Materials fabrication from *Bombyx mori* silk fibroin

Danielle N Rockwood, Rucsanda C Preda, Tuna Yücel, Xiaoqin Wang, Michael L Lovett & David L Kaplan

Department of Biomedical Engineering, Tufts University, Medford, Massachusetts, USA. Correspondence should be addressed to D.L.K. (david.kaplan@tufts.edu).

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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 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 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, as well as for drug delivery.

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 it has recently gained attention as a biomaterial because of several desirable properties. In particular, these properties include its biocompatibility, the ease with which it can be chemically modified[1–5], 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[6]. Because of large-scale cultivation of silkworms for the textile industry, there are abundant and reasonable cost sources for this natural polymer; however, for medical applications, adequate 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 *B. mori*[7]. These proteins cover the surface of fibroin, the silk filament core protein, in the cocoon filament. Once this adhesive protein is removed, 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 **Figure 1**. The following protocols can help with the design and implementation of a variety of silk-based biomaterials for a range of potential applications.

Native *B. mori* silk is composed of silk fibroin protein coated with 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 ~26 kDa) and a heavy chain (M_w ~390 kDa) linked by a disulfide bond[6]. Silk fibroin is a block copolymer rich in hydrophobic β-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 that are rich in glycine, alanine, serine and tyrosine[6]. The result is a hydrophobic protein that self-assembles to form strong and resilient materials. The dominance of the β-sheet-forming regimes within the fibroin structure imparts the protein-based materials with high mechanical strength and toughness. The toughness of silk fibers is greater than the best synthetic materials, including Kevlar[8]. 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 of *B. mori* silk fibers is 740 MPa. In contrast, collagen has an ultimate tensile strength of 0.9–7.4 MPa and PLA 28–50 MPa[9]. 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

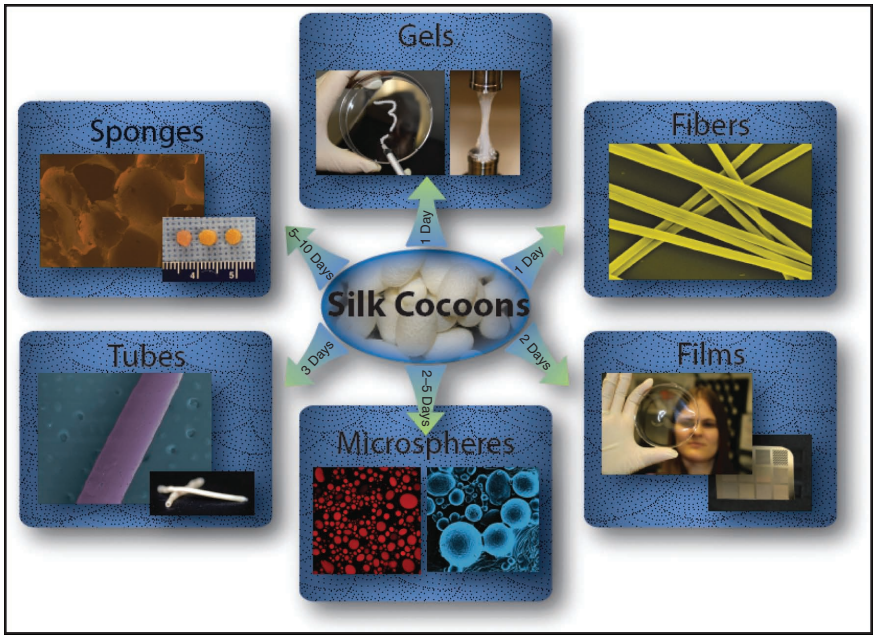

Figure 1 | Schematic of material forms fabricated from silk fibroin using both organic solvent– and aqueous-based processing approaches. Overall, the silk fibroin extraction process takes 4 d and the time within the arrows indicates the time required to process the silk fibroin solution into the material of choice.

TABLE 1 | Biomedical applications of silk scaffolds.

Application	Tissue type	Material format
Tissue engineering		
	Bone	HFIP sponges[41,43,44]
		Aqueous sponges[43,45,46]
		Electrospun fibers[36]
	Cartilage	HFIP sponges[47]
		Aqueous sponges[48–50]
		Electrospun fibers[51]
	Soft tissue	HFIP sponges[52]
		Aqueous sponges[52]
		Hydrogels[15]
	Corneal	Patterned silk films[31,53]
	Vascular tissues	Tubes[22]
		Electrospun fibers[54–56]
	Cervical tissue	Aqueous sponges[57]
	Skin	Electrospun fibers[58,59]
Disease models		
	Breast cancer	HFIP sponges[60]
		Aqueous sponges[61,62]
	Autosomal dominant polycystic kidney disease	Aqueous sponges[63]
Implant devices		
	Anterior cruciate ligament	Fibers[64,65]
	Femur defects	HFIP sponges[17]
	Mandibular defects	Aqueous sponges[66,67]
Drug delivery		
	Drug delivery	Spheres[34,68–70]
	Growth factor delivery	Spheres[71]
	Small molecule	Spheres[33]

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

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[9]. *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[10]. In addition, 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 β-sheet crystals and degree of organization of the noncrystalline domains. In general, the degradation rate decreases with an increase in overall β-sheet content. However, it has also been shown that the rate of degradation of silk biomaterials directly affected the metabolism of human mesenchymal stem cells (hMSCs) and consequently altered the rate of osteogenesis[11]. 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[9]. 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*[12]. The *in vitro* response to silk fibroin was similar to the response to collagen and tissue culture plastic controls. *In vivo*, silk showed a lower inflammatory response when compared with collagen and PLA[12]. The inflammatory potential has also been assessed *in vitro* with macrophages in which silk was shown to have levels of tumor necrosis factor-α, a cytokine indicative of an inflammatory response, similar to those of tissue culture plastic[13]. In addition, silk fibroin has been used in several *in vivo* studies for brain[14], soft tissue[15], subcutaneous[16] and bone[17] applications.

Overview of the procedures

The applications of silk to 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 used 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 by 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 in which the silk materials are incubated in a humid environment for several hours. For more detailed

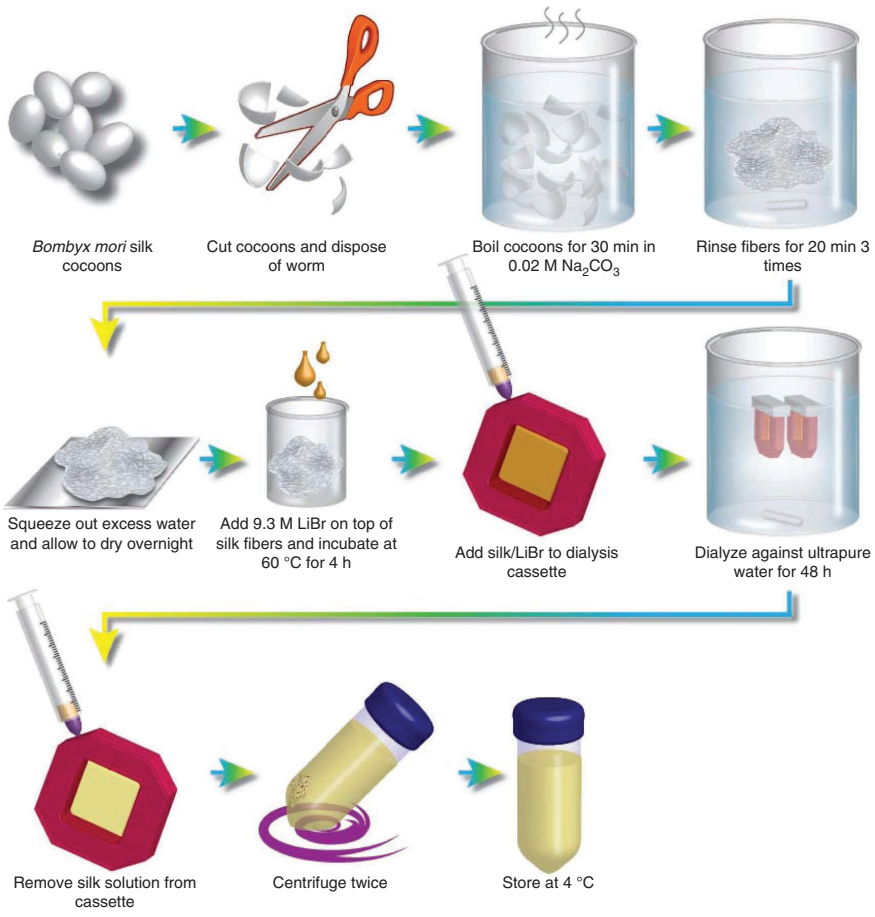

Figure 2 | Schematic of the silk fibroin extraction procedure. Going from the raw material (cocoons) to the final aqueous-based solution takes 4 d.

information, we refer the reader to the study by Hu *et al.*[18], which describes a systematic analysis of crystalline induction through 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. Our protocol is designed to produce one batch from 5 g of silk cocoons; however, 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 can be used without changes.

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 min, 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[13]. Wray and co-workers[13] have also established a simple method to quickly and reproducibly measure the amount of protein degradation via fluorescence spectroscopy. Briefly, dilute samples (0.1%, wt/vol) 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[13]. In addition, thin films of the silk solution can be prepared and thermally tested in a differential scanning calorimeter to determine the T_g (178 °C) and T_m (192–203 °C)[19].

