

Scalable Production of Functional Silk Fibroin with Tunable Mechanics by Fine Control of Secondary Structure

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Jeffery A. Roshko

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Advisor:

Dr. Fiorenzo Omenetto

Committee Members:

Dr. David L. Kaplan

Dr. Charles R. Mace

Dr. J. Devin MacKenzie

Abstract

Due to its tunable physical, chemical, and biological properties, silk fibroin has been studied extensively in the film format for applications ranging from drug delivery to wearable diagnostics. Silk fibroin derives its tunable nature from its ability to form secondary structure with both crystalline and amorphous domains, a phenomenon which can be modulated through heat, moisture, solvent, pH, and pressure. As silk fibroin's properties correspond to its secondary structure, managing its formation allows for fine control of silk constructs, but can introduce variation across samples if not managed. Prominent methods of spin coating or drop casting to create silk fibroin films and coatings do not readily translate to larger scales without a combination of long processing times, low yields, or variability in topology and crystallinity. This limits the viability of many silk-based innovations beyond the laboratory with a need for scalable, repeatable, and reliable manufacturing of silk films.

Roll-to-roll processing has emerged as an efficient, rapid, and consistent means of treating substrate as it is conveyed between a series of rollers. This technique is highly modular and enables manufacturing of a variety of precision materials, including optical coatings or multilayer electronic architectures. To fill the gap between the laboratory and commercialization of silk fibroin, I investigated roll-based processing to produce silk films at scale with controllable secondary structure and physical properties. I also present functionalization of these films through several unit operations compatible with roll-to-roll processing, including embossing, UV lithography, and inkjet printing. This scalable production technique for silk fibroin coatings can bridge the divide between discovery and industry, enabling widespread adoption of silk fibroin-based devices.

While silk fibroin films produced through roll-based processing offer a remarkable platform for the creation of silk-based devices, this technique produces films with a homogeneous secondary structure, and therefore homogeneous physical properties. The inability to control the spatial distribution of these properties constrains the versatility of silk fibroin-based devices. To fill this gap, I developed a method using direct inkjet printing of crystallinity-inducing solvents to control silk fibroin secondary structure, and by extension, its physical properties. Leveraging this technique, I produced

scalable monomaterial films with highly tunable physical properties which behave analogously to composite materials. I then demonstrated the functionality by creating film structures which mechanically actuate in response to humidity gradients. This approach has applications in tunable optics, biomedical devices, and environmental sensors, where fine control of material properties and shape are critical.

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Scalable Production of Functional Silk Fibroin
with Tunable Mechanics by Fine Control of Secondary Structure

Chapter 1: Introduction

1.1 Silk Fibroin as a Remarkable Biomaterial

Nature's ability to create hierarchically organized structures from the molecular level to macroscopic scales bestows many organisms with impressive structural and functional materials. This ranges in diversity from the hard, protective exoskeletons of arthropods to the iridescent flowers, leaves, and fruits of countless plants. Natural selection has provided millions of years of evolutionary pressure to create and refine functional materials at minimal energetic cost to their various hosts, whereas humans have had at best several millennia to develop metallurgy, and only centuries for modern chemistry and polymer science. Though progress in these fields has been impressive, scientists and engineers have started to take note of nature's prolific portfolio of materials, which has inspired a host of different biomimetic innovations. This can be seen through several examples of successful integration of nature's principles of enriching function through form, such as superhydrophobic stain resistant coatings modeled on the slippery lotus plant leaf, antimicrobial surfaces based on sharkskin, and adhesive surfaces inspired by gecko feet. Although emulating biology has enabled tremendous advancement in a diverse array of fields, the design of many useful devices is limited by currently available materials. Synthetic materials tend to be too stiff, too inert, too difficult to process safely, or some combination thereof, whereas few high-performing biologically derived materials have successfully been transferred from laboratory research to scales needed for widespread use.

Silk cultivated from *Bombyx mori* cocoons is a biomaterial that has been used by humans for thousands of years as a fiber and in textiles. As an agricultural product, silk is produced at a large scale – more than 150,000 metric tons of silk are processed a year by the sericulture industry¹. The main constituent of silk cocoons is silk fibroin, a biocompatible and biodegradable protein that has recently emerged with utility in tissue engineering, and biomedical, electronic, mechanical, and optical devices, amongst others^{2,3}. As a protein, many of the material's properties are derived from its amino acid sequence including alternating subunits of repeating GAGAGX motifs and nonrepetitive ~40 residue

linkers, which provides the ability for the protein to assemble into both amorphous and crystalline domains⁴⁻⁷ (Figure 1.1 a,b). When silk fibroin is produced by the *B. mori* silkworm, the protein is transformed in a complex process through shear, pH, and concentration gradients from an aqueous solution inside of the silk gland of the worm to a water insoluble, crystalline fiber⁸. These fibers are coated in a glue-like protein called sericin and are spun into the cocoon, which protects the insect during its pupation^{9,10}. If the insect is allowed to fully mature, it produces proteolytic enzymes to break down a portion of the fibers in the cocoon so it can exit and continue its lifecycle¹¹, but the textile industry desires continuous silk fibers, so the silk fiber is reeled from the cocoons before the insect reaches maturity, and the fiber is degummed to remove the sericin, leaving behind nearly pure silk fibroin fibers.

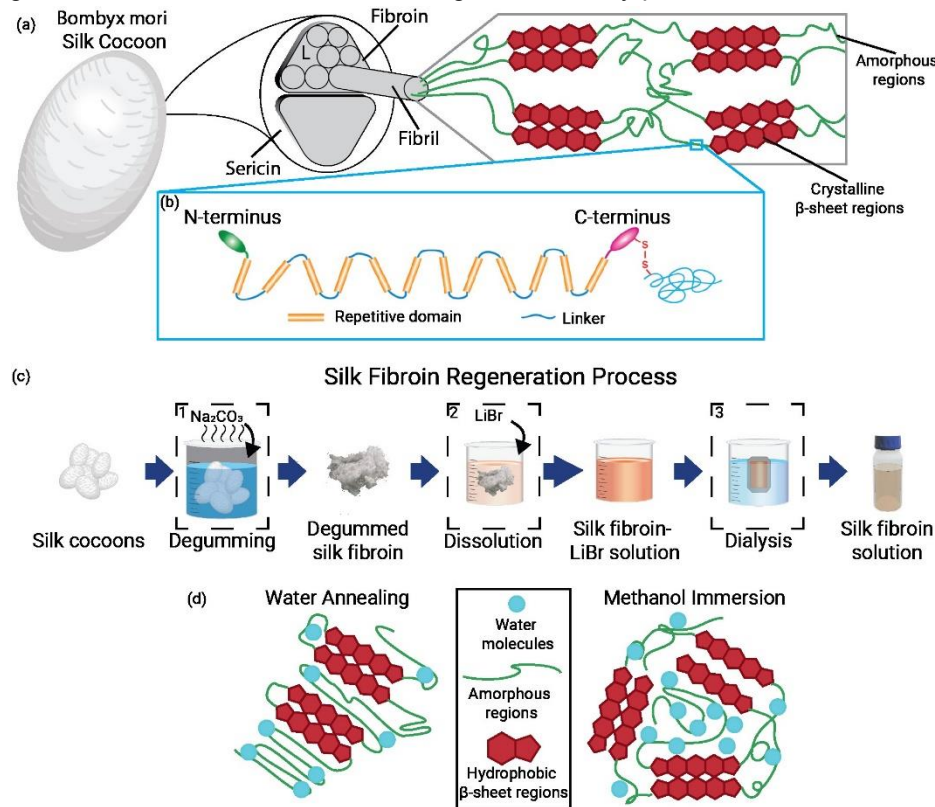


Figure 1.1 Native and regenerated *Bombyx mori* silk fibroin. (a) Overview of *Bombyx mori* silk structure. (b) Silk fibroin primary structure. (c) Silk fibroin regeneration process from cocoon to solution. (d) Comparison of water and methanol treatments

It was discovered that these silk fibroin fibers can be dissolved in a chaotropic solution that disrupts the hydrogen bonds of the water-insoluble fibers, allowing processing into an aqueous solution called regenerated silk fibroin (RSF) (Figure 1.1 c)^{12,13}. This dissolution process makes it possible for scientists and engineers to further transform RSF solution into useful forms including solid blocks,

hydrogels, scaffolds, fibers, and films³. During the process of transforming RSF solution into usable forms, the secondary structure of the protein can be modulated through means such as temperature, solvents, or even water vapor (Figure 1.1 d). The ability to form different crystalline domains at the microscopic scale ultimately dictates silk fibroin's macroscopic properties, and directing this assembly process is often utilized RSF device fabrication^{14–16}. Furthermore, it is possible to incorporate cells and other sensitive molecules into silk fibroin devices, and the material can even be an effective stabilizer of biological moieties^{17–20}. Silk fibroin's optical, electrical, biocompatible properties, along with its tunable nature, allow this incredible material to be readily transformed to meet the demands of many different applications.

1.2 Silk Fibroin in Film Format

Many silk fibroin devices developed at the lab scale utilize silk fibroin in the film format as it allows for utility as a substrate in multilayer architecture, conformability in planar electronics, and optical clarity in diagnostic devices. RSF films can take on fine resolution patterns via photo- and electron-beam lithography, where the material has been utilized as both a positive and negative resist^{21–24}. Silk films can also take on various shapes and forms through thermal nanoimprinting and casting, allowing the quick transformation of silk's optical performance or surface properties^{25,26,23}. Some examples of promising RSF film-based devices include biodegradable tissue scaffolds, controlled-release drug delivery vehicles, enzyme-based biosensors, and optoelectronics^{27–31,25}, a small selection which can be seen in Figure 1.2.

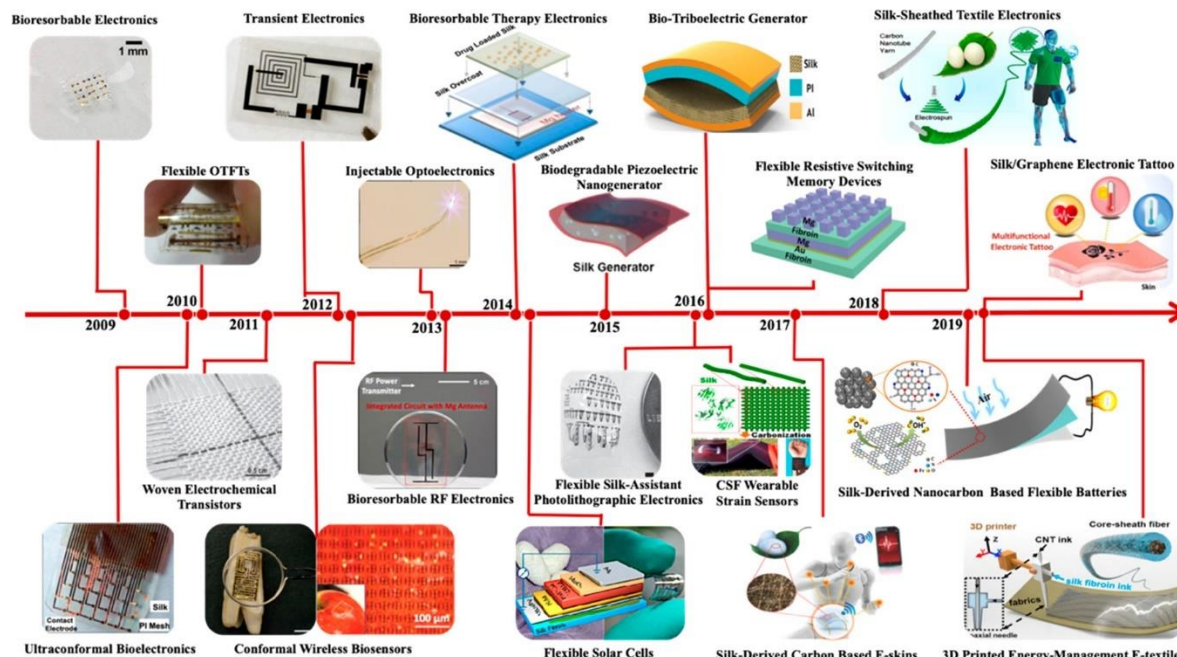


Figure 1.2 Timeline of development of silk-based advanced materials for soft electronics. Reproduced with permission ³²

1.3 Motivation

The silk fibroin layers in the devices illustrated in the previous section are routinely produced via methods such as drop casting and spin coating, which are well-suited for experimentation in the laboratory³³. However, due to presence of edge effects, material waste, long processing times, or inconsistencies illustrated in Figure 1.3, these techniques do not lend themselves to the scalability necessary to convert lab-born innovations into readily usable technology^{17,34,33,35,36}. Additionally, many of the formats produced in prior publications rely on long drying/processing times at precisely controlled environmental conditions, making these techniques difficult to translate beyond the lab bench. Given that the lab-centric techniques currently used to convert silk fibroin solution into functional film-based devices exhibit such drawbacks, and that silk is readily available in enormous quantities as a raw material, there is a gap in validated methods to viably translate silk devices from discovery to industry.

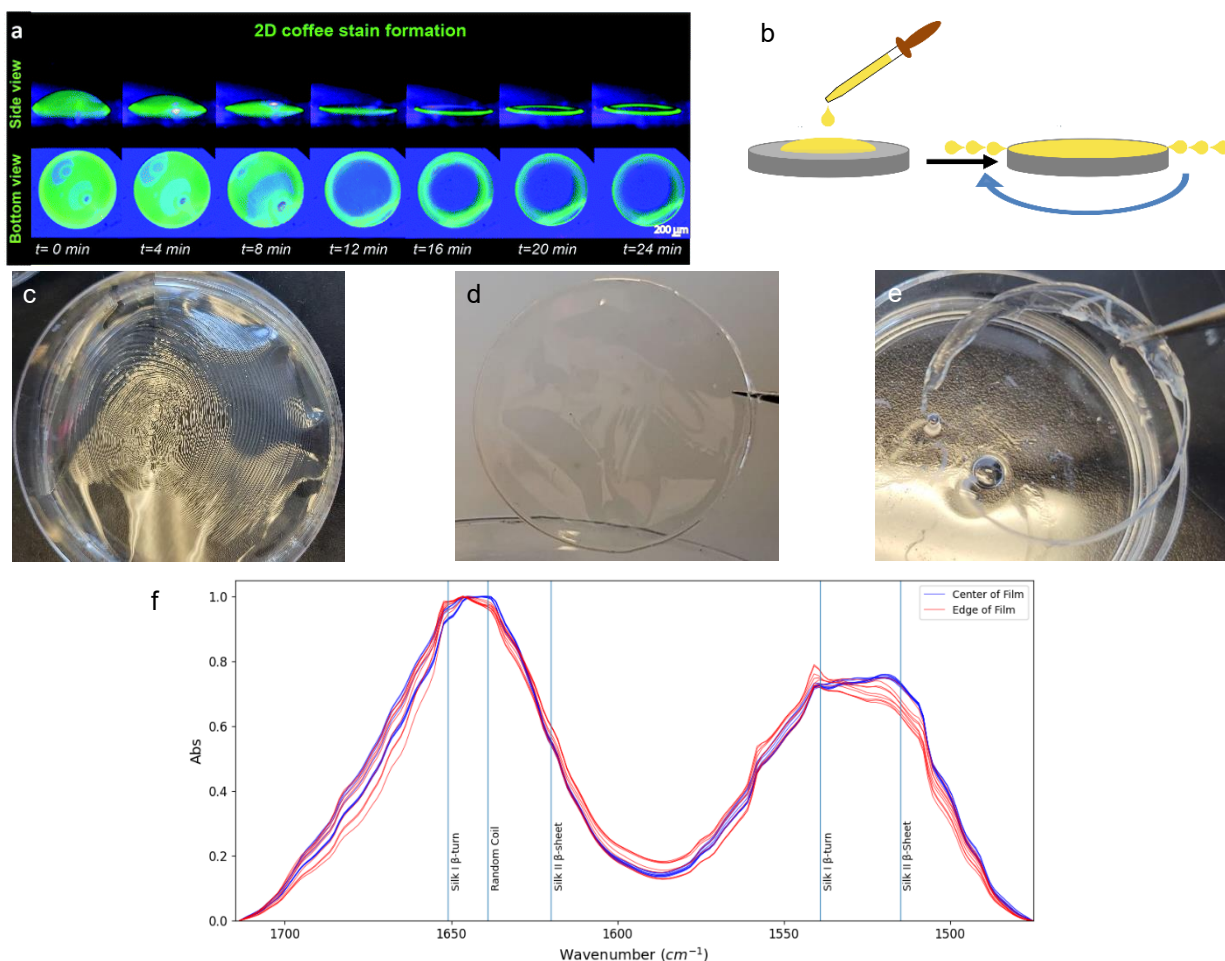


Figure 1.3 Examples of film forming techniques. (a) Formation of coffee ring of drop-casted silk films. Adapted with permission ³⁷. (b) Schematic of spin-coating process ejecting majority of material applied. (c) Heterogeneous silk film formed during the drying of drop-cast silk. (d) Drop cast silk fibroin film as dried and (e) after immersing in water (right). (f) FT-IR spectra of edge and center of film in d/e demonstrating measurable differences in secondary structure between locations of a drop-cast film

Meanwhile, many industries utilize roll-to-roll (R2R) manufacturing, a technique through which substrates are conveyed between a series of processes by spinning rolls. This approach enables both the low-cost production of simple printed products as well as the rapid conversion of dozens of raw materials to complex multi-layered devices with minimal loss and high precision. The approach is also readily reconfigurable as equipment can be added and subtracted from the path of the substrate's travel as needed. In Chapter 2, we explore the feasibility of producing silk fibroin films via R2R coating and demonstrate the utility of the technique via several unit operations compatible with R2R manufacturing to create functional devices.

While the R2R coating of silk at large scales would allow for control of secondary structure homogeneously across the bulk of the films, a method to spatially tune secondary structure and thereby physical properties of silk fibroin films could further enable innovative use of this material. In recent decades, inkjet printing has emerged as a technique to precisely control the deposition of inks and solvents. It is also readily compatible with roll-to-roll processing and is used routinely for the printing of a variety of materials from visible ink on newspaper to active photovoltaic layers in flexible solar cells. Numerous studies have demonstrated that various solvents can be used to homogeneously control crystallization within silk fibroin constructs, yet few methods exist that allow the spatial control of this crystallization. In Chapter 3, we will investigate the feasibility of using inkjet printing to control the deposition of inks to selectively modify the secondary structure of RSF films spatially. We then demonstrate the utility of this technique by tuning the mechanical characteristics of printed films.

Chapter 2: Scalable Roll-to-Roll Production of Silk Fibroin Film

2.1 Introduction to Roll-to-Roll Processing

Roll-to-roll processing broadly involves the large-scale, high speed, and efficient production of multi-layered film materials by modifying an extended length of substrate as it travels between multiple rollers (Figure 2.1 a). This technique allows otherwise economically or technologically unfeasible devices to be produced at greater scales compared with traditional batch/sequential processing. R2R processing has been applied in the polymer, paper, metal, and textile industries for several decades. More recently, this technique has supported many recent technological developments in the field of materials and electronics, notably flexible LED arrays, low-cost solar cells, and wearable devices^{37–41}.

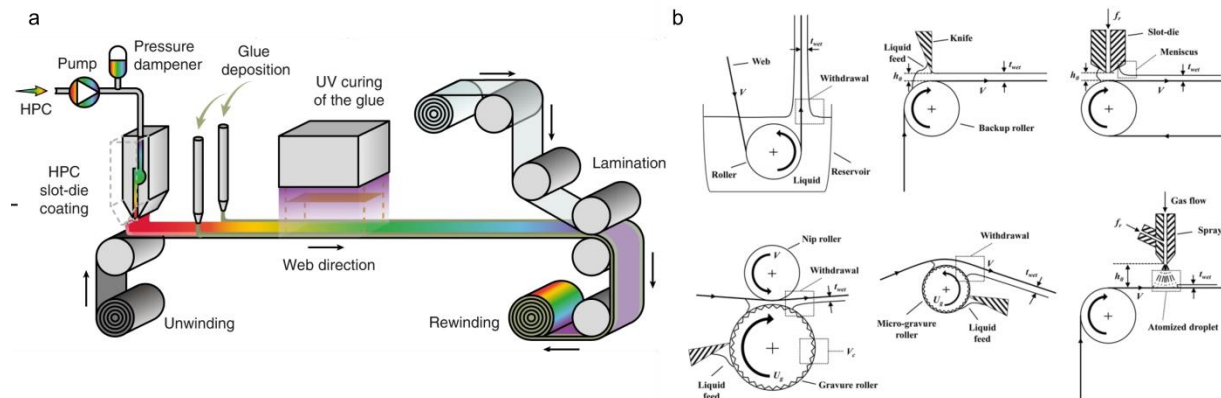


Figure 2.1 Overview of R2R Processing. (a) Example process coating pressure sensing cellulose composites. Adapted with permission³⁸ (b) Several coating technologies utilized in R2R. Adapted with permission⁴¹

There are numerous types of R2R techniques for applying materials on substrate rolls (Figure 2.1 b). These include slot-die coating, spray coating, gravure, inkjet printing, screen-printing, ribbon transfer printing, and knife-over coating⁴². Furthermore, it is also possible to produce layers with precise layers of molecules using techniques such as physical vapor deposition, chemical vapor deposition, plasma coating, sputtering, and Langmuir-Blodgett coatings^{43–45}. Various substrates can be utilized in R2R processing, ranging from paper, metal, and polymer-based webs. Combining these methods, materials,

and configurable techniques allows great flexibility in the types of materials and devices that can be produced with R2R.

Despite R2R's increasing importance in technology and manufacturing, few examples exist where functional biodegradable biomaterials such as RSF have been produced reproducibly at large scales using these techniques. The handling of silk fibroin does provide some unique challenges due to the sensitive nature of the self-assembly process as RSF transitions from a solution to a solid, and the relationship between the structure of silk fibroin and its function. Moreover, scalable substrate coating relies on a delicate balance between ink rheology, substrate wetting, and drying dynamics⁴⁶. However, R2R equipment has been designed to transform liquid inks into films quickly and to maintain precise control of each processing step for repeatable output. And whereas some roll-based techniques are complex to setup and run, others such as knife-over coating are quite simple, with laboratory equivalent methods of testing and validation that are directly transferable to full-scale coating techniques. Here we attempt to bridge the gap between laboratory experiments and scalable devices by investigating the processing space of silk solutions and substrates compatible with roll-based coating, producing silk fibroin films with this technique, and characterizing these films. We then demonstrate utility of this approach with functionalization via several unit operations and devices compatible with R2R manufacturing.

2.2 Laboratory-Scale Optimization of RSF Inks and Coating Parameters

2.2.1 Processing of Ink from Dried Silk Fibroin Powder

Extensive work has been done to characterize the process for transforming degummed silk fibroin fibers to RSF solution, and the transformation of this material into final formats³. However, experimentation with roll-to-roll processing requires large volumes of solution due to the large coating areas, losses in pumps and metering equipment, and sufficient material to try variations in coating parameters. Given that our lab capacity to produce RSF solution is currently limited to degumming 50 grams of untreated cocoons, which yields roughly 250ml of 8wt% concentration solution from a single degumming process and does not include additional time to concentrate solution and the associated

volume loss, another approach was needed to generate consistent solutions for coating. A silk fibroin powder produced via a similar process as lab-treated RSF solution was considered for this application to reduce the burden and increase consistency between experiments. For this material, the silk fibroin is degummed, dissolved, and then dried into pure silk fibroin powder through a spray drying process. As such a material has not been used extensively in prior silk fibroin research, understanding how to efficiently process this material, and determining whether there were any differences in materials produced with the raw material was necessary.

