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Materials Fabrication from *Bombyx mori* Silk Fibroin

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Abstract

Silk fibroin, derived from *Bombyx mori* cocoons, is a widely used and studied protein polymer for biomaterial applications. Silk fibroin has remarkable mechanical properties when formed into different materials, demonstrates biocompatibility, has controllable degradation rates from hours to years, and it can be chemically modified to alter surface properties or to immobilize growth factors. A variety of aqueous or organic solvent processing methods can be used to generate silk biomaterials for a range of applications. In this protocol we include methods to extract silk from *B. mori* cocoons in order to fabricate hydrogels, tubes, sponges, composites, fibers, microspheres and thin films. These materials can be used directly as biomaterials for implants, as scaffolding in tissue engineering and in vitro disease models, and for drug delivery.

Keywords

Bombyx mori; silk; fibroin; scaffolds; hydrogels; tubes; sponges; fibers; films; microspheres

Introduction

Bombyx mori (silkworm) silk is a unique material, which has historically been highly regarded for its strength and luster. Physicians have used silk as a suture material for centuries and recently it has gained attention as a biomaterial due to several desirable properties. In particular, these properties include its biocompatibility, the ease with which it can be chemically modified[4-8], its slow rate of degradation in vivo, and its ability to be processed into multiple material formats from either aqueous solution or an organic solvent[9]. Due to large-scale cultivation of silkworms for the textile industry, there are abundant and reasonable cost sources for this natural polymer, however, for medical applications proper extraction and preparation of the core protein is required. From the raw cocoons, the sericin component must be removed from the core fibroin fibers. Sericin is a group of soluble glycoproteins expressed in the middle silk gland of *Bombyx mori*[10]. These proteins cover the surface of fibroin, the silk filament core protein, in the cocoon filament. Once this adhesive protein is removed, then the fibroin fibers are dissolved into an aqueous solution that can be further processed into different materials. Some of the material formats that have been studied are shown in Fig 1. The following protocols can help with the design and implementation of a variety of silk-based biomaterials for a range of potential applications.

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Native *B. mori* silk is composed of silk fibroin protein coated by sericin proteins. Sericins are adhesive proteins that account for 25-30% of the total silkworm cocoon by weight. The silk fibroin consists of a light chain (M_w approximately 26 kDa) and a heavy chain (M_w approximately 390 kDa) linked by a disulfide bond[9]. Silk fibroin is a block copolymer rich in hydrophobic sheet forming blocks linked by small hydrophilic linker segments or spacers. The crystalline regions are primarily composed of glycine-X repeats, where X is alanine, serine, threonine, or valine. Within these domains lie subdomains rich in glycine, alanine, serine, and tyrosine[9]. The result is a hydrophobic protein that self-assembles to form strong and resilient materials. The dominance of the sheet-forming regimes within the fibroin structure impart the protein-based materials with high mechanical strength and toughness. The toughness of silk fibers is greater than the best synthetic materials, including Kevlar®[11]. In terms of strength, silkworm silk is superior to commonly used polymeric degradable biomaterials such as collagen and poly(L-lactic acid)(PLA). The ultimate tensile strength (UTS) of *B. mori* silk fibers is 740 MPa. In contrast, collagen has a UTS of 0.9-7.4 MPa and PLA 28-50 MPa[12]. Therefore, silk fibroin is an excellent candidate polymer for biomedical applications.

In addition to the impressive mechanical properties, silk fibroin is also a degradable material. Highly crystallized silk degrades slowly, but the rate *in vivo* depends on the implantation site, mechanical environment, and features of the processing used to prepare the silk material. Silk degradation is mediated by proteases, with the peptides generated metabolized by cells[12]. *B. mori* yarns have been incubated in protease XIV up to 12 weeks and it has been shown that with increasing incubation time the enzyme cleaved the silk protein at multiple locations along the chains; overall enzymatic degradation was mediated by surface erosion[13]. Additionally, the degradation rate of silk can be altered by the mode of processing the fibroin as well as post-processing treatments, related to the content of sheet crystals and degree of organization of the noncrystalline domains. In general, the degradation rate decreases with an increase in overall sheet content. In an aside, it has also been shown that the rate of degradation of silk biomaterials directly impacted the metabolism of human mesenchymal stem cells (hMSCs) and consequently altered the rate of osteogenesis[14]. Thus, links between silk fibroin processing, material properties such as degradation rate, and biological activity have been established, and provide a solid basis for utilizing silk as a biomedical material for many applications.

As a suture material, silk fibers are often coated in wax to prevent fraying and potential immune responses. Although silk was thought to cause allergies in some patients, subsequent research has shown that sericin was the cause of the immune responses[12]. Therefore, sericin must be removed from the fibroin to assure biocompatibility. The inflammatory response to silk films was evaluated *in vitro* with hMSCs or by seeding rat MSCs on the films and implanting them *in vivo*[15]. The *in vitro* response to silk fibroin was similar to the response to collagen and tissue culture plastic (TCP) controls. In vivo, silk showed a lower inflammatory response when compared to collagen and PLA[15]. The inflammatory potential has also been assessed *in vitro* with macrophages where silk was shown to exhibit a similar tumor necrosis factor-alpha (TNF-), a cytokine indicative of an inflammatory response, levels to that of tissue culture plastic[16]. In addition, silk fibroin has been used in several *in vivo* studies for brain[17], soft tissue[18], subcutaneous[19], and bone[20] applications.

Overview of the Procedures

The applications of silk for a range of biomaterials, cell, and tissue studies have been growing in recent years (Table 1). These applications include four categories: tissue engineering, disease models, implantable devices, and drug release. The methods used to generate the material formats used in these studies are included within this protocol. For

each of the silk material formats, the raw silk from the cocoons first must be treated to remove the sericin. These steps are included in the "Silk fibroin extraction" protocol. The end result is an aqueous solution of pure silk fibroin that can be used in many of the protocols. Alternatively, the solution can be lyophilized for long-term storage or to produce materials in organic solvent (1,1,1,3,3,3-hexafluoro-2-propanol, HFIP).

Once the extracted silk fibroin is processed into one of the several formats described here, the final step is often to induce crystallinity. Crystallinity can be induced via two methods, either immersion in an alcohol such as methanol or ethanol, or by water annealing. Alcohol immersion is simple and quick but if the researcher wants to avoid the use of an alcohol, water annealing can be used. Water annealing is the process where the silk materials are incubated in a humid environment for several hours. For more detailed information, we refer the reader to Hu et al.[21], which describes a systematic analysis of crystalline induction via water annealing.

Silk fibroin extraction

This protocol is the starting point in order to obtain any of the material types listed earlier – sponges, films, fibers, and gels. The protocol is described for one batch of 5 grams of silk cocoons but if more material is required, the volumes can be scaled appropriately (Fig. 2). Only cocoons that look undamaged should be used in the process. If necessary, silk cocoons can be replaced with bave fibers (raw silk textile yarn) in step 5 and the remainder of the protocol will continue unchanged.

The resulting solution can be characterized by a variety of techniques but some of the values presented here will vary depending on the length of the boiling time and the source of the silk. For example, the molecular weight has been determined by gel electrophoresis and gel permeation chromatography. For samples that have been degummed for 30 minutes, the molecular weight has a broad distribution centered near 100 kDa. This value can increase for shorter degumming times or decrease when degummed for longer times[16]. Wray and co-workers have also established a simple method to quickly and reproducibly measure the amount of protein degradation via fluorescence spectroscopy. Briefly, dilute samples (0.1 w/v%) were excited at 280 nm in a fluorescence spectrophotometer and the band at 307 nm was assessed. For samples that were degummed for 30 min, the emission value was 0.76. This value decreased for samples that were degummed for longer times and vice versa for shorter boiling times[16]. Additionally, thin films of the silk solution can be made and thermally tested in a differential scanning calorimeter (DSC) to determine the T_g (178°C) and T_m (192-203°C)[55].

The extraction process takes 4 days. If lyophilized silk is required then an additional 3 days will be needed. Aqueous solutions can also be concentrated within an additional 24 hours. Aqueous silk solutions can be sterilized by a number of methods including sterile filtration, gamma irradiation, and autoclaving. Unfortunately, the more rigorous procedures, such as gamma irradiation[56,57] and autoclaving (unpublished results), have been shown to degrade the molecular weight of the silk fibroin and therefore irradiation dosing or steam sterilization timing should be worked out for each system. In some cases, it is simpler to sterilize the final product before use. The preferred methods of sterilization for each material format will be noted within each protocol.

The purified silk can then be used for a variety of applications summarized in Figures 3-11.

Silk Tubes (step 24, options A and B)

There are two methods for preparing silk tubes. If sterilized tubes are required, incubate the tubes in a solution of 70 v/v% ethanol overnight and then rinse in sterile water prior to use.

Dip Method The first protocol focuses on a simple dip method to create thin-walled tubes (Fig 3)(Option A). With this method, silk tubes with various inner diameters can be obtained. The thickness of the tube walls can also be varied by increasing or decreasing the number of layers deposited on the mandrel. With this method, we have formed tubes with an approximate wall thickness of 200 μm[35]. In addition, if porous tubes are required, various amounts of polyethylene oxide (PEO, 900 kDa) can be added to the silk solution and then leached out in water after the tubes have been formed. These tubes have been previously characterized by the use of scanning electron microscopy (SEM) for overall morphology and the diffusion characteristics have been studied with the use of a fluorescent label and a confocal microscope[35].

Gel Spun Tubes The latter protocol describes a gel spinning technique where concentrated silk fibroin is extruded onto a rotating mandrel (Fig 4)(Option B). By applying high shear forces as the concentrated silk solution as it is extruded through a small diameter needle, the silk is induced to gel. Further gelation is completed by the addition of methanol once the tube is formed. Due to the nature of the spinning process, gel spun tubes can add texture to the resulting tubes[58]. Silk tubes can be tailored to individual applications by changing the mandrel size in order to change the inner diameter of the resulting tube, by creating different winding geometries, or by adding a sacrificial polymer such as PEO to create pores[58]. These tubes have been evaluated for use as vascular conduits and in bioreactor systems[2,35]. SEM can be used in order to determine tube structure and whether pores were formed (if applicable)[58].

Silk hydrogelation protocols (step 24, Options C, D, E and F)

Silk hydrogels can be produced through a variety of mechanisms[59]. Here we present protocols to gel aqueous silk solutions (Fig 5) through vortexing (step 24, Option C)[60], sonication (step 24, Option D)[61], the application of direct electrical current (step 24, Option E)[62], or by lowering the pH (step 24, Option F)[63]. Each method is relatively simple and weak gels can be achieved within a few minutes. In some cases, we recommend incubating the gels at 37°C overnight after the gelation process in order to obtain stiffer gels. If an adhesive gel is of interest, we refer the reader to the electrogel and pH gel protocols. In order to produce sterile gels, we suggest that the solution be sterilized and that the gelation protocol be carried out in a sterile field. Aqueous silk solutions can be sterilized by passing through a 0.2 μm filter. If it is difficult to filter the solution, we recommend diluting the solution and then subsequently concentrating it back to 8 w/v% after filtering, if necessary. In the case of sonicated gels, the silk solution can be autoclaved in a wet cycle. Once the gels have been prepared, they can be stored at either 4 °C or room temperature. Take care to prevent the gels from drying out by keeping them tightly capped or stored in a humid environment. Silk hydrogels can be characterized with a few techniques including DSC to verify the melting point (approximately 206°C for e-gels), FTIR for secondary structure, and rheology to determine the stiffness of the gel, for example the stiffness of a 5 w/v% silk gel is around 100 kPa[60]. In the case of silk adhesives, the adhesive properties were determined using a dynamic mechanical analyzer using stainless steel fixtures (the work of adhesion values, that is the area under the normal force-strain curve, for e-gels is around 1 mJ)[62,63].