The extraction process takes 4 d. If lyophilized silk is required, then an additional 3 d will be needed. Aqueous solutions can also be concentrated within an additional 24 h. Aqueous silk solutions can be sterilized by a number of methods, including sterile filtration, γ-irradiation and autoclaving. More rigorous procedures, such as γ-irradiation[20,21] 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 are noted within each protocol. Purified silk can then be used for a variety of applications, as summarized in **Figures 3–11**.

Silk tubes (Step 25, 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% (vol/vol) ethanol overnight and then rinse them in sterile water before 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

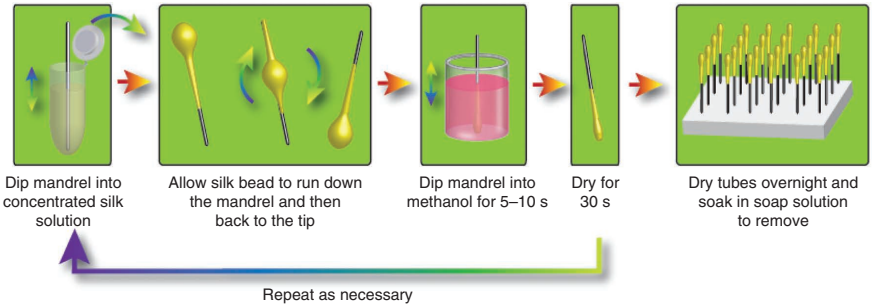

Figure 3 | Schematic for making dipped silk tubes.

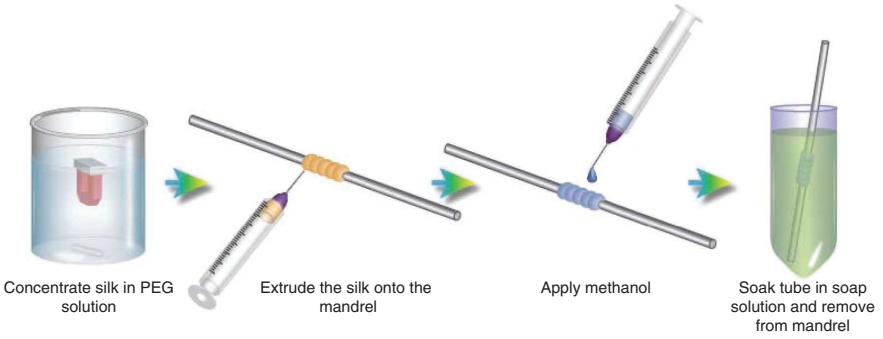

Figure 4 | Schematic for making gel-spun tubes.

number of layers deposited on the mandrel. With this method, we have formed tubes with an approximate wall thickness of 200 μm[22]. 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[22].

Gel-spun tubes. The latter protocol describes a gel spinning technique in which concentrated silk fibroin is extruded onto a rotating mandrel (**Fig. 4**; option B). By applying high shear forces to 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. Because of the nature of the spinning process, gel-spun tubes can add texture to the resulting tubes[23]. 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[23]. These tubes have been evaluated for use as vascular conduits and in bioreactor systems[22,24]. SEM can be used to determine tube structure and to determine whether pores were formed (if applicable)[23].

Silk hydrogelation protocols (Step 25, options C–F)

Silk hydrogels can be produced through a variety of mechanisms[25]. Here we present protocols to gel aqueous silk solutions (**Fig. 5**) through vortexing (Step 25, option C)[26], sonication (Step 25, option D)[27], the application of direct electrical current (Step 25, option E)[28] or by lowering the pH (Step 25, option F)[29]. Each method is relatively simple and weak gels can be obtained 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 (e-gel) and pH gel protocols. 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% (wt/vol) 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 at room temperature (20–25 °C). 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 differential scanning calorimetry to verify the melting point (~206 °C for e-gels), Fourier transform infrared spectroscopy for secondary structure, and rheology for determining the stiffness of the gel; for example, the stiffness of a 5% (wt/vol) silk gel is around 100 kPa[26]. 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)[28,29].

Vortexing. This protocol produces silk gels from simple equipment without needing to contact the solution with a probe[26]. 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[27]. As there is a lag time between the sonication and the onset of gelation, this method is also useful when cells or compounds 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% (wt/vol), and sonication time can be varied from 5 to 30 s at 10–20% amplitude.

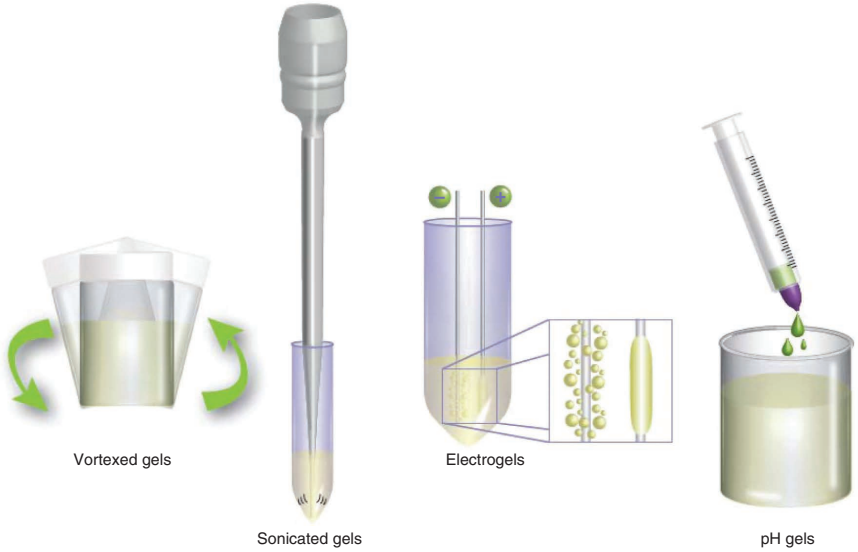

Figure 5 | Methods of preparing silk hydrogels.

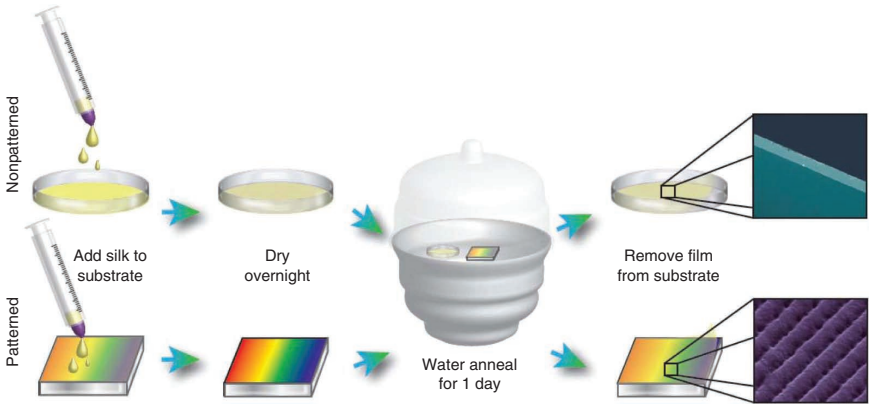

Figure 6 | Schematic for making patterned and nonpatterned silk films.

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% (wt/vol) 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 per ml. A volume of 50 µl of the cell suspension was added to the sonicated silk and then incubated at 37 °C and 5% CO_2 for 2 h before adding cell culture medium[27].

Electric current. The material produced in this protocol is an adhesive gel[28]. 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. Because of the reversible nature of this gel, it can be used in applications in which it is advantageous to have tunable adhesion.

Silk films (Step 25, 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 prepare both patterned (Step 25, option G) and nonpatterned (Step 25, option H) silk films from aqueous solutions (**Fig. 6**). As the processing is water based, these techniques offer the flexibility to incorporate bioactive molecules[30]. To sterilize silk films, they can be incubated in 70% (vol/vol) ethanol overnight and then rinsed in sterile PBS or water. Film thickness and morphology can be assessed by either manually measuring it with a micrometer or by visually measuring it by SEM.

The patterned silk films generated from the latter protocol are generally 40 µm thick[31]. The pattern depth and number of grooves can be modified by changing the grating used to prepare the polydimethylsiloxane (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 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.*[32].

Silk microspheres (Step 25, 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 uses 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 25, option I)[33]. 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 alcohol, PVA; Step 25, option J)[34] (**Fig. 8**). This second method is particularly useful because of 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[33]. The average particle size of spheres prepared with this method is ~2 µm, which can be determined with either optical microscopy or SEM[33]. As for drug loading, different drugs will interact differently with the silk and therefore the loading efficiency will differ among various molecules. We suggest that small batches be tested in the process before scaling up. Resuspended microspheres can also be mixed with silk solution and then be prepared into porous scaffolds, hydrogels or films.

The second protocol, which utilizes PVA, will generate silk spheres with a size range between 300 nm and 20 µm[34]. 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. To characterize these spheres, a few techniques have been used, 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.