To produce RSF solution with this silk fibroin powder, it can be incorporated into deionized water with sufficient shear to disperse the particles (Figure 2.2), and after dissolution, it can be treated much like RSF solution prepared directly from degummed fiber. While mixing low concentration solution from this starting material was straightforward, requiring a shake plate or stir bar to dissolve the powdered silk fibroin, mixing concentrations above 10wt% proved challenging given silk's propensity to stabilize air in the form of bubbles and the low density of the powder. For low concentrations, silk fibroin was added to a given volume of deionized water (Millipore MilliQ) and allowed to shake or stir until the solution appears homogeneous and free of undissolved particles. For higher concentrations of solution, the powder was mixed with an overhead mixer and 45-degree pitched-blade impeller. and required slow powder addition rate and longer mixing times to hydrate and dissolve the powder. Once all the powder for a solution was added in the liquid and fully wetted, the mixing rate was reduced to prevent further air incorporation while allowing the silk powder to continue to dissolve. Concentrations higher than 20wt% became highly aerated with stiff foam during powder dissolution, preventing additional solids from being readily incorporated.

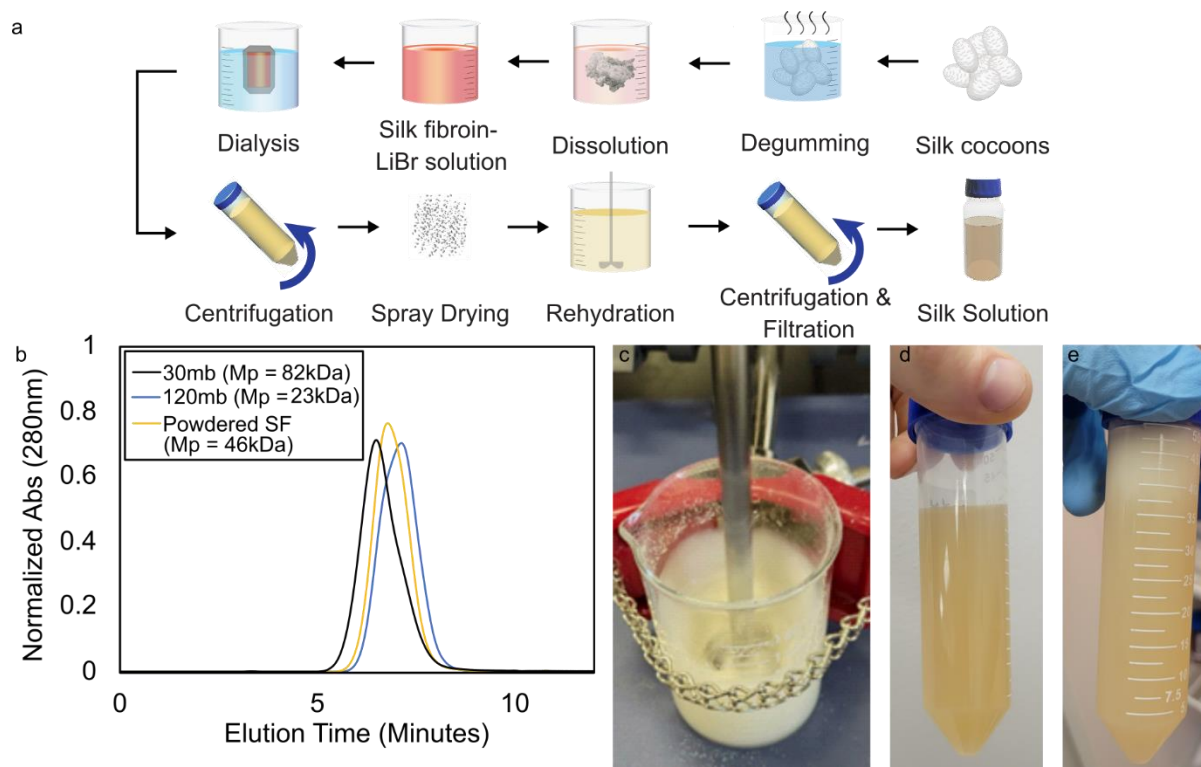


Figure 2.2 Processing of solution from dried RSF powder. (a) Overview of process for transforming silk cocoons to dried powder and redissolving to regenerated solution. (b) Gel-permeation chromatography for determining molecular weight distribution of spray dried silk fibroin. (c) Re-dissolution of spray dried powder. (d) Final RSF solution as prepared from this process. (e) Gelled silk after several weeks storage at 4°C

After mixing until no undissolved solids were visible (~30 minutes after last portion of powder was added, depending on volume and concentration), the solution was then centrifuged twice at 11200 RCF in a fixed angle rotor (Beckman Coulter FX6100, Allegra X-14R) in 100ml round bottom tubes (Beckman Coulter) for 2x20 minute cycles, and the supernatant was then filtered through Whatman 4 (20-25 μ m) filter paper (a kimwipe was used above these filters for solutions above 10wt% to avoid filter clogging) to remove remaining undissolved non-fibroin material that remained in the raw material. Due to losses in mixing equipment and inability to redissolve all silk fibroin powder, final concentration of solutions tended to be ~90-95% of starting concentration, and the total loss of solution during centrifugation and filtration was 10% due to multiple changes in centrifuge tubes and filter paper needed. Final solution concentration was measured via gravimetric analysis. While concentrations below 10wt% were stable for several weeks when stored at 4°C, higher concentrations solutions suffered from reduced storage stability, where sol-to-gel transition can occur quickly, on the order of days instead of weeks or months.

2.2.2 Ink Characterization

It has been suggested that the knife coating process benefits from low shear viscosities greater than 100cP⁴⁶. However, the high concentrations of RSF needed to produce viscosities in this range caused difficulty in processing from powder to solution. Several studies have suggested that while shear forces have distinct impact on native silk fibroin properties and secondary structure, the self-assembly process of regenerated silk fibroin is not shear sensitive^{47–49}. The shear forces experienced by fluids in knife-based coating are geometry and substrate speed dependent, and in the case of slot-die, also pump rate and geometry dependent. For knife-based coating, maximum shear rate occurs at the knife blade, and can be approximated with a simple planar Couette flow using Equation 2.1:

$$\gamma = \frac{v}{h} \quad \text{Equation 2.1}$$

where γ is shear stress, v is substrate speed, and h is knife-substrate gap. For gaps and coating rates applicable to this work, we would expect shear rates from 500 to 2000 sec⁻¹. For slot-die coating, the shear rates experienced in the slot-die head depend on geometry, but typically can be modeled as Hagen-Poiseuille flow in a rectangular channel with a length L , Width W , and flowrate Q ⁵⁰:

$$\gamma = \frac{6Q}{(WL^2)} \quad \text{Equation 2.2}$$

To determine the behavior of the silk solutions under shear conditions experienced while coating and during flow, viscosity vs shear rate was measured. Solutions of several concentrations of RSF were prepared to examine the impact of concentration on rheology. Silk solution rheology was characterized on a rheometer (TA HR-20) with a rotating cone (40mm, 2 degree)-flat plate geometry by performing a viscosity-shear rate sweep from 1 to 1000 s⁻¹, with 5 points at each shear rate and 30s initial temperature equilibration time at 25°C⁵¹ (Figure 2.3 a). All solutions of RSF prepared here exhibited a shear thinning rheology, with generally higher viscosities across shear rates with increasing concentration, as has been observed for RSF prepared from degummed fibers⁵². This behavior is ideal for substrate coating as it prevents undesirable flow, such as through the feed hopper in knife-blade coating and after a wet film has been spread, yet reduces the energy needed to spread the ink into film.

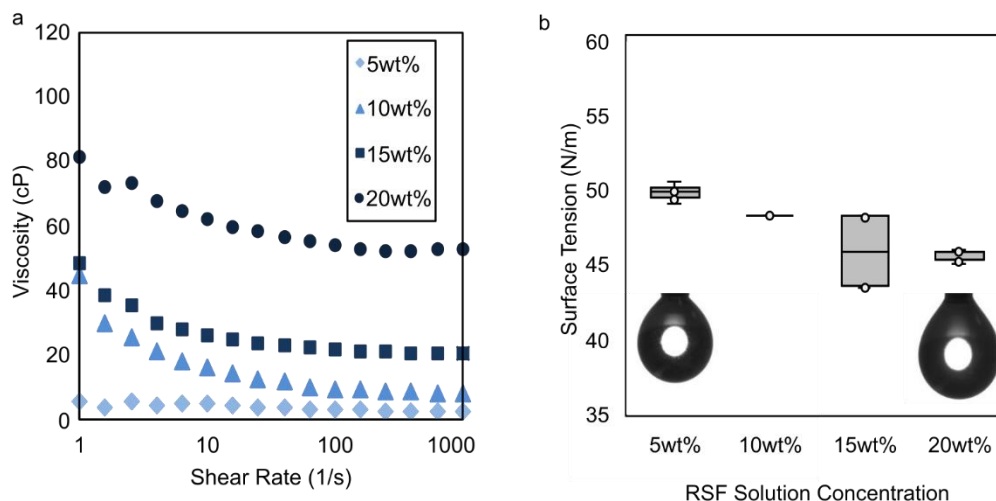


Figure 2.3 Characterizing potential silk solutions for coating. (a) Rheology shear sweep (representative). (b) Surface tension of RSF solutions with varying concentrations measured via the pendant drop method.

The impact of concentration on surface tension of the powdered RSF solutions was also investigated, as surface tension plays a significant role in determining how inks behave as they are deposited onto a substrate. Generally, a surface tension of an ink equal or less than the surface energy is desirable between substrate and ink in order to lower the contact angle of the ink and form films instead of separate droplets^{53,54}. The surface energy of the candidate silk solutions was characterized via pendant droplet measurement measurements via the sessile drop technique (Rame-Hart Model 250-F1, DROPImage Pro)⁵⁵ (Figure 2.3 b). Here, increasing RSF concentration also resulted in a reduction in surface tension due to silk fibroin's ability to act as surfactant (Figure 2.3 c), which matches with what has been observed in other work⁵⁶.

2.2.3 Ink-Substrate Interaction

As mentioned in 2.2.2 , a surface tension of an ink should be equal or less than the surface energy is desirable between substrate and ink. For a specific ink, the interaction with a substrate can also be changed by changing the substrate material, adding a surface coating, plasma treatment, and surface patterning. For this work, the contact angle of ink solution on several commercially available substrates (FLEXcon) was measured by carefully evacuating droplets of the same volume from a syringe onto a sample of a substrate and assessed via contact angle goniometry⁵⁵. As can be seen in Figure 2.4, RSF solution exhibited lower contact angles with substrates of higher surface energy, which is preferable to

improve spreading during the coating process. The lowest contact angle was observed with untreated cellulose-based substrate, however, this substrate absorbed moisture from the wet RSF coating and caused the substrate to deform while drying. The contact angle with the most promising substrates could be reduced further through corona treatment, but produces a transient change in surface energy, and increases adhesion between silk and plasma-treated substrates⁵⁷. PET-based substrate was chosen as the primary material for coating experiments given the balance of attributes.

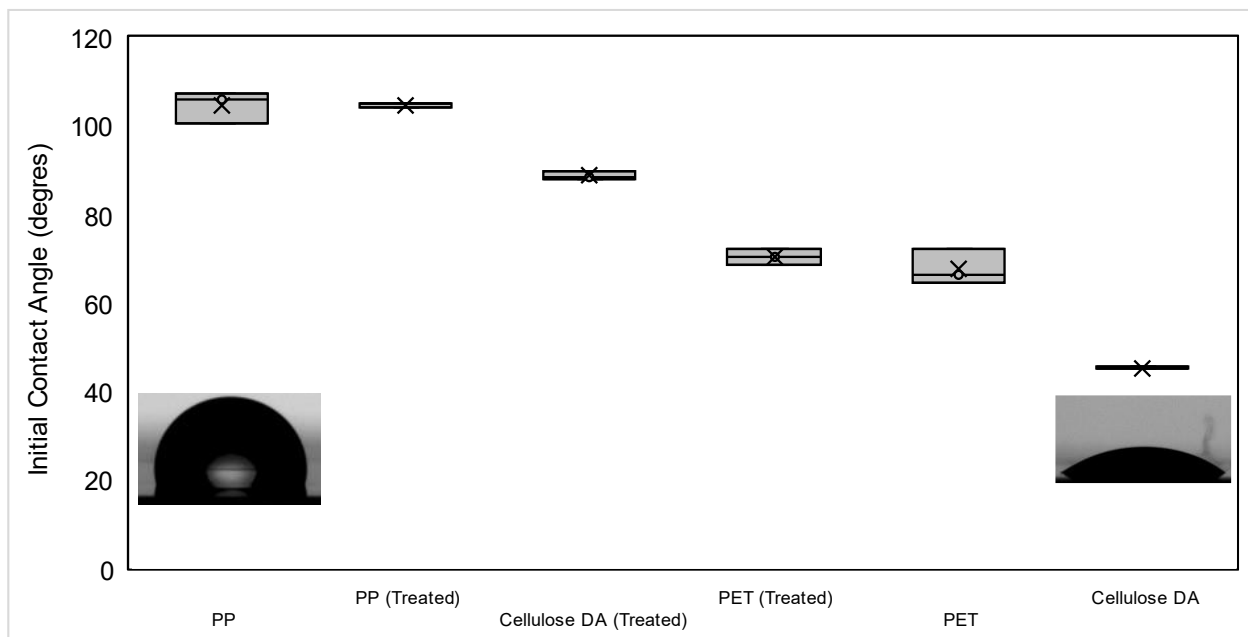


Figure 2.4 Initial contact angle of silk fibroin (20wt%) on selected available substrates. Inset: photos of droplets on substrate listed below taken for contact angle analysis in DROPImage Pro.

2.2.4 Drying Dynamics

Films were applied to a substrate via an adjustable height knife-over film coater (Gardco) for manual lab coating, and fixed height blades for the automated lab coating (BYK byko-drive XL), with substrate clamped flat over 305mm x 203mm x 4.5mm borosilicate glass sheets (McMaster-Carr) on a granite surface plate for manual coating, or vacuum plate for the automated coating (Figure 2.5). Ink solution of sufficient volume (calculated from blade gap x coating width x length, with overhead to prevent incorporating air; generally 3-10ml) was pipetted on the top of the substrate, and the knife-over coater was then drawn across the substrate by hand or via electronically controlled speed with the automated coater to create the wet film. Films were dried in a convection oven (Thermofisher Precision) with each

ink-coated substrate was suspended in a PMMA frame clamped to the borosilicate glass sheet to keep the substrate flat and placed onto a horizontal drying rack unless otherwise noted. These films were dried at varying temperatures of 25°C-140°C for investigating drying temperature, or 60°C for investigating coating thickness and concentration. After drying, films were allowed to equilibrate to ambient conditions.

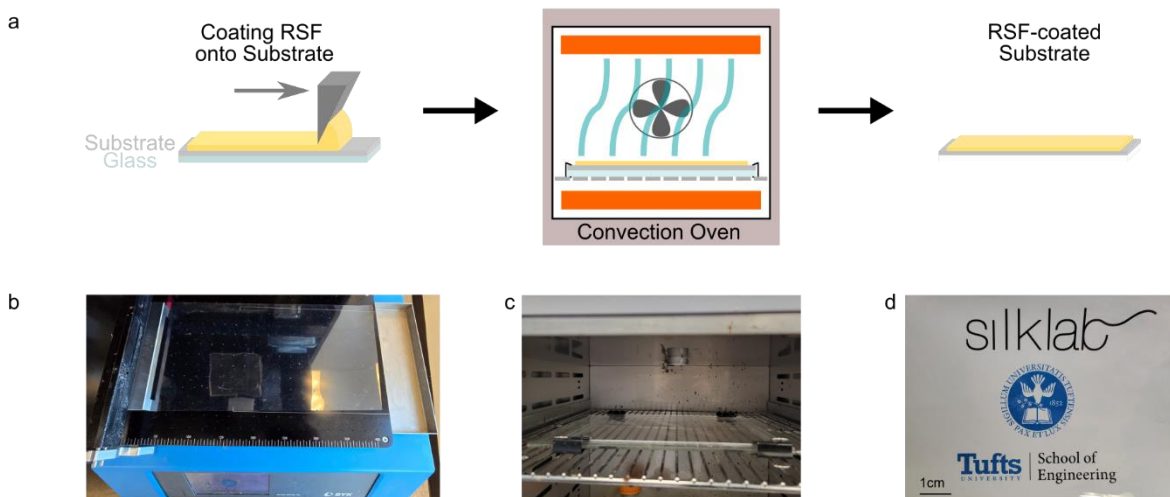


Figure 2.5 Overview of lab-based knife coating. (a) Illustration of process of coating, drying and final film. (b) Automatic lab-coater with substrate and fixed height blade. (c) Suspended RSF film on substrate while drying in convection oven on plate glass held in place by PMMA frame. (d) Example of highly transparent dried film held over letter-sized paper with printed logos.

As expected, lower concentrations formed thinner final dried films for a given coating knife gap³⁹ (Figure 2.5). Films prepared from lower concentration solutions also required longer drying times due to higher water content. The blade gap was found to have a strong influence on final film thickness as has been described previously³⁹. The relationship between final dry-film thickness and coating thickness has previously been described by Equation 2.3, where the thickness, d , should be determined by the density of the final film (ρ), the concentration of the solids in the ink, (c , in mass/volume), and the gap height (g).

$$d = \frac{1}{2} \left(g \frac{c}{\rho} \right) \quad \text{Equation 2.3}$$

Sheet coating followed a linear relationship (dry thickness = .1017*applied thickness with an $R^2 = 0.9197$, compared to an expected slope of 0.96 assuming $c = 250\text{g/cm}^3$ and $\rho = 1300\text{g/cm}^3$). The maximum thickness achieved with this technique and ink combination was $45\mu\text{m}$ (SD $11\mu\text{m}$) for a $400\mu\text{m}$ blade gap. Generally, as the wet coating thickness increased, standard error increased, potentially due to the low tension in the substrate and the movement of the ink after coating both before and during drying

(b). It should be noted that in preliminary trials, substrates were coated while flat on a lab bench but transferred to a convection oven where they were able to hang vertically while drying with a suspended mass. While this method can approximate the tension that might be experienced in a continuous drying process, lower viscosity or high thickness coatings were quite prone to flowing while drying, creating visible defects (Figure A.1).

To investigate the impact of drying temperature, we increased the temperature from 40°C, which made drying times quite long (upwards of 30 minutes for coating at 200µm) up to 140°C, which is above the T_g of the PET substrate but below that of non-plasticized Silk Fibroin⁵⁸. No difference was noted in the secondary structure of dried RSF films using the approach described in 3.1.4 (Figure 2.6 a). However, increased temperatures caused a mismatch between thermal expansion of RSF layer and the PET substrate, such that at low temperatures dried film curls toward the RSF coated surface, and at high drying temperatures, the trend was reversed (Figure 2.6 b,c). This is likely due to the contraction of the silk fibroin as it transforms from a liquid layer to a solid film⁵⁹. Substrate tension was difficult to control during batch experimentation to determine if this could be modulated to alleviate this issue. Other factors that might be relevant to control this phenomenon are the rates of heating and cooling during this process, and the glass transition temperature of the substrate⁵⁹.

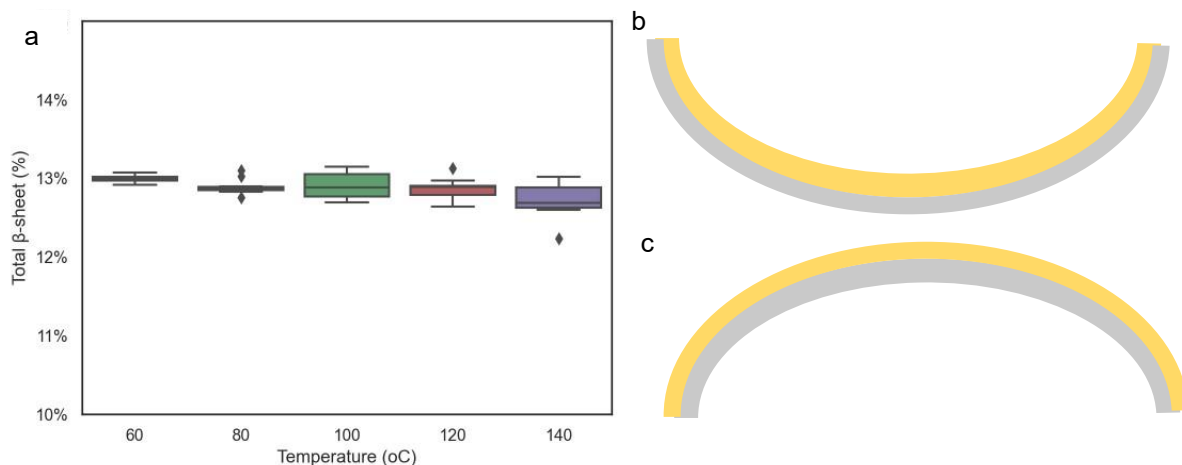


Figure 2.6 Influence of drying temperature. (a) secondary structure of films dried at different temperatures in batch convection oven. (b) Film dried at 60°C. (c) Film dried at 140°C.

2.3 Roll-to-Roll Processing

2.3.1 R2R Slot-Die Coating

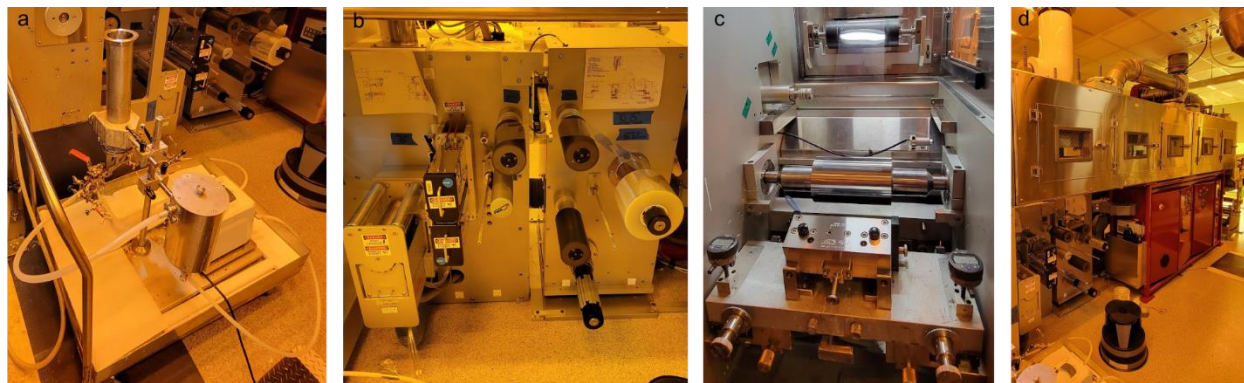


Figure 2.7 Overview of slot-die coating process equipment. (a) Feed tank and pump which feeds the slot-die head. (b) Substrate unwinding and optional corona pretreatment. (c) Slot-die coating of substrate being fed directly into the oven after slight incline. (d) Continuous three-zone drying oven with optional infrared lamps with rewinding on far right.

Though several of the variables for slot-die coating differ from knife-based coating, a pilot scale R2R slot-die coating system was used to examine several parameters for translating batch coating conditions to roll-based coating due to the relatively low volumes required per experiment compared to industrial scale equipment. A range of conditions were tested while determining useful operating parameters of this coating technique (Table A-1). Slot-Die coating was performed on pilot sized roll-coating equipment (Frontier), with a 15.25cm web width, optional in-line corona treatment, and a

convection drying oven with 3 zones, with the first also having IR capability. A 125 μ m slot shim was employed in a 14.5cm wide slot-die head, which was fed by a metering pump. A solution of 20wt% RSF was used first as a coating solution, and it was initially coated on 125 μ m thick polyethylene terephthalate (PET) substrate. During startup, a delicate balance between pumping rate, coating gap, oven temperature, and substrate speed is required to avoid flooding the slot-die head as it applies ink to the substrate. An initial substrate speed of 1 meter/minute was utilized, with a flowrate of 50ml/min, which caused flooding, so the feed pump speed was reduced to 30ml/min and below for the remainder of the trial. Figure 2.8 captures the solution being coated onto the substrate when the ink is aerated as its being poured into the feed tank to the pump, as well as when coating occurs with minimal defects.

