Published in final edited form as:

Acta Biomater. 2018 April 15; 71: 379–387. doi:10.1016/j.actbio.2018.02.035.

3D Freeform Printing of Silk Fibroin

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Abstract

Freeform fabrication has emerged as a key direction in printing biologically-relevant materials and structures. With this emerging technology, complex structures with microscale resolution can be created in arbitrary geometries and without the limitations found in traditional bottom-up or top-down additive manufacturing methods. Recent advances in freeform printing have used the physical properties of microparticle-based granular gels as a medium for the submerged extrusion of bioinks. However, most of these techniques require post-processing or crosslinking for the removal of the printed structures. [1,2] In this communication, we introduce a novel method for the one-step gelation of silk fibroin within a suspension of synthetic nanoclay (Laponite) and polyethylene glycol (PEG). Silk fibroin has been used as a biopolymer for bioprinting in several contexts, but chemical or enzymatic additives or bulking agents are needed to stabilize 3D structures. Our method requires no post-processing of printed structures and allows for *in situ* physical crosslinking of pure aqueous silk fibroin into arbitrary geometries produced through freeform 3D printing.

Graphical Abstract

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Keywords

silk; printing; freeform; biomaterials; laponite

1. Introduction

Silk fibroin is an attractive biopolymer for diverse applications due to its all-aqueous and ambient processing conditions, cytocompatible crosslinking methods, robust mechanical features, and full degradability to amino acids. [3,4] With the increase in research into additive manufacturing for biological and clinical applications over recent years, [5] several efforts are underway to create silk constructs through bioprinting methods. Such techniques allow the formation of custom geometries that would be difficult or impossible to manufacture through molding or other fabrication techniques. Notably, encapsulated cells in silk fibroin-gelatin hydrogels were reported and these bioprinted constructs deposited layer-by-layer supported the multilineage differentiation of stem cells. [6] A droplet-based approach for inkjet printing of silk using alginate as a sacrificial material with horseradish peroxidase (HRP) crosslinking was also reported. [7] Recently, further characterization of bioprinted silk-gelatin blends demonstrated potential for soft tissue implants. [8] However, despite these efforts that have propelled the field of silk-based printing methods, creating arbitrary geometries of silk fibroin through additive manufacturing without the use of crosslinking agents or sacrificial material has remained elusive. In this work, we describe a novel 3D freeform fabrication strategy and method of silk fibroin through the use of a nanoclay and PEG suspension, inducing the *in situ* physical crosslinking of silk fibroin without the need for additives or post-processing.

Several groups have recently demonstrated a novel technique for freeform fabrication by printing into a granular medium. [1,2,9] These techniques are based on using gels made from soft microparticles that can smoothly and rapidly transition between fluid and solid states. Using the same process for traditional 3D printing, materials can be printed into a granular gel whose physical characteristics allow it to fluidize at the point of injection and quickly recover into a solid support, trapping and holding the printing material behind the moving tip. However, biopolymeric substances printed generally need enzymatic, thermal, or UV crosslinking after the printing process. The process in this current work allows for printing of pure aqueous silk fibroin, which is physically cross-linked *in situ* with the use of PEG without any thermal, chemical, or enzymatic crosslinking. This one-step gelation of

silk fibroin into any geometry is made possible through submerged printing in a nanoclay colloidal suspension. The gel medium is composed of a synthetic nanoclay, Laponite XLG, a water dispersible sodium magnesium silicate. Laponite XLG is a gel-forming grade of synthetic clay often used industrially as a viscosity enhancer for polymers and as an emulsion stabilizer in lotions and creams.