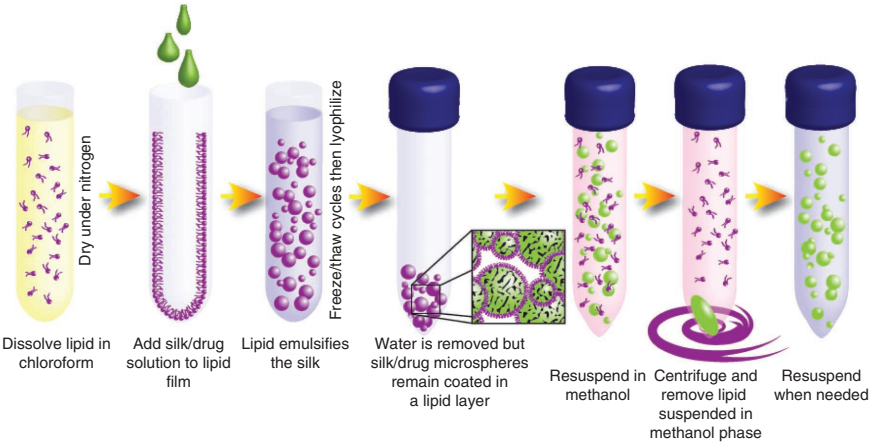

Figure 7 | Schematic of silk microsphere preparation using DOPC.

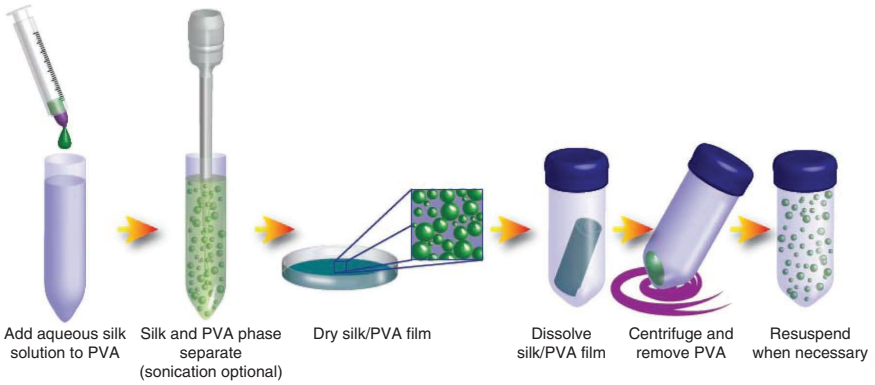

Figure 8 | Schematic of silk microsphere preparation using PVA.

Confocal microscopy was also used to determine model drug loading[34]. 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 depending on molecular weight, charge and hydrophobicity, and therefore it must be determined for each drug.

Electrospun silk fibers (Step 25, option K)

Electrospinning is a process by which small-diameter fibers with large surface areas can be produced (**Fig. 9**; Step 25, option K). The equipment required is relatively simple, but the fibers produced are much smaller in diameter compared with conventionally spun fibers[35], and the process offers the ability to incorporate molecules of interest[36]. In addition, fibers can be aligned either by using a rotating mandrel[37] or by inducing the Hall Effect[38]. Electrospun mats can be sterilized by immersing the mats in 70% (vol/vol) ethanol overnight and then subsequently rinsing them in either sterile water or PBS.

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

Silk sponges (Step 25, 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[39] (**Fig. 10**) or HFIP-based scaffolds[40] (**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 is incorporated into the silk matrix during processing. The HFIP-based sponges have smoother surfaces along the pores and have greater mechanical strength. Through this processing method, reinforcing agents can be added to the silk matrix to further increase its mechanical properties[41]. In addition, it has been shown that aqueous-based sponges degrade faster than HFIP-based sponges[11], 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% (vol/vol) ethanol overnight or by autoclaving. The first protocol will produce aqueous-based silk sponges that are 15 mm in diameter and 10 mm in height, and the second protocol will produce at least nine scaffolds that are ~12 mm in both height and diameter. SEM can be used to characterize the morphology and pore structure of these materials. Porosity can be determined via liquid displacement[40].

MATERIALS

REAGENTS

Extraction
- Silk cocoons (Tajima Shoji or equivalent)
- Sodium carbonate (Sigma-Aldrich, cat. no. 451614, http://www.sigmaaldrich.com/)
- Ultrapure water
- Lithium bromide (LiBr, Sigma-Aldrich, cat. no. 213225, http://www.sigmaaldrich.com/)

Concentrating silk fibroin
- Fibroin solution (7–8%, wt/vol); PROCEDURE Step 22)
- PEG (10,000 MW, Sigma-Aldrich, cat. no. P6667, http://www.sigmaaldrich.com/)
- Ultrapure water

Silk tubes and gel-spun tubes
- Concentrated silk fibroin (PROCEDURE Step 24B)
- Methanol (Acros Organics, cat. no. 176840010, http://www.fishersci.com/)
- Dish soap

Vortexed gels and sonicated gels
- Aqueous silk solution, 8% (wt/vol)
- Ultrapure water

Electrogelated gels
- Aqueous silk solution, 8% (wt/vol)
- pH gels
- Aqueous silk solution, 8% (wt/vol)
- Hydrochloric acid (0.3 M HCl, Sigma-Aldrich, cat. no. H1758, http://www.sigmaaldrich.com/)

Silk films
- Aqueous silk solution, 8% (wt/vol)

Patterned silk films
- Aqueous silk solution, 8% (wt/vol)
- Polydimethylsiloxane (PDMS; Sylgard 184, Ellsworth Adhesives, cat. no. 184, SIL ELAST KIT 0.5KG, http://www.ellsworth.com/)

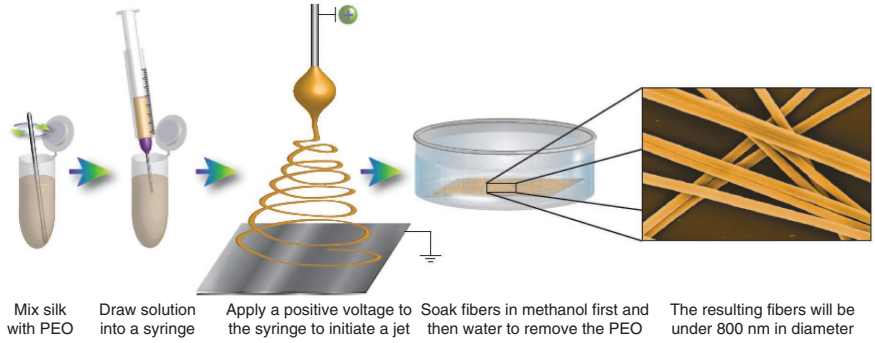

Figure 9 | Method to prepare electrospun silk fibers.

Figure 10 | Schematic for making aqueous-based silk sponges.

Silk microspheres using DOPC
- Aqueous silk solution, 8% (wt/vol)
- 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; Avanti Polar Lipids, cat. no. 850375P, http://www.avantilipids.com/)
- Chloroform (Fisher Scientific, cat. no. C298-1, http://www.fishersci.com/)
- Methanol (Sigma-Aldrich, cat. no. R4828000-4C, http://www.sigmaaldrich.com/)
- Nitrogen gas
- Drug, small molecule or protein of interest
- Ultrapure water

Microspheres (PVA)
- Aqueous silk solution, 8% (wt/vol)
- PVA (MW 30–70 kDa; Sigma, cat. no. P8136, http://www.sigmaaldrich.com/)
- Ultrapure water

Electrospinning
- Aqueous silk solution, 8% (wt/vol)
- Polyethylene oxide (PEO, 900 kDa; Sigma-Aldrich, cat. no. 189456, http://www.sigmaaldrich.com/)
- Ultrapure water
- Methanol (Acros Organics, cat. no. 176840010, http://www.fishersci.com/)

Silk sponges
- Aqueous silk solution, 6–8% (wt/vol)
- Salt (Fisher Scientific, cat. no. BP358-1, http://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, http://www.sigmaaldrich.com/)
- NaCl (Fisher Scientific, cat. no. BP358-1, http://www.fishersci.com/)
- Ultrapure water
- Methanol (Acros Organics, cat. no. 176840010, http://www.fishersci.com/)