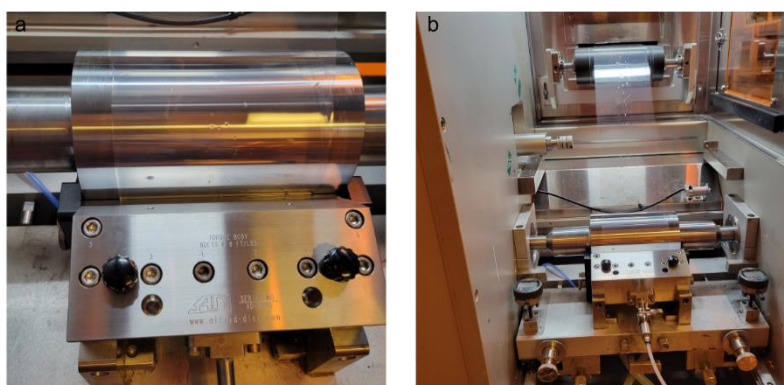


Figure 2.8 Slot-die coating issues. (a) Closeup of slot-die head while coating. (b) Slot-die coating silk solution with air entrapped.

Once steady state conditions were achieved for coating and drying, it was observed that the silk film was delaminating from the substrate before reaching the rewinder. To counter this effect, a corona treatment of 60 watts intensity (exposed area of 15cm X 5cm = 75cm², intensity of 0.8w/cm²) was applied to the substrate which increased adhesion between the silk film and the PET substrate, and delamination stopped, but removal from the substrate was no longer possible without dissolving the interfacial layer. A thinner substrate was used to determine if the mismatch in thermal expansion during drying would be reduced, but this thinner substrate curled excessively towards the coated silk surface during drying, a phenomenon that increased with the use of infrared lamps in the oven (Figure 2.9 c). The 125 μ m PET substrate was tested again, but with a higher oven temperature (80°C vs 60°C initial temperature) (Figure 2.9 d). This reduced silk film delamination, allowing coated substrate to be rewound, yet for the film to be removed from the substrate as needed. RSF plasticized with glycerol (25wt% glycerol:75wt% RSF solids,

25wt% total in *aqueous* solution) also reduced the delamination and bending issues during the drying and rewinding processes, but the high loading of glycerol caused the humectant to saturate the film and emerge on the surface of the RSF film after drying.

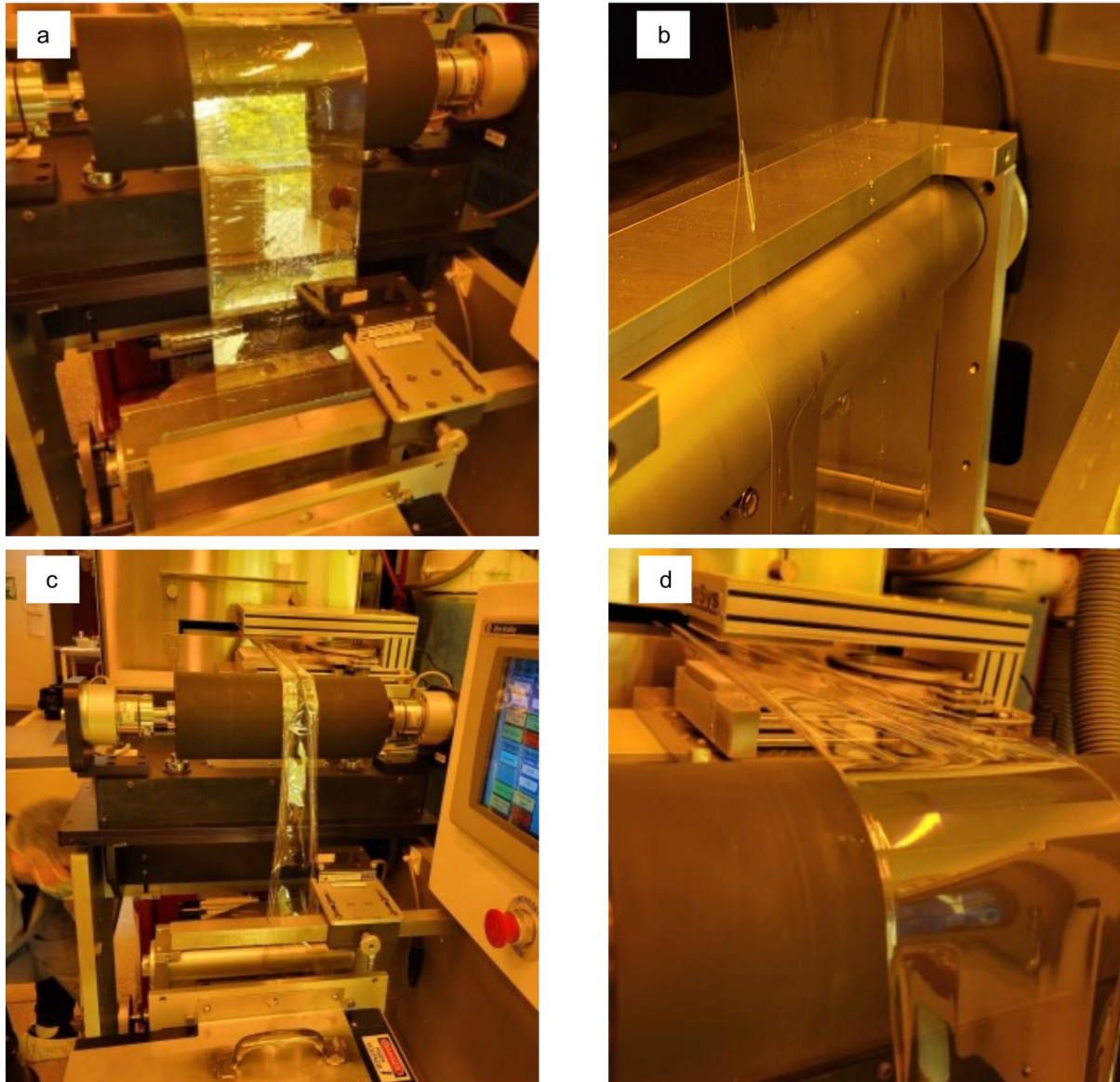


Figure 2.9 Drying phenomenon during Slot-Die Coating Trial. (a,b) Silk film delaminating from 125µm PET substrate. (c) Silk Film and 75µm PET substrate curling towards silk coating while drying. (d) Silk film dried on 125 µm PET produced without dimensional issues.

2.3.2 R2R Knife-Blade Coating

Parameters from batch-scale knife-blade roll coating and slot-die R2R experiments were applied to industrial knife-coating equipment, but due to the large scale and associated volumes required per experiment, a minimal number of conditions could be tested. Knife-over coating was conducted on manufacturing-sized machinery with a knife coating head featuring adjustable height and width, coupled to a 30-meter-long drying oven with 3 zones of temperature control. Coating was performed on non-treated PET-based substrate with a thickness of 125 μm (FLEXcon). The initial goal was to produce a 20 μm dried film which could be detached from the substrate and handled as a freestanding film. A starting knife-to-substrate gap of 100 μm was chosen for an RSF solution of 20wt% based on previous lab results. The coating width was set to 10cm, with the three oven zones set to 60, 100, and 115 $^{\circ}\text{C}$ respectively, substrate tension 25N, and the substrate speed was set to 10 meters/minute. Coating at this condition yielded a final dried film thickness of 15 μm , so the knife-gap was increased to 150 μm , and a final dried film thickness of 19 μm . At the same line speed, this film was not completely dry when reaching the rewinding roll, so the speed was reduced to 5 meters/minute, after which films were dried before reaching the rewinding stage. Forty meters of RSF-coated substrate were produced continuously at this condition. A second coating was produced with the same RSF solution, but with half of the wet thickness (75 μm), and a coating width of 15cm. This yielded a final dried film with a thickness of 10 μm , and 10 meters were coated at this condition. Coating of glycerol containing solution (15wt% glycerol:85wt% silk fibroin solids; 3.4wt% glycerol and 18.8wt% SF in final solution) was also tested. Conditions were the same as the first R2R knife experiment, and films measured ~20 μm microns, thinner than RSF solution alone. Nearly ten meters of this condition were coated. Photos of these experiments were not allowed due to company policies; however, an example film can be seen in Figure 2.10 b.

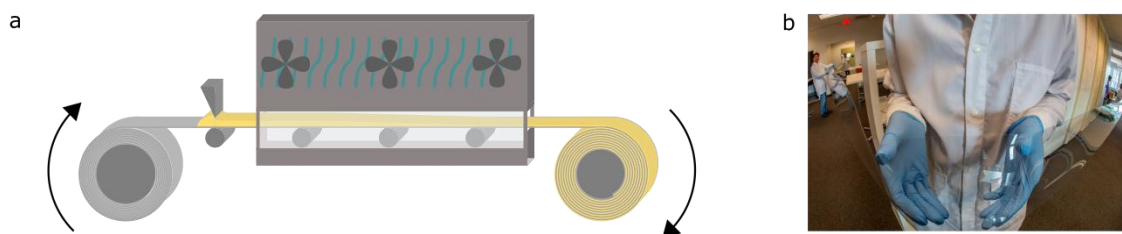


Figure 2.10 R2R Knife-blade coating. (a) Simplified schematic of coating, drying, and rewinding process. (b) Unrolled 40m film produced via R2R coating on industrial equipment.

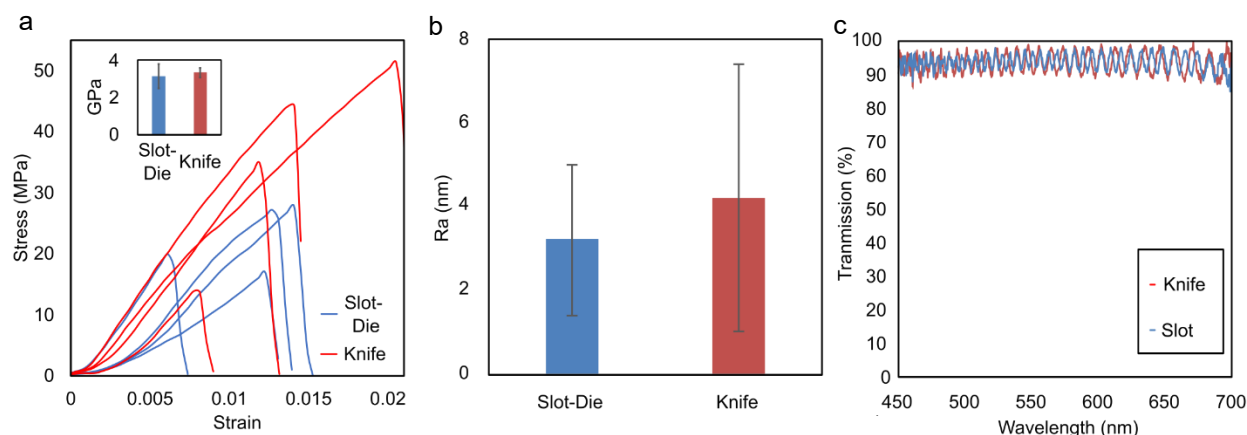


Figure 2.11 Comparison of films produced via slot-die and knife-based coating. (a) Mechanical tensile testing of films at 50% RH. (b) Surface roughness of the exposed face of the silk films. (c) Optical transmission in visible wavelengths.

Films produced with both methods were compared using several characterization methods.

Mechanical properties of films were tested for mechanical robustness using a Universal Testing Device (Instron 34SC-1) in tensile strain according to ASTM D882⁶⁰. RSF films were removed from the substrate by peeling with an acrylic frame coated with double sided tape and then the freestanding films were laser-cut with a 75w CO₂ laser (Trotec Speedy 300 75W, power=5%, speed=10%, pulse=1000Hz) into 100mm x 10mm strips. These strips were then clamped into the testing apparatus with a minimum of five film samples tested per condition. Atomic Force Microscopy (AFM) in contact mode (Bruker Innova, Respa cantilever) was used to analyze surface topology of plain and patterned films. A fiber-coupled UV-Visible spectrophotometer (Ocean Optics) coupled to an upright microscope (Olympus BX51, 10x objective) was used to measure optical transparency in the visible wavelength range, using a glass slide as a reference. It should be noted that this technique can also be used to approximate film thickness by quantifying the number of fringes between two given wavelengths if the refractive index of the material is known between these same wavelengths⁶¹. Overall, films produced in both roll-based trials exhibited similar mechanical, optical, and morphological properties, with differences being within error of margin for measurement techniques and for variability observed during production (Figure 2.11).

2.3.3 Thermal Embossing

It has previously been demonstrated that complex microstructures can be transferred from a rigid mold to amorphous silk fibroin films via thermal nanoimprint embossing performed by Amsden et al.²⁶ This is well characterized for single planar sheets, where there is only movement along the axis that applies pressure, and the contact time is in the tens of seconds or multiple minutes. However, the long cycle time and batch-based approach are potential bottlenecks for scalable production compared to continuous processes. For continuous thermal nanoimprinting via R2R methods, the throughput and consistency can surpass batch processes. However, the changes in thermal transfer, forces, angles, and residence time all differ sufficiently from batch processing, and likely require different conditions to successfully transfer patterns pattern^{62–64}. Here, we attempted to transfer a submicron resolution diffraction pattern onto R2R knife coated RSF films from 2.3.2 to investigate these differences.

The pattern used for this test was a symmetric in 2D with peak-to-peak spacing of 900nm and a profile depth of 100nm constructed of a patterned metallic nickel master with a thin layer of adhesive on the backing of the pattern. This pattern was adhered to a bare nickel cylinder that was then mounted onto the continuous nanoimprint processing line (Carpe Diem). The patterned nickel master was preheated to 100C and 150lbf (670N) of force was applied for a roughly 15cm wide and roughly 1.25cm tall contact patch, equivalent to 350kPa (50psi), matching prior batch-process embossing conditions²⁶. The silk film was loaded with exposed silk facing the nickel patterned cylinder and the silk-coated PET substrate was conveyed through the imprinting portion of the equipment at a rate of 1.5m/min. However, no pattern was transferred under these initial conditions. After increasing the temperature to 130C, the pattern was successfully transferred to the silk film (Figure 2.12a-d). While this temperature is below the T_g of pure silk fibroin, RSF films experience water's plasticizing effect on the protein and exhibit a reduced T_g unless thoroughly dried^{65–67}. Embossed films were imaged via AFM as detailed in the last section to inspect the resolution of the transferred pattern (Figure 2.12 e). The resulting symmetric 2D diffraction pattern can be seen when a 532nm laser source is applied to a sample of this patterned film after it was removed from the PET substrate (Figure 2.12 f).

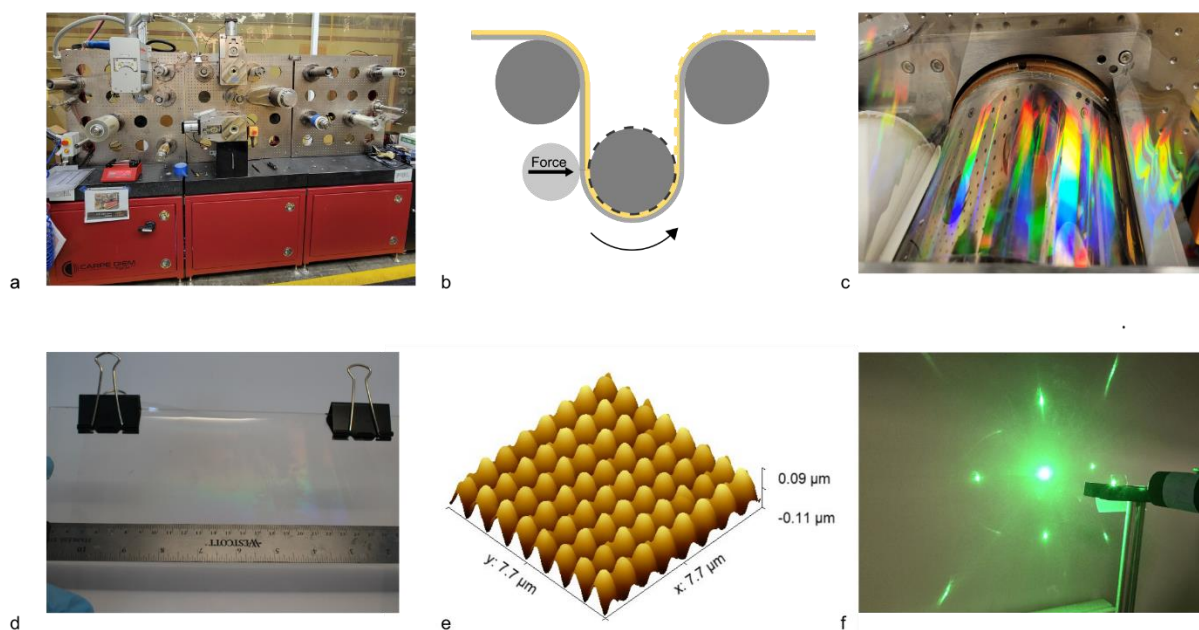


Figure 2.12 R2R thermal nanoimprinting. (a) Overview of thermal nanoimprinting R2R equipment. (b) Schematic of nanoimprinting process. (c) Nanoimprinting patterned nickel cylinder master with coated substrate being patterned (d) Image of sheet of patterned silk film. (e) AFM of patterned silk surface (f) Illumination with 532nm laser shows 2D symmetric diffraction pattern

2.3.4 Other Paths to Functionalization

RSF films produced in the R2R and batch coating trials were functionalized via unit operations compatible with roll-based processing. To demonstrate the potential for bulk crystallization of RSF films, several were immersed in solvent bath (100% Isopropyl Alcohol, Sigma) for set times (5 seconds, 5 minutes). As expected, increasing immersion time resulted in increasing β -sheet content (Figure 2.14 a). Interestingly, while freestanding films were homogeneously affected by the immersion, films that were well adhered to the PET substrate exhibited marked differences between the exposed side and the substrate-covered side (Figure 2.13).

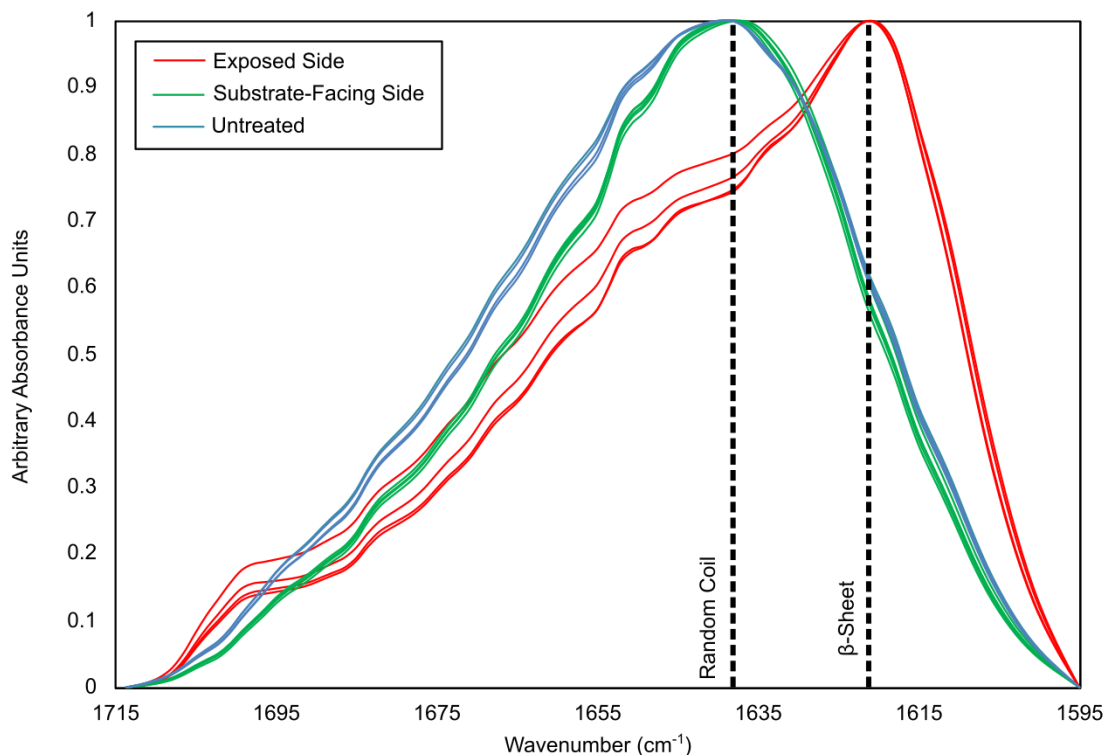


Figure 2.13 FT-IR spectra for 20-micron RSF film produced via knife-coating on PET substrate, immersed for 5 minutes in 100% Isopropanol.

UV lithography was used to modify surface properties by reducing crystallinity of insoluble fibroin film (Figure 2.14 b). Metal shadow masks were employed in a process analogous to positive resist photolithography, where patterns exposed to light turned from insoluble to soluble, and these exposed areas can be dissolved in water and removed⁶⁸. Light exposure from a 254nm UV-C lamp (VL-215.G UV, intensity of $76 \mu\text{W}/\text{cm}^2$) placed 2.5cm from the sample for 9 hours. The exposed surfaces showed a reduction in crystallinity when assessed by FT-IR. Films incorporating pH indicator (nitrazine yellow, bromocresol green, and phenol red pictured, $0.75\text{mg}/\text{mL}$ inclusion level) were produced and able to respond to pH changes after water vapor-induced crystallization (Figure 2.14 c). RF split-ring resonators (pattern dimensions: 10mm wide, 1mm line width, 1.25mm gap) were inkjet printed (Dimatix DMP-2831) with a 16 nozzle 10pl cartridge and an ink consisting of 30wt% silver nanoparticles dispersed in ethylene glycol (Sigma Aldrich) onto dyed silk fibroin film. Each printing step was followed by an annealing step at 160°C , and this process was repeated three times to create continuous electrically conductive traces in the designed pattern (Figure 2.14 d). A resistance of $R_s = 1.2 \pm 0.1 \Omega/\text{sq}$ of the printed traces was achieved, and a frequency response plot is shown.