Over the past few years, laponite has been incorporated into bioink formulations to add stretchable and tissue sealant properties to a variety of hydrogels. [10–12] However, the present work focuses on a new direction of the use of laponite, as a nanoclay gel medium for submerged extrusion/freeform printing. In its solid, anhydrous form, laponite nanoparticles consist of stacks of clay platelets. The platelets are 20 nm in diameter and 2–4 nm thick. [13] Clusters of laponite platelets become swollen in the presence of water and over time platelets separate (exfoliation) to form a colloidal suspension of nanoclay in water. The effective volume of a single laponite disk has been shown to increase by a factor of 60 during swelling. [14] Gel forming grades of laponite may exhibit thixotropic behaviors and high shear thinning at concentrations exceeding 2% in water. It is this thixotropic behavior that enables liquid-borne protein deposition inside the gel matrix. As the gel is sheared, the locally deformed solid region flows freely and then self-assembles back into a solid after the excitation has been removed. The microstructure of the gel may be a face-face aligned structure of platelets or a face-edge structure (“house of cards” structure). The structure depends on the chemical composition of the clay, ionic strength, and concentration in water and pH. [15] Chemically, laponite platelets (mainly composed of MgO sheets sandwiched between SiO₂ sheets) are charge-neutral when dry. When hydrated, the alkali or alkali earth metal (lithium, sodium, magnesium) cations would be replaced by water (due to osmotic pressure) and spacing between platelets increases over time. The form factor of the platelets results in a viscous suspension that has a very large surface area of charged suspended solids of over 300 m²/g at times. [15]

Nanocomposites of silk fibroin and nanoclays have been explored in the past, mostly in thin film applications, where layer by layer assembly of silk/nanoparticles yielded robust nanocomposite films of various optical properties [16,17] or where silk fibroin provided a supporting material for clay particles to facilitate bone remodeling. [18] The interaction mechanisms between clay and silk fibroin were suggested to be governed by ion-dipole interactions above the isoelectric point of silk and by electrostatic interactions below the isoelectric point. [19] This work also showed that the latter would yield a microarchitecture with a high content of beta-sheets with high clay dispersion. However, the experimental system in question involved a combination of silk fibroin and nanoclay to force an intercalated structure, whereas in the present work the nanoclay dispersion served to support and crosslink the silk fibroin. Even so, the prior work [19] provides an in depth analysis into the mechanisms, and in our present approach we offer an adaptation where the intercalation may only occur at the surface of the silk fibroin/DI-H₂O extrudate. This surface effect induces the fibroin to re-conform to a more stable, beta-sheet, solid phase and expel excess water while intercalating with the clay platelets at the interface, [19] resulting in stability of the extruded material. While robust mechanical testing of bioprinted constructs *in vivo* was beyond the scope of the current study, in a separate study (unpublished data), this submerged printing technique was used to create silk cantilevers in tissue culture wells. The cantilevers

were modelled to support contraction forces up to 10mN, as an upper limit to what bioartificial muscle has been shown to produce, and cell viability and differentiation were demonstrated after three weeks in culture [20]. While the present study used pure silk constructs as a proof-of-concept, it is suggested that only minor adjustments to the printing process are needed to support the range of published silk bioinks, including silk-gelatin [8,21], silk-polyols [22], hydroxyapatite-silk and silk films [23].

2. Materials and Methods

2.1. Printing medium (Gel) Formulation

Laponite nanoclay suspension was prepared by dissolving Laponite XLG (BYK additives, Southern Clay Products) in solvents. For initial characterization, deionized water was used as the solvent. The suspension was stirred at room temperature and left stirring at 60 RPM for 8 hours. The suspension was then allowed to settle for an additional 8 hours. The rheology of these formulations was tested as detailed below to determine optimal concentration for printing. The final printing medium of PEG-laponite was prepared by adding laponite into polyethylene glycol solutions (PEG 400 Sigma Aldrich) ranging from 25 to 100%. The suspensions were prepared as described above. The rheological characterization of these formulations was compared with those of laponite-only formulations to determine optimal ratios for printing.