EQUIPMENT

Extraction
- Titanium scissors (Staples, cat. no. 487905, http://www.staples.com/)
- Small stir bar (Fisher Scientific, cat. no. 14-513-62, http://www.fishersci.com/)
- Large stir bar (Fisher Scientific, cat. no. 14-513-56, http://www.fishersci.com/)
- Spatula (Fisher Scientific, cat. no. 14-373-25A, http://www.fishersci.com/)
- Glass beaker (50 ml; Kimble Chase Kimble, cat. no. 14000 50, http://www.fishersci.com/)
- Glass beaker (1 liter; Kimble Chase Kimble, cat. no. 14000 1000, http://www.fishersci.com/)
- Glass beaker (2 liters; Kimble Chase Kimble, cat. no. 14000 2000, http://www.fishersci.com/)
- Plastic beaker (2 liters; Fisher Scientific, cat. no. 02-591-33, http://www.fishersci.com/)
- Aluminum foil
- Hot plate (Fisher Scientific, cat. no. 11-100-100H, http://www.fishersci.com/)
- Large weigh boat (Fisher Scientific, cat. no. 08-732-115, http://www.fishersci.com/)
- Medium weigh boat (Fisher Scientific, cat. no. 08-732-113, http://www.fishersci.com/)
- Small weigh boat (Fisher Scientific, cat. no. 08-732-112, http://www.fishersci.com/)
- Analytical balance (A&D Weighing, model Orion HR-200, http://www.americanweigh.com/)
- Hot hand protectors (Bel-Art Products, cat. no. 380000000, http://www.fishersci.com/)
- Graduated cylinder (50 ml; Fisher Scientific, cat. no. 08-557C, http://www.fishersci.com/)
- Slide-A-Lyzer dialysis cassette 3500 MWCO, 3–12 ml capacity (Thermo Scientific, cat. no. 66110, http://www.fishersci.com/)
- Dialysis cassette buoy (Thermo Scientific, cat. no. 66432, http://www.fishersci.com/)
- Syringe (20 ml; BD Medical, cat. no. 309661, http://www.fishersci.com/)
- Needles (18G; BD Medical, cat. no. 305195, http://www.fishersci.com/)
- Conical tubes (50 ml; BD Medical, cat. no. 14-432-22, http://www.fishersci.com/)
- Centrifuge (Eppendorf 5804R, cat. no. 022623501, http://www.fishersci.com/)
- Fixed-angle rotor (Eppendorf, cat. no. 022637207, http://www.fishersci.com/)
- Lyophilizer (optional: use only if lyophilized silk is required, Labconco, cat. no. 7751030, http://www.fishersci.com/)
- Freezer (−80 °C; optional: use only if lyophilized silk is required, Thermo Scientific, cat. no. ULT2586-9SI-A, http://www.fishersci.com/)
- Kimwipes (Kimberly-Clark, cat. no. 34256, http://www.fishersci.com/)

Concentrating silk fibroin
- Syringe (10 ml; BD Medical, cat. no. 309604, http://www.fishersci.com/)
- Syringe (3 ml; BD Medical, cat. no. 309585, http://www.fishersci.com/)
- Needles (18G; BD Medical, cat. no. 305195, http://www.fishersci.com/)
- Slide-A-Lyzer dialysis cassette (0.5–3.0 ml; Thermo Scientific, cat. no. 66330, http://www.fishersci.com/)
- Beaker (100 ml; Kimble Chase Kimble, cat. no. 14000 100, http://www.fishersci.com/)
- Beaker (1 liter; Kimble Chase Kimble, cat. no. 14000 1000, http://www.fishersci.com/)

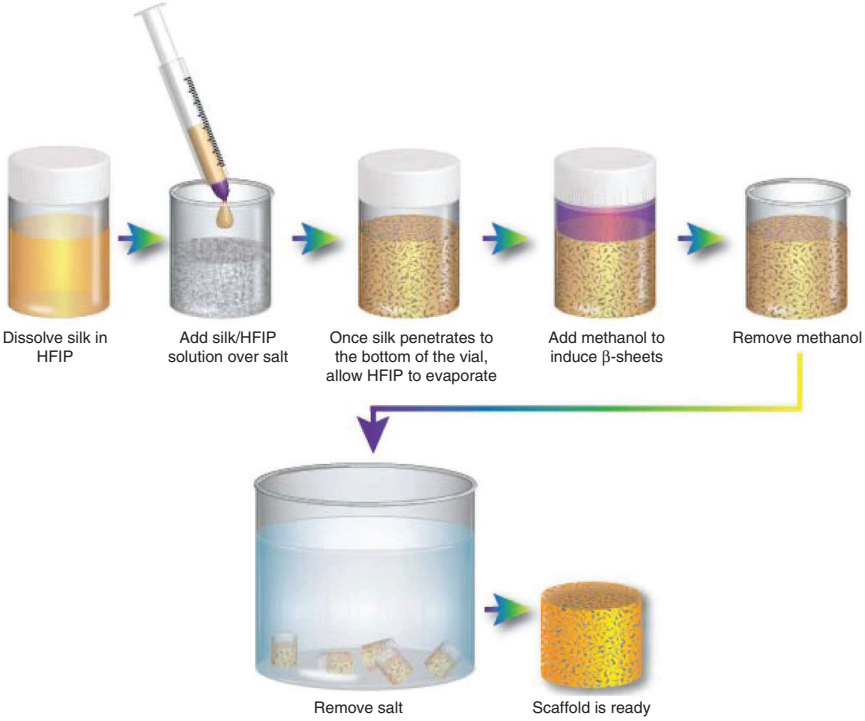

Figure 11 | Method for preparing HFIP-based silk sponges.

- Large stir bar (Fisher Scientific, cat. no. 14-513-56, http://www.fishersci.com/)
- Small dialysis cassette buoy (Thermo Scientific, cat. no. 66430, http://www.fishersci.com/)
- Aluminum foil
- Microcentrifuge tubes (2 ml capacity, Fisher Scientific, cat. no. 05-408-138, http://www.fishersci.com/)

Silk tubes

- Stainless steel wire cut to 3-inch lengths; gauge will depend on required inner diameter (e.g., 0.5 mm, McMaster-Carr, cat. no. 8908K21, http://www.mcmaster.com/)
- Feather scalpel (EMS, cat. no. 72042-20, http://www.fishersci.com/)
- Conical tube (15 ml; BD Medical, cat. no. 352096, http://www.fishersci.com/)
- Tweezers (Fisher Scientific, cat. no. 08-880, http://www.fishersci.com/)
- Styrofoam

Gel-spun tubes

- Mandrel rotation system, custom made to rotate and transverse[23]
- Luer-Lok syringe (1 ml; BD Medical, cat. no. 309628, http://www.fishersci.com/)
- Blunt-tip needle (14 gauge; McMaster-Carr, cat. no. 75165A672, http://www.mcmaster.com/)
- Blunt-tip needle (30 gauge; McMaster-Carr, cat. no. 75165A31, http://www.mcmaster.com/)
- Blunt-tip needle (27 gauge; McMaster-Carr, cat. no. 75165A688, http://www.mcmaster.com/)
- Stainless steel wire; gauge will depend on required inner diameter (e.g., 0.5 mm, McMaster-Carr, cat. no. 8908K21, http://www.mcmaster.com/)
- Kimwipes (Kimberly-Clark, cat. no. 34256, http://www.fishersci.com/)

Vortexed gels

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

Sonicated gels

- Ultrasonicator (Branson 450, cat. no. 101-135-022, http://www.fishersci.com/)
- Externally Threaded Disruptor Horn (1/2-inch, Branson, cat. no. 101-147-037, http://www.fishersci.com/)
- Tapered Microtip (1/8-inch diameter, Branson, cat. no. 101-148-062, http://www.fishersci.com/)
- Conical tubes (15 ml; BD Medical, cat. no. 352096, http://www.fishersci.com/)

Electrogelated gels

- DC Power supply (Agilent E3634A, http://www.agilent.com/)
- Platinum wires (0.032 in diameter, 1 foot; Surepure Chemetals, cat. no. 1987, http://www.surepure.com/)
- Conical tubes (50 ml; BD Medical, cat. no. 14-432-22, http://www.fishersci.com/)

pH gels

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

Silk Films

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

Patterned silk films

- Diffraction grating: size, number of grooves and depth of grooves can change depending on requirements; an example is shown here (Edmund Optics, 600 grooves mm^{-1}, 1,000 nm ruled diffraction grating, 50 × 50 mm, cat. no. NT43-208, http://www.edmundoptics.com/)
- Vacuum oven (Fisher Scientific, cat. no. 13-262-280A, http://www.fishersci.com/)
- Tweezers (Fisher Scientific, cat. no. 08-880, http://www.fishersci.com/)
- Kimwipes (Kimberly-Clark, cat. no. 34256, http://www.fishersci.com/)
- Paper cup (local grocery)
- Pipette (5 ml; BD Vacutainer Labware Medical, cat. no. 357543, http://www.fishersci.com/)
- Aluminum foil
- Ethanol (70%, vol/vol); Ricca Chemical, cat. no. 2546701, http://www.fishersci.com/)
- Needle (gauge unimportant; BD Medical, cat. no. 305195 (18G), http://www.fishersci.com/)

Silk microspheres using DOPC

- Round-bottomed glass tubes (Fisher Scientific, cat. no. 14-961-29, http://www.fishersci.com/)
- Microcentrifuge tube (1.5 ml; Fisher Scientific, cat. no. 054-08-129, http://www.fishersci.com/)
- Microcentrifuge tube (2.0 ml; Fisher Scientific, cat. no. 02-681-258, http://www.fishersci.com/)
- Conical tubes (15 ml; BD Medical, cat. no. 352096, http://www.fishersci.com/)
- Conical tubes (50 ml; BD Medical, cat. no. 14-432-22, http://www.fishersci.com/)
- Beaker (100 ml; Kimble Chase Kimble, cat. no. 14000 100, http://www.fishersci.com/)
- Small stir bar (Fisher Scientific, cat. no. 14-513-62, http://www.fishersci.com/)
- Freezer (−80 °C; Thermo Scientific, cat. no. ULT2586-9SI-A, http://www.fishersci.com/)
- Lyophilizer (Labconco, cat. no. 7751030, http://www.fishersci.com/)
- Microcentrifuge (Eppendorf 5417R, cat. no. 022621700, http://www.fishersci.com/)

Microspheres (PVA)