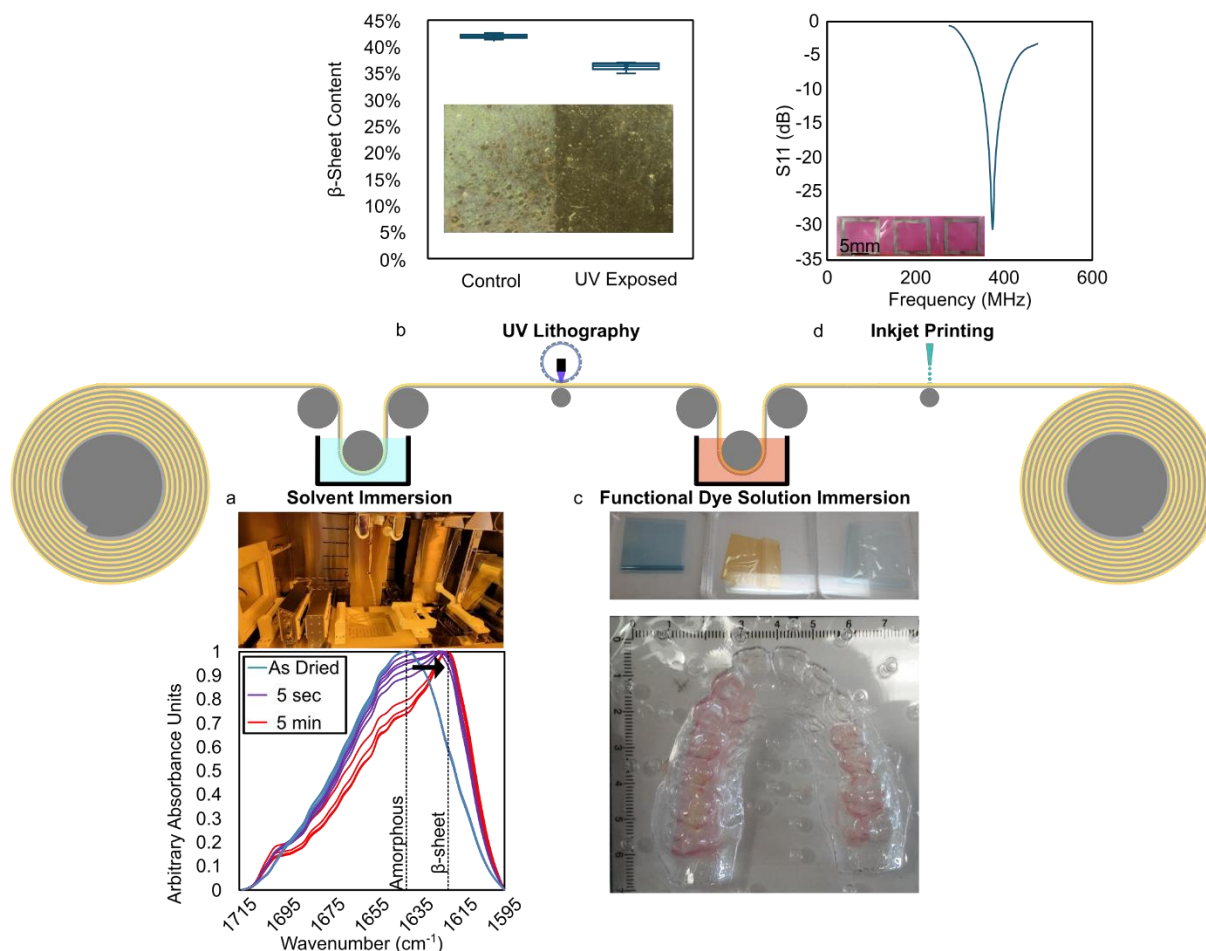


Figure 2.14 Examples of R2R compatible unit operations for functionalized films. (a) Solvent immersion used to modulate secondary structure through immersion time, with image of immersion process above and resulting secondary structure for immersion in Isopropyl Alcohol solution below. (b) UV Lithography with 254nm light used to reduce crystallinity of highly crystalline silk film in positive resist process. (c) Incorporation of dyes and other functional molecules through immersion; shown are pH indicators, with phenol red-dyed silk film applied to orthodontic device to monitor local oral conditions. (d) Inkjet printing, in this case printing wireless split ring resonators from conductive Ag ink on silk dyed with phenol red.

2.4 Alternative Approaches

While the results of roll-to-roll coating in this chapter are very promising for expanding the availability of consistent silk fibroin and devices built on such a platform, there are areas where additional work could help in understanding the limitations of this approach. Specifically, the drying process is both a critical and rate-limiting step, requiring either elevated temperatures or slow substrate speed. These conditions may limit the scope of different devices that can be produced with RSF substrate, notably those incorporating sensitive molecules such as enzymes or active pharmaceuticals, which may not be

stable at such elevated temperatures or in the solution state for extended lengths of time. Accordingly, it may be necessary to seek the use of alternative drying methods, such as pulsed light or microwave drying for inclusion of other electronic, biological, or chemical components, or to consider processing with different solvent systems to reduce time at unstable states⁶⁹.

In one specific example of difficulties experienced while incorporating sensitive molecules into silk films, we investigated the stability of the RT-LAMP (Reverse-transcriptase loop-mediated isothermal amplification) method for the detection of viruses during the outbreak of the SARS-CoV-2 virus in silk films (Figure 2.15). As RT-LAMP is performed at a constant temperature between 60 and 70°C, it can be conducted in a water bath or on a heating pad, making it a candidate to provide access to amplification and detection in situations where PCR (polymerase chain reaction) is not practical due to requirement of an expensive thermocycler, such as in home testing or remote clinics⁷⁰. Encapsulation of the reaction components into silk fibroin film has the potential to improve their shelf life and enable a diagnostic that can be shipped without any fluids that need to be handled by end users. The reaction requires a DNA polymerase that also exhibits reverse transcriptase activity (or a separate reverse-transcriptase), looped primers, dNTPs, Magnesium Sulfate, and a suitable running buffer. Incorporation of the reaction components into a silk solution or a silk lyophilized powder that was rehydrated both yielded results suggesting the reaction could take place in the presence of silk fibroin, though the visible colorimetric detection functionality of the supplied reaction mixture (New England Biolabs) was slightly hindered in the presence of the silk, requiring fluorescent quantification instead. However, more critically, while silk fibroin films have been shown to stabilize simple enzyme systems, performance of the DNA amplification step was hindered, and the reverse transcription severely hindered in the case of dried and rehydrated silk films containing various combinations of the reaction components. It is possible that proper modulation of the temperature and drying conditions could improve the activity of the reaction mixture, as could separation of the various reaction components and assessment of each in the presence of RSF to ensure compatibility.

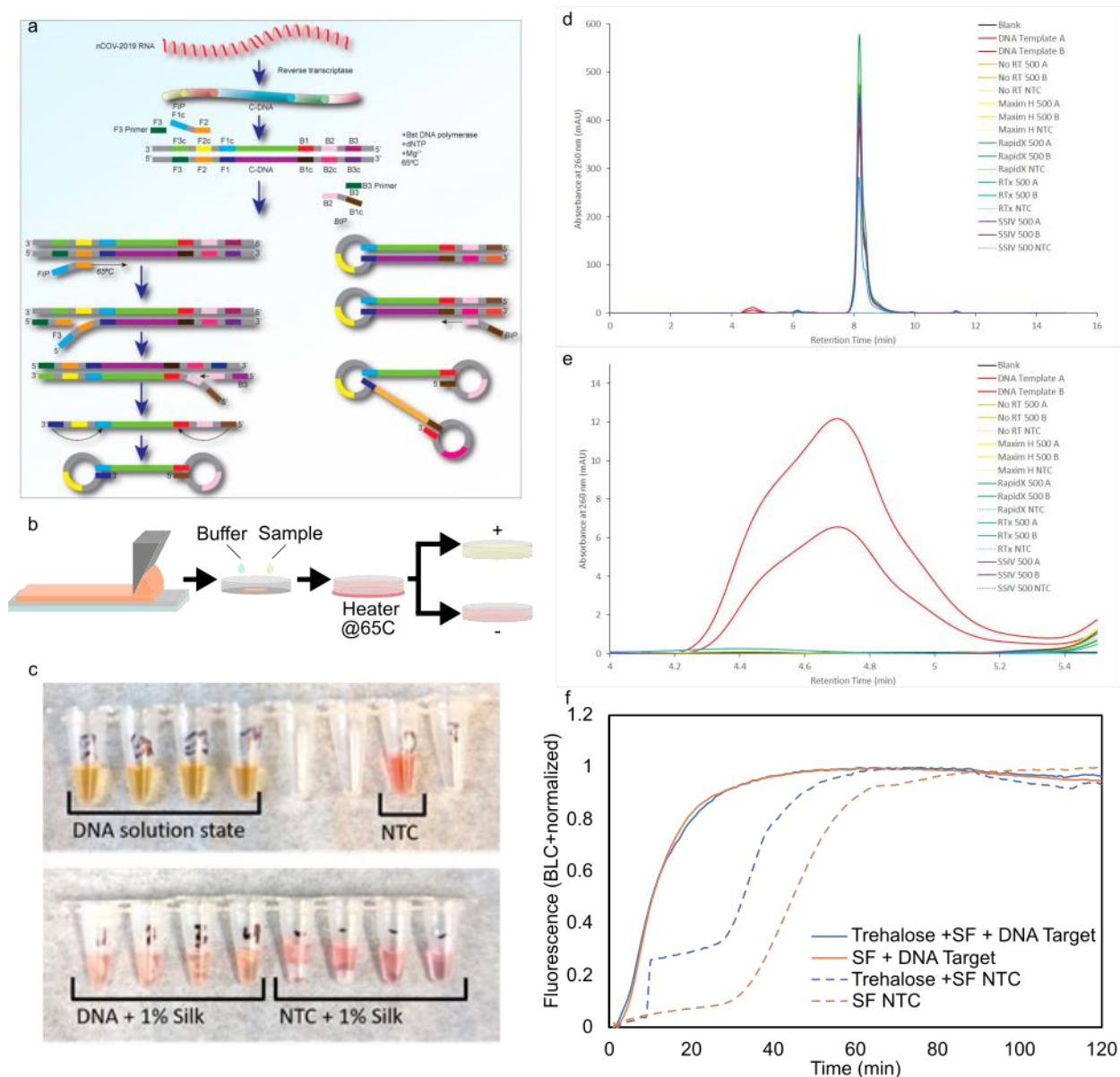


Figure 2.15 Investigation of RT-LAMP in RSF solution and films. (a) Overview of RT-LAMP amplification technique, adapted with permission ⁷¹. (b) Schematic of process of producing functional RT-LAMP silk films. (c) Image demonstrating result of colorimetric change with silk+RT-LAMP system. (d,e) GPC elution of reaction components. (f) DNA amplification as measured via fluorescence with and without trehalose.

To improve roll-based coating of silk fibroin, there are several other variables that could be useful to study in greater detail. Higher concentrations of silk fibroin solution would allow thicker dried films to be produced, but such solutions were difficult to handle with the properties derived from the spray-dried silk fibroin powder. A deeper dive into the influence of molecular weight or even combinations of specific molecular weights on the ease of solution preparation, maximum stable concentration, and associated

rheology could be insightful here. Additionally, several groups have worked with protein solutions containing chaotropic agents or other plasticizers in film-casting processes^{71,72}. It is possible that these additives allow easier handling and coating of silk fibroin, though potentially at the expense of adding additional steps to remove these agents after casting or changing the final bulk properties of silk substrates.

More broadly, roll-to-roll processing faces limitations in processing for on certain materials and geometries, such as silicon wafers or glass. While the percentage of solution ejected and during spin coating in laboratories is quite high, it is possible to improve yield for these processes through recapturing solution that is ejected while coating. Additionally, the technique is quite useful for quickly producing nanometer thickness coatings for devices such as optical and electronic applications. Similarly, while drop cast films may not be as homogeneous films produced with R2R manufacturing, techniques such as surface pre-treatments via plasma or other coatings, surface patterning, controlled drying, and incorporating surfactants can improve quality of drop casted materials.

2.5 Future Directions

Due to the flexibility of the roll-to-roll approach, as well as the versatility of silk fibroin as a substrate, there are numerous potential directions for this work. For example, adhesive films can be produced based on formulations previously developed with silk⁷³, with concentrations increased to improve flow characteristics while coating (Figure 2.16). While these films adhered to additional PET substrate and aluminum sheets, additional optimization is needed for the continuous process and for specific adhesive applications.

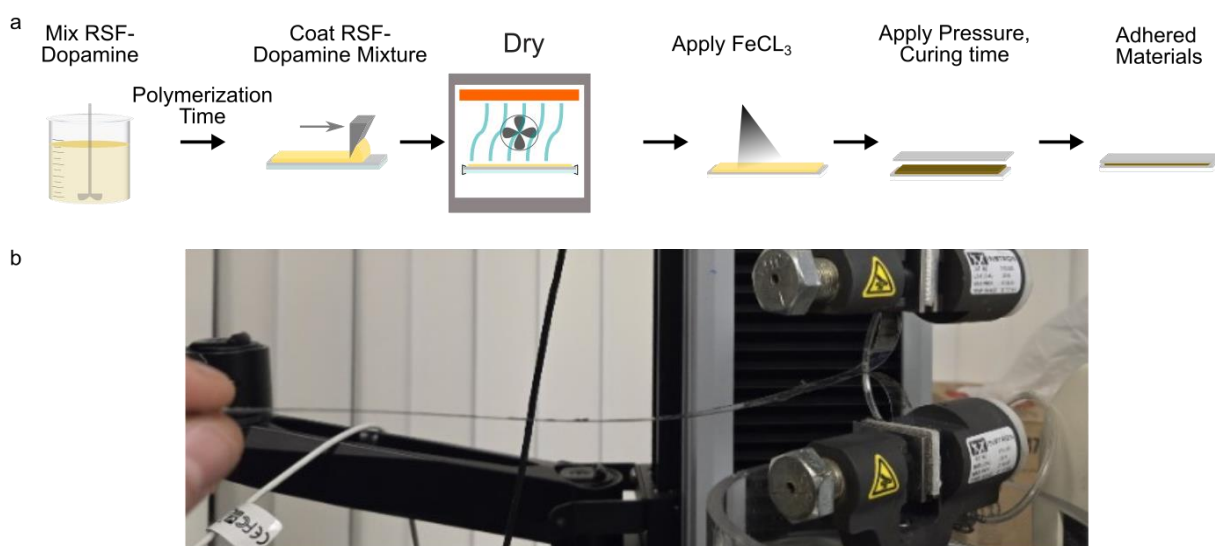


Figure 2.16 A silk-dopamine adhesive film. (a) Overview of process. (b) Testing in a 180-degree peel test.

The work conducted in this chapter utilized powdered silk fibroin to reliably screen and produce large volumes of silk fibroin solution for purposes of testing R2R coating. However, silk fibroin properties including viscosity, mechanical properties, and degradation time are also correlated with molecular weight. While a range of suitable coating conditions was determined using powder-based silk fibroin, molecular weight was not examined in this work due to the intensive nature of processing sufficient volumes of multiple molecular weight silk fibroin and the relative ease of processing water-soluble powdered silk fibroin, which requires a maximum molecular weight. Our lab has done extensive work studying the viability and variability of scaling the silk regeneration process building on top of previous characterization (Figure 2.17).

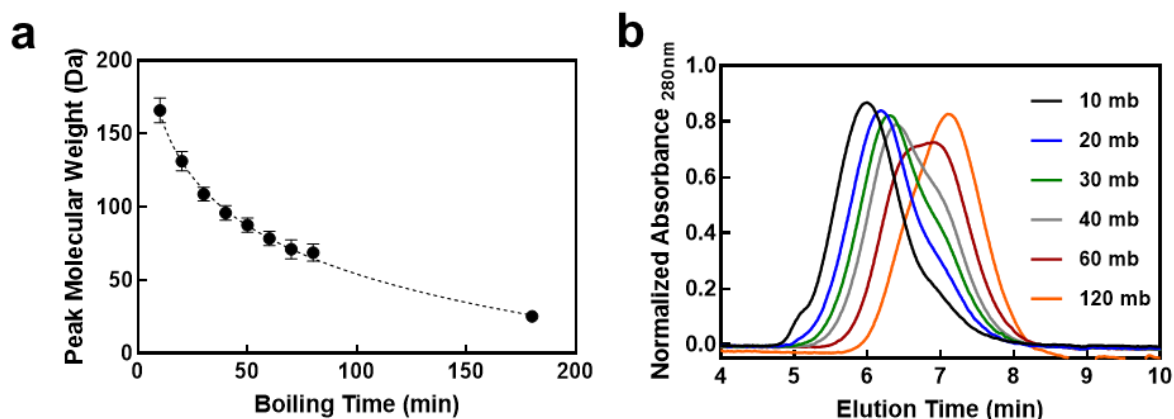


Figure 2.17 Characterization of degumming time via Gel-Permeation Chromatography. (a) Peak MW versus degumming time. (b) GPC Raw Data for degummed samples. Data from N.Ostrovsky-Snider

The largest time-based bottleneck in producing RSF solution from degummed fibers is the dialysis process, which traditionally requires changing the dialysate (deionized water) at set intervals for several days per batch and requires increasingly large volumes of water to achieve the same final processing time for larger dialysis geometries. This also becomes a point of quality concern as a change in dialysis batch size and geometry changes the diffusion length and time to reach osmotic equilibrium for a given stage in the process⁷⁴. To address these issues, we built a prototype system to automate the dialysis of silk fibroin and other molecules using stirred tanks with modular dialysis cassettes and automated inflow and outflow valves regulated by a script running on a raspberry pi which is constantly monitoring dialysate conductivity (Figure 2.18). This system saves considerable time by optimizing and automating the process of water changes, but also reduces water consumption by optimizing the time for each step and cycling water between steps when run in parallel (i.e. using dialysate with low salt concentration from a final dialysis step for the first dialysate in a second batch). This system will enable further work in investigating the impact of molecular weight on properties of films and other RSF formats.

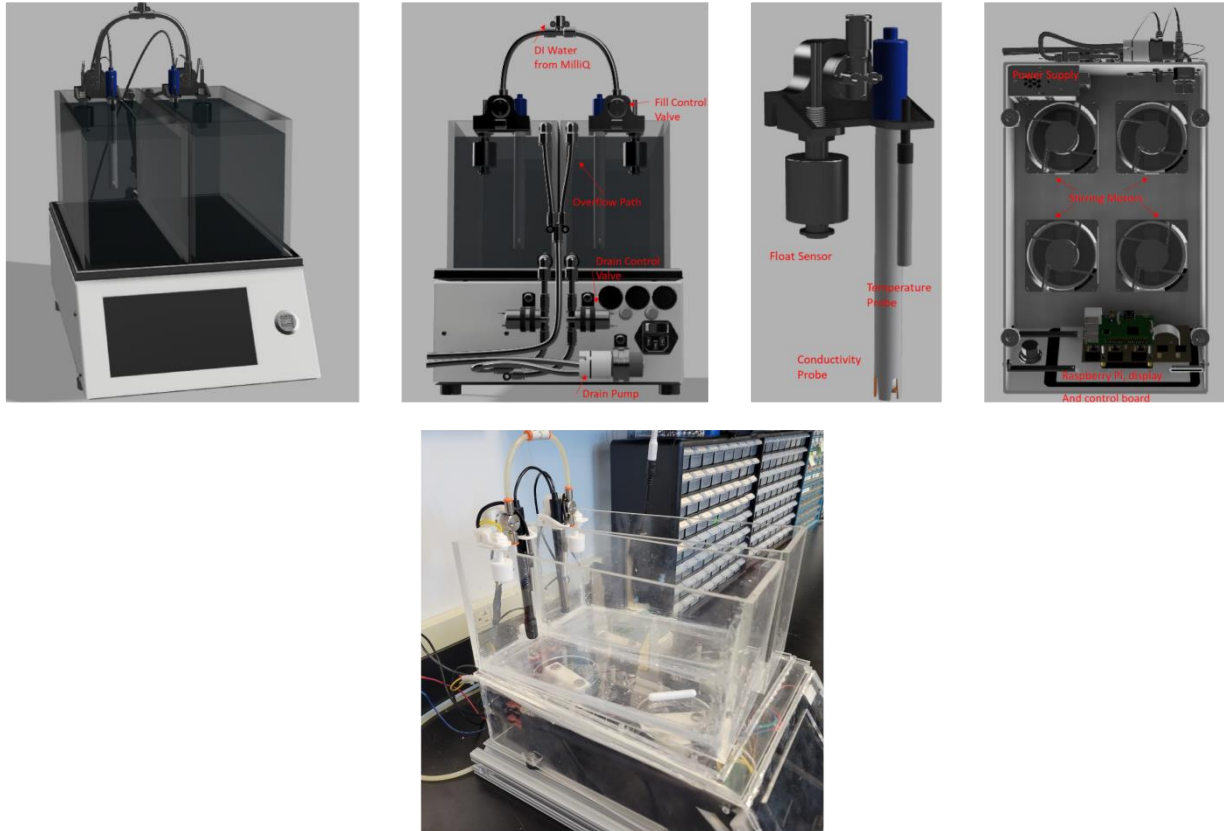


Figure 2.18 Lab-based auto-dialysis system. Top: Rendering. Bottom: Working Prototype

Chapter 3: Tunable Monomaterial Silk Fibroin Composites

3.1 Direct Patterning of Secondary Structure through Inkjet Printing

3.1.1 Background

As mentioned previously, alcohol solution is one means by which crystallinity can be induced in regenerated silk fibroin structures. We also know that many properties of silk fibroin are directly correlated with the material's secondary structure, including mechanical integrity of the films, vapor permeability, and biodegradability. Meanwhile, inkjet printing has been developed as a versatile technique for material deposition, compatible with both small scale bench-scale research as well as high-speed R2R manufacturing. It allows complex forms and functions to be imbued through the precise application of a variety of inks. Here, we hypothesize that spatial control over the secondary structure of silk fibroin can enhance the material's functionality by spatially modifying its physical properties, and that inkjet printing of alcohol solutions at designated locations on of an otherwise amorphous silk fibroin film could be a useful method for realizing such a tunable construct. By understanding the efficacy of inkjet printing for the targeted modulation of silk fibroin's secondary structure, this research aims to open new opportunities in the design of advanced functional materials that can be tailored for unique applications.

Inkjet printing requires a balance between ink characteristics (rheological properties, surface tension) and equipment parameters (piezoelectric waveform, voltage, and frequency) to reliably produce and deliver droplets of ink into desired patterns. Here we explore piezoelectric drop-on-demand printing given the previous success with the technique and silk fibroin constructs, its and versatility^{68,75,76}. Specifically, a Dimatix DMP-2831 printer featuring cartridges with 16 piezoelectric-controlled nozzles delivering 10pL droplets was used for this work. This printer provides the flexibility to print any ink compatible with the materials of construction of the print cartridges, in addition to offering a wide range of adjustable print parameters including waveform, voltage, frequency, print height, print resolution, and platen temperature. The printer also allows the printing of simple designs constructed of lines, rectangles, and dots with a built-in pattern editor, and the conversion of bitmap images to printable patterns for more

intricate designs. While several other inkjet printing methods (continuous, thermal) could also be considered for this work, continuous printing causes additional waste compared with drop-on-demand techniques, and thermal printing is not suitable for working with flammable high vapor pressure solvents such as low-carbon aliphatic alcohols. Given the flexibility of precisely depositing inks and solvents through direct inkjet printing, the approach was applied to directly pattern crystalline regions on amorphous RSF film in a process outlined in Figure 3.1. This work was carried out despite the difficulties involved in printing high vapor pressure or low viscosity fluids given that inkjet printing system are highly tunable in nature, and success with printing solvents of similar characteristics in other work^{77,78}.

This chapter will cover a series of experiments designed to test this hypothesis. Initial sections will discuss the formulation of the alcohol-based inks, tuning of the inkjet printing system to accommodate these inks, and an exploration of the effects of these inks combined with controlled deposition patterns. Subsequent sections will present the impact of this printing technique on the mechanical properties of films designed to exhibit directional behavior, as well as the resulting modulation of humidity-based actuation that the printing process imbues on silk films.

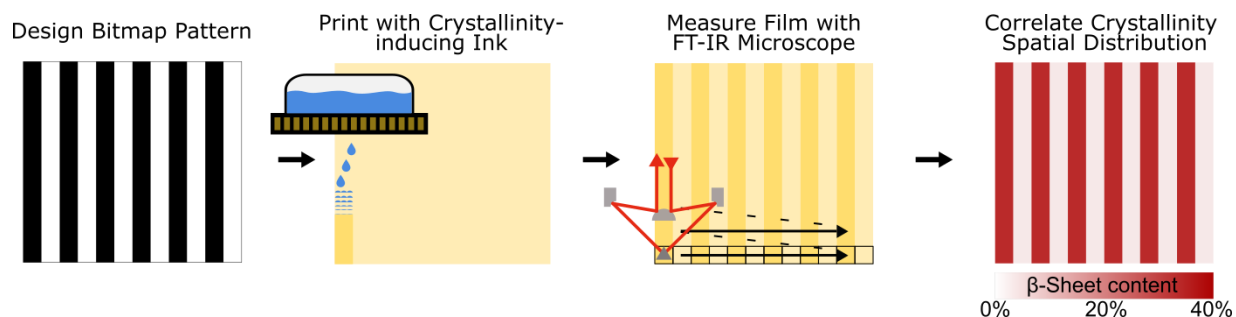


Figure 3.1 Schematic of spatial patterning of crystallinity using inkjet printing

3.1.2 Ink Screening

To begin, potential solvents for inducing secondary structure were screened for viscosity, surface tension, and ability to induce secondary structure into silk fibroin. It is well understood that low-carbon aliphatic alcohols can increase crystallinity of silk fibroin. However, those with the most effective capacity to induce crystallinity in silk fibroin also exhibit viscosity below recommended by theory as described by the inverse of the Ohnesorge number (Oh)⁷⁹, the dimensionless Z-number⁸⁰:

	$Oh = \frac{\sqrt{We}}{Re} = \frac{\eta}{(\sigma \rho a)^{1/2}} = Z^{-1}$	Equation 3.1
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Where:

η = dynamic viscosity

ρ = density of fluid

σ = surface tension

a = characteristic length scale (nozzle diameter)

which is based on two commonly used dimensionless numbers in fluid dynamics, the Reynolds number, which describes viscous versus inertial forces in fluid flow (i.e. turbulent versus laminar behavior):

$$Re = \frac{av\rho}{\mu} \quad \text{Equation 3.2}$$

(where v is the characteristic velocity) and the Weber number, which compares a fluids' inertia flow relative to its surface tension (i.e. the formation of droplets from a continuous stream of fluid):

$$We = \frac{v^2 \rho a}{\sigma} \quad \text{Equation 3.3}$$

A Z-number between 1 and 10 is recommended for inkjet printing by the printer manufacturer and other work for piezoelectric inkjet printing^{81,82}. Values above 10 often result in satellite droplet formation, whereas values below 1 suggest that viscous forces dominate, and that the system is unable to form droplets. Calculated values for methanol, ethanol, and isopropyl alcohol are listed in Table 3-1, and the window for ideal printing is visualized below in Figure 3.2.