Vortexing This protocol produces silk gels from simple equipment without needing to contact the solution with a probe[60]. From a 1 ml solution, generally around 0.75 ml is recovered to form the gel. Cells may be encapsulated into the gel after the vortexing step but prior to gelation. To ensure a proper time window for cell seeding, vortexing conditions should be optimized first without cells.

Sonication—This is a simple method to produce silk gels[61]. Since there is a lag time between the sonication and the onset of gelation, this method is also useful when cells are to be encapsulated in the gel. Once again, to ensure a proper time window for cell seeding, sonication conditions should be optimized first without cells. The silk concentration can be varied from 1 to 20 w/v% and sonication time can be varied from 5 to 30 s at 10-20% amplitude. Cells can be added after the sonication step and prior to the gelation, to provide homogeneous and effective encapsulation in the gel. As an example, we have encapsulated cells into sonicated gels by autoclaving 4 w/v% silk and sonicating 5 ml of it using the methods below. Separately, human mesenchymal stem cells were harvested and brought up to a concentration of 50×10^6 cells/ml. Fifty microliters of the cell suspension was added to the sonicated silk and then incubated at 37 °C and 5% CO2 for 2 hours prior to adding cell culture medium[61].

Electric Current—The material produced in this protocol is an adhesive gel[62]. This gel is reversible if the polarity of the voltage is switched. Thus, if a positive voltage is applied to the electrode that had a negative voltage previously, the gel will begin to form on the newly positive electrode and the gel that had formed earlier will begin to disassemble. Due to the reversible nature of this gel, it can be used in applications where it is advantageous to have tunable adhesion.

Silk Films (Step 24, Options G and H)

Films provide a way to screen materials for *in vitro* or *in vivo* testing, pattern cells, embed molecules of interest, and test degradation and release. Here we present protocols to make both patterned (Step 24, Option G) and non-patterned silk films (Step 24, Option H) from aqueous solutions (Fig 6). Since the processing is water based, these techniques offer the flexibility to incorporate bioactive molecules[64]. In order to sterilize, silk films can be incubated in 70 v/v% ethanol overnight and then rinsed in sterile phosphate buffered saline (PBS) or water. Film thickness and morphology can be assessed by either manually measuring it with a micrometer or by visually measuring it with an SEM.

The patterned silk films generated from the latter protocol are generally 40 □m thick[34]. The pattern depth and number of grooves can be modified by changing the grating used to make the PDMS mold. Film thickness can be controlled by changing the concentration of the silk solution, diluting for thinner films or concentrating for thicker films. In addition, pores can be induced within the silk films by mixing various amounts of poly(ethylene oxide) (PEO) into the aqueous silk solution prior to casting. Once the films have been dried and water annealed, the PEO can be removed by immersion in water overnight. For more information please refer to Jin et al.[65].

Silk Microspheres (step 24, Options I and J)

The following protocols describe the preparation of silk micro- and nanospheres that can be used to encapsulate and release growth factors, small molecules, or therapeutic compounds. The first process described utilizes an unsaturated fatty acid lipid (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) (DOPC) to encapsulate aqueous based-silk solutions with a molecule of interest (step 24, Option I)[54]. Once vesicles have been generated, the lipid is removed and the microspheres can be resuspended when required (Fig 7). The second procedure describes the formation of microspheres through the use of phase separation between silk and another polymer (polyvinyl alchohol)(PVA)(step 24, Option J)[52] (Fig 8). This second method is particularly useful due to its simplicity and avoidance of organic solvents in the process.

The first protocol is an example on how to incorporate molecules of interest into silk microspheres using DOPC as an emulsifier[54]. The average particle size of spheres made

with this method is approximately 2 μm, which can be determined with either optical microscopy or SEM[54]. As for drug loading, different drugs will interact differently with the silk and therefore the loading efficiency will differ between various molecules. We suggest that small batches are tested in the process before scaling up. Resuspended microspheres can also be mixed with silk solution and then be made into porous scaffolds, hydrogels or films.

The second protocol, that utilizes PVA, will generate silk spheres with a size range between 300 nm to 20 μm[52]. The size of the spheres can be controlled by changing the concentration of the silk or PVA solutions or by sonicating to the solution prior to casting the film. In order to characterize these spheres a few techniques of been employed including dynamic light scattering to determine the size of the nanometer spheres, laser light diffraction to determine the size of the microspheres, and SEM to image the morphology. Confocal microscopy was also used to determine model drug loading[52]. Drugs may be encapsulated in these spheres by adding the drug to the silk solution prior to mixing with the PVA. In general we start with loading a mass ratio of 100:1 silk to drug. Drug loading efficiency is specific to each case due to molecular weight, charge, and hydrophobicity and therefore must be determined for each drug.

Electrospun Silk Fibers (step 24, Option K)

Electrospinning is a process by which small diameter fibers with high surface area can be produced (Fig 9)(step 24, Option K). The equipment required is relatively simple but the fibers produced are much smaller in diameter than conventionally spun fibers[66] and the process offers the ability to incorporate molecules of interest[26]. In addition, fibers can be aligned by either using a rotating mandrel[67] or inducing the Hall Effect[68]. Electrospun mats can be sterilized by immersing the mats in 70 v/v% ethanol overnight and then subsequently rinsing them in either sterile water or PBS.

This protocol produces a non-woven isotropic mat of silk fibers. The fibers will have diameters less than 800 nm and should appear fairly uniform. SEM can be used to examine the fiber morphology, diameter, and orientation.

Silk Sponges (Step 24, Options L and M)

Silk sponges provide a versatile 3D porous scaffolding material for several applications as shown in Table 1. Here we provide protocols to form aqueous-based[69] (Fig 10) or HFIP-based scaffolds[70] (Fig 11). The aqueous-based sponges have excellent interconnectivity between the pores and have the benefit of not requiring an organic solvent. This may be of interest if a bioactive molecule will be incorporated into the silk matrix during processing. The HFIP-based sponges have smoother surfaces along the pores and have higher mechanical strength. Through this processing method, reinforcing agents can be added to the silk matrix to further increase mechanical properties[23]. In addition, it has been shown that aqueous-based sponges degrade faster than HFIP-based sponges[14] which enables the user to tailor these scaffolds to project specifications. Both of these sponge types may be sterilized by either immersing them in 70 v/v% ethanol overnight or by autoclaving. The first protocol will produce aqueous-based silk sponges 15 mm in diameter and 10 mm in height and the second protocol will produce at least 9 scaffolds approximately 12 mm in both height and diameter. SEM can be used in order to characterize the morphology and pore structure of these materials. Porosity can be determined via liquid displacement[70].

Materials

Reagents

Extraction

- Silk cocoons (Tajima Shoji Co., LTD., Yokohama, Japan or equivalent)
- Sodium carbonate (Sigma Aldrich, cat. no. 451614, www.sigmaaldrich.com)
- Ultrapure water
- Lithium bromide (Sigma Aldrich, cat. no. 213225, www.sigmaaldrich.com)

Concentrating Silk Fibroin

- 7-8w/v% fibroin solution (Procedure step 22)
- Polyethylene glycol (PEG 10,000 MW, Sigma-Aldrich, cat. no. P6667, www.sigmaaldrich.com)
- Ultrapure water

Silk Tubes and Gel Spun Tubes

- Concentrated silk fibroin (Procedure step 23B)
- Methanol (Acros Organics, cat. no. 176840010, www.fishersci.com)
- Dish soap (local grocery store)

Vortexed Gels and Sonicated Gels

- 8 w/v% Aqueous silk solution
- Ultrapure water

Electrogelated Gels

- 8 w/v% Aqueous silk solution

pH gels

- 8 w/v% Aqueous silk solution
- 0.3 M Hydrochloric acid (HCl, Sigma-Aldrich, cat. no. H1758, www.sigmaaldrich.com)

Silk Films

- 8 w/v% Aqueous silk solution

Patterned Silk Films

- 8 w/v% Aqueous silk solution
- Polydimethylsiloxane (PDMS) (Sylgard 184, Ellsworth Adhesives, cat. no. 184 SIL ELAST KIT 0.5KG, www.ellsworth.com)

Silk Microspheres using DOPC

- 8 w/v% aqueous silk solution
- 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC, Avanti Polar Lipids, Inc., cat. no. 850375P, www.avantilipids.com)

- Chloroform (Fisher Scientific, cat. no. C298-1, www.fishersci.com)
- Methanol (Sigma-Aldrich, cat. no. R4828000-4C, www.sigmaaldrich.com)
- Nitrogen gas (local gas vendor)
- Drug, small molecule, or protein of interest
- Ultrapure water

Microspheres (PVA)

- 8 w/v% Aqueous silk solution
- Polyvinyl alcohol (MW 30-70 kDa, Sigma, cat. no. P8136, www.sigmaaldrich.com)
- Ultrapure water

Electrospinning

- 8 w/v% aqueous silk solution
- Polyethylene oxide (PEO, 900 kDa, Sigma-Aldrich, cat. no. 189456, www.sigmaaldrich.com)
- Ultrapure water
- Methanol (Acros Organics, cat. no. 176840010, www.fishersci.com)

Silk Sponges

- 6-8 w/v% aqueous silk solution
- Salt (Fisher Scientific, cat. no. BP358-1, www.fishersci.com)
- Ultrapure water

HFIP-based Sponges

- Lyophilized silk fibroin
- 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma-Aldrich, cat. no. 105228-100G, www.sigmaaldrich.com)
- NaCl (Fisher Scientific, cat. no. BP358-1, www.fishersci.com)
- Ultrapure water
- Methanol (Acros Organics, cat. no. 176840010, www.fishersci.com)

Equipment

Extraction

- Titanium scissors (Staples, cat. no. 487905, www.staples.com)
- Small stir bar (Fisher Scientific, cat. no. 14-513-62, www.fishersci.com)
- Large stir bar (Fisher Scientific, cat. no. 14-513-56, www.fishersci.com)
- Spatula (Fisher Scientific, cat. no. 14-373-25A, www.fishersci.com)
- 50 ml glass beaker (Kimble Chase Kimble, cat. no. 14000 50, www.fishersci.com)
- 1 L glass beaker (Kimble Chase Kimble, cat. no. 14000 1000, www.fishersci.com)