- Small weigh boat (Fisher Scientific, cat. no. 08-732-112, http://www.fishersci.com/)
- Spatula (Fisher Scientific, cat. no. 14-373-25A, http://www.fishersci.com/)
- Glass beaker (50 ml; Kimble Chase Kimble, cat. no. 14000 50, http://www.fishersci.com/)
- Graduated cylinder (10 ml; Fisher Scientific, cat. no. 08-557A, http://www.fishersci.com/)
- Small stir bar (Fisher Scientific, cat. no. 14-513-62, http://www.fishersci.com/)
- Hot plate (Fisher Scientific, cat. no. 11-100-100H, http://www.fishersci.com/)
- Syringe filter unit (pore size 0.45 µm; Millipore, cat. no. SLHA033SS, http://www.fishersci.com/)
- Syringe (5 ml; BD Medical, cat. no. 309603, http://www.fishersci.com/)
- Conical tube (15 ml; BD Medical, cat. no. 352096, http://www.fishersci.com/)
- Conical tubes (50 ml; BD Medical, cat. no. 14-432-22, http://www.fishersci.com/)
- Petri dish, non–tissue culture treated (100 mm; Fisher Scientific, cat. no. 08-757-12, http://www.fishersci.com/)
- Parafilm (Pechiney Plastic Packaging, cat. no. PM996, http://www.fishersci.com/)
- Tweezers (Fisher Scientific, cat. no. 08-880, http://www.fishersci.com/)
- Ultrasonicator (Branson 450, cat. no. 101-135-022, http://www.fishersci.com/)
- Externally Threaded Disruptor Horn (1/2-inch, Branson, cat. no. 101-147-037, http://www.fishersci.com/)
- Tapered Microtip (1/8-inch diameter, Branson, cat. no. 101-148-062, http://www.fishersci.com/)
- Reciprocating shaker (MaxQ, Thermo Scientific, cat. no. 11-675-152, http://www.fishersci.com/)

Electrospinning

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

Silk Sponges

- Sieves (Fisher Scientific, various pore sizes, e.g., 1 mm, cat. no. 04-884-1AJ; 850 µm, 04-884-1AK; 600 µm, 04-884-1AM; 500 µm, 04-884-1AN; http://www.fishersci.com/)
- Sieve cover (Fisher Scientific, cat. no. 04-888B, http://www.fishersci.com/)
- Small weigh boat (Fisher Scientific, cat. no. 08-732-112, http://www.fishersci.com/)
- Analytical balance (A&D Weighing, model Orion HR-200, http://www.americanweigh.com/)
- Cylindrical molds (5 ml; Fisher Scientific, cat. no. 03-338-1E, http://www.fishersci.com/)
- Syringe (5 ml; Air Tite Products, cat. no. A5, http://www.fishersci.com/)
- Plastic beaker (2 liters; Fisher Scientific, cat. no. 02-591-33, http://www.fishersci.com/)
- Large stir bar (Fisher Scientific, cat. no. 14-513-56, http://www.fishersci.com/)
- Hot plate (Fisher Scientific, cat. no. 11-100-100H, http://www.fishersci.com/)
- Conical tubes (50 ml; BD Medical, cat. no. 14-432-22, http://www.fishersci.com/)

HFIP-based sponges

- Sieves (Fisher Scientific, various pore sizes, e.g., 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; http://www.fishersci.com/)
- Sieve cover (Fisher Scientific, cat. no. 04-888B, http://www.fishersci.com/)
- Small weigh boat (Fisher Scientific, cat. no. 08-732-112, http://www.fishersci.com/)
- Analytical balance (A&D Weighing, model Orion HR-200, http://www.americanweigh.com/)
- Glass scintillation vials (20 ml; Fisher Scientific, cat. no. 03-337-15, http://www.fishersci.com/)
- Glass vials (1.8 ml; Fisher Scientific, cat. no. 03-339-26A, http://www.fishersci.com/)
- Parafilm (Pechiney Plastic Packaging, cat. no. PM996, http://www.fishersci.com/)
- Syringe (5 ml; Air Tite Products, cat. no. A5, http://www.fishersci.com/)
- Plastic beaker (2 liters; Fisher Scientific, cat. no. 02-591-33, http://www.fishersci.com/)
- Large stir bar (Fisher Scientific, cat. no. 14-513-56, http://www.fishersci.com/)
- Hot plate (Fisher Scientific, cat. no. 11-100-100H, http://www.fishersci.com/)
- Fume hood
- Conical tubes (50 ml; BD Medical, cat. no. 14-432-22, http://www.fishersci.com/)

PROCEDURE

Fibroin extraction ● TIMING 2.5 h active, overnight drying (day 1)

1| Prepare a 2-liter glass beaker filled with 2 liters of ultrapure water, cover it with aluminum foil and heat until boiling.
! CAUTION Do not leave the beaker unattended while heating and boiling. Because of high temperatures, plastic beakers should not be used.

2| Meanwhile, cut cocoons with titanium scissors into dime-sized pieces and dispose of silkworms. Measure out 5 g 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 prepare 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 continue boiling for 30 min. Occasionally, stir 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 it by rinsing in ultrapure cold 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| Place fibroin in a 1-liter beaker filled with 1 liter of ultrapure water and a stir bar.
! CAUTION If you are using a plastic beaker, ensure that the hot plate has cooled.

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

9| Repeat Steps 7 and 8 twice for a total of three 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, in which the sericin has been removed, can be stored indefinitely at room temperature. For long-term storage, place it in a clean plastic bag or wrap it in aluminum foil. Be sure to indicate the length of the boiling step on the label.

Dissolve silk fibroin in LiBr ● TIMING 4.5 h (day 2)

12| Calculate the amount of 9.3 M lithium bromide needed to prepare a 20% (wt/vol) solution based on the amount of dried fibroin available. As 20% of the solution will be silk, 80% will be LiBr. That is, a ratio of 1:4 (1 g to 4 ml) silk to LiBr. Therefore, multiply the amount of the dried silk fibroin by 4 to obtain the total volume of 9.3 M LiBr needed (X).

13| Prepare a 9.3 M LiBr solution.

$$\left(86.85\frac{g}{\text{mol}}\right)\left(9.3\frac{\text{mol}}{L}\right)\left(\frac{1L}{1{,}000\,\text{ml}}\right)(X) = \_\_\_\_\_\ g\ \text{of LiBr}$$

! CAUTION Adding LiBr to water results in an exothermic reaction; be mindful of the heat generated. When preparing large volumes, we recommend carrying this out on ice.

▲ CRITICAL STEP LiBr has a low density and its volume should be taken into account while preparing 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.

14| Pack silk fibroin tightly into a 50-ml glass beaker and add the required amount of LiBr solution on top.
▲ CRITICAL 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 h. 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 assessment.
? TROUBLESHOOTING

Dialysis and centrifugation ● TIMING 49 h (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, and this step will be easier if the solution is kept warm before 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. Moreover, have an additional needle and insert it into another top port of the cassette to allow air to escape. Remove the extra needle once all the air has been purged.

18| Dialyze against 1 liter of ultrapure water per 12 ml cassette. To ensure mixing, use a large stir bar and place on a magnetic stir plate. Change the water after 1 h, 4 h, that evening, the next morning and night, as well as in the morning on the following day (i.e., six changes within 48 h).

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 it between two 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 r.p.m. (~12,700*g*) at 4 °C for 20 min.

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

22| Repeat Steps 20 and 21 again.

23| To determine the concentration of the silk in solution, measure the weight of a small weigh boat. Thereafter, 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 7–8% (wt/vol) 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.

Lyophilization and concentration
24| The fibroin solution (25 ml at concentration 7–8% (wt/vol)) can either be used as is or it can be lyophilized (option A) or concentrated (option B). For storage for longer than 1 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 HFIP. The concentrated solution (20–30%, wt/vol) can be used directly for preparing silk tubes.

(A) Lyophilization (optional) ● **TIMING 3 d**

(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 the 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 Kimwipe 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 d 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 (20–25 °C).

■ **PAUSE POINT** The lyophilized material can be stored at room temperature indefinitely.

(B) Concentrating silk fibroin (optional) ● **TIMING 21–25 h**

(i) Add 100 g of PEG to 900 ml of ultrapure water to prepare a 10% (wt/vol) 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 24B(ii).

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

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

(iv) Draw out 10 ml of 7–8% (wt/vol) 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 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 decrease.

(xi) Remove the needle/syringe from the cassette.

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

(xiii) Place the cassette into 10% (wt/vol) PEG solution and cover it 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 h. For the gel-spun tubes, the solution must be more viscous and therefore is concentrated for ~22 h.

(xv) Remove the concentrated silk solution after the desired amount of time using 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. Because of the high viscosity of concentrated silk, we suggest using either large-orifice pipette tips or cutting off the tip of the pipette. 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% (wt/vol) PEG for 20–22 h. 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 it is 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 reach the required concentration. It is suggested that the silk be checked daily, as 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 with 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, as 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 is intended to be used relatively soon.

25| The silk fibroin can now be used to prepare a number of different materials (**Fig. 1**). Using the concentrated solution (prepared in Step 24B) you can prepare silk tubes by either a simple dip method to create thin-walled tubes (option A, **Fig. 3**) or by gel spinning, wherein the fibroin is extruded onto a rotating mandrel (option B, **Fig. 4**). Alternatively, silk solution may be used

to prepare hydrogels via vortexing, sonication, electrical current or pH change (options C–F, **Fig. 5**); nonpatterned or patterned silk films (options G and H, **Fig. 6**); silk microspheres using the DOPC (option I, **Fig. 7**) or PVA (option J, **Fig. 8**) method; electrospun silk fibers (option K, **Fig. 9**); or silk sponges that are either aqueous-based (option L, **Fig. 10**) or HFIP-based (option M, **Fig. 11**).