Alcohol	Alcohol Concentration (Volume %) in Aqueous Solution					
	50	60	70	80	90	100
Methanol	16.0	16.1	17.7	21.5	23.9	36.0
Ethanol	9.9	9.0	9.5	9.6	11.3	16.0
Isopropanol	7.1	6.7	6.9	7.3	7.8	7.9

Table 3-1 Calculated Z-values for candidate alcohol inks and 21.5 μ m diameter inkjet nozzle at 20°C based on reference values of viscosity, surface tension, and density^{25,83–85}.

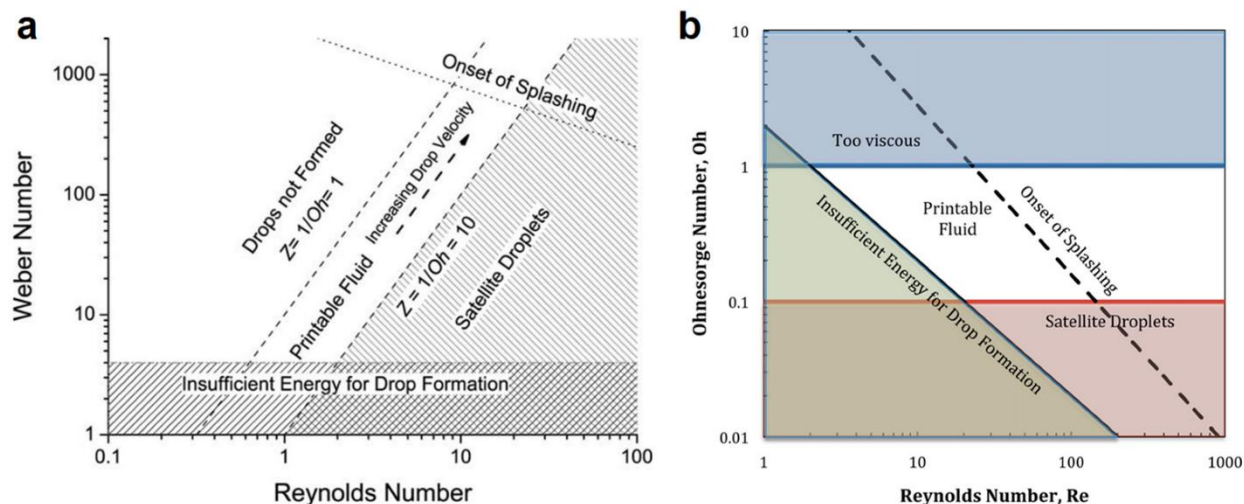


Figure 3.2 Ideal inkjet printing windows as determined by Weber Number, Reynolds Number, and Ohnesorge Number. (a) Weber Number vs. Reynolds Number. (b) Ohnesorge Number vs. Reynolds Number. Reproduced from Zhang et al. 2022⁸⁶

To improve the printing characteristics of inks for this work, several aqueous solutions of methanol, ethanol and isopropanol at varying concentrations were prepared. To screen the effect of these solvents without requiring optimization of the printer for all solutions, 2 μ L droplets were dispensed onto the surface of amorphous silk films with a micropipette and the effect of inducing secondary structure was investigated via FT-IR with a diamond ATR crystal. It was observed that pure solutions of these alcohols did not induce the strongest β -sheet content, with increasing effect upon the addition of water up to a maximum before declining with the addition of further water (Figure 3.3). Once the water content approached 50vol% for each of the solutions, reflow was a significant factor in the quality of the surface of the film as the solutions tended to partially dissolve the film before evaporating. Similarly, alcohol solutions with high water content were slow to evaporate, a potential issue during inkjet printing if multiple print layers were necessary. As a result, only alcohol concentrations above 50vol% were considered for printing. Additionally, while viscosity of all the alcohols examined in pure solutions were lower than that of pure water, mixed solutions had higher viscosities than both water and the alcohol component⁸⁶. However, surface tension reduced from a maximum of pure water towards the value of the alcohol component, suggesting that the inclusion of a surfactant was not necessary for printing alcohol inks with alcohol concentrations above 50vol%^{87,88}.

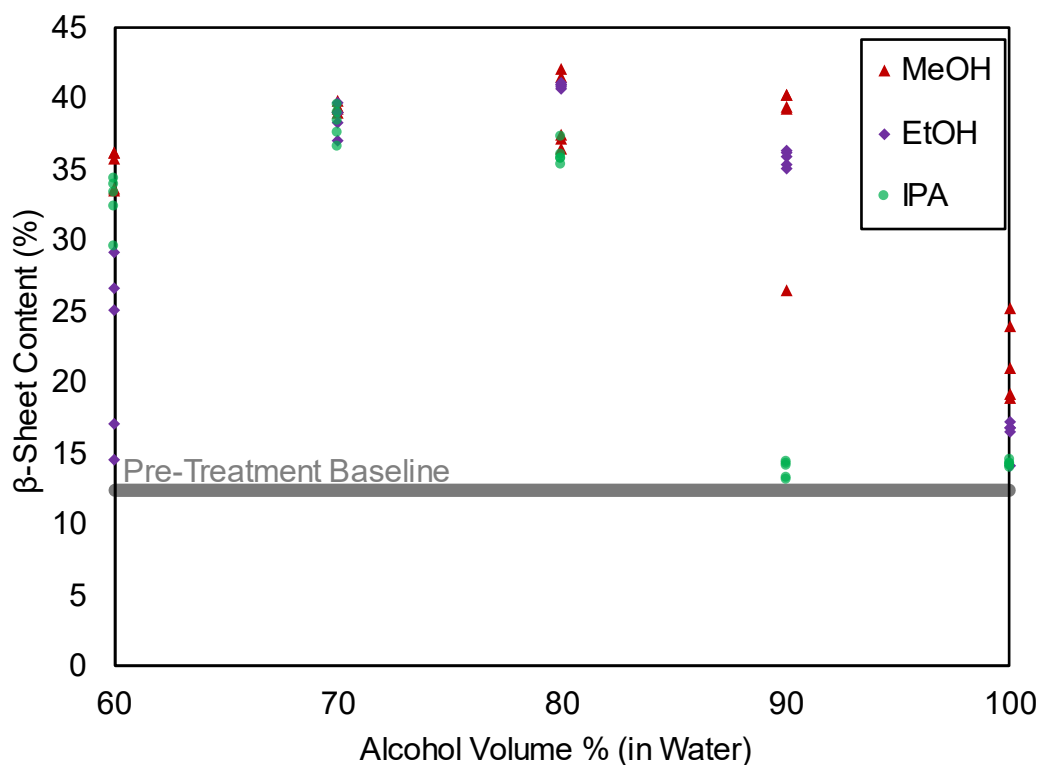


Figure 3.3 Alcohol Aqueous Solution Concentration vs. Crystallinity (2 μ L droplet on dried film delivered via Micropipette)

3.1.3 Direct Printing of Solvent onto Silk Films

Despite the fluid and physical properties of several inks being outside of the ideal printing range as predicted by the Z-factor determined by prior experimental work, piezoelectric printing heads provide substantial room to modify parameters, notable the applied waveform, the actuator voltage, and the pulse frequency. The main approach involves matching the waveform of the piezoelectric cartridge to the behavior of the fluids under the shear induced by the actuator⁸⁹. Each fluid will have an optimal waveform, though it is labor and time intensive to optimize for each fluid. This is a scenario where building an automated feedback loop where print parameters are adjusted while drop performance is recorded and analyzed could drastically improve print optimization and quality with minimal user input, and reduce time to print for new inks, though this is not currently possible with the Dimatix system⁹⁰.

To optimize the waveform manually, one should start with a waveform with three distinct regions: a filling portion, a printing portion, and a recovery portion (Figure 3.4). Additional regions can be added depending on the ink characteristics. As a rough guideline, one should adjust the length of the print portion of the waveform to maximize drop velocity and then adjust the overall printhead voltage to achieve a droplet velocity appropriate for the printer and ink. Then the relative voltage levels for each additional print phase can be adjusted, including the fill, recovery, and intermediate segments between them and the print region to minimize air entrainment and reduce ligament length/satellite drop formation. Once the voltages for each print stage are defined, the rate of change between segments can be adjusted to maintain drop velocity and improve drop formation. After this process, each nozzle individually needs to be tuned to provide matching velocities so that printing complex patterns at high nozzle firing rates is possible. Incorrectly tuned printer nozzles for a given solution can cause issues with droplet quality such as misdirection, satellite formation, and variable drop velocity (Figure 3.5).

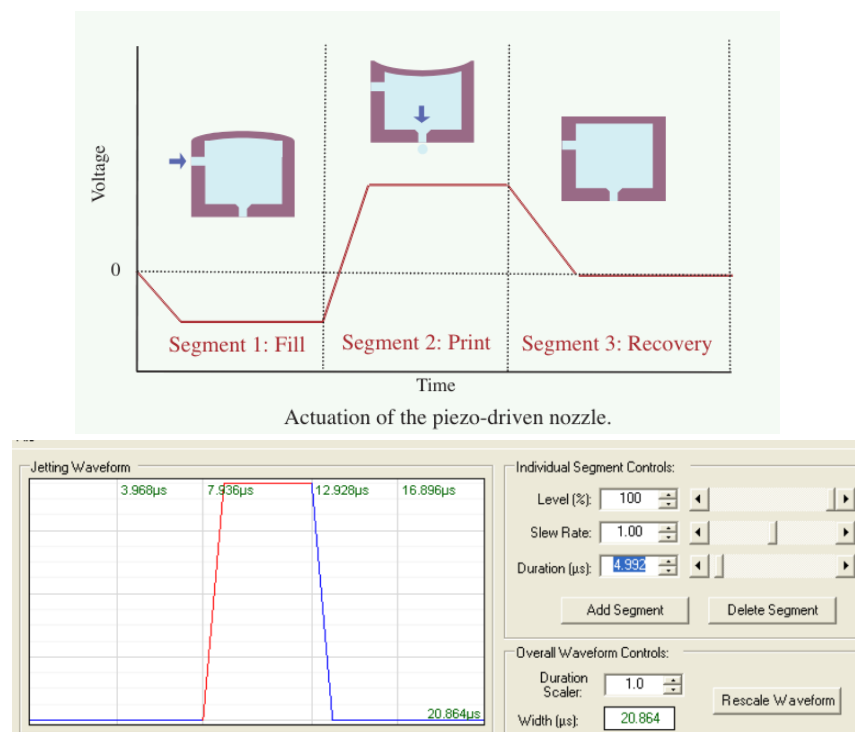


Figure 3.4 Waveform development. Top: Overview of waveform phases for a typical piezoelectric inkjet print; from Fujifilm Dimatix Manual. Bottom: Example waveform; Reproduced from Fujifilm Dimatix Drop Manager Version 2.1.1.0.

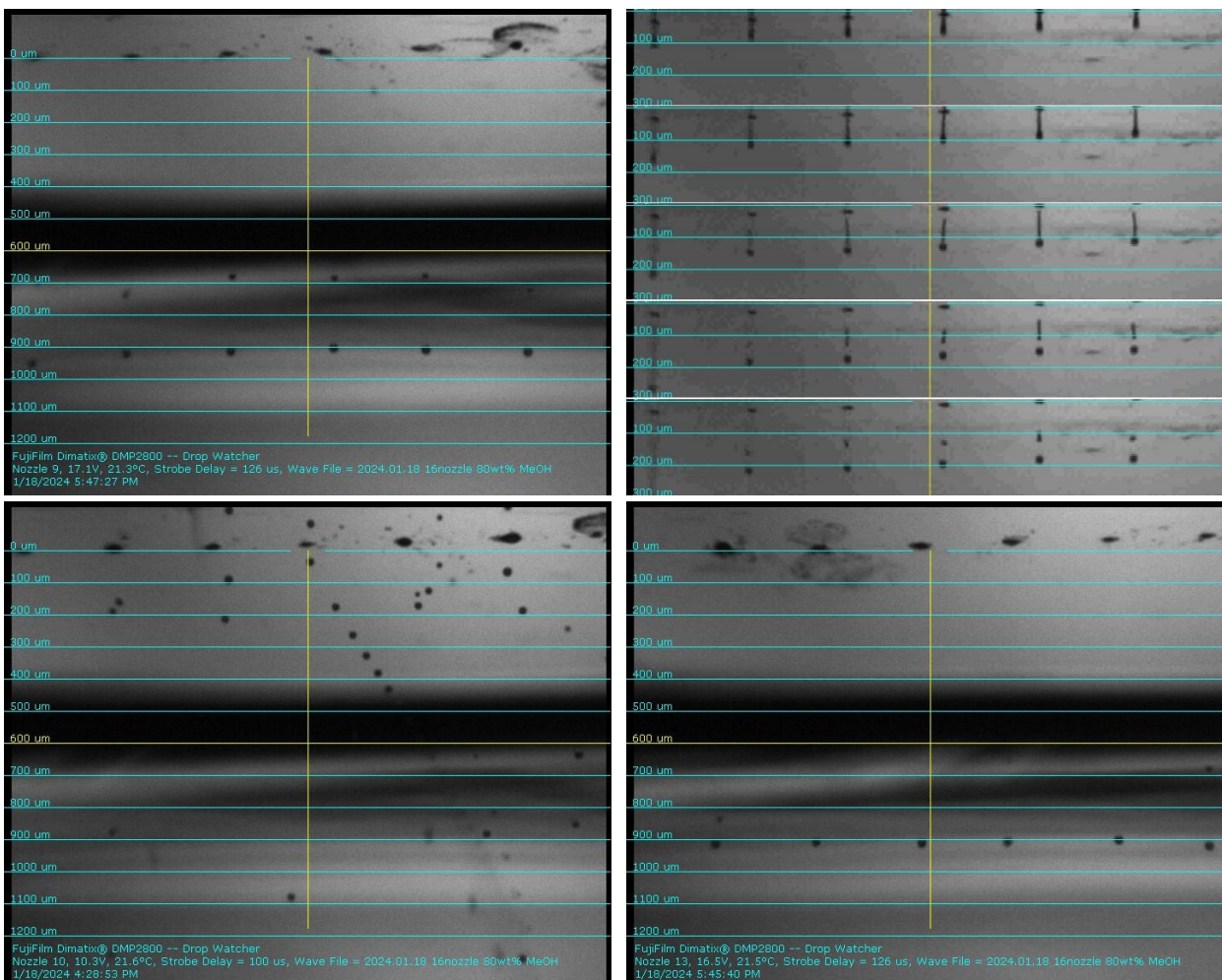


Figure 3.5 Example of inkjet droplet behavior. (a) Satellite droplets present at printing height of 900 μm . (b) Timelapse of formation of satellite droplet formation. (c) Example of jet misdirection. (d) Well-functioning printing with 6 active nozzles at 7m/s droplet velocity at printing height of 900 μm

Beyond print nozzle performance and ink composition, parameters that influence print quality include print resolution, print pattern, and number of print layers. Their effects were examined for several of the most promising optical inks for inducing secondary structure. To produce a pattern to print, one can create a print path through the conversion of a bitmap-based image to a dot matrix based on a selected print resolution/drop spacing. Each resolution requires manual setting of the printing head to a specific angle to produce a desired resolution, which is unfortunately a potential source of error with this printer system (Figure 3.6).

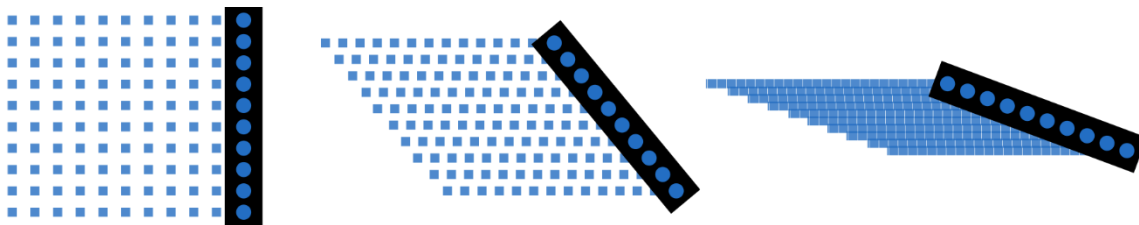


Figure 3.6 Overview of adjustment of print resolution/nozzle spacing by changing the print nozzle angle, with lowest resolution to highest resolution, left to right, with decreasing print cartridge angle.

To prepare an ink for printing, solutions of 1.5-3ml were diluted as needed, filtered through a 0.2 μ m syringe filter and filled into print cartridges, all in a chemical fume hood. Each cartridge was loaded into the printer and allowed to equilibrate for at least 10 minutes in this position to reduce presence of air bubbles in the printhead. Then the waveform for the cartridge was adjusted, and the voltage for each nozzle tuned to have similar droplet velocity and satellite characteristics for each individual experiment. To explore the impact of droplet spacing, several print resolutions for the same print pattern (a line one droplet tall) were printed (Figure 3.7a). At high resolutions, droplets coalesced before they finished crystallizing and evaporating from the silk substrate, so droplets are larger than those produced with lower resolution droplets. Dried droplet diameter increases until the resolution limit of 5 μ m droplet spacing is reached. As drop spacing increases beyond 30 μ m and resolution decreases, droplet size reaches and maintains a minimum diameter (Figure 3.7 b).

Another factor to consider which was not exhaustively tested here is print height, which can influence the speed of a drop impacting a substrate, as well as the presence of satellite droplets in the case of unstable print jetting⁹¹. Print height was kept to a minimum above the substrate to minimize evaporation of highly volatile print solutions, with a target of 1mm between the nozzle and substrate.

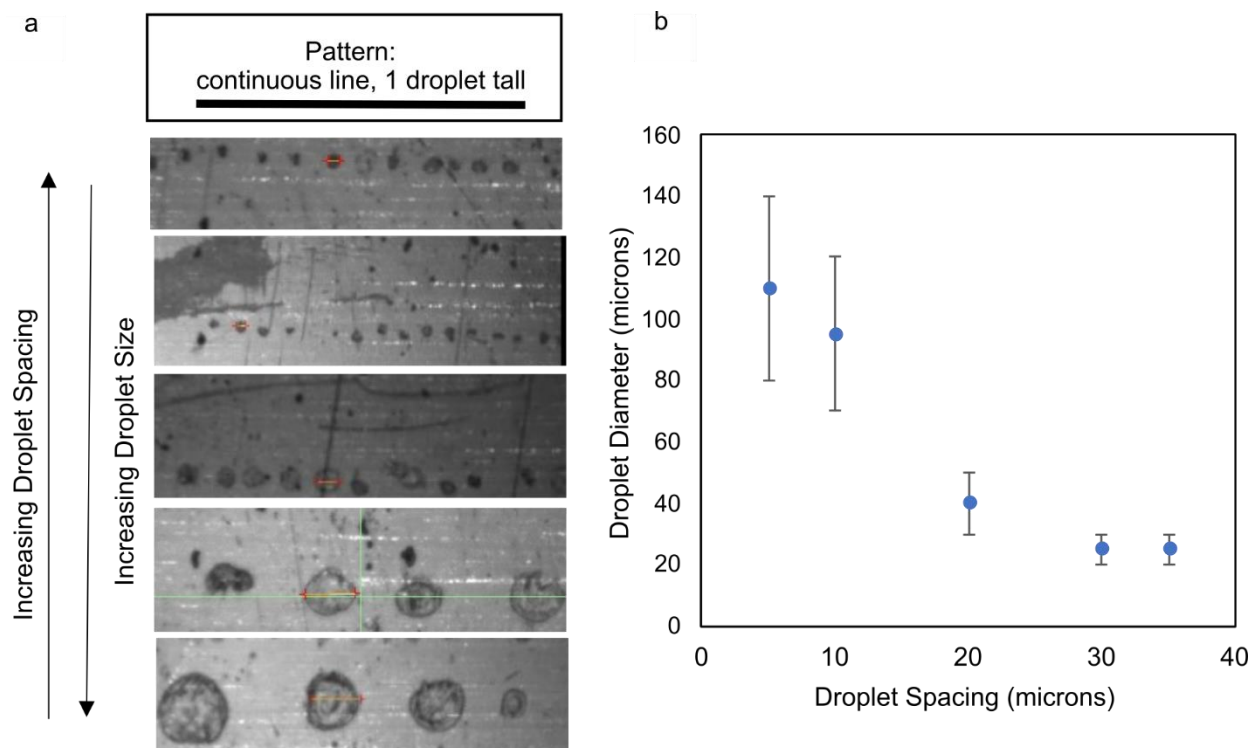


Figure 3.7 The impact of droplet spacing for a row of single-nozzle printing of 80vol% Ethanol. (a) Optical images of decreasing droplet spacing (top to bottom). (b) plot of droplet spacing from photo analysis.

3.1.4 Control of Secondary Structure on Silk

Printing of silk fibroin secondary structure was investigated by combining the results from solvent screening and print parameter optimization. To examine the effect of the solvents and the number of layers of printing these solvents, squares 3mm x 3mm were chosen as a print pattern to allow complete coverage of an FT-IR diamond ATR (Bruker Invenio) as well as assessment with a FT-IR microscope using an ATR objective (Bruker Hyperion 2000). For FT-IR analysis of microscopy data, spectra were taken from the microscope and extracted from the software (Bruker OPUS) into x vs. y data files. The spectra needed additional smoothing with the microscope that was not needed with the diamond ATR. Spectra were baseline corrected, normalized to 1, then smoothed using a Savitzky-Golay filter and a window length of five. These data points were then loaded into a python script to perform baseline correction, normalization, and deconvolution of the Amide I region of spectra for all microscope points measured in an automated fashion based on tools developed by KBI Pharma which utilize the Scipy optimize.minimize function for peak fitting and values of expected secondary structure peaks for silk

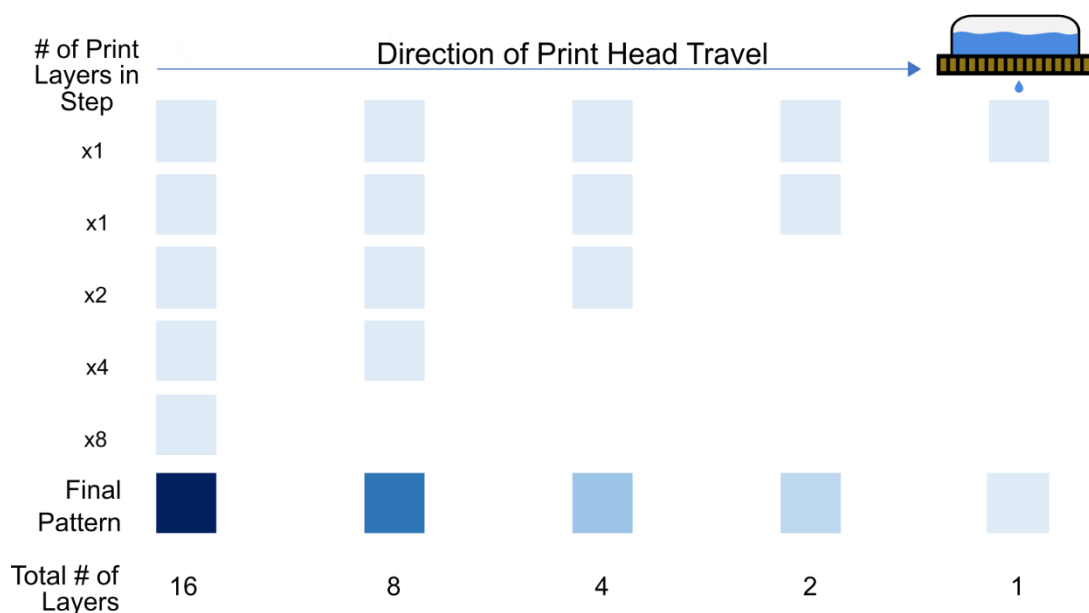


Figure 3.8 Example of multi-layer print achieved through tiling approach on a single row. The top five rows are the path of the printer, and the bottom row is the final printed design.

fibroin reported in literature⁶. This python script was then modified to allow extraction of the total β -sheet content in the form of an intensity heatmap was then overlaid on top of optical photographs taken with the microscope before each scan. Data from this analysis was also extracted and compared between multiple conditions via a two sample Student's-t test with Bonferroni correction for multiple comparisons, or Welch's t-test if sample sizes were different. A tiling approach was used to increase the rate of printing for multi-layer prints. Here, a pattern was repeated spatially for an initial print layer, and the number of spatial repeated patterns was adjusted for sequential print layers to reach the desired number of total print layers (Figure 3.8).