2.2. Bioink Formulations

Aqueous silk fibroin solution was prepared according to previously published methods.[3] Briefly, silkworm *Bombyx mori* silkworm cocoons, purchased from Tajima Shoji Co. (Yokohama, Japan), were extracted in 0.02 M sodium bicarbonate (boiling, 60 minutes, “degumming” process) to remove the sericin coating from the fibers. Fibers were then rinsed in distilled water, dissolved in 9.3 M lithium bromide, and dialyzed against distilled water to remove the salt. The resulting 6% w/v silk solution was concentrated from 20% w/v to 40% w/v and stored at 4°C until use.[4]

2.3. Secondary Structure Analysis

Silk fibroin secondary structures were determined through Fourier Transform Infrared Spectroscopy (FTIR, using a JASCO FTIR 6200 spectrometer (JASCO, Tokyo, Japan) combined with a MIRacle™ attenuated total reflection (ATR) germanium crystal. Background and spectral scans were measured from 4000-600 cm⁻¹ at a resolution of 2 cm⁻¹ for 32 scans per samples. The effect on the secondary structure of silk fibroin extruded into four different conditions was evaluated. As a control, untreated silk fibroin extruded and left to dry over a 12-hour period. The three test conditions consisted of the silk fibroin solution extruded into 100% w/v PEG 400, 2.5% w/v laponite solution, and a 50% w/v PEG +2.5% w/v laponite solution. An untreated silk cocoon and 60ME (minute-extracted) degummed fibers were also tested as additional controls. To preserve protein secondary structures, samples were air dried for 24 hours in a desiccator. All samples were run in triplicate. The secondary structures along the cross-section and the outer walls of printed silk fibers were determined by Bruker Lumos FTIR microscope (Billerica, MA, USA) with a liquid nitrogen cooled mercury–cadmium–telluride (MCT) detector at the Center for

Nanoscale Systems at Harvard University. The absorbance spectra were collected in ATR mode by averaging 16 scans with a resolution of 4.0 cm^{-1} . The background spectra taken in air were collected under the same conditions and subtracted from the sample scans. Fourier self-deconvolution (FSD) were performed over the amide I peaks on the IR spectra, using Opus 5.0 software with Lorentzian peak profile (half-bandwidth of 25 cm^{-1} and a noise reduction factor of 0.3). In this process, the broad and indistinct amide I band is transformed to a set of distinct self-deconvoluted bands. After FSD, the deconvoluted spectra were fitted with 11 Gaussian peaks to determine the secondary structure components. The details of this method were described in our previous works. [24–26]

2.4. Flow Behavior

The stress versus strain relationship under steady shear was measured using a Brookfield viscometer RVDV-II +P (TA Instruments, New castle, DE) with a 50-mm stainless steel conical plate (angle: 0.0402 rad). The samples were equilibrated at 25°C for 15 minutes prior to testing. A steady shear rate ramp was applied from $1 - 200\text{ s}^{-1}$, with 3 second equilibration at each point and the resulting shear stress was measured. Solutions with increasing concentration of laponite were tested ($n=3$) to establish the optimal concentration for a fluid-like behavior at high shear rates and a solid-like behavior at low shear rates indicative of the thixotropic behavior of laponite in its gel form. The same analysis was performed with the PEG –laponite gel medium to establish the right ratio that would give us the same thixotropic behavior. The thixotropic index was calculated as the difference between the area under the shear ramp up and the shear ramp down.

2.5. Sterile Printing Process

The 30–40% (w/v) of 60 ME silk fibroin solution was loaded into a 3mL Nordson syringe and connected to a CellInk Inkredible+ bioprinter via the pneumatic syringe holder. PEG-laponite solution (2.5% w/v Laponite XLG (BYK additives, Southern Clay Products) dissolved into 50% w/v PEG in dionized H_2O) was filled in the wells of the multiwell plates and the plates were loaded into bioprinter plate holders. Designed part files (STL) were converted to printing code (G-code) using open-source software (slic3r and Repetier-Host) which was further modified with custom Python scripts to allow for freeform printing. The constructs were printed with 20–28G syringe tips, depending on silk concentration used. Pressures were applied to syringes in the range of 50–90kPA, and printing speed was 2–200mm/s. After the printing process was complete, prints were left in nanoclay solution for 12 hours for silk crystallization. Depending on the silk concentration used, much shorter incubation times may be used. For example, with high concentration silk (>30%) gelling occurred in 30 to 60 minutes. Overnight (12 hour) incubations, at the concentrations of silk used in the present study, yielded consistently robust and removable prints and therefore, this incubation period was used for all printed structures. Prints were subsequently washed with phosphate buffer solution (PBS) three or more times and left on a shaker plate overnight for removal of nanoclay. Prior to cell seeding, the printed parts were incubated at room temperature with 0.1% Pluronic F127 for one hour to reduce gel adhesion to culture surface and then UV sterilized for 20 minutes. Primary Human skeletal muscle myoblasts (HSMs, Lonza, Portsmouth, NH, USA) were cultured in growth medium (GM) containing DMEM supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin $100\times$, and 1% (v/v)