(A) Dipped tubes ● TIMING 2 min per tube with overnight dry and 1 h soak

(i) (Optional) If porous tubes are required, prepare a solution of 7–8% (wt/vol) 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 as 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 the wire from the silk and invert the wire, allowing the beads of silk to evenly run down the wire.
When the beads coalesce at the bottom of the wire, flip it and allow the silk to run back down toward the end of the wire (try to coat evenly).
? TROUBLESHOOTING

(iii) Let excess silk beads drip off of the wire back into silk solution.

(iv) Place the wires in a vial of methanol for 5–10 s.
▲ CRITICAL STEP Remove the silk-coated wire from methanol and allow to dry for ~30 s.

(v) Repeat dipping process and methanol treatment 2–4 times or until the wire is sufficiently coated with silk. The silk may appear uneven, but it will shrink as it dries.

(vi) 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.

(vii) When silk is dry, place the wires coated with silk into a 15-ml conical tube filled with soapy water and soak for 1 h.

(viii) After soaking, remove the tubes from the soapy water and cut the end of the silk tube with a scalpel or a razor blade.

(ix) Remove the tube from the wire using tweezers. The tube should slide off easily. If it is difficult to remove without scrunching the tube, then place the tube back into soapy water and wait before trying again.
? TROUBLESHOOTING

(x) 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 h to remove residual soap.

(xi) Store silk tubes in a plastic Petri dish at room temperature. These tubes can be 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 the concentrated silk solution. Be sure to avoid including air bubbles.

(iii) Remove the needle and replace with a 30-gauge needle.

(iv) Start the 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. To obtain a uniform fiber, a generous amount of pressure is required. If solution does not come out, replace the needle with a 27-gauge needle.
▲ 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 one 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.
? TROUBLESHOOTING

(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, and then place the mandrel into a solution of soapy water.

(viii) When the tube has softened, grab the tube uniformly with a Kimwipe and gently pull it 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 water and incubate in a shaking water bath for 1 h to remove residual soap.

(x) Store the silk tube in a plastic Petri dish at room temperature for up to 1 year (depending on tube treatment and storage conditions).

(C) Vortexed gels ● TIMING 1 d

(i) Adjust the concentration of an 8% (wt/vol) silk solution to 2–5% (wt/vol).

(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 ~7 min at 3,200 r.p.m. (maximum speed setting). This treatment should increase the solution turbidity.
▲ CRITICAL STEP Silk solutions may vary between batches and as a solution ages; therefore, the vortex time may need to be increased or decreased.
? TROUBLESHOOTING
(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 in a well plate to allow 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% (wt/vol) gel through a 21-gauge needle. If injection requires substantial 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 d

(i) Dilute 8% (wt/vol) aqueous silk solution to 4% (wt/vol).
(ii) Add 5 ml of 4% (wt/vol) silk solution to a 15-ml conical tube.
(iii) Sonicate the solution at 50% amplitude (21 W) for 30 s.
! 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 earmuffs 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 overnight, if necessary, to allow hydrogelation.

(E) Electrogels (e-gels) ● TIMING 15 min

(i) Add 10 ml of 7–8% (wt/vol) 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 ~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 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% (wt/vol) 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 d

(i) Add 4 ml of 8% (wt/vol) 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 covering 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 1 d.
▲ 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 to adding methanol but to a lesser extent.

(vi) Gently remove the film from the dish.
? TROUBLESHOOTING
(vii) Store at room temperature for up to 1 year (depending on film treatment and storage conditions).

(H) Patterned silk films ● TIMING 6 h for generating a mold, 2d for casting

(i) Prepare work area by laying down a sheet of aluminum foil and collecting the required materials (a plastic pipette, a box of Kimwipes, a paper cup, a needle, a Sylgard base and curing agent, a bottle of 70% (vol/vol) ethanol and diffraction grating).
▲ CRITICAL STEP If you are reusing a mold, skip to Step 25H(xii).
▲ CRITICAL STEP Ensure that the diffraction grating is free of debris. Spray with ethanol, wipe and blow-dry with pressurized air from the bench top.
(ii) Place paper cup on balance, weigh out an appropriate amount of base and then curing agent from the Sylgard kit. For 1 mold (5 cm × 5 cm) use 4.5 g of base and 0.5 g of curing agent (9:1 ratio).
(iii) Mix with a plastic pipette for 1 min.
(iv) Place PDMS in a vacuum oven for 30–60 min to remove air bubbles.
(v) Place the diffraction grating in a Petri dish.
(vi) Slowly pour the PDMS over the diffraction grating; try not to introduce bubbles.
(vii) Once the grating is covered, remove any bubbles with a needle.
(viii) Gently move the Petri dish to a 60 °C oven for 4 h or leave at room temperature for over 24 h.
(ix) Remove the PDMS from the diffraction grating. Be careful not to rip the PDMS.
(x) Clean the mold with a Kimwipe 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.
(xi) (Optional) the PDMS mold can be punched to varying diameters depending on need. For this protocol, punch out 14-mm disks from the PDMS.
(xii) Place the PDMS molds in a Petri dish.
(xiii) Add 100 µl of 8% (wt/vol) silk solution onto each 14-mm disk.
(xiv) Allow the films to dry overnight.
(xv) Fill the bottom of the vacuum desiccator with water.
▲ CRITICAL STEP Place the dry films in the desiccator and apply vacuum to the vacuum port.
(xvi) Allow the films to water anneal for 1 d.
(xvii) Allow the films to dry for 10 min and gently peel films from the mold using tweezers.
(xviii) To indicate the patterned side of the film, place an edge of the nonpatterned side onto a Post-it note. Store at room temperature until use.

(I) Microspheres (DOPC) ● TIMING 4.5 d

(i) Add 200 mg of DOPC lipid powder to a glass test tube.
(ii) In a 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^{-1} solution of the drug, protein or chemical to be encapsulated to 1 ml of 8% (wt/vol) silk solution. If desired, also prepare a solution of 1 ml of 8% (wt/vol) 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 rehydrated, 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 min 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 min (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 a 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 the 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 it at −80 °C for at least 5 h. Do not exceed 40 ml of solution per tube as the solution may expand during freezing.
(xv) Lyophilize for 2–3 d.
(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; we recommend using the proper safety equipment. In addition, methanol is a flammable solvent; therefore, sources of ignition should be removed before use.
(xvii) Incubate the sample for 30 min 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 r.p.m. 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 10,000 r.p.m. for 5 min at 4 °C.
(xxii) Remove the water and resuspend microspheres in the desired water or buffer. If the microspheres have aggregated, they can be dispersed by sonicating the solution for about 10 s at 30% amplitude (~20 W).

(J) Microspheres (PVA) ● TIMING 2 d

(i) Prepare a 5% (wt/vol) 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 adjust the volume of the solution up to 5 ml with ultrapure water.
(iii) Filter the PVA solution with a 0.45-μm filtration unit.
■ PAUSE POINT Store the PVA solution at room temperature for no more than 3 months.
(iv) Dilute silk solution to 5% (wt/vol) with ultrapure water.
(v) Add 1 ml of 5% (wt/vol) silk and 4 ml of 5% (wt/vol) PVA into a 15-ml conical tube and gently mix. While slowly moving the probe up and down, sonicate the solution for 30 s 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 earmuffs is highly recommended.
(vi) Pour the solution into a Petri dish and allow it to cover the bottom of the dish evenly.
(vii) 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 it at room temperature.
(viii) Peel off the film from the dish and place one film in a 50-ml conical tube.
(ix) Add 20 ml of deionized water to the tube; shake for 30 min at room temperature to dissolve the film.
? TROUBLESHOOTING
(x) Centrifuge at 11,000 r.p.m. for 20 min at 4 °C and discard the supernatant.
(xi) Resuspend the pellet in 5 ml of ultrapure water. Sonicate for 15 s at 15% amplitude, if necessary.
Optional: use the suspension (containing both nano- and microspheres) or lyophilize and store the dry material.

(K) Electrospun fibers ● TIMING 1 d

(i) Prepare a solution of 5% (wt/vol) PEO in 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 1 year.
(ii) Add 5 ml of the 5% (wt/vol) PEO (900,000 g mol^{-1}) solution into 20 ml of 8% (wt/vol) silk solution to generate an aqueous solution of 6.4% (wt/vol) silk/1% (wt/vol) PEO.
▲ CRITICAL STEP A high viscosity, because of 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 ~10 min 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 7–20 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 A. 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 (0.01–0.03 ml min^{-1}), electric potential (8–15 KV) and the distance between the capillary tip and the collection screen (7–20 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 because of 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. Finally, the flow rate should be adjusted so that the solution is constantly being supplied to the tip without allowing excess solution to drip[42].
(xi) Collect the silk fibers until the desired thickness is achieved.
(xii) Immerse the fiber mats in a 90% (vol/vol) 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 before 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 d

(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.
▲ CRITICAL STEP It is important to note that the pore sizes will be slightly smaller than the salt particles used, as 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.
? TROUBLESHOOTING
(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 per mold. Note: For salt particles 750 µm and larger, use 8% (wt/vol) silk solution. For smaller salt particles, use 6% (wt/vol) 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 settle overnight at room temperature. The silk fibroin should gel in 1–2 d.
(vii) Once the silk has gelled, remove the lids and place the molds in a 2-liter beaker with ultrapure water (three containers per 2 liters of water).
(viii) Transfer the beaker to a stir plate and stir.
(ix) Change the water 2–3 times per day for 2 d (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 them at room temperature.
(xii) Before use, cut hydrated scaffolds into the desired dimensions. Autoclave to sterilize. To cut the scaffolds to smaller dimensions, we suggest disposable biopsy punches to obtain uniform disks.