Screening of several solutions of methanol, ethanol, and isopropanol was performed using the tiled approach. Printing was repeated between 1 and 16 layers for each ink, and solution concentrations were varied for each alcohol with the same multi-layer print pattern. For methanol, it was found that concentrations of 90vol% and above caused significant cracking often experienced with this solvent¹⁷, perhaps due to a high rate of crystallization (Figure A.2). Solutions below 70vol% produced limited effect on secondary structure with 16 print layers or fewer, so only 70 and 80vol% were considered in detail (Figure 3.9).

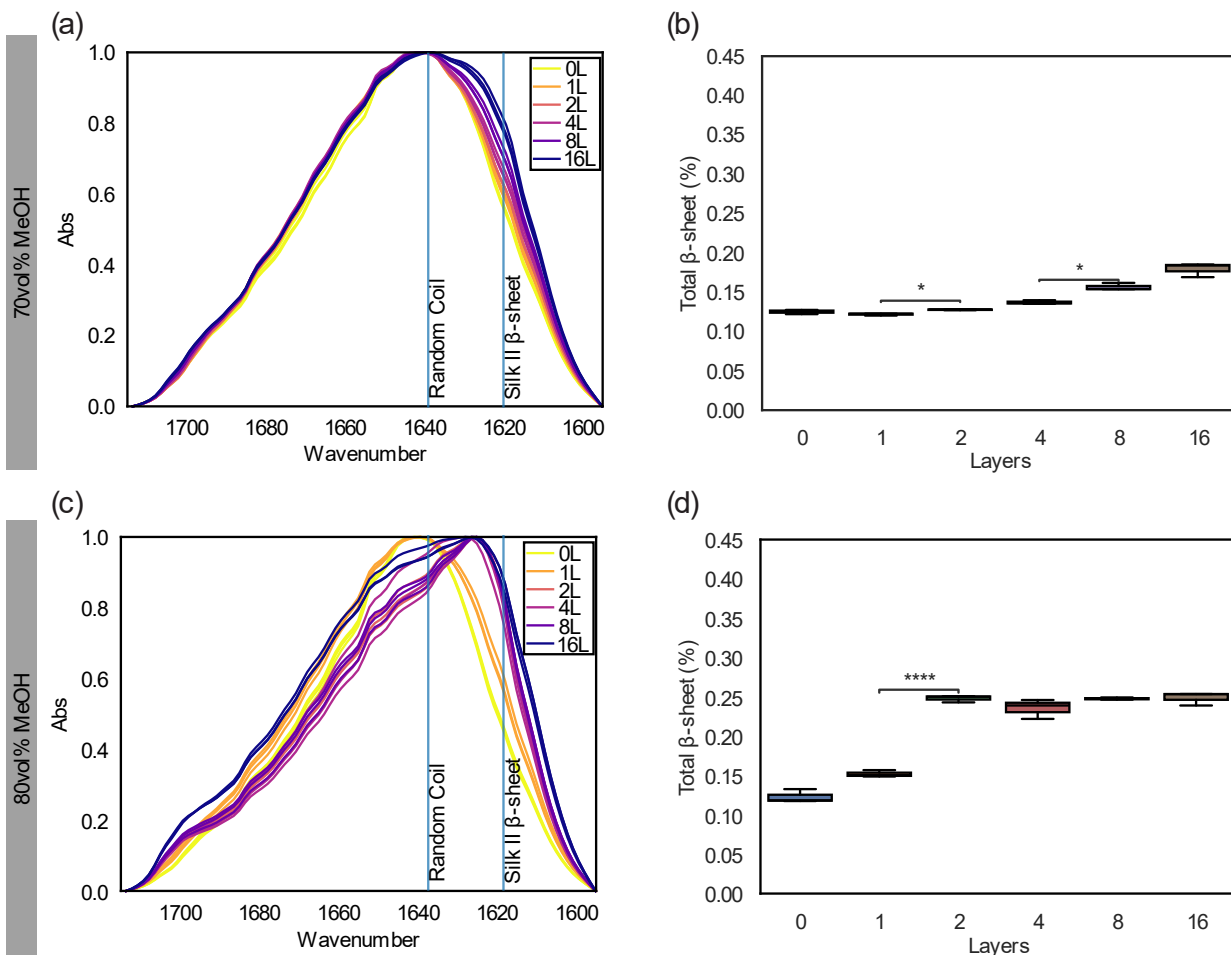


Figure 3.9 Screening of methanol printed inks on amorphous silk films. (a) Normalized baseline corrected Amide I spectra (left) and (b) beta-sheet content (right) of 70vol% printed Methanol (a,b) and 80vol% printed Methanol (c,d).

For ethanol, 70vol% and 90vol% had minimal impact on secondary structure (Figure 3.10). A solution of 80vol% ethanol had similar impact on secondary structure as methanol, with a slightly reduced

response of crystallinity to the number of layers for methanol. Isopropanol exhibited a strong effect for 60- and 70vol%, with 70vol% demonstrating increased crystallinity comparatively (Figure 3.11).

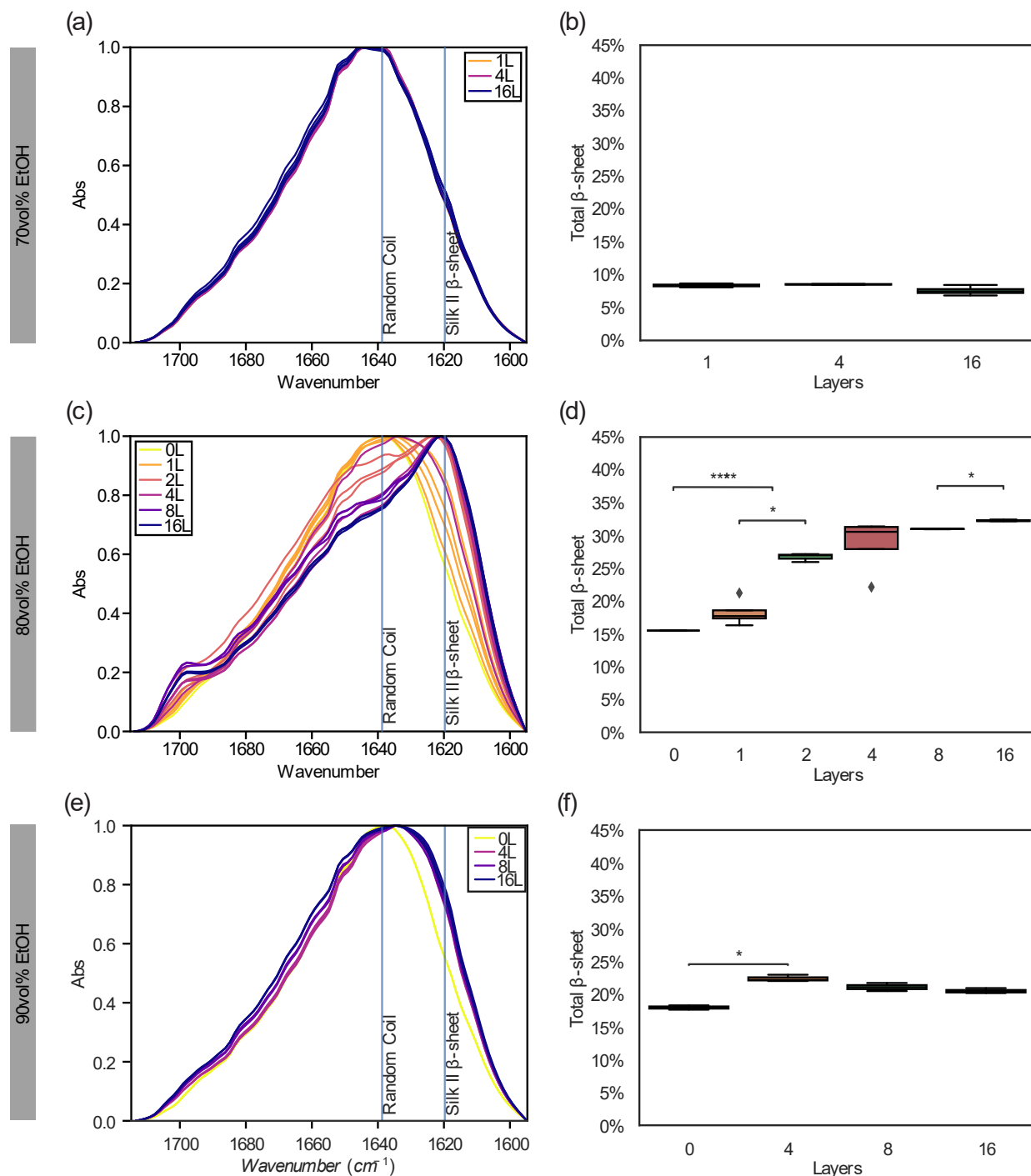


Figure 3.10 Screening of ethanol printed inks on amorphous silk films. Normalized baseline corrected Amide I spectra (left) and calculated β -sheet content (right) for 70vol% (a,b), 80vol% (c,d), and 90vol% (e,f).

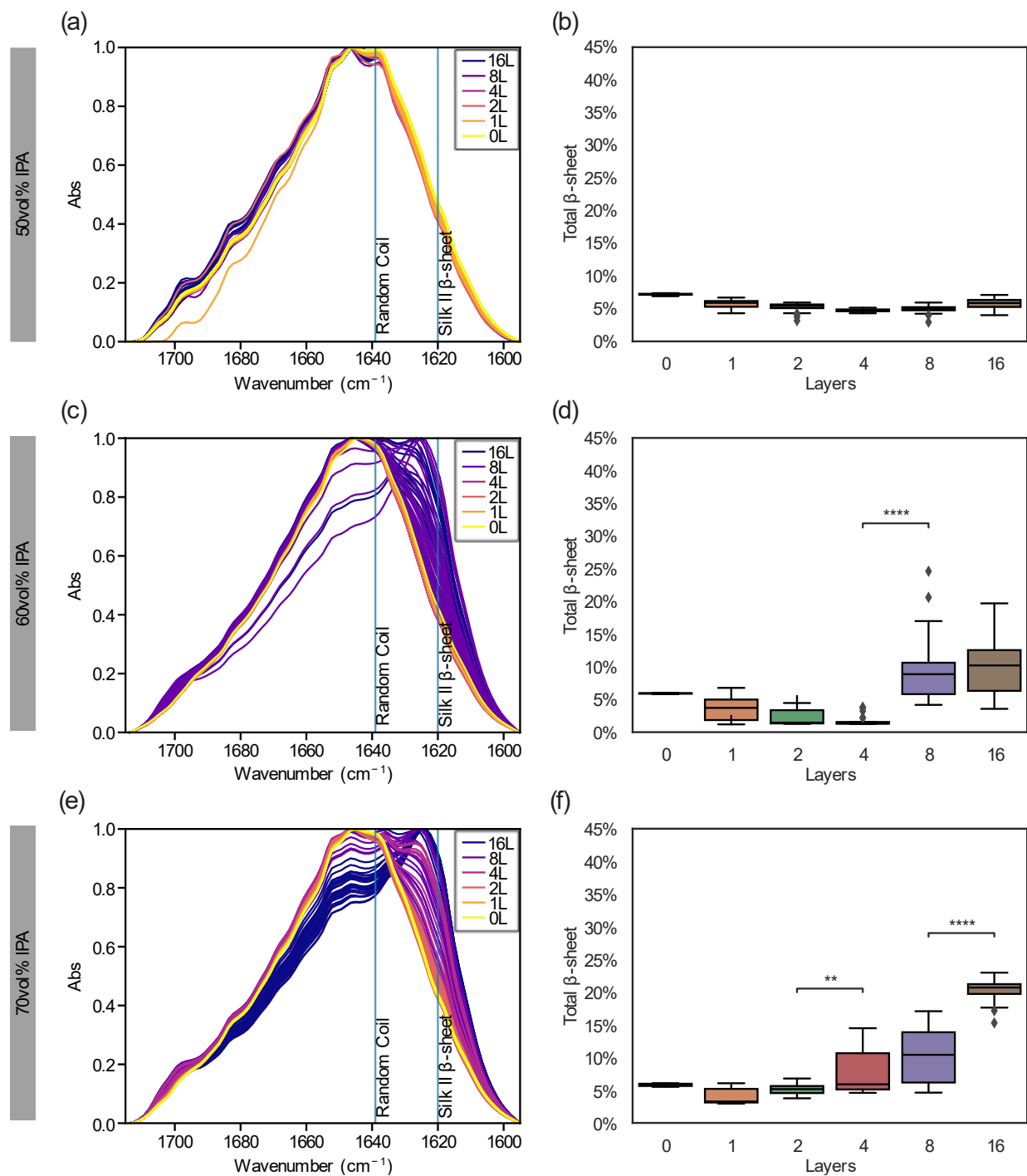


Figure 3.11 Screening of isopropanol printed inks on amorphous silk films. Normalized baseline corrected Amide I spectra (left) and calculated β -sheet content (right) for 50vol% (a,b), 60vol% (c,d), and 70vol% (e,f).

These experiments confirm that the number of printed layers impacts the secondary structure of silk fibroin films. However, as printing patterns become larger, or for the case of multiple passes in a roll-based process, the elapsed time between multi-layer printing creates a potential source of variability if time impacts the process of crystallization. As a result, the impact of time between printing of two given layers was assessed. Here, a single alcohol solution was selected (80vol% methanol), and the time between printing layers was varied from zero to 5 minutes. A moderate time dependency was observed (Figure 3.12). This is potentially due to phenomenon involving residence time of solution on the film, changes in permeability of the film to the solvent due to crystallization from a single layer influencing the next layer, or physical cracking on the surface of the film increasing surface area after the first exposure to solvents. Additional work is needed to fully understand this behavior. The impact of printer platen temperature was also briefly explored, but increasing this temperature to 40°C reduced the degree of crystallinity observable with 80vol% methanol ink compared with printing at room temperature and increased the risk of using high vapor pressure inks, so additional work with this variable was discontinued. It is possible that refrigeration of the alcohol inks while printing could be beneficial to the printing process, but equipment modification would be necessary to test this approach.

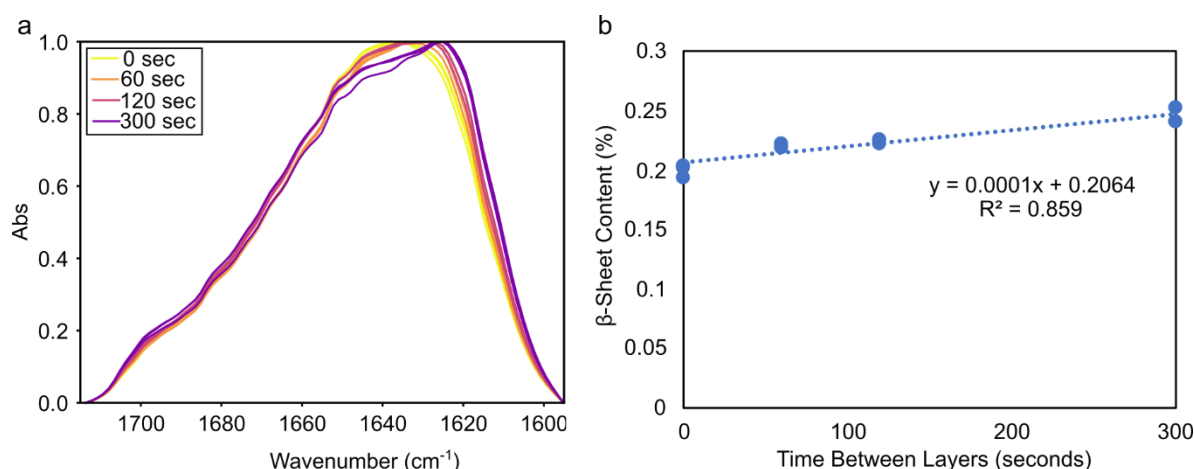


Figure 3.12 Assessment of time between printing layers and secondary structure for 2-layer print of 80vol% methanol. Amide I spectra (a) and calculated β -sheet content (b).

3.1.5 Printing-Induced Crystallization Resolution

Given the ability of 80vol% methanol to quickly induce β -sheet secondary structure into silk film via a minimal number of layers of printing with a 10pl droplet, it was selected as the primary ink for

patterning. Before functional films could be created with this ink, the resolution limit for detecting secondary structure was examined by printing simple lines with a controlled number of nozzles. Patterns were printed at a drop spacing of 30 μm and lines from 1 to 5 droplets tall with the same number of nozzles engaged from the print head as the number of droplets in height for the pattern. These patterns of lines were also printed with 1, 2, 4, and 8 layers to understand the influence of print layers on feature resolution (Figure 3.13). A linear relationship was observed for printing layers and number of droplets/nozzles, with a slight increase in line width with increasing layer count. Two-layer prints were 10 μm ($\pm 7.2 \mu\text{m}$) wider than one-layer prints, and eight-layer prints were 38 μm ($\pm 11.2 \mu\text{m}$) wider than one-layer prints.

Correlating the visible patterns to secondary structure was done via FT-IR microscope scanning across larger areas with a germanium crystal in contact with the film substrate for each designed data location. The spectra were recorded with the same settings as described in the previous section (16 scans per sample, 4 cm^{-1} resolution, 15Hz scanner velocity, 6mm aperture). In Figure 3.14, we can see that in the case of a 16-layer print of 80vol% methanol the secondary structure heatmap closely resembles that of the visible images of the printed line. The increase of secondary structure occurs over a width of 100 μm for the highest observed value, with a transition to an intermediate state of crystallinity for the next row of data points over 20 μm on either side, then levels off to the baseline value of the untreated film. This is compared to the designed pattern of lines with a specified height of 100 μm . Viewing the printed lines under higher magnification optical microscopy, we can see that the edges of these rows with 16 layers are quite irregular in areas where the layers of droplets overlapped. Single layer printing exhibits cleaner edges, but secondary structure could not be discerned between untreated and printed areas. Nonetheless, it is possible to visible see the printed pattern with the naked eye (Figure A.4).

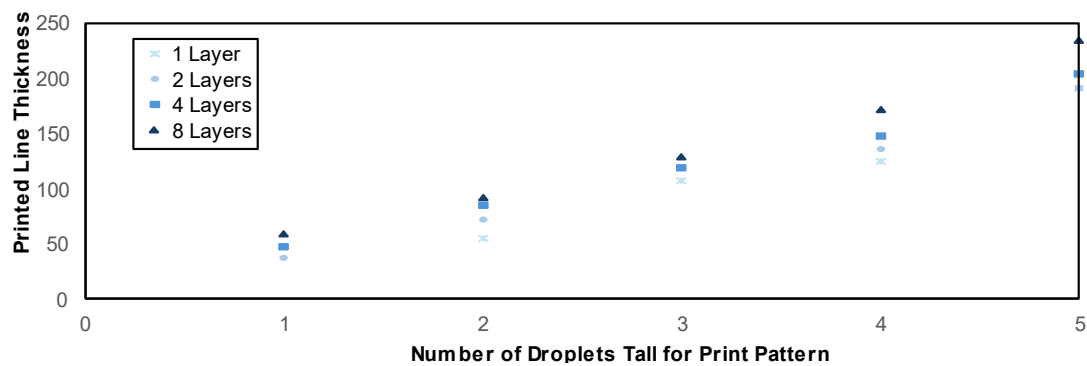
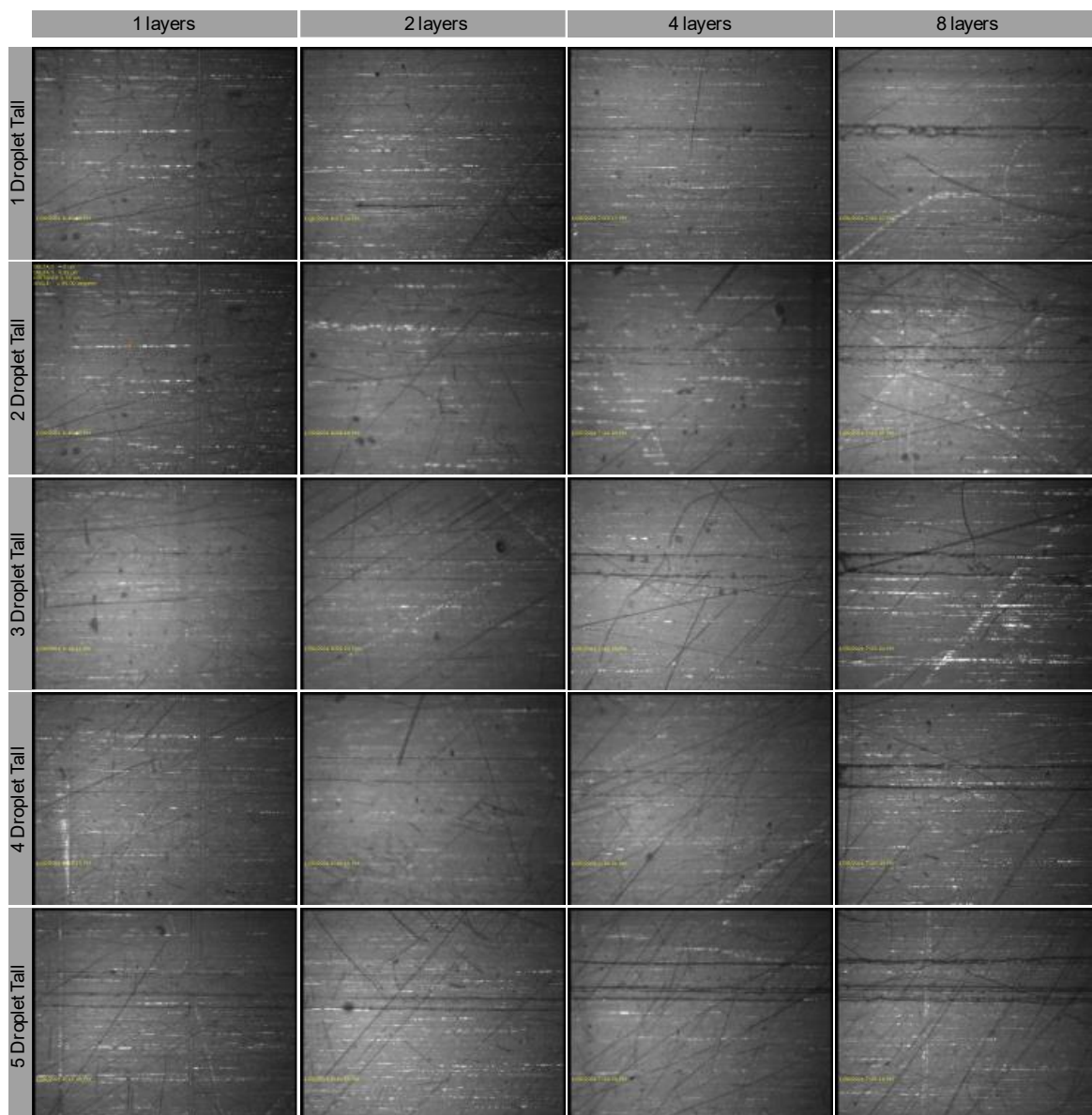


Figure 3.13 Printing Resolution of 80vol% Methanol Ink. (a.) Printed lines from 1 to 5 droplets tall (top to bottom, and 1 to 8 layers of print (left to right). (b.) Plot of extracted line widths from printing.