penicillin/streptomycin (materials from Thermo Fisher, Waltham, MA). Human skeletal muscle myoblasts were then seeded at a concentration of 1×10^6 cells/mL and grown for up to two weeks. Cells were visualized for cytoplasmic growth and expansion with staining for actin through FITC- and rhodamine- conjugated phalloidin stains and visualized at three and seven days. Differentiation of myoblasts was demonstrated through fluorescence microscopy of anti-Desmin antibody (Alexa 555) and alpha bungarotoxin (Alexa 488 conjugate) with TO-PRO-3 nuclear stain. Further demonstration of myoblast differentiation was performed in separate work [20].

2.6. CT Scan of printed structure

Three dimensional images of printed constructs were taken using Micro-CT images (SkyScan 1176, Bruke, Allentown, PA). The scans were set to 9 μ m pixel sections with a 0.5 mm AL filter. Constructs were placed into 50 mL conical test tubes and loaded into the machine for imaging. The region of interest, size slice, and resolution of image were input via the operating software. The x-ray images were reconstructed into 3D models using SkyScan's reconstruction software, CTVol.

2.7. Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX)

Scanning Electron Microscopy was used to visualize the printed silk filaments. Sections were prepared by freeze-fracturing under liquid nitrogen. All samples for SEM were sputter-coated with 102–20 nm gold coating using a Polaron SC502 Sputter Coater (Fisons, VG Microtech, East Sussex, England) and imaged using a Zeiss EVO MA10 electron microscope (Carl Zeiss AG, Germany).

For EDX: Samples were coated with gold and then observed using a Carl Zeiss (Carl Zeiss SMT, Germany) Supra 55 field emission scanning electron microscope (FESEM) at an accelerated voltage of 20 kV. EDX was performed using Zeiss Supra 55 SEM coupled with an EDAX EDX system at the Center for Nanoscale Systems at Harvard University.

3. Results and Discussion

3.1. Characterization of printing medium

As previously mentioned, the thixotropic behavior of gel-forming grades of laponite is concentration-dependent. To be used as a gel medium, the colloidal suspension should be at an appropriate concentration at the dispensing needle tip, where the moving needle tip shears the medium. The suspension should rapidly transition from solid to fluid so that the bioink can be dispensed. After deposition of the ink, the colloidal suspension needs to quickly recover into a solid to provide structural support for the object being printed. A thixotropic material will lose structure during shear, and rebuild it on standing. To characterize this behavior, we analyzed the material with up and down shear ramps. The area between the shear ramp up and down curves is an indication of the extent of the thixotropy, referred to as the thixotropy index [27].

The linear velocities during printing are specific to each printing system. For pneumatic systems such as the one we used the printing speed is inversely proportional to the pressure