- 2 L glass beaker (Kimble Chase Kimble, cat. no. 14000 2000, www.fishersci.com)
- 2 L plastic beaker (Fisher Scientific, cat. no. 02-591-33, www.fishersci.com) . Aluminum foil (Costco Warehouses)
- Hot plate (Fisher Scientific, cat. no. 11-100-100H, www.fishersci.com)
- Large weigh boat (Fisher Scientific, cat. no. 08-732-115, www.fishersci.com)
- Medium weigh boat (Fisher Scientific, cat. no. 08-732-113, www.fishersci.com)
- Small weigh boat (Fisher Scientific, cat. no. 08-732-112, www.fishersci.com)
- Analytical balance (A&D Weighing, model Orion HR-200, www.americanweigh.com)
- Hot hand protectors (Bel-Art Products, cat. no. 380000000, www.fishersci.com)
- 50 ml graduated cylinder (Fisher Scientific, cat. no. 08-557C, www.fishersci.com)
- Slide-A-Lyzer Dialysis cassette 3500 MWCO, 3-12 ml capacity (Thermo Scientific, cat. no. 66110, www.fishersci.com)
- Dialysis cassette buoy (Thermo Scientific, cat. no. 66432, www.fishersci.com)
- 20 ml syringe (BD Medical, cat. no. 309661, www.fishersci.com)
- 18G needles (BD Medical, cat. no. 305195, www.fishersci.com)
- 50 ml conical tubes (BD Medical, cat. no. 14-432-22, www.fishersci.com)
- Centrifuge (Eppendorf 5804R, cat. no. 022623501, www.fishersci.com)
- Fixed angle rotor (Eppendorf, cat. no. 022637207, www.fishersci.com)
- Lyophilizer (Optional: only if lyophilized silk is required, Labconco Corp., cat. no. 7751030, www.fishersci.com)
- −80 °C Freezer (Optional: only if lyophilized silk is required, Thermo Scientific, cat. no. ULT2586-9SI-A, www.fishersci.com)
- Kim wipes® (Kimberly Clark, cat. no. 34256, www.fishersci.com)

Concentrating Silk Fibroin

- 10 ml syringe (BD Medical, cat. no. 309604, www.fishersci.com)
- 3 ml syringe (BD Medical, cat. no. 309585, www.fishersci.com)
- 18G needles (BD Medical, cat. no. 305195, www.fishersci.com)
- 0.5 – 3.0 ml Slide-A-Lyzer dialysis cassette (Thermo Scientific, cat. no. 66330, www.fishersci.com)
- 100 ml beaker (Kimble Chase Kimble, cat. no. 14000 100, www.fishersci.com)
- 1 L beaker (Kimble Chase Kimble, cat. no. 14000 1000, www.fishersci.com)
- Large stir bar (Fisher Scientific, cat. no. 14-513-56, www.fishersci.com)
- Small dialysis cassette buoy (Thermo Scientific, cat. no. 66430,www.fishersci.com)
- Aluminum foil (Costco Warehouses)
- Microcentrifuge tubes (2 ml capacity, Fisher Scientific, cat. no. 05-408-138, www.fishersci.com)

Silk Tubes

- Stainless steel wire cut to 3 inch lengths, gauge will depend on required inner diameter (eg. 0.5 mm, McMaster-Carr, cat. no. 8908K21, www.mcmaster.com)
- Feather™ Scapel (EMS, cat. no. 72042-20, www.fishersci.com)
- 15 ml conical tube (BD Medical, cat. no. 352096, www.fishersci.com)
- Tweezers (Fisher Scientific, cat. no. 08-880, www.fishersci.com)

Gel Spun Tubes

- Mandrel rotation system, custom made to rotate and transverse[58]
- 1 ml Luer-Lok syringe (BD Medical, cat. no. 309628, www.fishersci.com)
- 14 gauge blunt-tip needle (McMaster-Carr, cat. no. 75165A672, www.mcmaster.com)
- 30 gauge blunt-tip needle (McMaster-Carr, cat. no. 75165A31, www.mcmaster.com)
- 27 gauge blunt-tip needle (McMaster-Carr, cat. no. 75165A688, www.mcmaster.com)
- Stainless steel wire, gauge will depend on required inner diameter (eg. 0.5 mm, McMaster-Carr, cat. no. 8908K21, www.mcmaster.com)
- Kim wipes® (Kimberly Clark, cat. no. 34256, www.fishersci.com)

Vortexed Gels

- Vortexer (Fisher Scientific, cat. no. 02-215-365, www.fishersci.com)
- 1 ml syringe (Air Tite Products Co., cat. no. A1, www.fishersci.com)
- Glass vials (Fisher Scientific, cat. no. 03-339-26A, www.fishersci.com)
- Duct tape (3M, cat. no. 3939, www.fishersci.com)

Sonicated Gels

- Ultrasonicator (Branson 450, cat. no. 101-135-022, www.fishersci.com)
- ½” Externally Threaded Disruptor Horn (Branson, cat. no. 101-147-037, www.fishersci.com)
- Tapered Microtip, 1/8” diameter (Branson, cat. no. 101-148-062, www.fishersci.com)
- 15 ml conical tubes (BD Medical, cat. no. 352096, www.fishersci.com)

Electrogelated Gels

- DC Power supply (Agilent E3634A, www.agilent.com)
- Platinum wires (0.032 in dia., 1 ft, SurePure Chemetals, Inc., cat. no. 1987, www.surepure.com)
- 50 ml conical tubes (BD Medical, cat. no. 14-432-22, www.fishersci.com)

pH gels

- Glass vials (1.8 ml, Fisher Scientific, cat. no. 03-339-26A, www.fishersci.com)

Silk Films

- 100 mm Petri dish non-tissue culture treated (Fisher Scientific, cat. no. 08-757-12, www.fishersci.com)
- Tweezers (Fisher Scientific, cat. no. 08-880, www.fishersci.com)
- Vacuum desiccator (Bel-Art Products, cat. no. 420200000, www.fishersci.com)

Patterned Silk Films

- Diffraction grating: size, number of grooves, and depth of grooves can change due to requirements, an example is shown here (Edmund Optics, 600 grooves/mm, 1000 nm ruled diffraction grating, 50 × 50 mm, cat. no. NT43-208, www.edmundoptics.com)
- Vacuum oven (Fisher Scientific, cat. no. 13-262-280A, www.fishersci.com)
- Tweezers (Fisher Scientific, cat. no. 08-880, www.fishersci.com)
- Kim wipes® (Kimberly Clark, cat. no. 34256, www.fishersci.com)
- Paper cup (local grocery)
- 5 ml pipet (BD Vacutainer Labware Medical, cat. no. 357543, www.fishersci.com)
- Aluminum foil (Costco Warehouses)
- 70 v/v% Ethanol (Ricca Chemical, cat. no. 2546701, www.fishersci.com)
- Needle (gauge unimportant, BD Medical, cat. no. 305195 (18G), www.fishersci.com)

Silk Microspheres using DOPC

- Round-bottom glass tubes (Fisher Scientific, cat. no. 14-961-29, www.fishersci.com)
- 1.5 ml microcentrifuge tube (Fisher Scientific, cat. no. 054-08-129, www.fishersci.com)
- 2.0 ml microcentrifuge tube (Fisher Scientific, cat. no. 02-681-258, www.fishersci.com)
- 15 ml conical tubes (BD Medical, cat. no. 352096, www.fishersci.com)
- 50 ml conical tubes (BD Medical, cat. no. 14-432-22, www.fishersci.com)
- 100 ml beaker (Kimble Chase Kimble, cat. no. 14000 100, www.fishersci.com)
- Small stir bar (Fisher Scientific, cat. no. 14-513-62, www.fishersci.com)
- −80°C Freezer (Thermo Scientific, cat. no. ULT2586-9SI-A, www.fishersci.com)
- Lyophilizer (Labconco Corp., cat. no. 7751030, www.fishersci.com)
- Microcentrifuge (Eppendorf5417R,cat.no.022621700, www.fishersci.com)

Microspheres (PVA)

- Small weigh boat (Fisher Scientific, cat. no. 08-732-112, www.fishersci.com)
- Spatula (Fisher Scientific, cat. no. 14-373-25A, www.fishersci.com)
- 50 ml glass beaker (Kimble Chase Kimble, cat. no. 14000 50, www.fishersci.com)

- 10 ml graduated cylinder (Fisher Scientific, cat. no. 08-557A, www.fishersci.com)
- Small stir bar (Fisher Scientific, cat. no. 14-513-62, www.fishersci.com)
- Hot plate (Fisher Scientific, cat. no. 11-100-100H, www.fishersci.com)
- Syringe filter unit (pore size 0.45 □m, Millipore, cat. no. SLHA033SS, www.fishersci.com)
- 5 ml syringe (BD Medical, cat. no. 309603, www.fishersci.com)
- 15 ml conical tube (BD Medical, cat. no. 352096, www.fishersci.com)
- 50 ml conical tubes (BD Medical, cat. no. 14-432-22, www.fishersci.com)
- 100 mm Petri dish non-tissue culture treated (Fisher Scientific, cat. no. 08-757-12, www.fishersci.com)
- Parafilm® (Pechiney Plastic Packaging, cat. no. PM996, www.fishersci.com)
- Tweezers (Fisher Scientific, cat. no. 08-880, www.fishersci.com)
- Ultrasonicator (Branson 450, cat. no. 101-135-022, www.fishersci.com)
- ½" Externally Threaded Disruptor Horn (Branson, cat. no. 101-147-037, www.fishersci.com)
- Tapered Microtip, 1/8" diameter (Branson, cat. no. 101-148-062, www.fishersci.com)
- Reciprocating shaker (MaxQ, ThermoScientific, cat. no. 11-675-152, www.fishersci.com)

Electrospinning

- 20 ml glass scintillation vials (Fisher Scientific, cat. no. 03-337-15, www.fishersci.com)
- Small stir bar (Fisher Scientific, cat. no. 14-513-62, www.fishersci.com)
- Hot plate (Fisher Scientific, cat. no. 11-100-100H, www.fishersci.com)
- 10 ml syringe (BD Medical, cat. no. 309604, www.fishersci.com)
- Blunt tip needle (16 gauge, McMaster-Carr, cat. no. 75165A552, www.mcmaster.com)
- Syringe pump (Fisher Scientific, cat. no. 14-831-200, www.fishersci.com)
- High voltage supply (Glassman Series EH, cat. no. EH30P3, www.glassmanhv.com)
- Non-stick aluminum foil (Reynolds Wrap, local grocery)
- Reciprocating shaker (MaxQ, ThermoScientific, cat. no. 11-675-152, www.fishersci.com)
- Insulated electrical wire (local hardware store)
- Alligator clips (McMaster-Carr, cat. no. 7236K252, www.mcmaster.com)

Silk Sponges

- Sieves (Fisher Scientific various pore sizes, eg. 1 mm cat. no. 04-884-1AJ, 850 □m 04-884-1AK, 600 □m 04-884-1AM, 500 □m 04-884-1AN, www.fishersci.com)

- Sieve cover (Fisher Scientific, cat. no. 04-888B, www.fishersci.com)
- Small weigh boat (Fisher Scientific, cat. no. 08-732-112, www.fishersci.com)
- Analytical balance (A&D Weighing, model Orion HR-200, www.americanweigh.com)
- Cylindrical molds (5 ml, Fisher Scientific, cat. no. 03-338-1E, www.fishersci.com)
- 5 ml syringe (Air Tite Products Co, cat. no. A5, www.fishersci.com)
- 2 L plastic beaker (Fisher Scientific, cat. no. 02-591-33, www.fishersci.com)
- Large stir bar (Fisher Scientific, cat. no. 14-513-56, www.fishersci.com)
- Hot plate (Fisher Scientific, cat. no. 11-100-100H, www.fishersci.com)
- 50 ml conical tubes (BD Medical, cat. no. 14-432-22, www.fishersci.com)

HFIP-based Sponges

- Sieves (Fisher Scientific various pore sizes, eg. 1 mm cat. no. 04-884-1AJ, 850 μm cat. no. 04-884-1AK, 600 μm cat. no. 04-884-1AM, 500 μm cat. no. 04-884-1AN, www.fishersci.com)
- Sieve cover (Fisher Scientific, cat. no. 04-888B, www.fishersci.com)
- Small weigh boat (Fisher Scientific, cat. no. 08-732-112, www.fishersci.com)
- Analytical balance (A&D Weighing, model Orion HR-200, www.americanweigh.com)
- 20 ml glass scintillation vials (Fisher Scientific, cat. no. 03-337-15, www.fishersci.com)
- Glass vials (1.8 ml, Fisher Scientific, cat. no. 03-339-26A, www.fishersci.com)
- Parafilm® (Pechiney Plastic Packaging, cat. no. PM996, www.fishersci.com)
- 5 ml syringe (Air Tite Products Co, cat. no. A5, www.fishersci.com)
- 2 L plastic beaker (Fisher Scientific, cat. no. 02-591-33, www.fishersci.com)
- Large stir bar (Fisher Scientific, cat. no. 14-513-56, www.fishersci.com)
- Hot plate (Fisher Scientific, cat. no. 11-100-100H, www.fishersci.com)
- Fume hood
- 50 ml conical tubes (BD Medical, cat. no. 14-432-22, www.fishersci.com)

Procedure

Fibroin Extraction TIMING: 2.5 hrs active, overnight drying (Day 1)

1. Prepare a 2 L glass beaker filled with 2 L of ultrapure water, cover with aluminum foil, and heat until boiling. CAUTION: Do not leave beaker unattended while heating and boiling! Due to high temperatures plastic beakers should not be used.
2. Meanwhile, cut cocoons with titanium scissors into dime-sized pieces and dispose of silk worm. Measure out 5g of cocoon pieces into a large weigh boat.
3. Measure 4.24 g of sodium carbonate in a medium weigh boat.

