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Fabrication of precise shape-defined particles of silk proteins using photolithography

Ramendra K. Pal [a], Nicholas E. Kurland [a], Chenyang Jiang [b], Subhas C. Kundu [c], Ning Zhang [b], Vamsi K. Yadavalli [a,*]

[a] Department of Chemical and Life Science Engineering, 601 W Main Street, Virginia Commonwealth University, Richmond, VA, United States
[b] Department of Biomedical Engineering, 601 W Main Street, Virginia Commonwealth University, Richmond, VA, United States
[c] Department of Biotechnology, Indian Institute of Technology (IIT) Kharagpur, West Bengal 721302, India

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ABSTRACT

Non-spherical particles of different shapes have unique properties potentially beneficial in self-assembly, biosensing, therapeutic delivery and optical applications. Forming particles with precisely controlled physical and chemical characteristics, particularly using bioinspired or bio-derived materials can open up applications inaccessible to synthetic polymers. Here, a high throughput fabrication process of different shapes of protein-based particles at high resolution using photolithography is demonstrated. In contrast to synthetic polymers, the particles shown herein are comprised of the two silk proteins – fibroin and sericin. The demonstrated technique of silk protein lithography allows fabrication of monodisperse biopolymer particles with precise geometries ranging from a few to hundreds of microns. Large numbers of particles of controllable aspect ratios can be easily formed, collected and mixed. The particles themselves are mechanically robust and biocompatible, but can be proteolytically degraded over a period of weeks. Owing to the facile fabrication technique that uses benign solvents, bioactive molecules can be encapsulated within these protein matrices. By control of shape, size, thickness and surface properties, particles that may be harvested for optics, delivery or presentation of biologically functional agents, among other applications.

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1. Introduction

Micro- and nanofabrication techniques can enable the large scale manufacture of high fidelity particles of diverse shapes [1]. Typically fabricated from synthetic polymers, particles ranging from a few nm to hundreds of μm can function as imaging or contrast agents, vehicles to encapsulate and deliver therapeutics with precise control [2,3], or intracellular biosensors [4]. To achieve higher efficacy in these tasks, key factors include particle size, surface chemistry, mechanical properties, and permeability [5,6]. Several conventional fabrication techniques have been reported for the formation of particles, ranging from bottom-up approaches guided by self-assembly [7], to top-down approaches including spray drying [8], atomization [9], milling, grinding [10], and emulsion solvent evaporation [11]. However, such methods often result in large variations in particle sizes and distributions. Significantly, control of the shape of the particles formed is challenging, with spherical or near-spherical particles being most commonly formed. A recent addition to considering particle fabrication is the shape

* Corresponding author.
E-mail address: vyadavalli@vcu.edu (V.K. Yadavalli).

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of the particle [12]. *Non-spherical* and anisotropic micro and nanoparticles are of great interest to provide functionality not accessible to spherical geometries [13]. For example, engineering particle shape provides the ability to affect cellular interactions and uptake, vascular dynamics and pharmacokinetics [5,14]. Advantages include improved packing, controllable responses to external stimulus, and unique optical behavior [15]. Particles with unique shape and illustrative patterns can be used to tag various chemistries for multiplexed sensing and bioassays [1,16,17].