and for our purposes it ranged from 2–200 mm/s. To relate these velocities to the shear rates tested in the cone and plate viscometer we can relate the shear rate ($\dot{\gamma}$) to angular velocity (ω) by $\dot{\gamma} = (1/\rho) \times \omega$ where ρ is the cone angle in radians [28]. The linear velocities (v) expected at the edge of the cone viscometer would be $v = \omega \times r$, where r is the radius of cone. We tested the material at shear rates ranging from 0 to 200 s⁻¹ which corresponds to linear velocities of 0 to 400 mm/s. Low shear rates would be defined as those under 50 s⁻¹ corresponding to linear speeds of 100 mm/s and high shear rates as those above 100 s⁻¹ corresponding to linear speeds of 200 mm/s. The ideal material would exhibit higher viscosity at low shear rates (below 50 s⁻¹) and shear thinning behavior at higher shear rates (100–200 s⁻¹). The ideal material would also exhibit thixotropy with a balance between high enough viscosity to stabilize printed structures and low thixotropy index, indicating good recovery. We confirmed that at low concentrations 1% the colloidal suspension behaved like a Newtonian fluid with a steady viscosity around 1 cP (Figure 1A and B). At this low viscosity the material cannot stabilize the printed structures and does not exhibit thixotropic behavior. At concentrations between 1.5–3% there was a change to a gel-like behavior with an increase in viscosities of an order of magnitude (100–1000 cP) increasing linearly with concentration (Figure 1A, E). At these concentrations we saw thixotropy and good recovery indicated by thixotropic index (Figure 1F). Above these concentrations we saw poor recovery with a significant increase in thixotropic index, which indicated that at these concentrations the colloidal suspensions would not recover fast enough, and the resolution of the printed strands would be affected. We established that the optimal viscosity (at low shear rates) to stabilize the printed strands would be in the range of 500 cP with a thixotropic index of 900 corresponding to the 2.5% w/v laponite concentration.

Based on this characterization, we used a 2.5% laponite solution as the basis for further experiments for printing silk fibroin in the gel medium. We demonstrated that we could print complex structures inside this medium with filament widths of approximately 300 μ m and minimum reproducible space between filaments of 100 μ m (Figure 2A, S1A). This medium was ideal for printing due to its transparency and *in situ* visualization. However, we discovered that at these concentrations laponite was not able to physically crosslink and stabilize the aqueous silk solution within the time frame needed for printing (1–2 minutes) (Figure 3F(d)) and we looked for alternative crosslinking mechanisms.

3.2. Introduction of PEG to facilitate cross-linking

A large body of work already exists regarding phase transitions in silk solutions; the consensus is that the solidification of silk fibroin from solution involves the formation of crystalline beta sheets when no chemical crosslinking agents are intentionally involved. Mechanisms of gelation and structure-property relationships have been examined [29–32] and have concluded that beta sheet crystallization of silk fibroin's [GAGAGVGY]_n, [GAGAGS]_n and [GAGAGY]_n regions is the source of the emergence of a solid (or gel) material out of a silk solution, that the solution pH in relation to the protein isoelectric point plays a role in the process, and the existence of cation moieties in the solution has an effect on the solution concentration and saturation points [29–32].

