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# Nano- and Micropatterning of Optically Transparent, Mechanically Robust, Biocompatible Silk Fibroin Films**

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Biopolymers, such as silk fibroin, collagen, and chitosan, are promising candidates for a variety of applications that merge the fields of biomedical optics and biomaterials. Biocompatible silk fibroin, in particular, shows promise as a biomaterial, based on a number of attributes.[1] Silk fibroin is the strongest and toughest natural fiber known and is easily formed into robust films of thermodynamically stable beta-sheets of a controllable range of thicknesses (between tens of nanometers and hundreds of micrometers).[1–3] These films have excellent (ca. 95%) optical transparency across the visible range and can be easily characterized and biochemically functionalized because of the all-aqueous processing, broadening their overall value.

It is possible to form such silk fibroin films, having intricate 2D or 3D nano- or micropatterns, through a soft-lithography-based simple casting technique. This technique enables the fabrication of (at least) sub-30 nm transverse features in silk fibroin films, when cast at ambient conditions from an aqueous silk solution. The elegance of this method is in its simplicity; the fabrication of such features is completed in the absence of additional harsh chemicals, salts, or high pressures that traditionally accompany most micro- and nanofabrication techniques. By employing this simple casting technique, high-quality films that contain a wide spectrum of nano- and micropatterns can be fabricated. These films are of great consequence for use in a variety of studies based in biomedical optics.

In this Communication we report on a process developed for the construction of silk fibroin-based nano- and micropatterned films. This process includes the methods for producing ultrapure silk fibroin solution, the aqueous casting process for patterning silk fibroin films, and the characterization of the smallest transverse nanopatterns realized in silk fibroin films to date.

Production of the silk fibroin solution begins with the purification of harvested *Bombyx mori* cocoons. Sericin, a water-soluble glycoprotein that binds fibroin filaments, is removed from the fibroin strands by boiling the cocoons in a 0.02 M aqueous solution of sodium carbonate for 45 min.[4] Upon completion of this step, the remaining fibroin bundle is rinsed thoroughly in Milli-Q water and allowed to dry overnight. The dry fibroin bundle is then dissolved in a 9.3 M aqueous solution of lithium bromide at 60 °C for 4 h. The lithium bromide salt is then extracted from the solution over the course of three days, through a water-based dialysis process. The resulting solution is extracted from the dialysis cassette (Slide-a-Lyzer, Pierce, molecular weight cut-off (MWCO) 3500) and remaining particulates are removed through centrifugation and syringe-based microfiltration (5 μm pore size, Millipore Inc, Bedford, MA). This process enables the production of 8–10% w/v silk fibroin solution of excellent quality and stability. The purification step is particularly important for the generation of high-quality optical films with maximized transparency and, consequently, minimized scattering.

The patterning of silk fibroin films occurs through a modified soft-lithography casting process. Two sets of masters were employed for this purpose. The first masters were fabricated by electron beam lithography on silicon substrates.

The areas of the masks range from 0.5–1 $cm^2$. These masks are formed such that chromium or titanium nanoparticles that are 100 nm in diameter and 35 nm in height are arranged according to different geometries, not necessarily periodic. Our fabrication process flow starts with 180 nm of poly(methyl methacrylate) (PMMA) 950 spin-coated on top of the substrates, which consist of 10 nm thick indium tin oxide (ITO) films on quartz. After spin-coating, the substrate was soft baked at 180 °C for 20 min. The patterns were written using a Zeiss SUPRA 40VP SEM equipped with Raith Beam Blanker and a Nano Pattern Generation System (NPGS) for the nanopatterning stage. After developing and rinsing the resist, a 50 nm thin Cr film was deposited on the patterned surface by e-beam evaporation, and the lift-off process using acetone resulted in an array of cylindrical Cr nanoparticles. As a proof-of-concept demonstration, here we report our results on the nanoimprint of periodic square patterns and nonperiodic Thue–Morse designs directly transferred at the nanoscale

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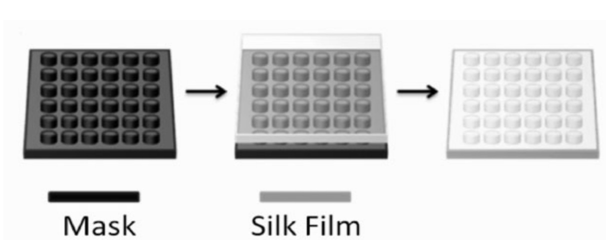
on transparent silk substrates.[5,6] Each square is either approximately $25 \times 25\ \mu m^2$ or approximately $10 \times 10\ \mu m^2$ in area, and the spacing between the individual nanoparticles ranges from 20–250 nm. The second masters were diffractive optics cards, displaying a variety of 3D diffractive micropatterns (Digital Optics Corp., Tessera Technologies).