In order to fabricate large numbers of monodisperse, non-spherical particles with precisely controlled physical and chemical characteristics, new methods have been devised such as soft lithography, microfluidic synthesis, stop flow lithography, contact flow lithography and optofluidic techniques [17–20]. The novel particle replication in nonwetting templates (PRINT®) technique has been one of the most successful and versatile at producing freestanding, high fidelity particles at high throughput [21]. Particles of various synthetic biopolymers like poly(ethylene glycol) (PEG), poly(ethylene glycol) di-acrylate (PEGDA), poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA) have been demonstrated [13,17,22]. However, to date, free-standing, high fidelity particles made from natural and bioinspired biopolymers, have been relatively unexplored [23]. Natural materials including proteins (silk, collagen, gelatin, elastin, keratin), polysaccharides and nucleotides present alternatives to form particles for various biomedical applications [24–26]. Among these biomaterials, the two core silk proteins from silkworms - fibroin and sericin, have been recognized for their outstanding mechanical performance, optical transparency, physical and chemical stability, biocompatibility, biodegradability, processability and ease of functionalization [27–30]. Micro and nanostructures of both silk fibroin and sericin have been widely reported for drug delivery and regenerative medicine. However, these have been restricted to films, foams, rods, and spherical particles formed using self-assembly, emulsification, desolvation, coacervation or electrospray drying [30–35]. Thus far, formation of controlled and non-spherical shapes of proteins has not been possible due to the significant mismatch between lithographic processing and the fragile biomaterials.

Our group reported on a versatile silk protein lithography (SPL) strategy to potentially exploit these proteins in non-traditional ways at the micro and nanoscales. The basis of this technique lies in the formation of photo-crosslinkable variants (similar to negative tone photoresists) of the two core proteins - fibroin and sericin [36,37]. Silk micropatterns on both rigid and flexible substrates were shown using photolithography. Here, it is shown how SPL can be used to form precise microscale architectures of silk proteins in the form of designed shapes and 3D objects. Freestanding shapes can be formed from both fibroin and sericin (separately), to achieve unabridged use of silk. Using a non-adhesive monolayer, independent, unattached shapes can be formed at the microscale via photolithography. While this is a mask-based process, the relatively simple processing involved, the rapid nature of particle synthesis, monodisperse and high fidelity architectures produced, makes this suitable to produce large quantities of precise protein shapes. The patterns produced are easily reproducible over large numbers, and the shape of the architectures is only limited to the mask. Silk protein particles possess an important advantage in that they can break down to physiologically compatible amino acids when resorbed, making them suitable choices for *in vivo* systems over synthetic polymers [38]. Both the protein matrix and the lithography strategy employing benign solvents, are suited for encapsulation of biologically functional agents This provides a route to design of monodisperse protein shapes that may be harvested for the potential delivery or presentation of biologically functional agents, among other applications [39].

2. Experimental

2.1. Materials

Silk fibroin was extracted and purified from *Bombyx mori* silkworm cocoons (Mulberry Farms, CA) using established protocols [40]. Briefly, 5 g of silk cocoons were finely shredded and degummed by boiling in 2 l of 0.02 M aqueous sodium carbonate solution for 30 min under continuous agitation. The degummed silk was washed thoroughly in running DI water followed by 3 times rinsing in 1 l DI water for 20 min. Following the third wash, the fibroin was dried overnight followed by dissolution in 9.3 M LiBr solution. The dissolved fibroin solution was then dialyzed for 48 h with periodic exchange of water. The dialyzed solution was lyophilized to obtain pure fibroin powder. Sericin was purchased from Wako Chemicals (Richmond, VA) and used as received. The reagent 2-isocyanatoethyl methacrylate, 98% with <0.1% BHT inhibitor (IEM, MW = 155.15 Da) was utilized for chemical modification of fibroin. Anhydrous dimethyl sulfoxide, anhydrous lithium chloride (LiCl) were purchased from Sigma-Aldrich (St. Louis, MO). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was obtained from Oakwood Chemical (West Columbia, SC). Irgacure 2959 (1-[4-(2-Hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one, Ciba, Tarrytown, NY) was employed as a photoinitiator. All chemicals were used as received without further purification.

2.2. Surface functionalization of silicon and glass substrates

Silicon and glass substrates were used for patterning of silk microshapes. Squares of silicon (1×1 cm^2) and glass (2×2 cm^2) were initially washed thoroughly with ethanol and deionized water to remove surface contaminants. Substrates were treated with Piranha solution (3:1 98% H_2SO_4:30% H_2O_2) for 30 min to remove organic contaminants and hydroxylate the surface for further modification (*Caution: Piranha solution reacts violently with organic materials and must be handled with*

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