PEG is a biocompatible inert synthetic polymer, both water-soluble and charge neutral in solution, as well as a known facilitator of fibroin crystallization. Based on these previous findings and our initial characterization we hypothesized that a low molecular weight PEG-laponite medium that exhibited a high recovery and thixotropic behavior would allow for silk to be printed as a fluid and be physically crosslinked, while suspended in the colloidal system. Work in our lab had previously demonstrated that low molecular weight polyethylene-glycols (PEG) can be used to induce silk gelation (while higher molecular weight PEG has been shown to induced the self-assembly of silk into fibers and particles). [14] We tested silk only controls (amorphous films and crystalline degummed fibers), and silk films treated with laponite only, PEG only, and PEG-laponite. The self-assembly process for the silk fibroin protein that produced an insoluble material was accompanied by an increase in β -sheet (silk II) crystalline structure and a reduction of random coil structure (silk I) [33]. We hypothesized that the PEG in the receiving matrix would induce the formation of the beta sheet secondary structure in the silk protein. FTIR analysis of the amide I region ($1600 - 1700 \text{ cm}^{-1}$) provides insight into protein secondary structure specific to intermolecular protein order and bonding in comparison to disordered amorphous structures. For silk fibroin, the β -sheet structure is typically observed with peaks between $1616-1637 \text{ cm}^{-1}$, while amorphous elements such as random coils and α -helices occur with peaks between $1638-1662 \text{ cm}^{-1}$ [24]. Figure 1C shows the fluid characterization of the PEG-laponite medium, with the 2.5 % laponite dissolved in 50% v/v PEG showing the same thixotropic behavior as the 2 to 3% laponite only colloidal suspensions. With this formulation we saw higher viscosities at low shear rates (2800 cP) which helped stabilize the printed structures further and the same shear thinning behavior at high shear rates (thixotropy index of 1000). We saw that at ratios above 50% v/v there was a phase separation of the colloidal suspension and the PEG leading to disruptions in the gel-like structure and loss of thixotropic behavior. At ratios lower than 50% v/v we saw poor recovery (reference to timescale) between ramp up and ramp down. These results along with analysis of the silk protein secondary structure (Figure 3F) helped to establish an optimal ratio of 50% v/v that would both crosslink the silk and be suitable for freeform fabrication. As controls, air-dried silk films which had not been physically crosslinked (showing random coil peak at 1630 cm^{-1}) and a solid degummed fiber (showing a beta sheet peak at 1620 cm^{-1}) were utilized. The introduction of PEG and the introduction of PEG-laponite caused the emergence of a peak at 1630 cm^{-1} which indicated an increase in beta sheet content (indicated by the dashed line in Figure 3F). This peak was absent in both the amorphous silk film and silk exposed to only laponite. This result indicated that the PEG was responsible for the physical crosslinking of the silk, thus stabilizing the structure as it is suspended in the laponite medium during printing. This two-component formulation allows for crosslinking of the liquid silk due to PEG, while retaining the thixotropic behavior of the laponite nanocolloidal suspension needed for freeform printing.

3.3. Suggested microstructure and mechanism

We hypothesized that a core-shell structure of silk fibroin/PEG emerged when silk fibroin was extruded into the PEG-laponite solution, where the outermost layers experienced significant beta sheet formation and osmotic pressure drove water away from the silk extrudate and into the laponite dispersion. Following the free volume collapse due to water

leaving the extrudate, silk fibroin chains migrated to intercalate with the laponite platelets to form a structure consisting of a crystallized outer shell of laponite/silk fibroin and an internal core of silk fibroin, possibly including an interpenetrating network of co-crystallized silk fibroin and PEG. The proposed mechanism is depicted in Figure 2B.

To confirm the proposed mechanism, FTIR was performed on cross-sections of printed filaments (Figure 3A–E). This analysis revealed that there was an increased beta-sheet content on the outer shell of the filament than in the inner core, confirming our initial assumptions. We also performed SEM-EDX on these filaments to determine the composition of the printed structures (Figure 4A). These images showed that silicon remains in the outer shell and does not penetrate inside the silk filament, further supporting the proposed mechanism.

Using these formulations, we demonstrated we could print complex structures into the PEG-laponite gel that were physically crosslinked within the medium. Macro-scale images of single filament structures designed as a continuous single layer (Figure 4C, left) showed the results of the printing process. During printing, the structure was suspended in laponite-PEG, after crosslinking the suspension was removed, and the resultant printed silk structure remained (Figure 4C right). Micro-CT images of printed structures and SEM images showed both the macro- and micro- level features that could be printed (Figure 2A, 4B, S1B and S2) with a resolution of fine structures limited to 100µm. Depending on printing system available, the resolution could theoretically be higher. In our experience, to increase resolution, efforts would need to focus on optimization and characterization of flow in high gauge dispensing tips (>26G), as well as finer motor controls of the dispensing nozzle.