4. Add the measured sodium carbonate to the water and let it completely dissolve (to make a 0.02 M solution of Na_2SO_3). CAUTION: If water is boiling, add sodium carbonate slowly to avoid boiling over!

5. Add the cocoon pieces once the water starts to boil and cook it for 30 minutes. Occasionally, stir it with a spatula to promote good dispersion of fibroin.

CRITICAL STEP To increase reproducibility, boil for exactly 30 min every time. If boiling for longer or shorter times, indicate this on the batch label. Increasing the boiling time will degrade the fibroin.

6 Remove the silk fibroin with a spatula and cool by rinsing in ultrapure water. Squeeze excess water out of the silk. Discard the sodium carbonate solution in the sink. CAUTION: Silk fibroin and solution will be hot, use hand protectors!

7 Put fibroin in a 1 L beaker filled with 1 L of ultrapure water and a stir bar. CAUTION: If using a plastic beaker, make sure that the hot plate has cooled.

8 Rinse the fibroin in water for 20 minutes while gently stirring on a stir plate.

9 Repeat steps 7-8 twice for a total of 3 rinses.

10 After the third wash, remove the silk, squeeze it well, and then spread it out on a clean piece of aluminum foil.

11 Allow the silk fibroin to dry in a fume hood overnight.

Pause Point: Degummed silk fibroin, where the sericin has been removed, can be stored indefinitely at room temperature. For long-term storage, keep in a clean plastic bag or wrap in aluminum foil. Be sure to indicate the length of the boiling step on the label.

Dissolve silk fibroin in LiBr TIMING: 4.5 hrs (Day 2)

12 Calculate the amount of 9.3 M lithium bromide needed to make a 20 w/v% solution based off the amount of dried fibroin available. Since 20% of the solution will be silk, 80% will be LiBr. That is a ratio of 1 to 4, silk to LiBr. Therefore, multiply the amount of the dried silk fibroin by 4 to get the total volume of 9.3 M LiBr needed (X).

13 Prepare a 9.3 M LiBr solution.

$$\left(86.85\frac{g}{mol}\right)\left(9.3\frac{mol}{L}\right)\left(\frac{1L}{1000ml}\right)(X)=\_\ g\ \text{of LiBr}$$

CRITICAL STEP LiBr has a low density and its volume should be taken into account while making the solution. We suggest adding only 60% of the calculated volume of water and then bringing the solution up to the final volume. Stir with a small stir bar. CAUTION: Adding LiBr to water results in an exothermic reaction, be mindful of the heat generated! When making large volumes it is suggested to carry this out on ice.

14 Pack silk fibroin tightly into a 50 ml glass beaker and add the required amount of LiBr solution on top.

CRTICAL STEP The LiBr must be added to the silk rather than adding silk to the LiBr so that the silk will eventually be covered and dissolved by the LiBr. It is also helpful to use the smallest glass container that will still hold the silk and LiBr solution.

15 Let fibroin dissolve in an oven at 60°C for 4 hours. Once the silk fibroin is completely dissolved, it will appear amber in color and will be transparent. Black bits

from the silkworm may be visible but will be removed later. This solution will be highly viscous but should not contain any intact fibers as determined by visual assesment.

Dialysis and Centrifugation TIMING: 49 hrs (Days 2-4)

16 Hydrate dialysis cassettes in water for a few minutes.

17 With a 20 ml syringe and an 18-gauge needle, insert 12 mL of the silk-LiBr solution into a 3-12 ml dialysis cassette.

CRITICAL STEP Be careful not to puncture or touch the dialysis membrane. The solution will be very viscous so this step will be easier if the solution is kept warm prior to adding to the cassette. It is important to avoid shearing the solution whenever possible to avoid the induction of β-sheet within the silk. Therefore, only use the needle when injecting into the cassette. Also, have an additional needle and insert into another top port of the cassette to allow air to escape. Remove extra needle once all the air has been purged.

18 Dialyze against 1L of ultrapure water per 12 ml cassette. To ensure mixing, use a large stir bar and place on a magnetic stir plate. Change water after 1 hr, 4 hrs, that evening, the next morning and night, and in the morning on the following day (6 changes within 48 hours).

19 Remove silk from the cassettes with another 20 ml syringe and an 18-gauge needle. Place silk in a 50 ml conical tube. Depending on the volume, either split between 2 tubes (if more than 40 ml) or fill one tube and use a counterbalance of water.

20 Centrifuge to remove impurities. Place in a centrifuge and spin at 9,000 rpm (approximately 12,700 g) at 4°C for 20 minutes.

21 Carefully remove tubes from the centrifuge and either pour or transfer the silk solution with a 25 ml pipet into another centrifuge tube. Be sure to leave any white flocculent or brown matter behind.

22 Repeat steps 20-21 again.

23 In order to determine the concentration of the silk in solution, measure the weight of a small weigh boat. Then add 0.5 ml of the silk solution to the boat and allow it to dry at 60 °C. Once the silk is dry, determine the weight of the silk and divide it by 0.5 ml. This will yield the weight per volume percentage.

CRITICAL STEP A batch of 5 g of silk cocoons generally yields 25 ml of 7 to 8 w/v% silk solution. The solution will be tinted yellow but should be relatively clear and slightly more viscous than water. If there are impurities such as white flocculents or dark particulates it is best to recentrifuge to remove them.

PAUSE POINT The silk solution can be stored at 4°C for at least a month. Depending on the purity, stored silk will eventually gel but gelation times will vary. Once the silk has gelled, it cannot be used for protocols that require solution and therefore another batch will need to be extracted.

24 The fibroin solution (25 ml at concentration 7-8 % (w/v)) can either be used as is or it can be lyophilized (Option A) or concentrated (Option B). For storage for longer than one month, the silk solution should be lyophilized. In this form, the silk will be stable for years at room temperature and can be reconstituted in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). The concentrated solution (20-30 w/v%) can be used directly for making silk tubes.

(A) Lyophilization (Optional) TIMING: 3 days

i. Divide the aqueous silk solution into 50 ml conical tubes with no more than 20 ml per tube.

ii. Place vertically in a −80°C freezer for several hours until solution is completely frozen. If a −80 °C freezer is not available, silk can also be frozen at −20 °C overnight or placed in liquid nitrogen until the solution is frozen.

iii. Fold a Kim wipe® over the top of the tube and attach with either a rubber band or tape. Keep caps for later use.

iv. Place frozen samples on a lyophilizer for 2-3 days until all of the water is removed from the solution. The tube will no longer feel cold.

v. Remove from lyophilizer, cap, and store at room temperature (20 to 25 °C).

<PAUSE POINT> The lyophilized material can be stored at room temperature indefinitely.

(B): Concentrating Silk Fibroin TIMING: 21-25 hrs

i. Add 100 g of PEG to 1 L of ultrapure water to make a 10 w/v% PEG solution. Mix with a large stir bar on a magnetic stir plate. The solution should be clear once the PEG is completely dissolved. While waiting for the PEG to dissolve, move to step 2.

ii. Hydrate dialysis cassette in a 100 ml beaker filled with ultrapure water for a few minutes.

iii. Remove cassette from the water and tap the bottom of the cassette on paper towel to dry. Be sure to hold the cassette at the edges and do not touch membrane.

iv. Draw out 10 ml of 7-8w/v% silk solution with the 10 ml syringe *without* a needle.

v. Attach the 18 gauge needle to the syringe filled with fibroin solution.

vi. Insert the tip of the needle into a top port of the cassette.

<CRITICAL STEP> Be careful to insert just the tip of the needle in order to avoid puncturing the membrane.

vii. Slowly insert the fibroin solution into the cassette.

CRITICAL STEP Avoid high shear that may allow premature alignment of the β-sheets in the silk fibroin.

viii. As the cassette becomes inflated, purge excess air by inserting another 18 gauge needle into the other top port to allow the air to escape.

ix. After air is removed, withdraw the extra needle from the cassette.

x. Finish filling the cassette with the remaining fibroin solution. Although the cassette indicates a maximum volume of 3.0 ml, 10 ml can be inserted. During the concentration process this volume will go down.

xi. Remove needle/syringe from cassette.

xii. Attach the dialysis buoy onto the top of the cassette.

xiii. Place cassette into 10 w/v% PEG solution and cover with aluminum foil.

xiv. Indicate the time and date that the cassette was added to solution. For dip method tubes, the silk is generally concentrated for 20 hours. For the gel spun tubes, the solution must be more viscous and therefore is concentrated for around 22 hours.

xv. Remove the concentrated silk solution after the desired amount of time via a 3 ml syringe and an 18-gauge needle.

xvi. Measure the solution concentration by weighing out a small weigh boat and then adding 0.1 ml of concentrated silk solution to the boat. Due to the high viscosity of concentrated silk we suggest to use either large orifice pipette tips or cut the tip of the pipette off. Dry the solution at 60 °C and then weigh the dried film. The weight of the silk divided by the volume used (0.1 ml) will yield the weight per volume percent.

<CRITICAL STEP> Depending on the initial silk concentration, 10 ml of silk fibroin will yield 2-4 ml of concentrated silk solution after dialyzing against 10 w/v% PEG for 20-22 hours. The amount of time used to concentrate the solution may need to be altered for each batch. The concentration time is not linear, so care must be taken to avoid gelling the silk in the cassette if left in the PEG solution for too long. With experience, the researcher will be able to visually determine whether the silk is concentrated enough. If the silk is collected too early, it can either be replaced into a cassette and allow the dialysis to proceed for more time or it can be stored at 4 °C. We find that storing the silk for a few days will allow it to come to the required concentration. It is suggested that it is checked daily since the solution can gel after it has been stored for too long (approximately 1-2 weeks). The solution can be checked by attempting to use it in the intended protocol. For example, for dipped tubes a mandrel can be dipped into the solution and then it can be visually determined whether multiple beads form without coalescing. If this occurs, then the solution is not ready. The resulting silk solution should be very viscous and will appear slightly cloudy when compared to the starting solution.

xvii. Remove the needle and transfer the solution to 2 ml microcentrifuge tubes.

CRITICAL: STEP Be careful not to introduce bubbles to the solution, they are very difficult to remove later.