During the casting process, 200 μL–1 mL of silk fibroin solution is deposited onto a clean, dry master, as illustrated in Scheme 1. This solution is then allowed to crystallize in free air at ambient temperature and pressure. Under these settings, dry films are produced after approximately 16 h. Alternative post-processing (such as water vapor annealing or exposure to methanol) can be used to shorten the time necessary for film formation, as previously described.[7]

Removal of the film can be accomplished by loosening at one corner of the master and subsequent levering-off using a thin razor blade or scalpel.

Once the film has been removed from the master, the silk fibroin can be further cross-linked through exposure to vacuum-induced methanol vapor (100% methanol at 26 mmHg; $1\ \text{mmHg} = 1.333 \times 10^2\ \text{Pa}$), or water vapor (less than $10^{-3}$ mmHg), for a period of 24–36 h.[7–9] This step is optional, based on the use for the films. Beyond making the films water-insoluble, this step influences the toughness of the films.

Characterization of the films was accomplished through the combination of multiple imaging modalities. Scanning electron microscopy (SEM) images of the 2D features patterned onto a silk fibroin film, formed from the e-beam etched master, can be seen in Figure 1A and B. From these images it was determined that the smallest feature capable of replication was the transverse spacing between adjacent holes. This feature was determined to be 40 nm in length. In Figure 1B the result of the drying and lifting processes associated with formation of silk fibroin films is visible as the smudged lower left-hand region of each patterned hole. This constitutes an artifact of the simple casting process, which ultimately could affect the precision obtainable when patterning on a scale of a few tens of nanometers. This artifact is being addressed by exploring different approaches for the detachment of the silk film which do not impose mechanical stress on the surface. Such approaches include the floating of the film off of the master through the use of surfactants.




**Scheme 1.** Schematic procedure for the described casting process. Masters employed in this process have patterns that range in size from $10 \times 10\ \mu m^2$ to $1 \times 1\ cm^2$.

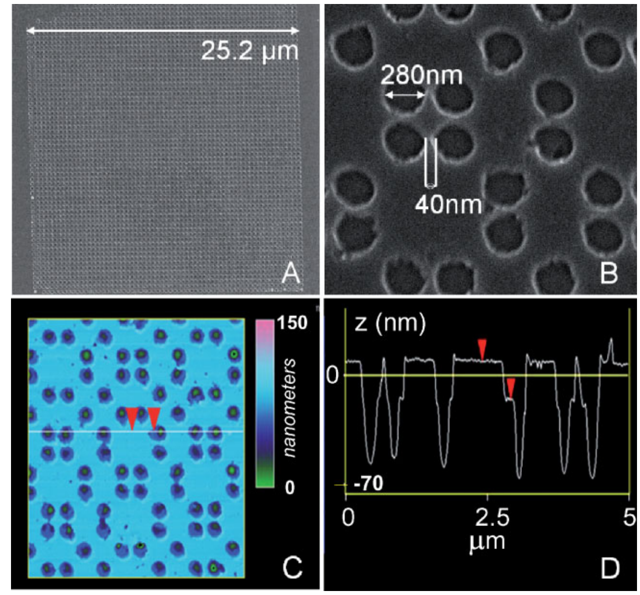



**Figure 1.** A) SEM images of the surface of a silk fibroin film with a periodic square pattern of air holes. The diameter of the holes is 100 nm; the lattice constant is 200 nm. B) SEM detail of a nonperiodic decoration motif (called Thue–Morse lattice) of air holes printed in a silk fibroin film. The dimensions are indicated in the Figure. The minimum measured spacing between adjacent holes throughout the entire patterned surface is approximately 30 nm at present. This represents the smallest known nanopatterning silk-fibroin achieved. C) Atomic force microscopy (AFM) images of larger ($25\ \mu m \times 25\ \mu m^2$) pattern on silk fibroin with a data scale of 110 nm. D) Cross-Section analysis of AFM image (C), corresponding to the white horizontal line and red arrows indicated in the figure.

