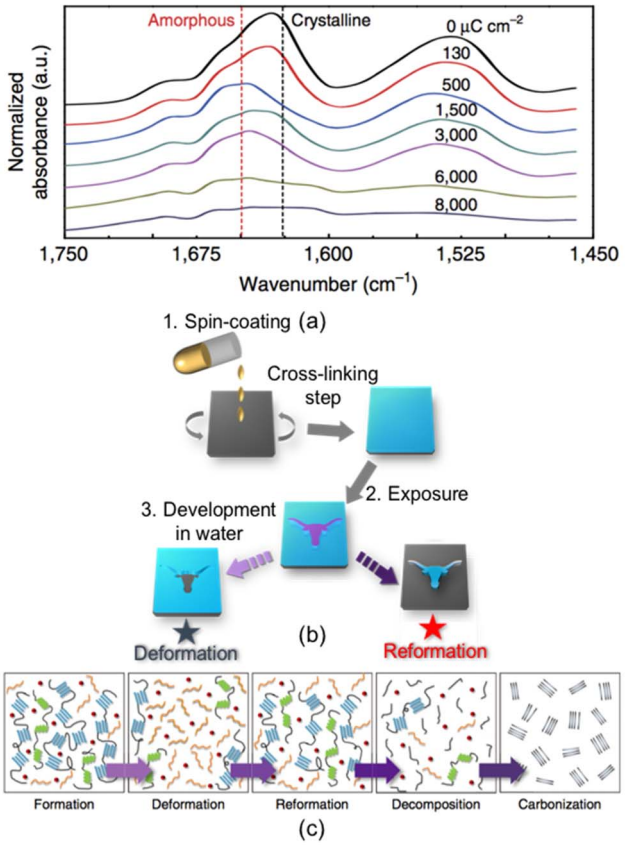

Figure 4: (a) the conformational evolution of silk protein secondary structure manifested by IR spectra. (b) the schematic of fabrication step for using the conformational change of silk to make dual tone EBL resist (c) schematic of the conformational evolution of silk protein.

The ability to locally probe nanoscale protein structural transitions combined with nanometer-precision EBL allows us to finely control the structure of silk proteins. The fine spectral resolution (can be as high as 1 cm^{-1}) and spatial resolution (~ 20 nm) offers important insight into the nanoscale structural change of silk fibroin with unprecedented details. To systematically investigate the electron-protein interaction, we prepared a set of samples (starting with crystalline silk film) exposed under different dosages of electron and acquired their IR spectra using AFM IR (a variant of SNOM) [15]. As shown in Figure 4a, the characteristic peak of β-sheets (~ 1625 cm^{-1}) structure becomes weaker as the electron dosages increase from 0 to 500 μC cm^{-2}. This indicates the deformation of the β-sheets structure under small amount of electron exposure. By increasing the dosage up to 500 μC cm^{-2}, the absorption peak corresponding to β-sheets re-appears, although with much lower intensity. This indicates a partial reformation of β-sheets structure [14]. The IR spectra for the further electron exposure shows that the β-sheets absorption peak becomes weaker again until a dosage of 6000 μC cm^{-2} and disappears with a dosage of 8000 μC cm^{-2} [6, 13]. This reveals that the β-sheets has gradually decomposed and partially carbonized, therefore losing its structure and functionality as a protein.

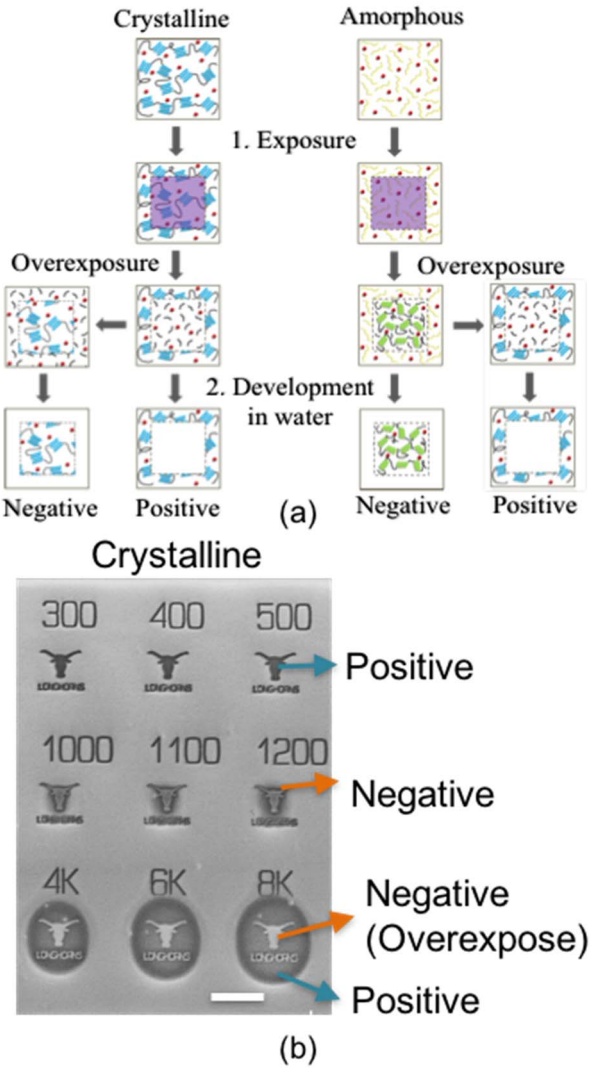

Figure 5: (a) the proposed mechanism for using silk fibroin protein as both positive and negative tone EBL resist regardless of the starting material. (b) SEM images of silk fibroin protein as both positive and negative tone EBL resist starting with crystalline silk protein. Scale bar: 5 μm.