<PAUSE POINT> Label and store at 4°C for a few days. Concentrated silk can only be stored for a few days, possibly up to a week, before it gels. Only concentrate silk when it will be used relatively soon.

24 The silk fibroin can now be used to make a number of different materials (Figure Scheme). Using the concentrated solution (prepared in 23A) you can make silk tubes by either a simple dip method to create thin walled tubes (Option A, Figure 3) or by gel spinning where the fibroin is extruded onto a rotating mandrel (Option B, Figure 4).

(A) Dipped Tubes TIMING: 2 min per tube with overnight dry and 1 hr soak

i. Optional. If porous tubes are required, make a solution of 7-8w/v% PEO and carefully add the appropriate amount to the concentrated silk solution. Gently stir with a stainless steel wire.

<CRITICAL STEP> It is important not to introduce bubbles to either solution since both are highly viscous and bubbles are difficult to remove. If bubbles are present, they will create holes in the resulting silk tubes.

ii. Dip stainless steel wire into concentrated silk solution. Remove wire from silk and invert wire, allowing the beads of silk to evenly run down the wire.

When the beads coalesce at the bottom of the wire, flip and allow the silk to run back down towards the end of the wire (try to coat evenly).

Troubleshooting

iii. Let excess silk bead drip off of wire back into silk solution.

iv Place in a vial of methanol for 5-10 seconds.

v CRITICAL: Remove silk-coated wire from methanol and allow to dry for approximately 30 seconds.

vi Repeat dipping process and methanol treatment 2-4 times total or until wire is sufficiently coated with silk. The silk may appear uneven but it will shrink as it dries.

vii. Dry tubes by sticking the inverted silk-coated wire into styrofoam and place it in a fume hood overnight. We suggest making an excess number of tubes as some of the tubes may crack during the drying process.

PAUSE POINT: Dried tubes can be stored for several days at room temperature.

viii. When silk is dry, place the wires coated with silk into a 15 ml conical tube filled with soap water and soak for one hour.

ix. After soaking, remove the tubes from the soap water and cut the end of the silk tube with a scalpel or razor blade.

x. Remove the tube from the wire using tweezers. The tube should slide off easily. If it is difficult to remove without scrunching the tube, return tube to soapy water and wait before trying again. TROUBLESHOOTING

xi. Fill a plastic Petri dish (10 cm) with ultrapure water. Place the tubes in the water and incubate on a shaking water bath for 1 hr to remove residual soap.

xii. Store silk tubes in a plastic Petri dish at room temperature. These tubes have been stored for several months up to a couple of years. They are brittle so we suggest that they be hydrated in water before being manipulated.

B: Gel spun tubes Timing: 5 min per tube plus overnight dry

i. Place the mandrel in the mandrel rotation system.

ii. Attach a 14 gauge needle on a 1 ml syringe and slowly draw up concentrated silk solution. Be sure to avoid including air bubbles.

iii. Remove needle and replace with a 30 gauge needle.

iv. Start mandrel rotation system.

v. Squeeze the gel solution out of the needle and onto the mandrel. The fiber should be uniform without any beads or discontinuities. In order to have a uniform fiber, it will require a generous amount of pressure. If solution does not come out, replace the needle with a 27 gauge needle. TROUBLESHOOTING

<CRITICAL STEP> The winding pattern can be altered through the use of the mandrel rotation system. If the rotation is constant and the mandrel oscillates back and forth, a cross-hatched design can be generated. If a fiber running in the same direction is required, the mandrel rotation system should be stopped after 1 layer and then the rotation reversed. The wall thickness obtained with this method will depend on the number of layers and the speed of the transverse motor.

vi. Once the silk has been laid onto the mandrel, apply methanol on top of the silk with a needle and syringe in order to induce β-sheet formation within the silk fibroin.

vii. Allow the methanol to dry then place the mandrel into a solution of soap water.

viii. When the tube has softened, grab the tube uniformly with a Kim wipe® and gently pull off. If the tube does not readily move, continue to soak it in the soap solution.

ix. Fill a plastic Petri dish (10 cm) with ultrapure water. Place the tube in the water and incubate on a shaking water bath for 1 hr to remove residual soap.

x. Store silk tube in a plastic Petri dish at room temperature.

C: Vortexed gels TIMING: 1 Day

i. Adjust the concentration of an 8w/v% silk solution to 2-5 w/v%.

ii. Transfer 1 mL of silk solution into a glass vial and close tightly.

<CRITICAL STEP> This protocol is for 1 ml of low concentration silk solution. Larger volumes may be used but the vortex time and speed may need to be altered. It is not necessary to use glass vials.

iii. Secure the vial in an upright position on the vortexer with duct tape.

iv. Vortex the solution in the glass vial for approximately 7 min at 3,200 rpm (maximum speed setting). This treatment should increase the solution turbidity. TROUBLESHOOTING

<CRITICAL STEP> Silk solutions may vary between batches and as a solution ages, therefore the vortex time may need to be increased or decreased.

v. Collect the turbid solution using a 1 mL slip-tip syringe avoiding any possible solid, sticky phase.

vi. Incubate turbid solution overnight in the syringe or a well-plate to allow for gelation. Incubation at 37°C will decrease the gelation time. For long-term storage, store at 4°C.

vii. Gels can be injected through 21 gauge (or larger diameter) needles if necessary. Minimal force should be sufficient to inject a 5 w/v% gel through a 21 gauge needle. If injection requires significant force, the tip of the syringe can be cut using a blade (before connecting the needle) to remove the skin layer at the tip.

D: Sonicated gels TIMING: 1 Day

i. Dilute 8 w/v% aqueous silk solution to 4 w/v%.

ii. Add 5 ml of 4 w/v% silk solution to a 15 ml conical tube.

iii. Sonicate the solution at 50% amplitude (21 watts) for 30 seconds. CAUTION: The sonicating horn must be immersed in solution while the power is applied in order to prevent damage to the instrument. Be careful not to touch the horn on the sides of the tube during sonication. CAUTION: Extended exposure to ultrasonication may cause damage to one's hearing. Use of protective ear muffs is highly recommended. TROUBLESHOOTING

iv. If required, the turbid solution can be pipetted into a well plate or a Petri dish immediately after sonication, prior to gelation.

v. Incubate the sonicated solutions at 37°C to allow for hydrogelation.

E: Electrogels (E-gels) TIMING: 15 min

i. Add 10 ml of 7-8 w/v% aqueous silk solution to a 50 ml conical tube.

ii. Immerse a pair of platinum wire electrodes into the silk solution. If the electrodes do not reach the solution, cut off the top portion of the tube.

iii. Apply approximately 25 V_{DC} to the platinum electrodes over a few minutes. Within seconds of the application of the voltage, a visible gel will form at the positive electrode, locking in some oxygen bubbles at the electrode surface as the gel emanates outward. TROUBLESHOOTING.

iv. Turn off the power supply when additional gel no longer collects on the positive electrode.

v. Collect the gel collected on the positive electrode.

vi. Optional. If a stiffer gel is required, collect the e-gel into a syringe by removing the plunger and placing the silk in the open end. The β-sheet content of the silk is increased when a rapid shear force is applied to the gel.

F.: pH gels Timing:10 min

i. Add 0.9 ml of 8 w/v% aqueous silk solution to a glass vial. This protocol has been established for a total solution volume of 1 ml but the volumes can be scaled up if the volumetric ratio of silk to acid is maintained at 10:1.

ii. Add 0.1 ml of 0.3 M HCl to the vial. The molarity of the HCl can be increased or decreased depending on the needs of the user. We have studied gels formed with 0.1, 0.3, and 1.0 M HCl and have found that the stiffness increased with increasing acid concentration. In addition, we found that the 0.3 M HCl gels were the most adhesive.

iii. Close cap and stir by inverting the vial.

G: Silk Films TIMING: 2 Days

i. Add 4 ml of 8 w/v% aqueous silk solution into a 100 mm Petri dish. The films produced by this method are generally 50 μm thick and can be easily removed from the polystyrene dish. For thicker films, either increase the amount of silk solution or the concentration of silk.

ii. Allow to dry overnight without the lid of the dish. Any modifications to either the silk concentration or volume may increase drying times.

iii. Fill the bottom of the vacuum desiccator with water.

iv. Place the dry films in the desiccator and apply vacuum to the vacuum port.

v. Allow the films to water anneal for one day.

<CRITICAL STEP> The water annealing step is critical to prevent the films from dissolving in water. We have found that water annealing induces β-sheet similarly as adding methanol would, but to a lesser extent.

vi. Gently remove the film from the dish. TROUBLESHOOTING

vii. Store at room temperature.

H: Patterned Silk Films – Generating a Mold TIMING: 6 hours—

<CRITICAL STEP> If reusing a mold, skip to step xii.

i. Prepare work area by laying down a sheet of aluminum foil and collecting the required materials (a plastic pipet, a box of Kim wipes®, a paper cup, a needle, Sylgard base and curing agent, bottle of 70 v/v% ethanol, and diffraction grating).

ii. Critical: Ensure that the diffraction grating is free of debris. Spray with ethanol, wipe, and blow dry with pressurized air from bench top.

iii. Place paper cup on balance, weigh out appropriate amount of base and then curing agent from the Sylgard kit. For 1 mold (5 cm × 5 cm) use 4.5 g base and 0.5 g of curing agent (10:1 ratio).

iv. Mix with plastic pipet for 1 min.

v. Place PDMS in vacuum oven for 30 to 60 min to remove air bubbles.

vi. Place the diffraction grating in a Petri dish.

vii. Slowly pour the PDMS over the diffraction grating, try not to introduce bubbles.

viii. Once the grating is covered, remove any bubbles with a needle.

ix. Gently move the Petri dish to a 60°C oven for 4 hours or leave at room temperature for over 24 hrs.

x. Remove the PDMS from the diffraction grating. Be careful not to rip the PDMS.

xi. Clean mold with a Kim wipe® and ethanol.

PAUSE POINT: PDMS molds can be stored at room temperature and used indefinitely. Discontinue use when there is damage present on the casting face.

Patterned Silk Films -Casting TIMING: 2 Days

xii. Optional: The PDMS mold can be punched to varying diameters depending on need. For this protocol, punch out 14 mm disks from the PDMS.

xiii. Place the PDMS molds in a Petri dish.

xiv. Add 100 □ of 8w/v% silk solution onto each 14 mm disk.

xv. Allow the films to dry overnight.

xvi. Fill the bottom of the vacuum desiccator with water.

xvii. Critical: Place the dry films in the desiccator and apply vacuum to the vacuum port.

xviii. Allow the films to water anneal for one day.

xix. Allow the films to dry for 10 min and gently peel films from the mold using tweezers.

xx. In order to indicate the patterned side of the film, place an edge of the non-patterned sided onto a Post-it® note. Store at room temperature until use.

I -DOPC: TIMING: 4.5 Days

i. Add 200 mg of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) lipid powder to a glass test tube.

ii. In hood, add 1 ml of chloroform and wait until the DOPC is completely dissolved, the solution will turn clear. CAUTION: Chloroform is a hazardous substance known to be carcinogenic, wear proper safety equipment and work inside a chemical fume hood!

iii. Roll the tube to evenly coat the interior of the tube with a thin, homogenous layer. While rolling the tube, dry out solution with nitrogen gas flow. When the film is nearly dry, the flow rate can be increased.

iv. Leave the tube to dry in the hood for 30 min.