(M) HFIP-based sponges ● TIMING 8 d

(i) Prepare 17% (wt/vol) 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 the Hazardous Materials Information System as a health hazard level 3; it can cause burns both topically and if inhaled. Wear proper safety equipment and work inside a chemical fume hood.
? TROUBLESHOOTING
(ii) Wrap the lid with Parafilm and keep it 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 at the bottom. Add salt and shake vigorously. Repeat until the desired amount of salt is collected.

(iv) Weigh 3.4 g of NaCl and place it in a glass shell vial. Repeat for eight 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 the bench top surface to level the salt in the 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 d).

(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 d.

! 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 the methanol to seep through the scaffold for 1 d.

(xi) Remove extra methanol and dispose of it in a proper waste container. Transfer the containers to a 2-liter beaker of ultrapure water (~6 containers per 2 liters of water).

? TROUBLESHOOTING

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

(xiii) Change the water 2–3 times a day for 2–3 d.

(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 be stored for 2–6 months) or dry the scaffolds and store them dry at room temperature.

(xvi) When needed for use, cut them 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**.

TABLE 2 | Troubleshooting table.

Step	Problem	Solution
15 (silk processing)	Silk does not dissolve after 4 h 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 min? If you boiled for a shorter time, you may need to allow the silk to dissolve in LiBr for longer. Make 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
	LiBr and silk fibroin solution is not amber colored	Change or clean glassware
25A(ii) (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 as the silk will gel if it is stored for too long

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Solution
25A(ix) (tubes)	Tubes crack after drying	Try a solution of 70% (vol/vol) 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 s for methanol treatment and 30 s for the tube to dry between layers
	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
25B(v) (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 as the silk will gel if it is stored for too long
25C(iv) (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
25D(iii) (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
25E(iii) (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
25G(vi) (thin films)	Films will not lift off of the plate	Use non–tissue culture–treated dishes
25J(ix) (PVA microspheres)	The film will not completely dissolve after 30 min	Sonicate the solution for 30 s at 15% amplitude
25L(i),25M(i) (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
25M(xi) (HFIP-based sponges)	HFIP-based sponges dissolved while in water	Methanol was not able to penetrate properly. Repeat but extend the methanol treatment time
25M(xvi) (HFIP-based sponges)	HFIP-based sponges have voids in them	Try pushing the scaffolds down or centrifuge them before drying

● TIMING

Steps 1–11, Fibroin extraction: 2.5 h to overnight (day 1)
Steps 12–15, Dissolve silk fibroin: 4.5 h (day 2)
Steps 16–23, Dialysis and centrifugation: 49 h (days 2–4)
Step 24A, Lyophilization: 3 d
Step 24B, Concentration: 21–25 h
Step 25A, Preparation of dipped tubes: 2 min per tube, plus overnight dry and 1 h soak
Step 25B, Preparation of gel-spun tubes: 5 min per tube, plus overnight dry
Step 25C, Preparation of vortexed gels: 1 d
Step 25D, Preparation of sonicated gels: 1 d
Step 25E, Preparation of electrogels: 15 min
Step 25F, Preparation of pH gels: 10 min
Step 25G, Preparation of silk films: 2 d
Step 25H, Generating molds and casting of patterned silk films: 6 h for generating molds, 2 d for casting
Step 25I, Preparation of microspheres (DOPC): 4.5 d
Step 25J, Preparation of microspheres: 2 d
Step 25K, Electrospinning: 1 d

Step 25L, Preparation of aqueous-based sponges: 5 d
Step 25M, Preparation of HFIP-based sponges: 8 d

ANTICIPATED RESULTS

Expected outcomes for the various options described in the PROCEDURE are summarized in the INTRODUCTION.

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1. Murphy, A.R. & Kaplan, D.L. Biomedical applications of chemically-modified silk fibroin. *J. Mat. Chem.* **19**, 6443–6450 (2009).
2. Murphy, A.R., John, P.S. & Kaplan, D.L. Modification of silk fibroin using diazonium coupling chemistry and the effects on hMSC proliferation and differentiation. *Biomaterials* **29**, 2829–2838 (2008).
3. Wenk, E. *et al.* The use of sulfonated silk fibroin derivatives to control binding, delivery and potency of FGF-2 in tissue regeneration. *Biomaterials* **31**, 1403–1413 (2010).
4. Vepari, C., Matheson, D., Drummy, L., Naik, R. & Kaplan, D.L. Surface modification of silk fibroin with poly(ethylene glycol) for antiadhesion and antithrombotic applications. *J. Biomed. Mat. Res. A* **93A**, 595–606 (2010).
5. Sofia, S., McCarthy, M.B., Gronowicz, G. & Kaplan, D.L. Functionalized silk-based biomaterials for bone formation. *J. Biomed. Mat. Res.* **54**, 139–148 (2001).
6. Vepari, C. & Kaplan, D.L. Silk as a biomaterial. *Prog. Poly. Sci.* **32**, 991–1007 (2007).
7. Craig, C. & Riekel, C. Comparative architecture of silks, fibrous proteins and their encoding genes in insects and spiders. *Comp. Chem. Physiol. B Biochem. Mol. Biol.* **133**, 493–507 (2002).
8. Omenetto, F.G. & Kaplan, D.L. New opportunities for an ancient material. *Science* **329**, 528–531 (2010).
9. Altman, G.H. *et al.* Silk-based biomaterials. *Biomaterials* **24**, 401–416 (2003).
10. Horan, R.L. *et al. In vitro* degradation of silk fibroin. *Biomaterials* **26**, 3385–3393 (2005).
11. Park, S.H. *et al.* Relationships between degradability of silk scaffolds and osteogenesis. *Biomaterials* **31**, 6162–6172 (2010).
12. Meinel, L. *et al.* The inflammatory responses to silk films *in vitro* and *in vivo*. *Biomaterials* **26**, 147–155 (2005).
13. Wray, L.S., Hu, X. & Kaplan, D.L. Effect of processing on silk-based biomaterials: reproducibility and biocompatibility. *J. Biomed. Mater. Res. B Appl. Biomater.* published online, doi:10.1002/jbm.b.31875 (21 June 2011).
14. Kim, D.-H. *et al.* Dissolvable films of silk fibroin for ultrathin conformal bio-integrated electronics. *Nat. Mater.* **9**, 511–517 (2010).
15. Etienne, O. *et al.* Soft tissue augmentation using silk gels: an *in vitro* and *in vivo* study. *J. Periodont.* **80**, 1852–1858 (2009).
16. Wang, Y. *et al. In vivo* degradation of three-dimensional silk fibroin scaffolds. *Biomaterials* **29**, 3415–3428 (2008).
17. Meinel, L. *et al.* Silk based biomaterials to heal critical sized femur defects. *Bone* **39**, 922–931 (2006).
18. Hu, X. *et al.* Regulation of silk material structure by temperature-controlled water vapor annealing. *Biomacromolecules* **12**, 1686–1696 (2011).
19. Hu, X., Lu, Q., Kaplan, D.L. & Cebe, P. Microphase separation controlled beta-sheet crystallization kinetics in fibrous proteins. *Macromolecules* **42**, 2079–2087 (2009).
20. Sung, N.-Y. *et al.* Effect of gamma irradiation on the structural and physiological properties of silk fibroin. *Food Sci. Biotechnol.* **18**, 228–233 (2009).
21. Kojthung, A. *et al.* Effect of gamma radiation on biodegradation of *Bombyx mori* silk fibroin. *Int. Biodeterior. Biodegrad.* **62**, 487–490 (2008).
22. Lovett, M. *et al.* Silk fibroin microtubes for blood vessel engineering. *Biomaterials* **28**, 5271–5279 (2007).
23. Lovett, M.L., Cannizzaro, C.M., Vunjak-Novakovic, G. & Kaplan, D.L. Gel spinning of silk tubes for tissue engineering. *Biomaterials* **29**, 4650–4657 (2008).
24. Lovett, M.L., Rockwood, D.N., Baryshyan, A. & Kaplan, D.L. Simple modular bioreactors for tissue engineering: a system for characterization of oxygen gradients, human mesenchymal stem cell differentiation, and prevascularization. *Tissue Eng. Part C Methods.* **16**, 1565–1573 (2010).
25. Kim, U.J. *et al.* Structure and properties of silk hydrogels. *Biomacromolecules* **5**, 786–792 (2004).
26. Yucel, T., Cebe, P. & Kaplan, D.L. Vortex-induced injectable silk fibroin hydrogels. *Biophys. J.* **97**, 2044–2050 (2009).
27. Wang, X.Q., Kluge, J.A., Leisk, G.G. & Kaplan, D.L. Sonication-induced gelation of silk fibroin for cell encapsulation. *Biomaterials* **29**, 1054–1064 (2008).
28. Leisk, G.G., Lo, T.J., Yucel, T., Lu, Q. & Kaplan, D.L. Electrogelation for protein adhesives. *Adv. Mater.* **22**, 711–715 (2010).
29. Yucel, T., Kojic, N., Leisk, G.G., Lo, T.J. & Kaplan, D.L. Non-equilibrium silk fibroin adhesives. *J. Struct. Biol.* **170**, 406–412 (2010).
30. Hofmann, S. *et al.* Silk fibroin as an organic polymer for controlled drug delivery. *J. Control Release* **111**, 219–227 (2006).
31. Lawrence, B.D., Marchant, J.K., Pindrus, M.A., Omenetto, F.G. & Kaplan, D.L. Silk film biomaterials for cornea tissue engineering. *Biomaterials* **30**, 1299–1308 (2009).
32. Jin, H.J., Park, J., Valluzzi, R., Cebe, P. & Kaplan, D.L. Biomaterial films of *Bombyx mori* silk fibroin with poly(ethylene oxide). *Biomacromolecules* **5**, 711–717 (2004).
33. Wang, X. *et al.* Silk microspheres for encapsulation and controlled release. *J. Control Release* **117**, 360–370 (2007).
34. Wang, X.Q., Yucel, T., Lu, Q., Hu, X. & Kaplan, D.L. Silk nanospheres and microspheres from silk/pva blend films for drug delivery. *Biomaterials* **31**, 1025–1035 (2010).
35. Jin, H.J., Fridrikh, S.V., Rutledge, G.C. & Kaplan, D.L. Electrospinning *Bombyx mori* silk with poly(ethylene oxide). *Biomacromolecules* **3**, 1233–1239 (2002).
36. Li, C., Vepari, C., Jin, H.-J., Kim, H.J. & Kaplan, D.L. Electrospun silk-BMP-2 scaffolds for bone tissue engineering. *Biomaterials* **27**, 3115–3124 (2006).
37. Rockwood, D.N., Akins, R.E., Parrag, I.C., Woodhouse, K.A. & Rabolt, J.F. Culture on electrospun polyurethane scaffolds decreases atrial natriuretic peptide expression by cardiomyocytes *in vitro*. *Biomaterials* **29**, 4783–4791 (2008).
38. Kakade, M.V. *et al.* Electric field induced orientation of polymer chains in macroscopically aligned electrospun polymer nanofibers. *J. Am. Chem. Soc.* **129**, 2777–2782 (2007).
39. Kim, U.J., Park, J., Kim, H.J., Wada, M. & Kaplan, D.L. Three-dimensional aqueous-derived biomaterial scaffolds from silk fibroin. *Biomaterials* **26**, 2775–2785 (2005).
40. Nazarov, R., Jin, H.J. & Kaplan, D.L. Porous 3-D scaffolds from regenerated silk fibroin. *Biomacromolecules* **5**, 718–726 (2004).
41. Rockwood, D.N. *et al.* Ingrowth of human mesenchymal stem cells into porous silk particle reinforced silk composite scaffolds: an *in vitro* study. *Acta Biomater.* **7**, 144–151 (2011).
42. Zhang, X.H., Reagan, M.R. & Kaplan, D.L. Electrospun silk biomaterial scaffolds for regenerative medicine. *Adv. Drug Del. Rev.* **61**, 988–1006 (2009).
43. Kim, H.J., Kim, U.J., Vunjak-Novakovic, G., Min, B.H. & Kaplan, D.L. Influence of macroporous protein scaffolds on bone tissue engineering from bone marrow stem cells. *Biomaterials* **26**, 4442–4452 (2005).
44. Hofmann, S. *et al.* Control of *in vitro* tissue-engineered bone-like structures using human mesenchymal stem cells and porous silk scaffolds. *Biomaterials* **28**, 1152–1162 (2007).
45. Kim, H.J. *et al.* Bone tissue engineering with premineralized silk scaffolds. *Bone* **42**, 1226–1234 (2008).