An atomic force microscopy (AFM) image in Figure 1C shows a slightly larger section of a Thue–Morse nonperiodic pattern directly transferred to silk fibroin. The cross-section measurement corresponding to the white horizontal line and red arrows of the image in Figure 1C can be seen in Figure 1D. Here, it can be observed that the replicated longitudinal features realized in the silk film are approximately 25 nm in depth. The remaining depth visible in the cross-section analysis is also an artifact of the simple casting process, which is currently being addressed.

Furthermore, 3D micropatterning was achieved through the casting of silk fibroin solution onto diffractive optics surfaces (Digital Optics Corp., Tessera Technologies). These surfaces are etched on a polycarbonate card encoded with multiple phase levels to produce fine detail and high-quality projected images. The AFM images of a replicated diffractive surface in silk fibroin can be seen in Figure 2A and B. These images clearly illustrate the 3D patterning capabilities of the described casting technique. Figure 2C demonstrates the capability of detailed silk-fibroin-based diffractive patterns to produce complex and elaborate projected images. The patterned films formed with this technique hold much promise for future experiments involving biocompatible biopolymers and the formation of diffractive optical images.

The sub-40 nm transverse resolution achieved in silk fibroin biopolymer is of particular significance when contrasted with

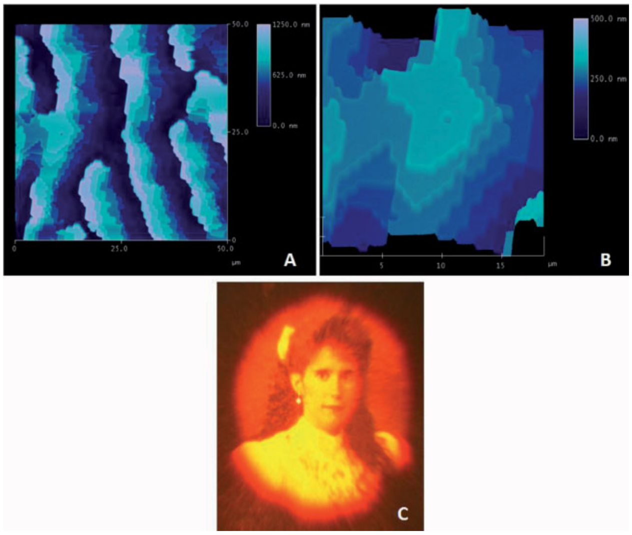



**Figure 2.** AFM images of a silk fibroin film that has 3D diffraction micropatterning. A) Large-scale view of diffraction patterned silk. Data scale is 1250 nm. B) Detail of 3D geometry associated with the micropatterning. Data scale is 500 nm. C) High-quality projected image from a 3D diffraction pattern made in silk fibroin.

the capabilities of patterning in other biopolymers. Currently, published data on patterning of gelatin films reports a transverse resolution of 5 μm, when using a modified glutaraldehyde (GA)-crosslinked gelatin.[9] Successful patterning at this resolution necessitates the use of techniques traditional to photolithography, coupled with chemical-based crosslinking, performed at 45 °C.[10,11] Furthermore, current literature describing patterning techniques in chitosan employ nanoimprinting lithography for the formation of nanopatterned chitosan films.[12] This technique necessitates the use of conditions at 90 °C and 5–25 psi (1 psi $= 6.895 \times 10^3$ Pa) to realize patterns limited to 150 nm in the transverse plane. The formation of micropatterns on these types of biopolymers holds limitations not only in achievable transverse resolution, but also in the casting process without the same level of optical clarity that is offered by silk fibroin. The process described above for the patterning of silk fibroin enables the formation of nanometer-scale patterns under ambient conditions of temperature and pressure. The employment of an all-aqueous silk solution, coupled with a simple and repeatable casting technique, results in optically clear, biocompatible, and mechanically tough films.[13]

In conclusion, we report a simple modified soft-lithography process developed for the construction of silk-fibroin-based nano- and micropatterned films. This process includes methods for producing ''optical-grade'' ultrapure silk fibroin solution, the casting process for patterning silk fibroin films, and the characterization of the smallest nanopatterns realized in silk fibroin films to date. This novel technique enables the formation of mechanically robust, optically transparent biopolymer films capable of sub-40 nm transverse pattern resolution. By employing this simple casting technique, high quality films containing intricate 2D and 3D nano- and micropatterns can be fabricated. Future applications for such nano- and micropatterned silk fibroin films include scaffolding for cell growth, substrates for cell culture, experiments in biomedical-related diffractive optics and optical interfaces, and substrates for tensegrity studies.[14] Furthermore, these patterned silk fibroin films can be easily characterized and biochemically functionalized, further broadening their scope and value.

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