PAUSE POINT: Glass tubes can be coated with DOPC and stored at −20°C until needed.

v. In a 1.5 ml microcentrifuge tube, add 200 μl of 10 mg/ml solution of drug, protein or chemical to be encapsulated to 1 ml of 8 w/v% silk solution. If desired, also prepare a solution of 1 ml 8 w/v% silk with 200 μl of ultrapure water for a control sample.

vi. Hydrate the lipid film with the silk and drug solution (or control solution, if applicable). By adding the aqueous solution, the water will slowly dissolve the lipid film from the glass tube surface. The solution will become more turbid as the lipid film is hydrated and eventually the lipid film will disappear from the glass, leaving it clear.

vii. Once the layer is re-hydrated, dilute with 3 ml of ultrapure water making sure to get the entire lipid layer off the wall of the glass tube.

viii. Transfer the solution from your glass tube to a 15 ml conical tube for freeze thawing.

ix. Submerge the tube in liquid nitrogen for 15 minutes or until the liquid nitrogen stops boiling. CAUTION: Liquid nitrogen is extremely cold and exposure to it may cause burns. Be careful when handling it and wear proper safety equipment!

x. Quickly move the tube to a 37°C water bath for 15 minutes (check that the contents are totally thawed at the end of each 15 min thaw).

xi. Repeat steps 9-10 twice more to complete three freeze-thaw cycles (in order to create more homogenous size distribution of spheres).

xii. Fill a 100 ml beaker with 50 ml of ultrapure water. Add a stir bar and move the beaker to a stir plate.

xiii. Pipette diluted solution into the 100 ml of ultrapure water very slowly (drip by drip) with fast stirring.

xiv. Transfer the diluted solution to a 50 ml conical tube and freeze at −80°C for at least 5 hours. Do not exceed 45 ml of solution per tube as the solution may expand during freezing.

xv. Lyophilize for 2-3 days

xvi. After lyophilization, transfer 30 mg of lyophilized product into a large 2 ml microcentrifuge tube and add 2 ml of pure methanol. CAUTION: Methanol is a hazardous substance, it is suggested to use proper safety equipment. Additionally, methanol is a flammable solvent therefore sources of ignition should be removed prior to use!

xvii. Incubate the sample for 30 minutes at room temperature (an incubation time of only 10 min is recommended if a protein or methanol soluble drug is being encapsulated).

xviii. Transfer the soluble portion to a new 2 ml microcentrifuge tube.

xix. Centrifuge the tubes at 10,000 rpm for 5 min at 4°C in a microcentrifuge.

xx. Discard the methanol supernatant into a proper waste bottle and dry the pellet overnight in the hood. Store at 4°C.

xxi To resuspend the microspheres, first wash the microspheres by adding 2 ml of water to the pellet and then centrifuge at 10,000rpm for 5 min at 4°C.

xxii. Remove the water and resuspend in the desired water or buffer. If the microspheres have aggregated, they can be dispersed by sonicating the solution for about 10 sec at 30% amplitude (approximately 20W).

J: Microspheres (PVA) TIMING: 2 Days

i. Prepare a 5 w/v% PVA solution by adding 0.25 g of PVA to 4 ml of ultrapure water. Heat to 60 °C to dissolve the PVA.

ii. Transfer the solution into the 10 ml graduated cylinder and bring the solution up to 5 ml with ultrapure water.

iii. Filter the PVA solution with a 0.45 □m filtration unit.

PAUSE POINT: Store the PVA solution at room temperature for no more than 3 months.

Dilute silk solution to 5 w/v% with ultrapure water.

Add 1 ml of 5% silk and 4 ml of 5% PVA into a 15 ml conical tube and gently mix.

While slowly moving the probe up and down, sonicate the solution for 30 seconds at 25% amplitude. CAUTION: The sonicating horn must be immersed in solution while the power is applied in order to prevent damage to the instrument. Be careful not to touch the horn on the sides of the tube during sonication. CAUTION: Extended exposure to ultrasonication may cause damage to one's hearing. Use of protective ear muffs is highly recommended.

Pour the solution into a Petri dish and allow it to cover the bottom of the dish evenly.

Dry the solution overnight in a fume hood.

PAUSE POINT: After drying, the film can be stored for a few weeks. Cover and seal the dish with Parafilm® and store at room temperature.

viii. Peel off the film from the dish, place 1 film in a 50 ml conical tube.

ix. Add 20 ml DI water to the tube, shake for 30 min at room temperature to dissolve the film. TROUBLESHOOTING

x. Centrifuge at 11,000 rpm for 20 min at 4°C, discard the supernatant.

xi. Resuspend the pellet in 5 ml of ultrapure water. Sonicate for 15 seconds at 15% amplitude, if necessary. Optional: use the suspension (containing both nano- and microspheres) or lyophilize and store the dry material.

K: Electrospinning Timing:1 day

i. Prepare a solution of 5 w/v% PEO in water by adding 30 g of PEO to 6 ml of ultrapure water in a 20 ml glass scintillation vial. Use a small stir bar to ensure proper mixing.

PAUSE POINT: The PEO will take time to dissolve into solution so this should be done ahead of time. This solution can be stored at 4°C for a year.

ii. Add 5 ml of the 5 w/v% PEO (900,000 g/mol) solution into 20 ml of 8 w/v% silk solution to generate an aqueous solution of 6.4 w/v% silk/1 w/v% PEO.

<CRITICAL STEP> A high viscosity, due to polymer concentration and molecular weight, must be met in order for the polymer molecules to entangle and form a jet. If either the concentration or molecular weight of the polymer is too low, only beads will form. Hence, we have included PEO in this protocol to promote fiber formation by increasing the overall polymer concentration.

iii. Slowly stir the blended solution for approximately 10 minutes at 4 °C to obtain a homogenous solution. Avoid high shear during mixing that may cause premature crystallization of the silk.

iv. Draw up 10 ml of the silk/PEO solution into a 10 ml syringe.

v. Attach a 16 gauge needle to the syringe.

vi. Mount the syringe on the syringe pump.

vii. Place a collection surface at a distance of 7 to 20 cm from the tip of the needle. Many surfaces can be used to collect electrospun fibers as long as they can be electrically ground. A simple surface that consists of a piece of cardboard covered with aluminum foil is sufficient.

viii. Attach the positive voltage lead to the needle on the syringe and the ground lead to the collection surface.

ix. Set the current to slightly above 0 amps. Turn on the high voltage and syringe pump. CAUTION: Electrospinning is performed at a high voltage. Be careful while the high voltage is on and do not touch charged surfaces such as the needle or positive voltage lead!

x. Adjust the solution flow rate (0.01 to 0.03 ml/min), electric potential (8 to 15 KV), and the distance between the capillary tip and the collection screen (7 to 20 cm) to obtain a stable jet.

<CRITICAL STEP> The electric field strength is required to initiate the jet and at the lower limit bead defects are evident on the fibers. At the higher limit, silk fibers transition from a round morphology to a ribbon-shaped cross-section. It has also been noted that with an increase in voltage the fiber diameter increases due to additional mass transport with the higher electrical force. By increasing the distance between the spinneret and the collecting surface, the fiber diameter can be reduced. And finally, the flow rate should be adjusted so that solution is constantly being supplied to the tip without allowing excess solution to drip[71].

xi. Collect the silk fibers until the desired thickness is achieved.

xii. Immerse the fiber mats in a 90 v/v% methanol/water solvent for 20 min to obtain water insoluble fiber mats. CAUTION: Methanol is a hazardous and flammable substance, use proper personal safety equipment and remove all sources of ignition prior to use!

xiii. Incubate the methanol treated fiber mats in ultrapure water on a reciprocating shaker overnight to remove the PEO from the mats.

xiv. Dry the fiber mats in a chemical fume hood.

L: Aqueous-based sponges TIMING: 5 days

i. Prepare the salt with the particle sizes of interest. Stack the sieves with the largest mesh on top and the smallest mesh on the bottom. Add salt and shake vigorously. Repeat until the desired amount of salt is collected. Troubleshooting

<CRITICAL STEP> It is important to note that the pore sizes will be slightly smaller than the salt particles used since the salt is partially dissolved while the silk gels. Therefore it is important to start with salt particles that are slightly larger than the required pore volume.

ii. Weigh 4 g of salt in weighing boats, one for each mold.

iii. With a 5 ml syringe, aliquot fibroin solution into plastic containers, 2 ml/mold.

Note: For salt particles 750 μm and larger, use 8 w/v% silk solution. For smaller salt particles, use 6 w/v% silk solution. The size and shape of the scaffolds may be varied by changing the molds. The ratio of salt to silk in solution should be maintained at 25:1. The number of scaffolds can easily be scaled up by filling additional molds.

iv. Slowly pour the salt on top of the fibroin solution in the mold while rotating the container so that the salt is uniform.

v. Tap the container gently on the bench top to remove air bubbles.

vi. Close the cap and allow the solution to sit overnight at room temperature. The silk fibroin should gel in 1-2 days.

vii. Once the silk has gelled, remove the lids and place the molds in a 2 L beaker with ultrapure water (3 containers per 2 L water).

viii. Transfer the beaker to a stir plate and stir.

ix. Change the water 2-3 times per day, for 2 days (4-6 washes in total).

x. Remove the scaffolds from the molds and replace them in fresh water for an additional day.

xi. Store the scaffolds in ultrapure water in 50 ml tubes at 4°C until needed for use. For long-term storage, dry the scaffolds and store at room temperature.

xii. Prior to use, cut hydrated scaffolds into the desired dimensions. Autoclave to sterilize. In order to cut the scaffolds to smaller dimensions, we suggest disposable biopsy punches to obtain uniform disks.

M: HFIP-based sponges TIMING: 8 days

i. Prepare 17 w/v% silk/HFIP solution. For a volume of 10 ml final solution, pour 9 ml of HFIP over 1.7 g of lyophilized silk. CAUTION: HFIP is listed by HMIS at health hazard level 3 and can cause burns both topically and if inhaled. Wear proper safety equipment and work inside a chemical fume hood!

ii. Wrap the lid with Parafilm® and keep at room temperature overnight or until all the silk is dissolved.

iii. While the silk is dissolving, prepare the salt with the particle sizes of interest. Stack the sieves with the largest mesh on top and the smallest mesh on the bottom. Add salt and shake vigorously. Repeat until the desired amount of salt is collected. TROUBLESHOOTING

iv. Weigh 3.4 g NaCl and place in a glass shell vial. Repeat for 8 more vials.

<CRITICAL STEP> If larger scaffolds are required, the mold can be exchanged with a larger one and the salt weight can be scaled with a 20:1 ratio with the silk mass in solution. Be careful to choose a mold material that will not interact with HFIP. HFIP is highly corrosive so metal should be avoided. In addition, for larger scaffolds the methanol treatment and dialysis times may need to be scaled up to ensure penetration of the solvent molecules into the center of the scaffolds.

v. OPTIONAL: If you are adding a reinforcing agent such as hydroxyapatite, weigh it and add it to the salt here. Mix well, both in the weigh dish and in the mold.

vi. Tap the container gently on benchtop surface to level the salt in container.

vii. In a fume hood, add 1 ml of silk solution to each container of salt very quickly using a 5 ml syringe. Immediately cap each container to reduce solvent evaporation.

viii. Wrap each cap with Parafilm® and allow silk solution to penetrate the salt (1-2 days).

ix. Once the salt appears “wet” and the silk solution has reached the bottom of the vial, uncap the vials to allow HFIP to evaporate for 1 day. CAUTION: Keep the scaffolds in the hood!