Additionally, with an ATR tip diameter of 100 μm , features smaller than this are challenging to detect. A gap between measurement locations smaller than the ATR tip diameter can provide information about the secondary structure of finer resolution features, but the spectral data will be averaged across material in contact with the ATR crystal. A limitation also occurs in the depth orientation of ATR measurements, as the depth of penetration with a diamond ATR in the Amide I region can be calculated using Equation 3.4, where n_1 is the refractive index of refraction of the ATR crystal, n_2 is the index of the material being measured, θ is the angle of incidence, and λ = wavelength of interest⁹²:

$$d_p = \frac{\lambda}{2\pi n_1 \sqrt{\sin^2 \theta - \left(\frac{n_2}{n_1}\right)^2}} \quad \text{Equation 3.4}$$

Using this equation, the depth of measurement was calculated as 0.98 μm for silk fibroin measured with the diamond ATR, and 0.41 μm with the Germanium ATR of the FT-IR microscope⁹³. It should be noted that the refractive index of the material being measured must be lower than that of the ATR crystal. Similarly, it has been demonstrated that there are slight distortions between spectra obtained with Germanium and Diamond ATR crystals, so direct comparison between results from these two materials should not be made without adjusting for this observed difference⁹³. In the case of a multi-layer print of 80vol% methanol with a width of only 1 droplet, no difference in secondary structure between printed and surround area could be distinguished (Figure 3.15).

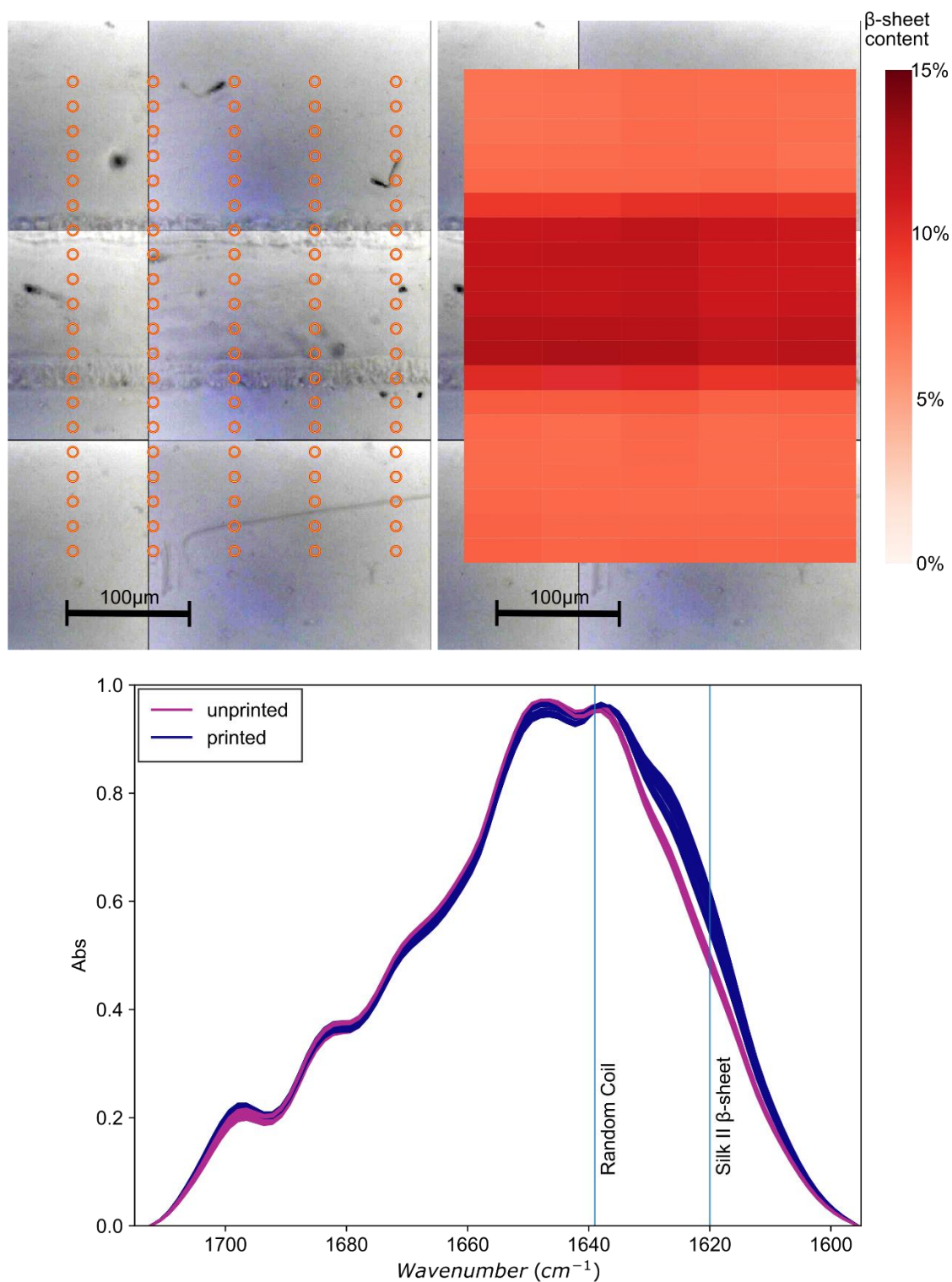


Figure 3.14 Heatmap overlay of 16-layer printed line of 80vol% methanol, 4 nozzles/drops (100 μm) wide. Above left shows the original optical image with designated measurement location for ATR spectra. Above right is the heatmap produced by fitting spectra for each measurement location. Below shows the spectra comparing those in the printed area and those outside/unprinted.

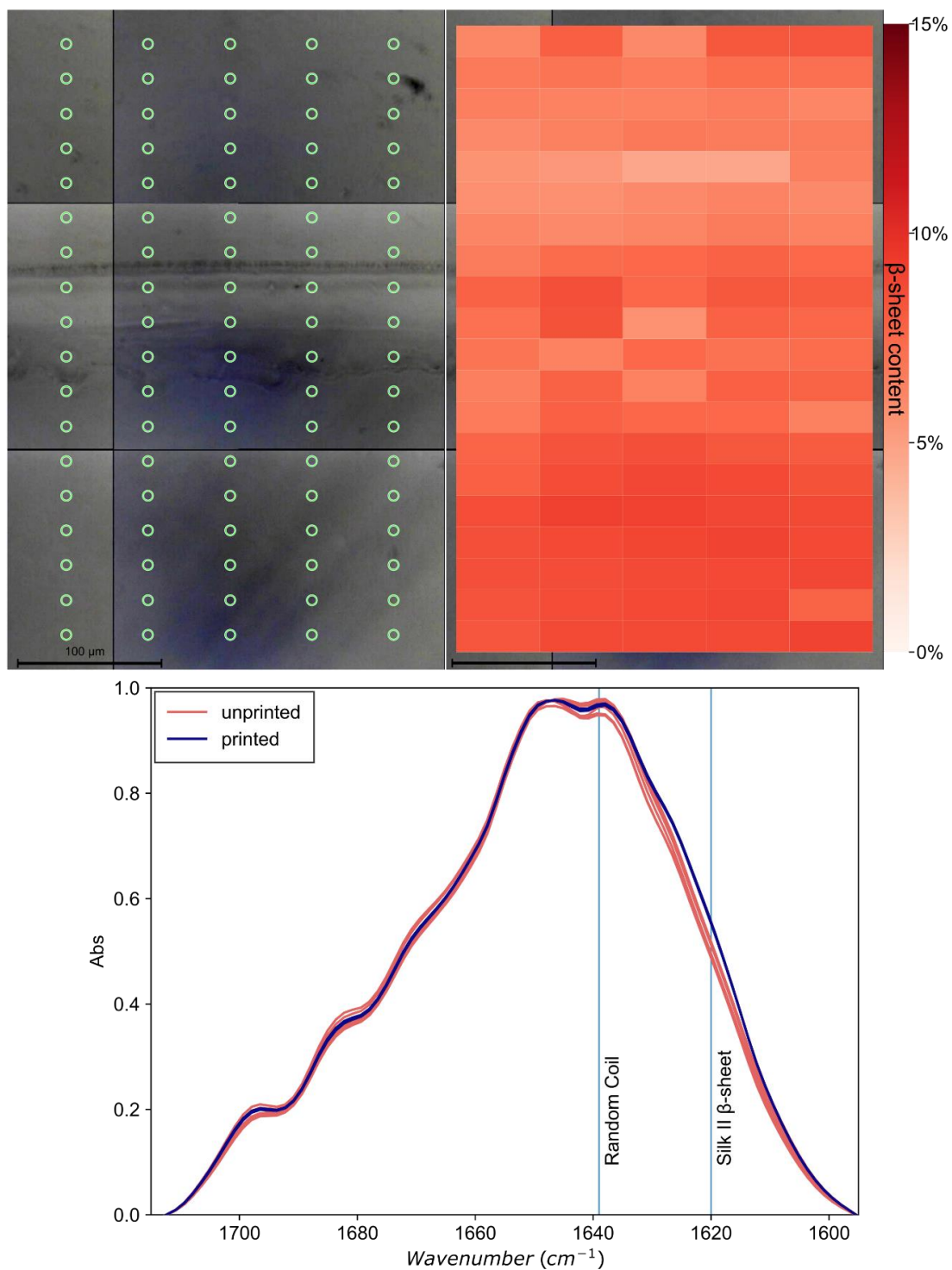


Figure 3.15 Heatmap overlay of 16-layer printed line of 80vol% methanol, 1 nozzle/drop (25 μm) wide. Above left shows the original optical image with designated measurement location for ATR spectra. Above right is the heatmap produced by fitting spectra for each measurement location. Below shows the spectra comparing those in the printed area and those outside/unprinted.

Additionally, the border region between crystalline and amorphous portions of the silk was examined via a scan performed on a microscope with finer spatial resolution (Bruker Lumos, 1 μm pixel size)Figure 3.16. While the dataset was not baseline corrected or deconvoluted at each pixel for quantitative analysis as in prior examples due to the large number of datapoints involved, plotting the wavenumber of maximum intensity in the Amide I region suggests that the transition of secondary structure closely follows the printed pattern that is optically visible, with the transition from highly crystalline (maximum of $\sim 1620\text{cm}^{-1}$) to amorphous (maximum at $\sim 1640\text{cm}^{-1}$) occurring over a distance of 20 μm laterally.

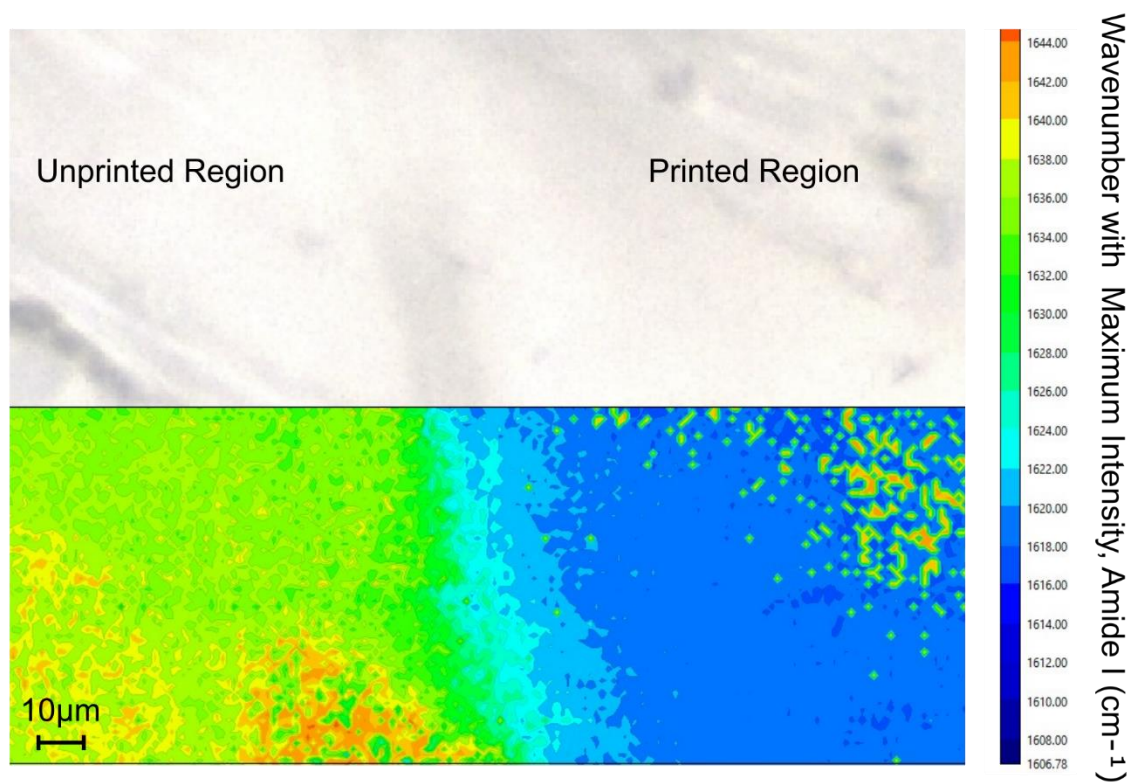


Figure 3.16 Heatmap overlay of border of printed area (80vol% methanol, 8 layers), taken with FT-IR microscope equipped with FPA detector and germanium hemisphere (resolution 1 μm).

3.1.6 Individual Printed Droplet Characterization

To determine the impact of printing these crystallinity inducing inks on the surface topology of silk films, we spin coated silk fibroin solution onto thin films produced via spin coating. Silk solution (5wt%) was spin coated onto silicon substrate at various speeds (2000-6000rpm) and dried. A single or two layers of ink in the form of individual droplets spaced by 150 microns were printed onto these thin films. Figure 3.17 Individual printed droplets of 80vol% ethanol on previously spin-coated RSF film for three different spin-coating speeds. There is a decrease in film thickness with increased spin coating speed, as expected, and the diameter of each drop droplet also increases with decreasing film thickness (Figure 3.17).

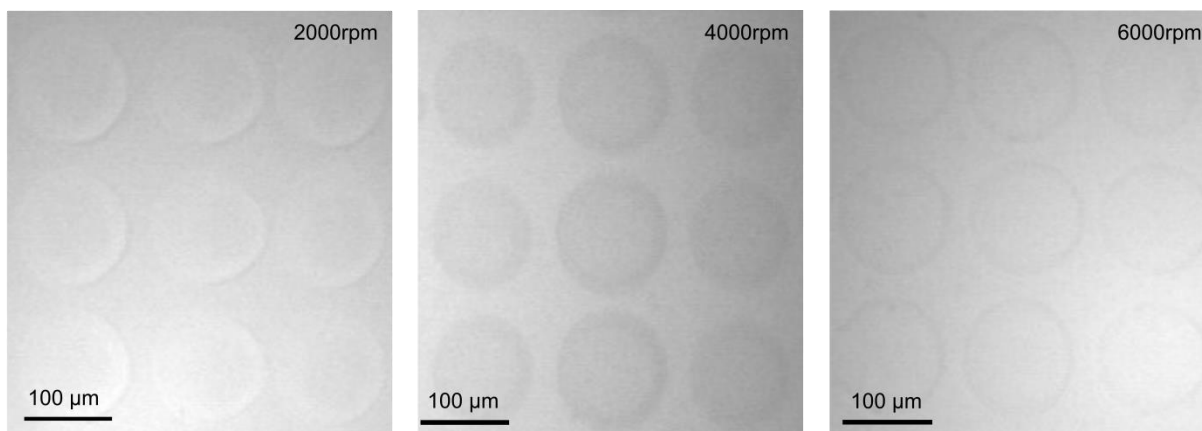


Figure 3.17 Individual printed droplets of 80vol% ethanol on previously spin-coated RSF film for three different spin-coating speeds

These dried droplets were readily measured via AFM (Figure 3.18). Here we can clearly see the formation of round printed surface patterns from each individual droplet, with an increase in thickness of 1-2nm near the edge of the printed area for the case of single layer, with a drop-off below that of the film baseline before returning to the baseline film thickness as you move farther from the center. For two-layer printing, the height of the edges becomes slightly more pronounced, between 5-10nm, with similar shape as 1-layer printed drops. Overall, this suggests that reflow of printed alcohol solutions influence surface topology in addition to crystalline morphology, but that the reflow phenomenon is considerably less than that of films printed with water^{94,95}.

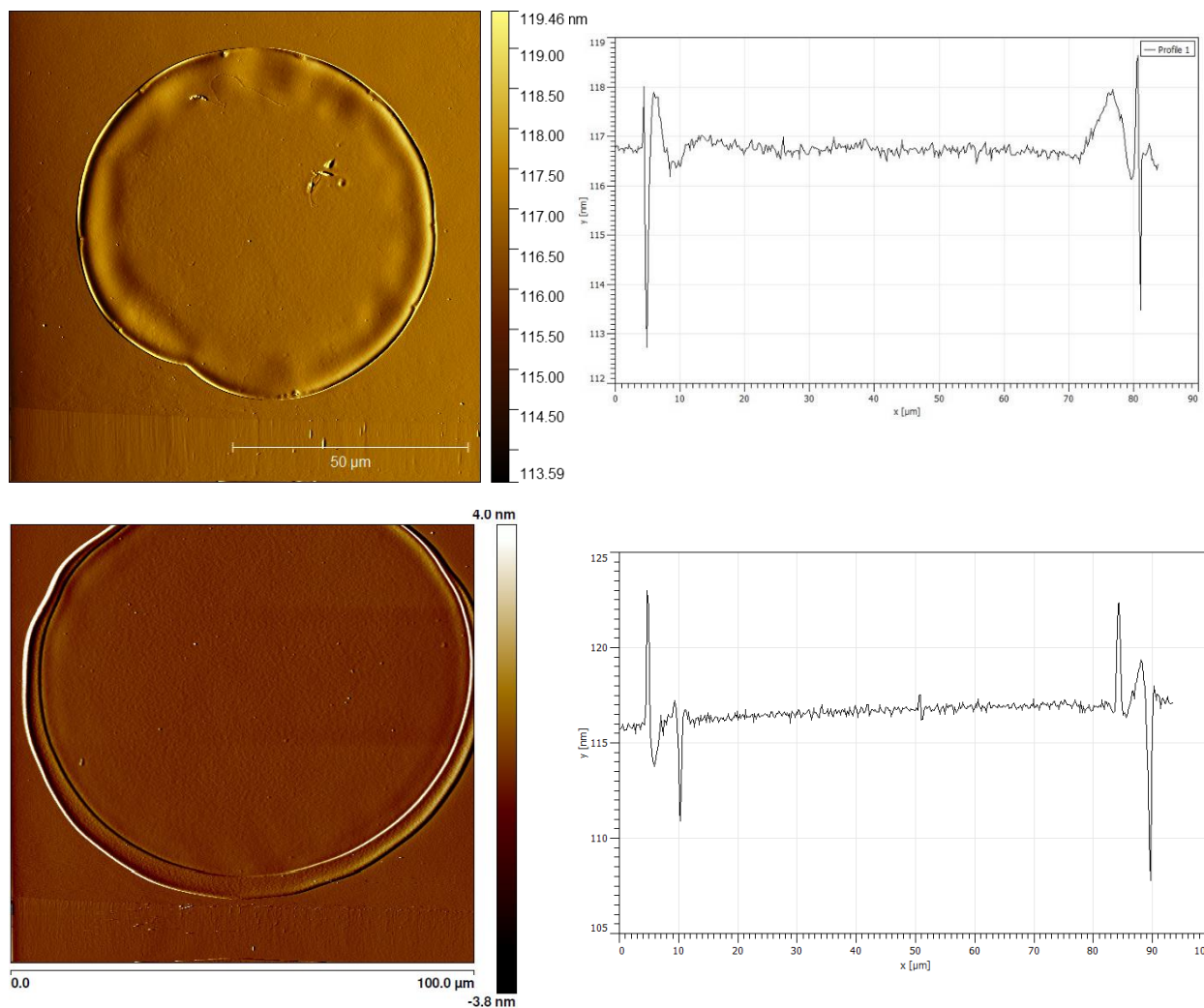


Figure 3.18 AFM scan (contact mode) and height profile across diameter of individual droplets. 1-layer (above) and 2-layer (below) printed 80vol% Ethanol, $\sim 10\text{pl}$ droplet size

3.2 Control of Physical Properties Through Printing

3.2.1 Background on Film Composites

The concept of tuning mechanical properties of materials such as films by combining multiple materials with distinct mechanical properties is quite widespread. Depending on the desired properties, composites are frequently constructed from fibers, fabrics, sheets, particles, or bulk solids, and usually contain a stiffer material held together by a matrix. A common example of this type of material is fiber reinforced polymer, which allows the inclusion of strong and stiff fibers into a matrix that can transfer load

between fibers. Composite materials allow material properties to be tuned by changing the composition and orientation of components, and the influence the orientation and volume fraction of components are both understood and readily utilized in countless applications (Figure 3.19)⁹⁶.

Continuous fibers can be oriented along a single axis of tension to create objects of maximum directional stiffness at the expense of mechanical properties in other axes, or the same fiber can be chopped into small strands and dispersed randomly in the same matrix material to create a material more resistant to forces along any axis at the expense of maximum directional stiffness⁹⁷. Thin flexible composites can be tuned similarly. A common example is filament tape, which often combines a traditional polymer substrate such as polyester, with fibers such as glass, resulting in increased tensile strength and increased resistance to tearing^{98,99}.