3.4. Cytocompatibility

We aimed to demonstrate that the freeform printed silk fibroin constructs could be extruded into standard tissue culture well plates using sterile technique and subsequently combined with primary human cell culture. Silk, PEG, and Laponite have all been demonstrated to have a reasonable degree of biocompatibility [3,34–36] and recently 3T3 fibroblasts were shown to have greater than 90% cell viability after being printed into a 2.0 (w/v) Laponite EP bath [37]. Silk proteins have also been tested extensively *in vivo* and silk has been found to be controllably degraded when implanted, in a manner allowing for tissue ingrowth and remodeling with low immunogenicity [38]. Other work has demonstrated silk-PEG-based materials to have comparable tissue sealant and hemostatic performance to the commercially available CoSeal in both *in vivo* and *in vitro* studies [39]. Initial proof-of-concept experiments in our work used nanoclay solution autoclaved in liquid cycle and then pipetted into multiwell plates, from 6 to 24 wells per plate. Custom-designed silk constructs were then printed and allowed to crosslink in laponite/PEG solution for at least 12 hours at room temperature in biosafety cabinets. These structures were then washed with phosphate buffer saline (PBS) sterilized through UV irradiation in biosafety cabinets. If silk was printed suspended in baths on non-adherent plates, prints would be free floating in aqueous solution. These prints were then seeded with human skeletal muscle myoblasts and grown for two weeks, after which they could be visualized with fluorescence microscopy. Some cells could be visualized extending on linear areas on printed silk although further visualization and

quantification was difficult due to opacity and thickness of the print. Initial proof-of-concept of cytocompatibility was demonstrated as cells that grew on cell culture surfaces directly adjacent to printed constructs were viable and showed advanced myogenic differentiation through Desmin and alpha-bungarotoxin staining after two weeks in cell culture (Figure 4). Further characterization of human myoblasts on freeform silk printed constructs is included in separate work [20]. Future steps towards creating truly one-step printing with live cells and integrated PBS washing post printing are under development.

Conclusions

Various techniques in freeform printing at or near room temperature have been demonstrated since 2015, all using a variation of thixotropic gel media such as a granular gel media of Carbapol [1], FRESH-printing gelatin [9], and most recently Laponite [37]. In this work we described a novel approach whereby the natural biopolymer silk fibroin can be reconstituted from raw cocoons into an aqueous solution, and then printed into a Laponite/PEG bath with no additional crosslinkers. We have established an optimal formulation of 2.5% w/v laponite 50% v/v PEG granular gel medium that can crosslink the silk solution due to presence of PEG and support structures while printing due to the laponite. In further work we will explore time dependent crosslinking of the silk solution and optimize the time dependent thixotropic properties of this gel media by performing dynamic oscillatory and thixotropy studies. This freeform method needs no support besides the aforementioned bath and produces beta-sheeting and gelation of silk into arbitrary structures, difficult to create using molding, top-down, or bottom-up manufacturing methods. These gelled silk structures can be removed from the support bath with gentle manipulation and washed and sterilized using traditional methods.

In future work we would like to explore additional modifications to printed silk, including RGD functionalization. We would like to further explore the use of post-printing cell culture with prints in micro-scale features in attempts to align cells. Until individual cells interacting with micro-scale printed substrate can be visualized it will be difficult to fully detect differences in cell behavior as opposed to other microscale substrates. Live-cell printing is another goal, which will need carefully controlled temperature, printing environment, and osmotic balance of support bath. Live-cell printing may also benefit from cooled syringes for collagen encapsulation, as well as use of sterile culture hoods with downstream fluid handling robotics to preserve cultures and minimize risks of contamination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH (NIH/NIBIB P41 EB002520, R01AR068048) and the AFOSR. A portion of this research was conducted at the Harvard University Center for Nanoscale Systems. FGO would like to acknowledge support from the Office of Naval Research (N00014-13-1-0596)

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Statement of Significance

3D bioprinting has emerged as a technology that can produce biologically relevant structures in defined geometries with microscale resolution. Techniques for fabrication of free-standing structures by printing into granular gel media has been demonstrated previously, however, these methods require crosslinking agents and post-processing steps on printed structures. Our method utilizes one-step gelation of silk fibroin within a suspension of synthetic nanoclay (Laponite), with no need for additional crosslinking compounds or post processing of the material. This new method allows for *in situ* physical crosslinking of pure aqueous silk fibroin into defined geometries produced through freeform 3D printing.