46. Kim, H.J. *et al.* Bone regeneration on macroporous aqueous-derived silk 3-D scaffolds. *Macromol. Biosci.* **7**, 643–655 (2007).
47. Hofmann, S. *et al.* Cartilage-like tissue engineering using silk scaffolds and mesenchymal stem cells. *Tissue Eng.* **12**, 2729–2738 (2006).
48. Tigli, R.S. *et al.* Comparative chondrogenesis of human cell sources in 3D scaffolds. *J. Tiss. Eng. Regen. Med.* **3**, 348–360 (2009).
49. Wang, Y.Z., Blasioli, D.J., Kim, H.J., Kim, H.S. & Kaplan, D.L. Cartilage tissue engineering with silk scaffolds and human articular chondrocytes. *Biomaterials* **27**, 4434–4442 (2006).
50. Wang, Y.Z., Kim, U.J., Blasioli, D.J., Kim, H.J. & Kaplan, D.L. *In vitro* cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells. *Biomaterials* **26**, 7082–7094 (2005).
51. Ghosh, S., Laha, M., Mondal, S., Sengupta, S. & Kaplan, D.L. *In vitro* model of mesenchymal condensation during chondrogenic development. *Biomaterials* **30**, 6530–6540 (2009).
52. Mauney, J.R. *et al.* Engineering adipose-like tissue *in vitro* and *in vivo* utilizing human bone marrow and adipose-derived mesenchymal stem cells with silk fibroin 3D scaffolds. *Biomaterials* **28**, 5280–5290 (2007).
53. Gil, E.S., Park, S.H., Marchant, J., Omenetto, F. & Kaplan, D.L. Response of human corneal fibroblasts on silk film surface patterns. *Macromol. Biosci.* **10**, 664–673 (2010).
54. Soffer, L. *et al.* Silk-based electrospun tubular scaffolds for tissue-engineered vascular grafts. *J. Biomat. Sci.- Poly. Ed.* **19**, 653–664 (2008).
55. Zhang, X.H., Baughman, C.B. & Kaplan, D.L. *In vitro* evaluation of electrospun silk fibroin scaffolds for vascular cell growth. *Biomaterials* **29**, 2217–2227 (2008).
56. Zhang, X.H. *et al.* Dynamic culture conditions to generate silk-based tissue-engineered vascular grafts. *Biomaterials* **30**, 3213–3223 (2009).
57. House, M., Sanchez, C.C., Rice, W.L., Socrate, S. & Kaplan, D.L. Cervical tissue engineering using silk scaffolds and human cervical cells. *Tissue Eng. Part A* **16**, 2101–2112 (2010).
58. Schneider, A., Wang, X.Y., Kaplan, D.L., Garlick, J.A. & Egles, C. Biofunctionalized electrospun silk mats as a topical bioactive dressing for accelerated wound healing. *Acta Biomater.* **5**, 2570–2578 (2009).
59. Wharram, S.E., Zhang, X.H., Kaplan, D.L. & McCarthy, S.P. Electrospun silk material systems for wound healing. *Macromol. Biosci.* **10**, 246–257 (2010).
60. Moreau, J.E. *et al.* Tissue-engineered bone serves as a target for metastasis of human breast cancer in a mouse model. *Cancer Res.* **67**, 10304–10308 (2007).
61. Wang, X.L. *et al.* A complex 3D human tissue culture system based on mammary stromal cells and silk scaffolds for modeling breast morphogenesis and function. *Biomaterials* **31**, 3920–3929 (2010).
62. Wang, X.L. *et al.* Preadipocytes stimulate ductal morphogenesis and functional differentiation of human mammary epithelial cells on 3D silk scaffolds. *Tissue Eng. Part A* **15**, 3087–3098 (2009).
63. Subramanian, B. *et al.* Tissue-engineered three-dimensional *in vitro* models for normal and diseased kidney. *Tissue Eng. Part A* **16**, 2821–2831 (2010).
64. Altman, G.H. *et al.* Silk matrix for tissue engineered anterior cruciate ligaments. *Biomaterials* **23**, 4131–4141 (2002).
65. Moreau, J.E., Bramono, D.S., Horan, R.L., Kaplan, D.L. & Altman, G.H. Sequential biochemical and mechanical stimulation in the development of tissue-engineered ligaments. *Tissue Eng. Part A* **14**, 1161–1172 (2008).
66. Jiang, X.Q. *et al.* Mandibular repair in rats with premineralized silk scaffolds and BMP-2-modified bMSCs. *Biomaterials* **30**, 4522–4532 (2009).
67. Zhao, J. *et al.* Apatite-coated silk fibroin scaffolds to healing mandibular border defects in canines. *Bone* **45**, 517–527 (2009).
68. Pritchard, E.M., Szybala, C., Boison, D. & Kaplan, D.L. Silk fibroin encapsulated powder reservoirs for sustained release of adenosine. *J. Control Release* **144**, 159–167 (2010).
69. Szybala, C. *et al.* Antiepileptic effects of silk-polymer based adenosine release in kindled rats. *Exp. Neurol.* **219**, 126–135 (2009).
70. Wilz, A. *et al.* Silk polymer-based adenosine release: therapeutic potential for epilepsy. *Biomaterials* **29**, 3609–3616 (2008).
71. Wang, X.Q. *et al.* Growth factor gradients via microsphere delivery in biopolymer scaffolds for osteochondral tissue engineering. *J. Control Release* **134**, 81–90 (2009).