PAUSE POINT: The dried scaffolds can be stored at room temperature for several days.

x. When the silk appears dry and detached from the container walls, add 1 ml of methanol to each container and recap. Allow methanol to seep through scaffold for 1 day.

xi. Remove extra methanol and dispose of it in the proper waste container. Transfer the containers to a 2 L beaker of ultrapure water (approximately 6 containers per 2 L of water). TROUBLESHOOTING

xii. Place the beaker on a stir plate and stir.

xiii. Change water 2-3 times a day for 2-3 days.

xiv. Remove the scaffolds from the molds and replace them in fresh water for an additional day. Scaffolds without reinforcement will begin to float when the salt is removed.

xv. Store scaffolds in ultrapure water in 50 ml tubes at 4°C until needed for use (can store for 2-6 months) or dry scaffolds and store dry.

xvi. When needed for use, cut in water at desired dimensions and autoclave to sterilize. We suggest slicing off the top and bottom layers and discarding them as the surface has a skin that is less porous. TROUBLESHOOTING

Troubleshooting: Troubleshooting advice can be found in Table 2.

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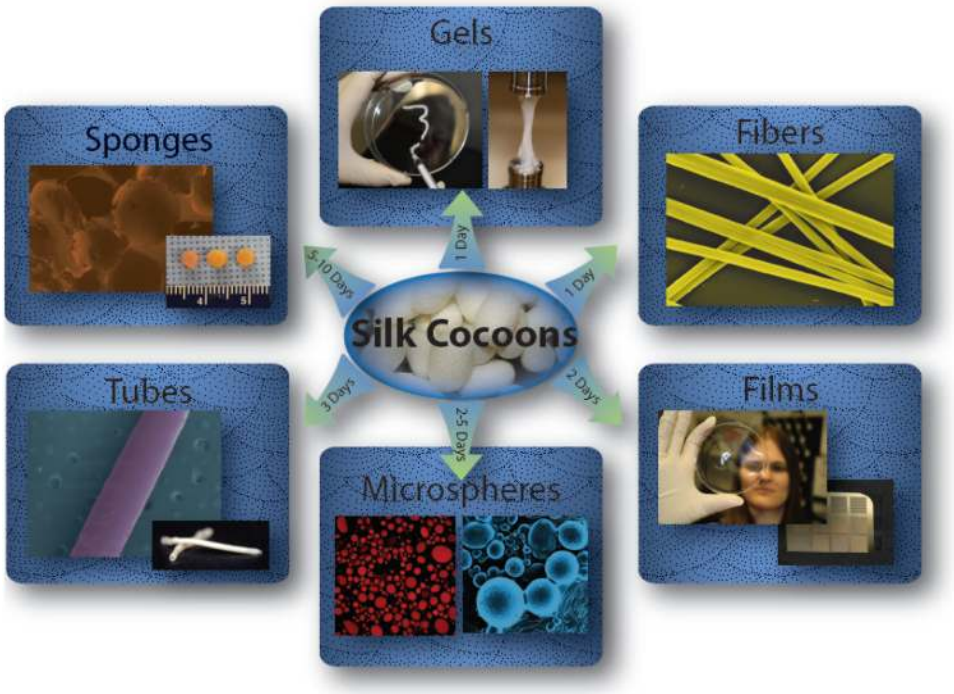

Fig 1.

Schematic of material forms fabricated from silk fibroin using both organic solvent and aqueous-based processing approaches. Overall, the silk fibroin extraction process takes 4 days and the time within the arrows indicates the time required to process the aqueous silk fibroin solution into the material of choice.

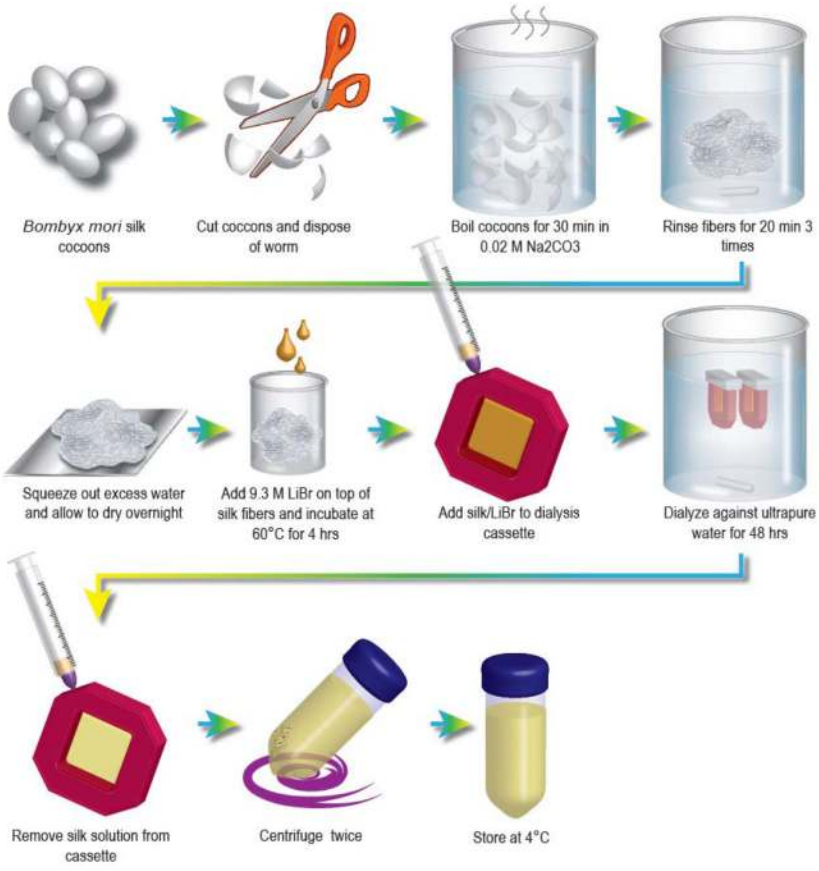

Fig 2.
Schematic of the silk fibroin extraction procedure. Starting from the raw material (cocoons) to the final aqueous-based solution will take 4 days.

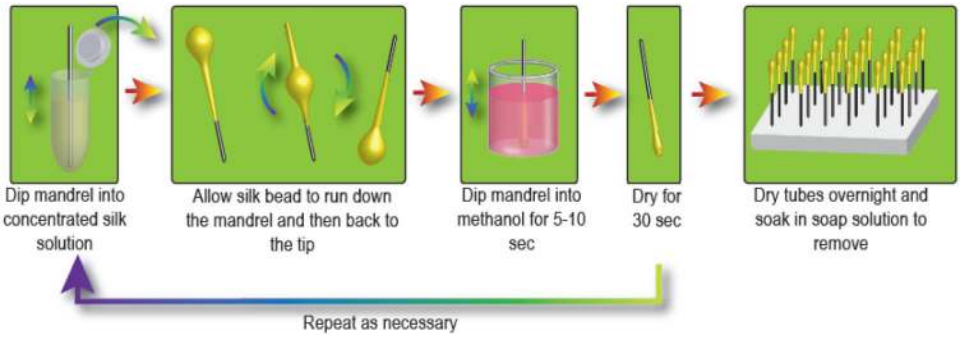

Fig 3.
Schematic for making dipped silk tubes.

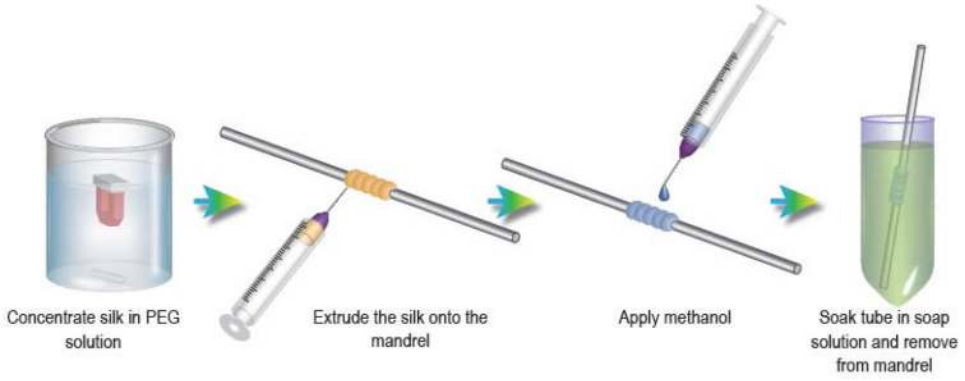

Fig 4.
Schematic for making gel spun tubes.

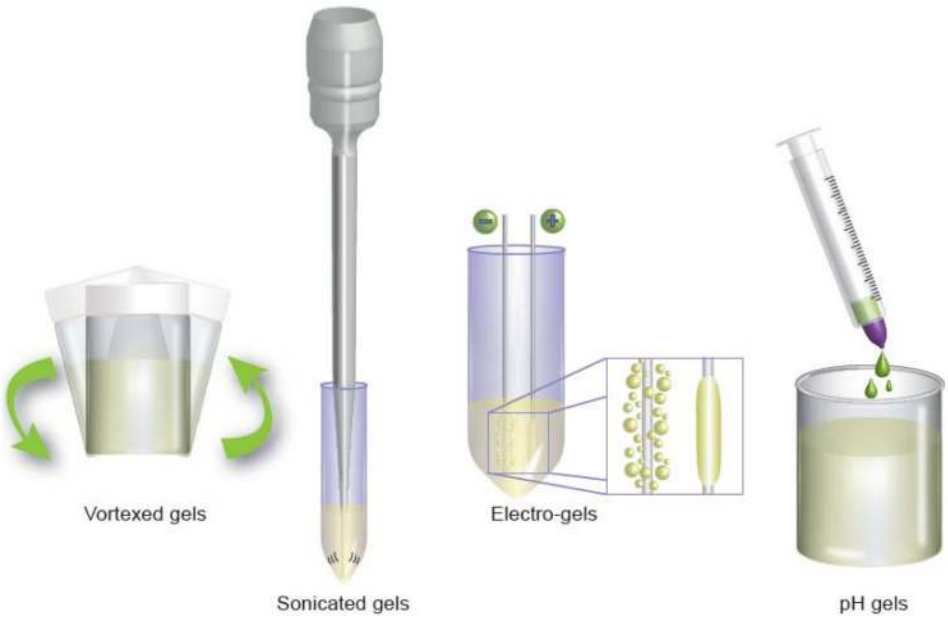

Fig 5.
Methods of preparing silk hydrogels.

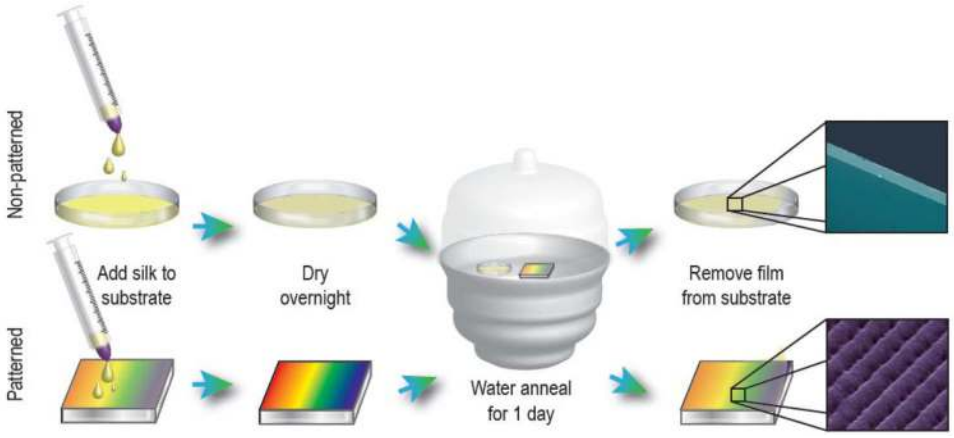

Fig 6.
Schematic for making patterned and non-patterned silk films.