Figure 1. Rheology of 3D freeform printing gel medium

The resulting viscosity (A) and shear stress (B) plotted against shear rate for suspensions with increasing concentration of laponite. Suspensions 1% w/v laponite exhibiting Newtonian fluid like behavior, 1.5 to 4.5% w/v laponite exhibiting shear thinning and different levels of thixotropic behavior. The flow behavior of the gel medium tested under steady shear rates from 1 – 200 s^{-1} . (C) Shear ramps (left) and summary table (right) for suspension blends with increasing PEG concentration (25 to 50% v/v) with a fixed concentration of laponite of 2.5% w/v. Range of shear rates highlighted by dotted box represent transition between low and high shear rates where gel medium most transition from fluid like as needle moves to solid like as filament is deposited as depicted in schematic (D). Viscosity (E) and thixotropic index (F) at a shear rate of 50 s^{-1} plotted against increasing laponite concentration (w/v%). The optimal concentration chosen (2.5 % w/v) for free form printing in terms of hysteresis and viscosity of laponite is highlighted.

Figure 2. Mechanism of 3D freeform printing

(A) Design of printed structures (left) and CT-scan images of printed structures (right) showing hollow structure (vase) tissue scaffold and free-standing object (helix). Scale bars represent 1 mm. (B) Suggested phase change mechanism for silk solution extruded into PEG-Laponite medium. (a) Steady state PEG-Laponite medium exhibits solid-like behavior. (b) Extrudate of Silk fibroin introduced into the medium. (c) Osmotic pressure drives water away from extrudate and locally increases the concentration of silk fibroin. (d) Silk fibroin chain segments migrate into PEG domains and co-crystallize with PEG and intercalate with clay particles to form a stable solid material.

Figure 3. Secondary structure analysis of printed dried silk filament by FTIR microscope

(A) Optical image of the cross-sectional area of the printed silk fiber, (B) Representative IR spectra for region a, b and c on the cross-sectional area, as marked in the optical image. (C) Fourier self-deconvolution and curve fitting of amide I spectra for the core phase (average of region a and b). (D) Fourier self-deconvolution and curve fitting of amide I spectra for the shell phase (average of region C and outer wall). (E) Secondary structure component for the core and shell phase of the printed silk fiber. (F) The effect of laponite and PEG on silk solution was also studied by FTIR. Amide I region of controls were tested a) degummed silk fiber which is crystalline (beta sheet structure) and (e) non-cross-linked silk film (amorphous structure). Aqueous silk solution (40% w/v) was treated with three conditions (b) 100% w/v PEG (c) 2.5% w/v Laponite 50% PEG (d) 2.5% w/v Laponite. The positive control degummed fiber (a) 100% PEG (b) and PEG-laponite blend (c) showed the characteristic beta sheet peak at 1630 cm^{-1} which was absent from the just laponite treated sample (d) and the non-crosslinked

Figure 4. Elemental analysis and biocompatibility of the printed silk fiber

(A) SEM image of fiber cross-sectional area (top left). Elemental maps are shown for carbon – C, oxygen – O and silicon - Si. (B) Solid printed filament viewed in high resolution scanning electron microscopy. Scale 20um. (bottom) Printed silk coil viewed in scanning electron microscopy (top). Scale bar 1mm. (C) (left) CAD design, visualized in G-code from Repeater Host V1.6.2, of a single layer lattice designed for continuous fiber printing in six-well plates. Scale bar 8mm. (right) Printed design of single silk filament incubated in phosphate-buffered saline in glass bottom six-well plate. Scale bar 1 cm. (D) Human skeletal muscle myoblasts (hSMMs) differentiated in dishes with printed constructs imaged with FITC-phalloidin and DAPI with inset of 10X (bottom left). Scale bar 50 µm. (E) Printed silk construct with seeded hSMMs imaged on glass slide with fluorescence microscope after 1 week, scale bar 1 mm (left). HSMMs stained with rhodamine phalloidin visualized on silk constructs after 3 days incubation in growth media, scale bar 200 µm. (F) HSMMs differentiated for two weeks demonstrating advanced differentiation with alpha bungarotoxin (green) and desmin (red) expression, with nuclear stain TO-PRO-3 (white). Scale 50 µm.