It is revealed that under electron irradiation, the protein undergoes a conformational revolution (formation -> deformation -> reformation -> decomposition) with increasing dosages, as schematically shown in Figure 4b and 4c. Since the solubility of silk is determined by the formation or deformation of β-sheets, which can be tuned by simply varying the electron irradiation dosage, this result provides important guidelines to engineer silk fibroin using electrons (Figure 5a). If the starting material is crystalline silk fibroin protein, then with small dosage of electron irradiation, the film can be simply used as a

positive tone EBL resist due to the deformation of β-sheets. The exposed area then become soluble in water and can be washed away in the following development step. However, with higher electron beam dosages, both positive and negative patterns can be realized on the same substrate as the β-sheets starts to reform at high dosage and those areas will become insoluble in water. For example, Figure 5b shows both positive and negative structure obtained on the same piece of crystalline silk film. Note that under excessive exposure, negative pattern will be surrounded by a positive "aura" due to proximity effects. Similarly, the dual tone effect can be achieved by using amorphous silk fibroin protein as the starting material. Using small exposure dosage, amorphous silk (soluble in water) serves simply as the negative tone EBL resist featured by the increase of crystalline content as the exposed area become insoluble in water. And under larger exposure dosage, the crystallized silk protein deforms again, and thus becomes soluble in water. This exemplifies its use as a negative tone resist. With such flexibility to switch between positive and negative tone, more complex structures can be fabricated, providing exciting opportunities for biologically compatible, eco-friendly nanofabrication.

CONCLUSION

We report on the ability to reshape extracted natural silk fibroin with energetic electrons and on the application of advanced spectroscopic imaging for nanoscale structural analysis. Silk fibroin films under electron irradiation at various dosages has been performed using infrared scattering near-field optical microscopy (s-SNOM) for the first time to decipher the electron-regulated structural transitions of silk fibroin and its solubility in water, which provides important guidelines for utilizing silk fibroin as a dual-tone protein resist for all-water-based eco-friendly electron beam lithography (EBL), with no hazards used or generated during the process.

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REFERENCES

[1] Tao, Hu, David L. Kaplan, and Fiorenzo G. Omenetto. Silk materials–a road to sustainable high technology. Advanced materials 24.21 (2012): 2824-2837.

[2] Shao, Z. Z. & Vollrath, F. Materials: surprising strength of silkworm silk. Nature 418, 741–741 (2002).

[3] Omenetto, F. G. & Kaplan, D. L. New opportunities for an ancient material. Science 329, 528–531 (2010).

[4] Kim, Sunghwan, et al. All-water-based electron-beam lithography using silk as a resist. Nature nanotechnology 9.4 (2014): 306-310.

[5] Morikawa, J. et al. Silk fibroin as a water-soluble bio-resist and its thermal properties. RSC Adv. 6, 11863–11869 (2016).

[6] Hu, X., Kaplan, D. & Cebe, P. Determining beta-sheet crystallinity in fibrous proteins by thermal analysis and infrared spectroscopy. Macromolecules 39, 6161–6170 (2006).

[7] Yang, H. Y., Yang, S. N., Kong, J. L., Dong, A. C. & Yu, S. N. Obtaining information about protein secondary structures in aqueous solution using Fourier transform IR spectroscopy. Nat. Protoc. 10, 382–396 (2015).

[8] Rockwood, D. N. et al. "Materials fabrication from Bombyx mori silk fibroin." Nat. Protoc. 6, 1612–1631 (2011).

[9] Fei, Z. et al. Gate-tuning of graphene plasmons revealed by infrared nano-imaging. Nature 487, 82–85 (2012).

[10] Amenabar, I. et al. Structural analysis and mapping of individual protein complexes by infrared nanospectroscopy. Nat. Commun. 4, 2890 (2013).

[11] Brehm, M., Taubner, T., Hillenbrand, R. & Keilmann, F. Infrared spectroscopic mapping of single nanoparticles and viruses at nanoscale resolution. Nano Lett. 6, 1307–1310 (2006).

[12] Ruggeri, F. S. et al. Infrared nanospectroscopy characterization of oligomeric and fibrillar aggregates during amyloid formation. Nat. Commun. 6, 7831 (2015).

[13] Vollrath, F., Hawkins, N., Porter, D., Holland, C. & Boulet-Audet, M. Differential Scanning Fluorimetry provides high throughput data on silk protein transitions. Sci. Rep. 4, 5625 (2014).

[14] Bai, S. et al. Controllable transition of silk fibroin nanostructures: an insight into in vitro silk self-assembly process. Acta Biomater. 9, 7806–7813 (2013).

[15] Jones, A. C. & Raschke, M. B. Thermal infrared near-field spectroscopy. Nano. Lett. 12, 1475–1481 (2012).

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