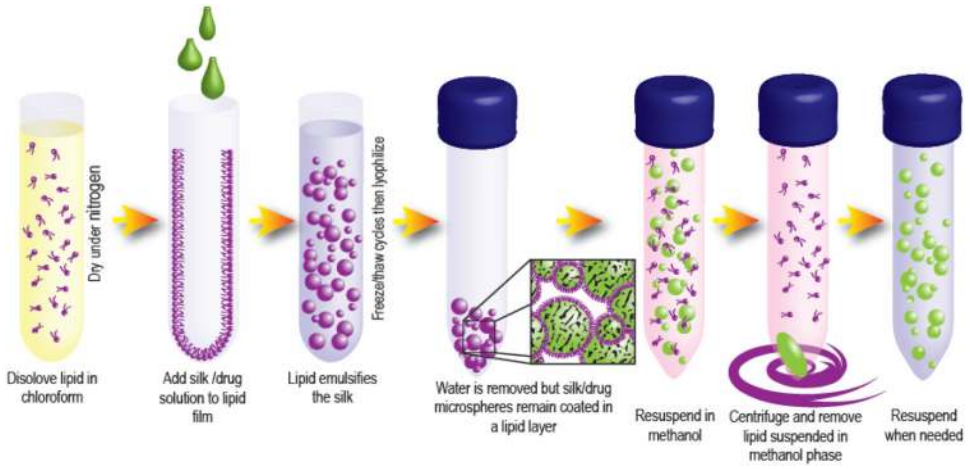

Fig 7.
Schematic of silk microsphere preparation using DOPC.

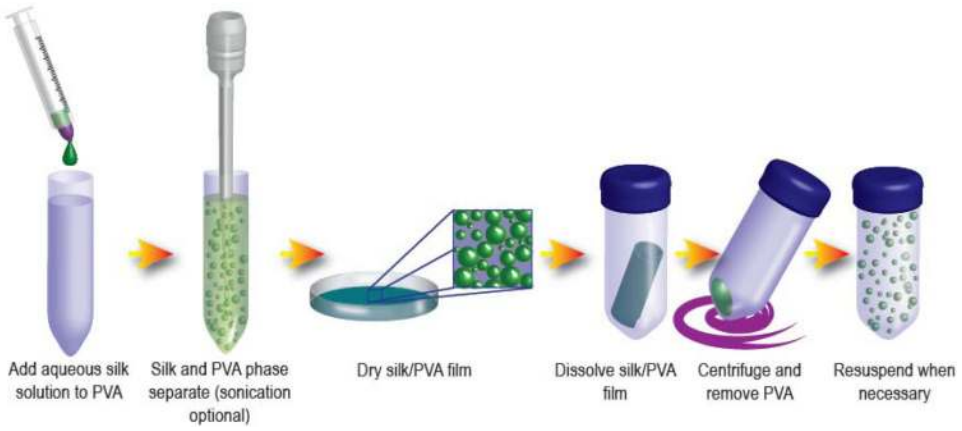

Fig 8.
Schematic of silk microsphere preparation using PVA.

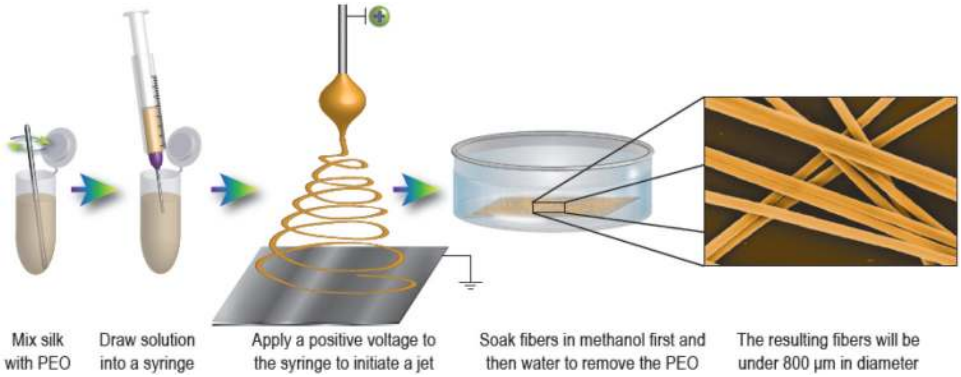

Fig 9.
Method to prepare electrospun silk fibers.

Fig 10.
Schematic to make aqueous-based silk sponges.

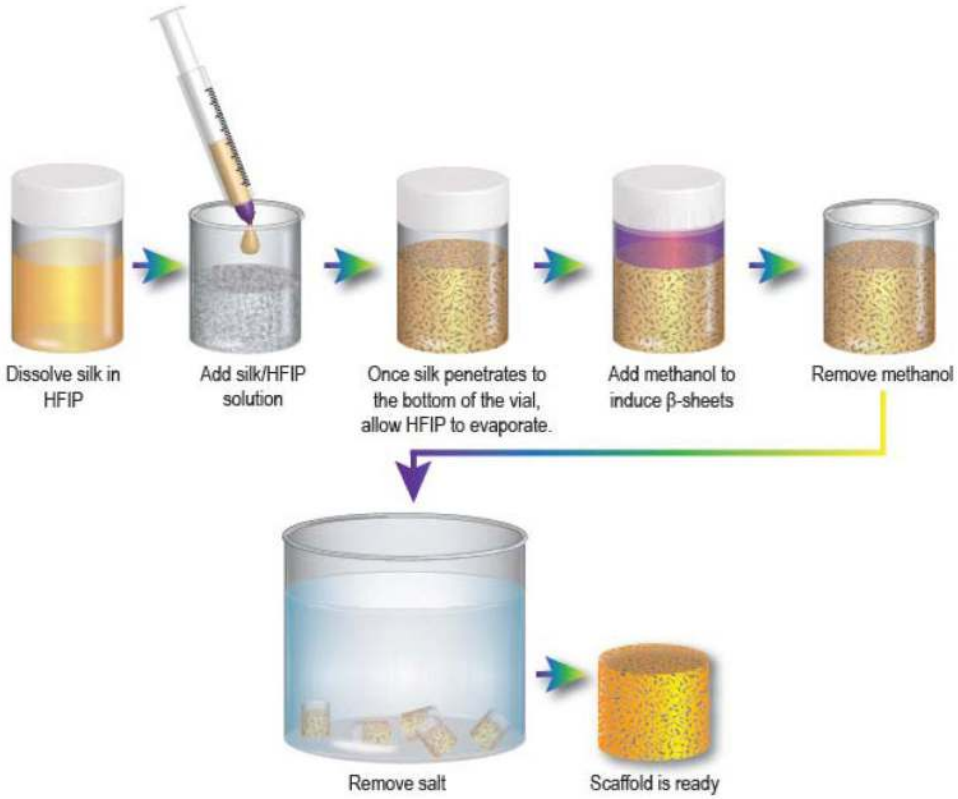

Fig 11.
Method to prepare HFIP-based silk sponges.

Table 1

Biomedical applications of silk scaffolds.

Application	Tissue Type	Material Format
Tissue Engineering		
	Bone	HFIP-based sponges[1,22,23]
		Aqueous-based sponges[1,24,25]
		Electrospun fibers[26]
	Cartilage	HFIP sponges[27]
		Aqueous sponges[28-30]
		Electrospun fibers[31]
	Soft Tissue	HFIP sponges[32]
		Aqueous sponges[32]
		Hydrogels[18]
	Corneal	Patterned silk films[33,34]
	Vascular Tissues	Tubes[35]
		Electrospun fibers[36-38]
	Cervical Tissue	Aqueous sponges[39]
	Skin	Electrospun fibers[40,41]
Disease Models		
	Breast cancer	HFIP sponges[42]
		Aqueous sponges[43,44]
	Autosomal dominant polycystic kidney disease (ADPKD)	Aqueous sponges[45]
Implant Devices		
	Anterior cruciate ligament (ACL)	Fibers[46,47]
	Femur defects	HFIP sponges[20]
	Mandibular defects	Aqueous sponges[48,49]
Drug Delivery		
	Drug delivery	Spheres[50-53]
	Growth factor delivery	Spheres[3]
	Small molecule	Spheres[54]

Note: The sources for the reagents and equipment described in these protocols are given only as an example. Equivalent materials can be used unless otherwise noted.

Table 2

Troubleshooting

Protocol	Problem	Suggestions
1: Silk processing	Silk does not dissolve after 4 hours in lithium bromide (LiBr)	• Did you account for the volume of LiBr when preparing the 9.3 M solution? If not, add the appropriate amount of LiBr. • Did you boil the cocoons in sodium carbonate for 30 minutes? If you boiled for a shorter time, you may need to allow the silk to dissolve in LiBr for longer. Take note of the boiling time as batches may vary if the boiling times are different. • Did you pour the LiBr over the silk fibers? If not, you will need to mix the solution to ensure contact between the silk and the LiBr solution. Be sure to cover the beaker to reduce evaporation. • Check that the oven is set to 60°C.
1: Silk processing	LiBr and silk fibroin solution is not amber colored	• Change or clean glassware.
2: Tubes	Silk will not evenly coat the wire and several beads are present along the mandrel.	• Silk is not concentrated enough, store at 4°C for a few days, check it periodically since the silk will gel if it is stored for too long.
2: Tubes	Tubes crack after drying	• Try a solution of 70 v/v% methanol in water or add more layers next time. If the problem continues, follow the protocol carefully allowing the bead to go up and down the mandrel. Also, allow 10 sec for methanol treatment and 30 sec for the tube to dry between layers.
2: Tubes	Large gaps are present on the tubes	• Bubbles are present in the silk solution. Be careful not to induce bubbles when pipetting or mixing in PEO (if applicable). Degas the solution in a vacuum oven before use.
3. Gel spinning	The silk gel fiber being wrapped around the mandrel has beads along it.	• If the fiber is not uniform as it is being wrapped around the mandrel, it will result in an uneven tube. Beads along the gel fiber indicate that the silk solution is not concentrated enough. Store the solution at 4°C for a couple of days but check it periodically since the silk will gel if it is stored for too long.
4. Vortex gels	Gel will not form	• Vortexing time and amplitude may need to be adjusted depending on the batch of silk, age of the solution, and volume.
5. Sonicated gels	Gel will not form	• Sonication time and amplitude may need to be adjusted depending on the batch of silk, age of the solution, and volume.
6. Electrogelated gels	Gel will not form	• Gelation time may need to be adjusted depending on the batch of silk, age of the solution, and volume. Electrogelation works best on silk solutions that are approximately 1 week old.
7. Thin Films	Films will not lift off of the plate.	• Use non-tissue culture treated dishes.
8. Microspheres (PVA)	The film will not completely dissolve after 30 min.	• Sonicate the solution for 30 seconds at 15% amplitude.
10. Sponges	When shifting the salt, there are very few crystals within the size range of interest.	• The sieve may be clogged. Invert the sieve and try to dislodge particles trapped within the mesh.
10. Sponges	HFIP-based sponges have voids in them.	• Try pushing the scaffolds down or centrifuge them prior to drying.

Protocol	Problem	Suggestions
10. Sponges	HFIP-based sponges dissolved while in water	• Methanol was not able to penetrate properly. Repeat but extend the methanol treatment time.