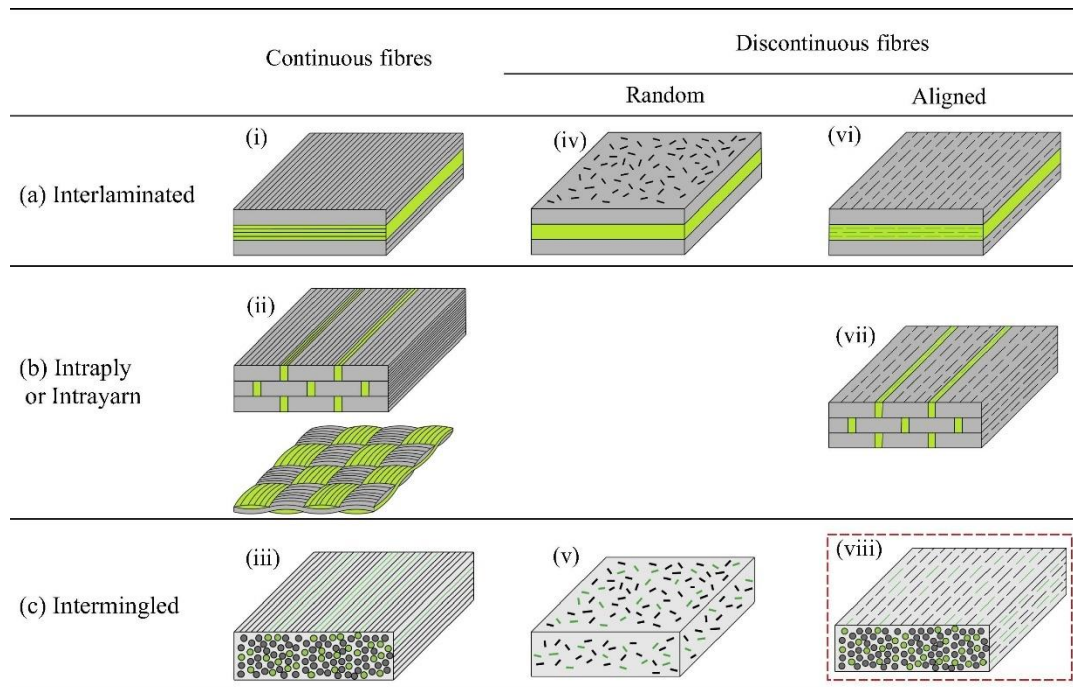


Figure 3.19 Hybrid configurations for continuous and discontinuous fiber reinforced composites. Reproduced with permission⁹⁶

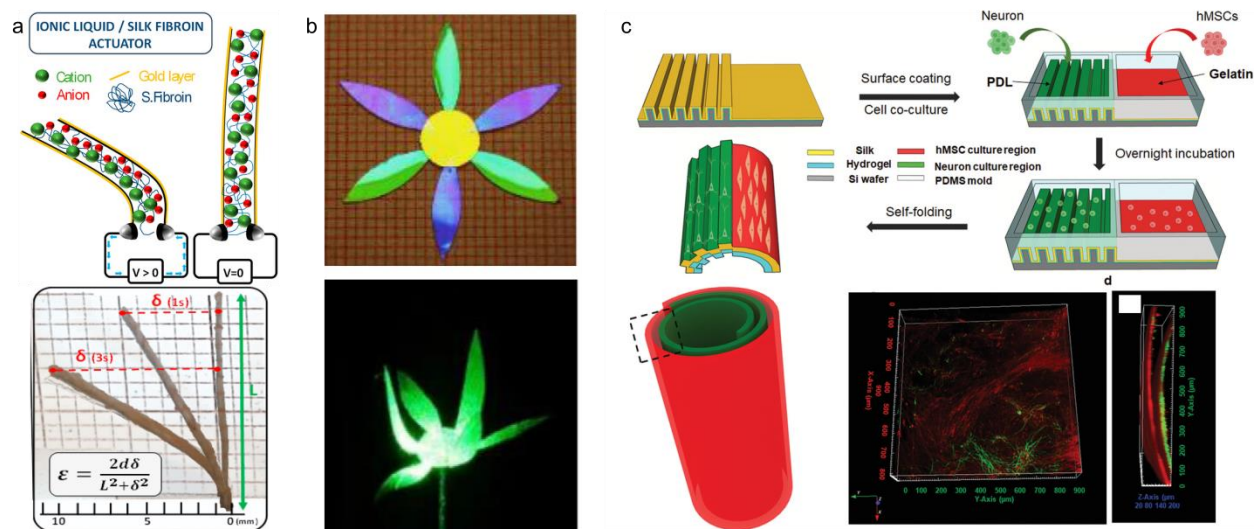


Figure 3.20 Examples silk-containing composite materials. (a) Silk ionic hydrogel actuator. Adapted with permission¹⁰⁰. (b) Light-actuated silk plasmonic PDMS composite. Adapted with permission¹⁰² (c) Self-folding 3d silk/hydrogel cell culture system. Adapted with permission¹⁰¹.

While most composites are produced through the combination of multiple materials, as is also the case with silk fibroin for use as ionic or light-activated actuators, and self-assembling tissue scaffolds (Figure 3.20), several methods exist that allow the production of structures that act like composites but are produced solely from a single material. This has the added benefit of reduced material handling during manufacturing, reduced number of materials to consider for chemical, environmental, or biological compatibility, and the potential to improve material interface that are a commonly failure point in composites. One such method to achieve single material constructs that can be spatially tuned akin to composites involves 3d printing, where the shape, direction, and density of material in certain regions can be used to modulate the properties of the bulk structure. This includes macroscopic approaches, such as an internal truss structure and direction of extrusion, or density of crosslinking in the case of photo-cured polymers^{103–105}. Recently, microscopic-scale approaches have also been incorporated to modulate material properties, such as the alignment of nanofibrils, liquid crystals, polymer chairs, or even isomer distribution of polymers during processing^{106–112} (Figure 3.21). These provide scientists and engineers with a means to produce structures with excellent biological compatibility in the case of 3d printed scaffolds, or ease of manufacture in the case of structural components.

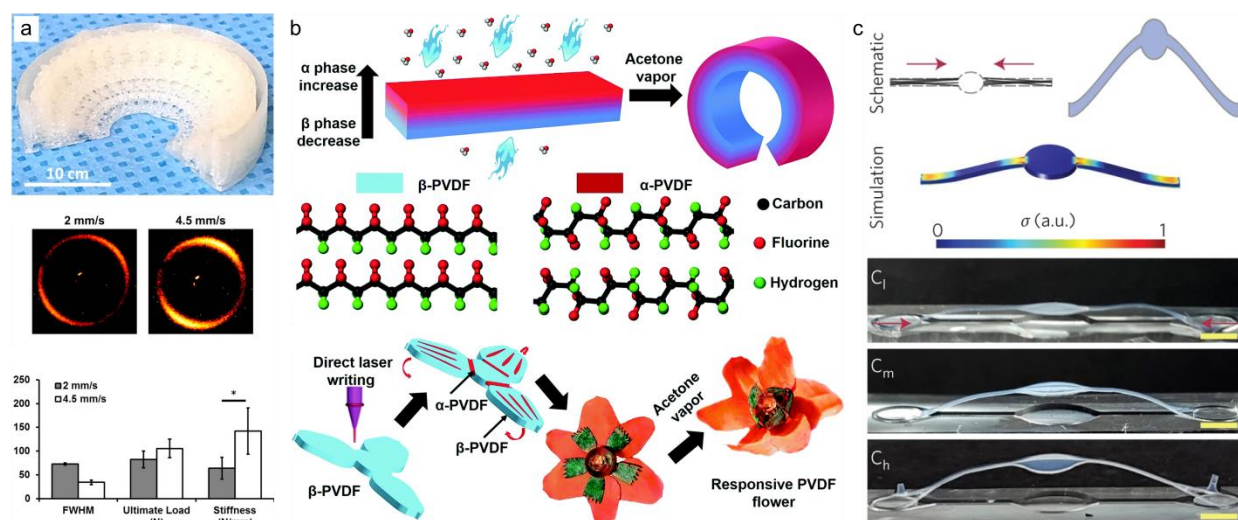


Figure 3.21 Techniques for production of monomaterial composites. (a) Extrusion-based 3d printing with controlled rates to modulate polymer crystallinity. Adapted with permission¹⁰⁸. (b) Laser sintering process to control crystallinity of otherwise homogeneous polymer film. Adapted with permission¹¹². (c) Confined sol-gel transition of silk materials allows for nanofibrillar arrangement. Adapted with permission¹¹¹

In the case of silk fibroin, it is well understood that material properties vary directly with changes in secondary structure^{17,33,113,114}. Accordingly, it seems plausible that selectively patterning crystallinity to create regions with variable physical properties could effectively create a composite material out of a single homogeneous film. Several studies have created single-material silk fibroin composites through the direct printing of both silk and solvent inks^{77,115}, layer-by-layer coating¹⁵, lamination of several films of differing secondary structure¹¹⁶, or through selective dissolution¹¹⁷. However, no one has previously demonstrated the use of inkjet printing to create composite biopolymer through the direct spatial modification of secondary structure of homogenous cast films.

3.2.2 Printing patterns for mechanical control

To compare the impact of printed secondary structure on silk films, patterns of printed secondary structure with increased crystallinity were printed with continuous lines either parallel or perpendicular to the axis of strain. Mechanical testing was conducted in a universal testing machine as described in 2.3.2. Here, however, humidity was controlled for each specimen by heating water underneath each specimen

in a tank designed for submerged sample testing (BioBath) with a custom fabricated lid to maintain humidity and a wireless humidity sensor inside of the tank. Each sample was allowed to equilibrate for 20 minutes at the desired humidity before testing. Silk fibroin films with a thickness of 20 microns were used for printing and mechanical testing in these experiments. Based on optimization work previously described in section 3.1, printing was done with 80vol% methanol as the ink. Line width was maintained at 225 μm wide lines, 2mm spacing between lines, and a drop spacing of 20 μm , with 8 layers of printing for these experiments to ensure significant depth of the secondary structure pattern. Testing was completed at strain rates of 10.00 mm/min with varied relative humidity (RH) based on ASTM D882 based on strain at failure. At a relative humidity of 65%, both films exhibited brittle failure, as do unprinted silk fibroin films, but films printed with crystallinity parallel to the axis of strain demonstrated higher a Young's modulus and failure at lower strains (Figure 3.22b). This is consistent with the trend of silk fibroin materials which are treated to induce crystallinity in literature¹¹⁸. When the same measurement was performed on printed films at a higher relative humidity of 75%, they exhibited a considerably larger plastic deformation before failing compared with the brittle failure mode of lower humidities films (Figure 3.22c). At this condition, the films with a parallel printed pattern again exhibited higher Young's moduli than those with perpendicular printed pattern, but the magnitude of the contrast between the Young's moduli of the two orientations was much greater.

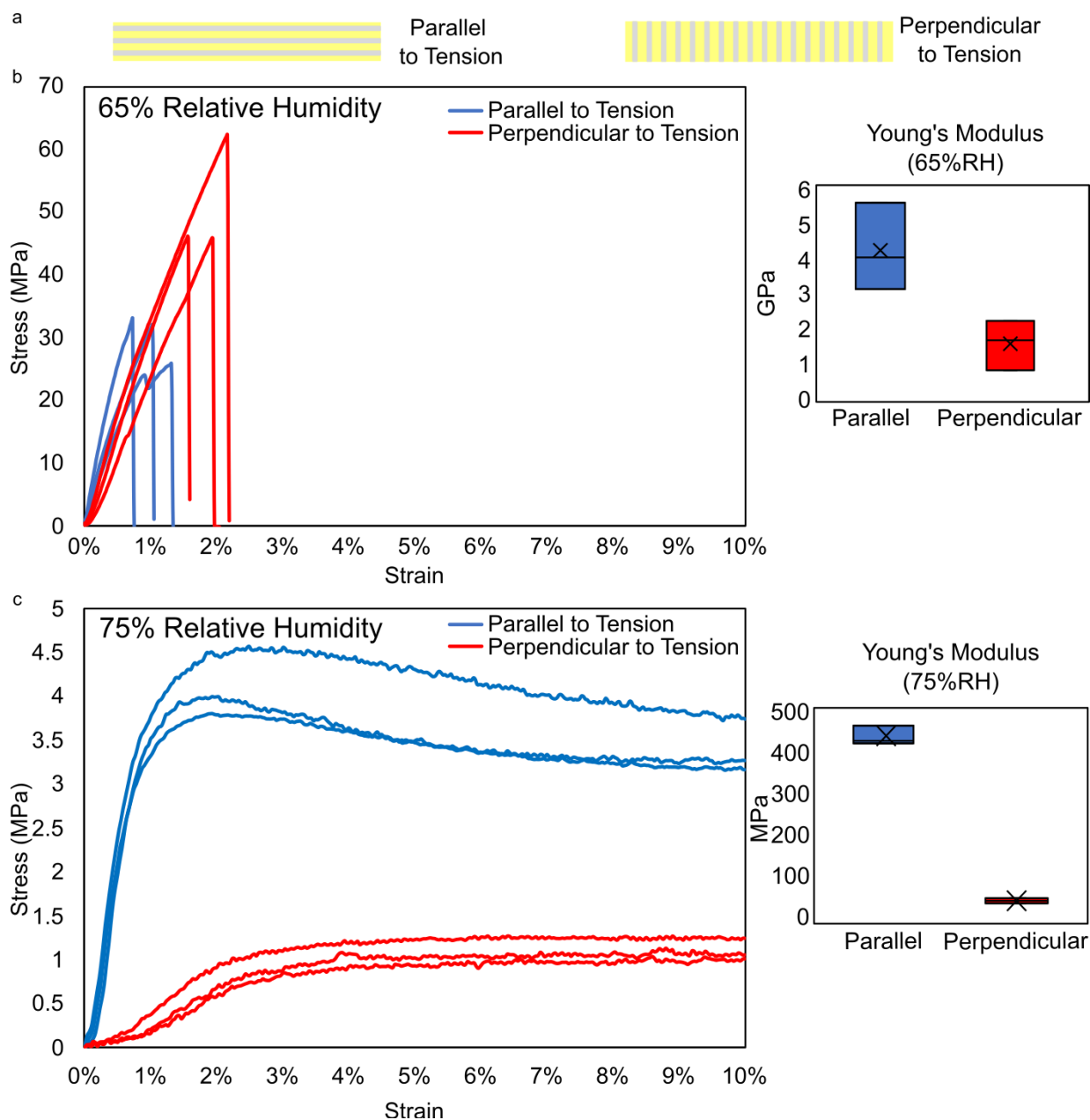


Figure 3.22 Tensile performance for printed samples. (a) Illustration of printed samples (b) Stress vs strain for films at 65% relative humidity. (c) Stress vs Strain for films at 75% relative humidity. (d) Comparison of Young's modulus calculated from linear portion of curves

Similar experiments were conducted with constant stress instead of constant strain by clamping weights to silk films and exposing them to controlled levels of humidity, measuring a creep-like phenomenon. In these experiments, films with parallel printed patterns demonstrated increased resistance to deformation at 75% relative humidity, but they tended to fail earlier than those with a perpendicular printed pattern (Figure 3.23). These align with prior work demonstrating that humid

environments cause an increase of plasticity in silk fibroin, and a subsequent reduction in Young's modulus and tensile strength¹¹⁹.

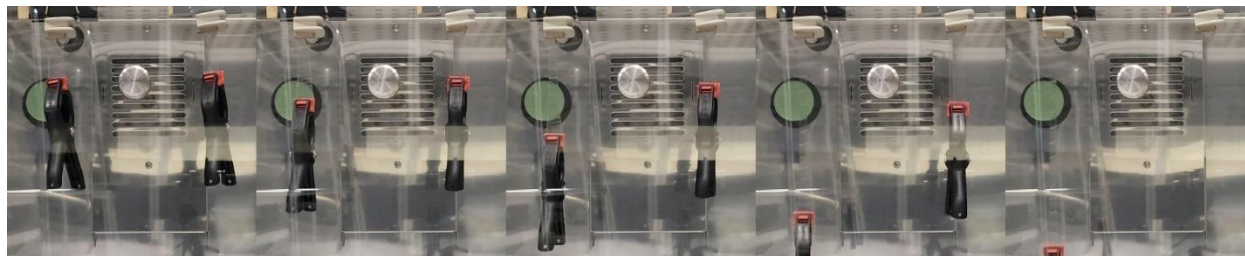


Figure 3.23 Timelapse of printed films under constant stress, 75% relative humidity, 25°C. From left to right, initial position to 10 minutes, in 2-minute intervals. Left-most sample is a film printed with a pattern perpendicular to stress, right is parallel printed pattern.

3.3 Humidity Driven Actuation of Printed Films

3.3.1 Actuator Background

An additional area of composite material research and functionalization involves utilizing material property mismatch to create actuators which exhibit a controlled mechanical force or displacement in response to an applied external stimulus. The ability to apply force, change shape, or create motion can be utilized for many different applications in the realm of robotics and automation, from implantable devices which expand only after reaching a target area to minimize surgical risks, to garments and structures that can help regulate temperature and humidity, to self-powered reconfigurable optics. Stimuli commonly applied to drive actuation include temperature, pH, solvents, magnetic fields, or light. Many actuators are made by combining two materials with different responses to a particular stimulus, for example, bimetallic strips made of two metals with differing thermal expansion coefficients for thermally regulated circuits, fibers aligned in specific paths in a matrix for 4-D printing^{120,121}.

Several examples exist of silk-containing composite actuators, including electronically responsive ionic liquid-silk films and silk/silk-polypyrrole films, humidity-responsive aligned-fiber silk/polyimide, aqueous solution self-folding silk/agarose films, light responsive gold-doped silk-inverse-opal/PDMS, and both silk/PDMS and silk/paraffin wrinkling bilayers^{68,100–102,122–125}. Furthermore, silk film has been shown to be responsive to the application of vapor, both aqueous and solvent-based, exhibiting controllable

actuation^{126,127}. Exposure to vapor gradients caused one side of films to expand (humidity) or contract (alcohol) preferentially compared to the un-exposed side, causing motion to occur in the direction of the side of reduced relative thickness (i.e. away from water vapor or towards alcohol vapor, Figure 3.24). It is suggested that water molecules diffuse into the fibroin chains by breaking the existing inter/intramolecular hydrogen bonds and form hydrogen bonds with the polar groups such as C=O, -NH, and -OH.

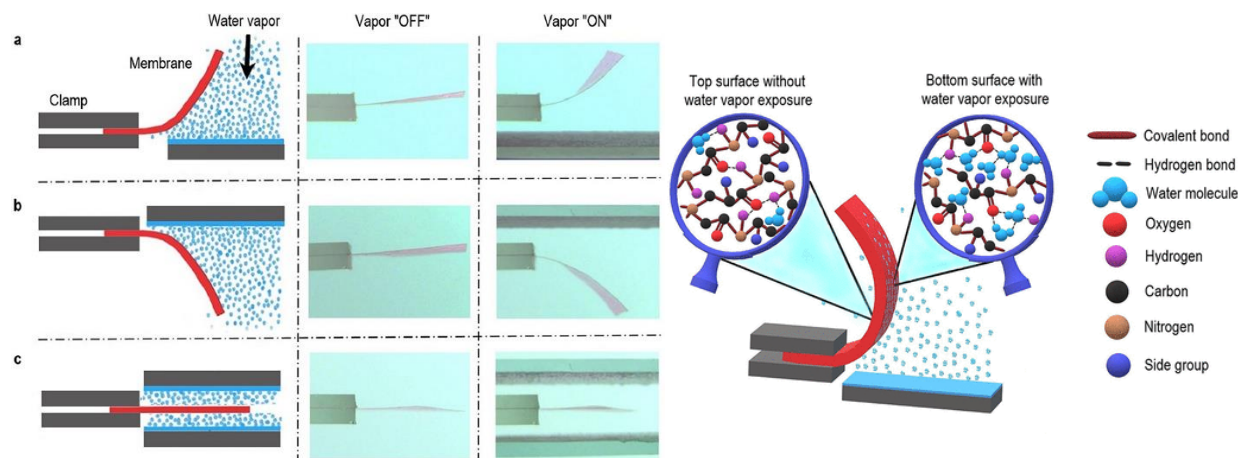


Figure 3.24 Example of a silk fibroin film actuator produced using trifluoroacetic acid as a solvent. (Left) Schematics and images of the film before and during exposure to water are shown for three different exposure conditions. (a) Bottom surface is exposed to water vapor. (b) Top surface is exposed to water vapor. (c) Homogeneous exposure to water vapor. (Right) Schematic representation of the presence and absence of water molecules at the hydrophilic domains. Adapted with permission¹²⁶

Here we expand on this work by combining spatial control over secondary structure to create similar water vapor responsive actuators made from silk fibroin, but with composite-like control over this actuation using a single material.

3.3.2 Modulation of Actuation with Printed RSF Constructs

To create the monomaterial actuators, printing was conducted based on results from solvent and printing optimization described in 3.2. Patterns were printed on one side of amorphous silk fibroin films with 80vol% methanol solutions. In a simple example, printing lines along the long axis of a film caused the film to curve along the long axis of the film towards the printed side, and printing lines across the short axis of the film caused the film to bend along the short axis of the film, again towards the printed side (Figure 3.25a). Spacing was varied intentionally to determine the effect on humidity responsiveness. Curvature increased with reduced print line spacing (Figure 3.25b). This appears to be due to the

increased crystallinity causing increased density locally in printed areas, leading to creation of stress concentrations^{66,128,129}. This behavior could also be partially attributed to the reduced absorbance of water by highly crystalline silk and the associated reduced swelling in the presence of water vapor^{130,131}. While the patterns are being printed, the silk films are held flat by the vacuum of the printing platen. After removing the printed portions from the larger film sheet through cutting with a surgical scalpel, the film actuators can freely move and reach an equilibrium state for a given relative humidity effectively creating 4D behavior. Applying humidity gradients to the film caused the films to actuate, moving away from the humidity by rolling and curling as seen in previous silk film actuator work^{126,127}. Here, however, the films bend along the paths dictated by the printed regions (Figure 3.25c).

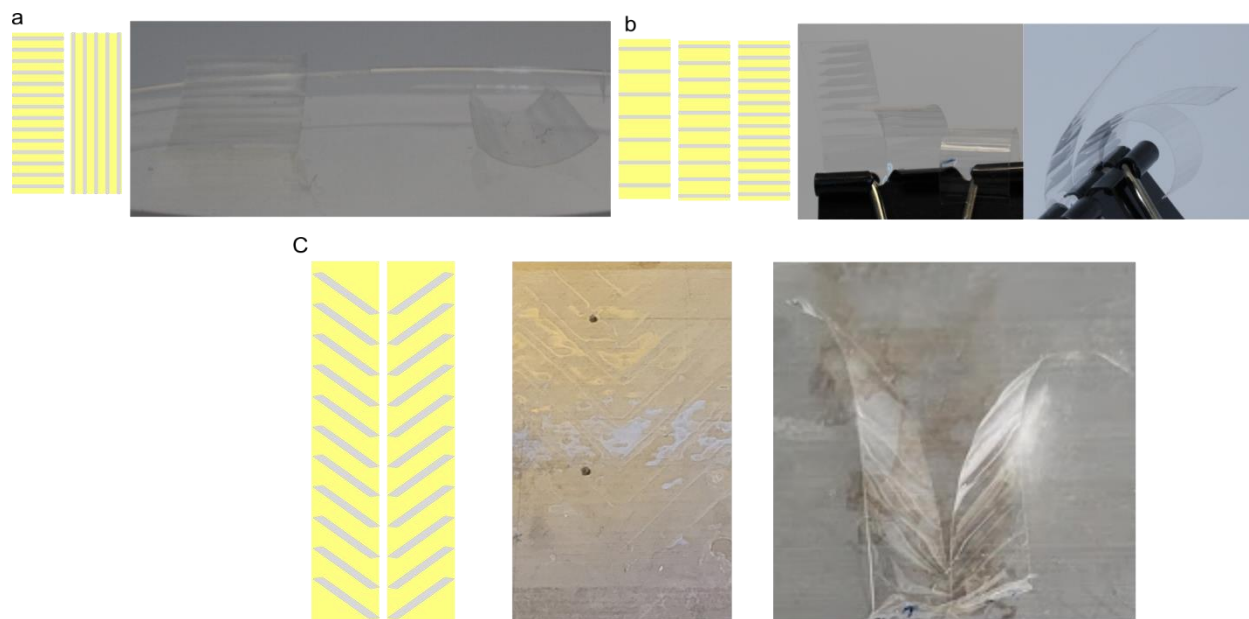


Figure 3.25 Printed Structures and resulting forms after exposure to humidity. (a) Parallel vs perpendicular prints, and corresponding equilibrium states for same starting substrate. (b) Variable print line spacing, and corresponding front and side views. (c) Diagonal pattern, with printed film still on printer platen, and final actuated films

3.4 Alternative Approaches

Several other approaches might be useful in achieving similar final structures and intended functionality as described here without requiring inkjet printing equipment. One that was briefly explored is the controlled application of heat spatially to silk constructs during heating to induce higher crystallinity selectively. When cast films were dried on patterned aluminum blocks, regions of increased crystallinity were observed near areas of contact with the thermally conductive metals (Figure 3.26). However, the final films were not as flat as the films lifted off the substrate while drying. Additionally, local variations in thickness were observed near the patterned areas, likely due to local heating gradients.

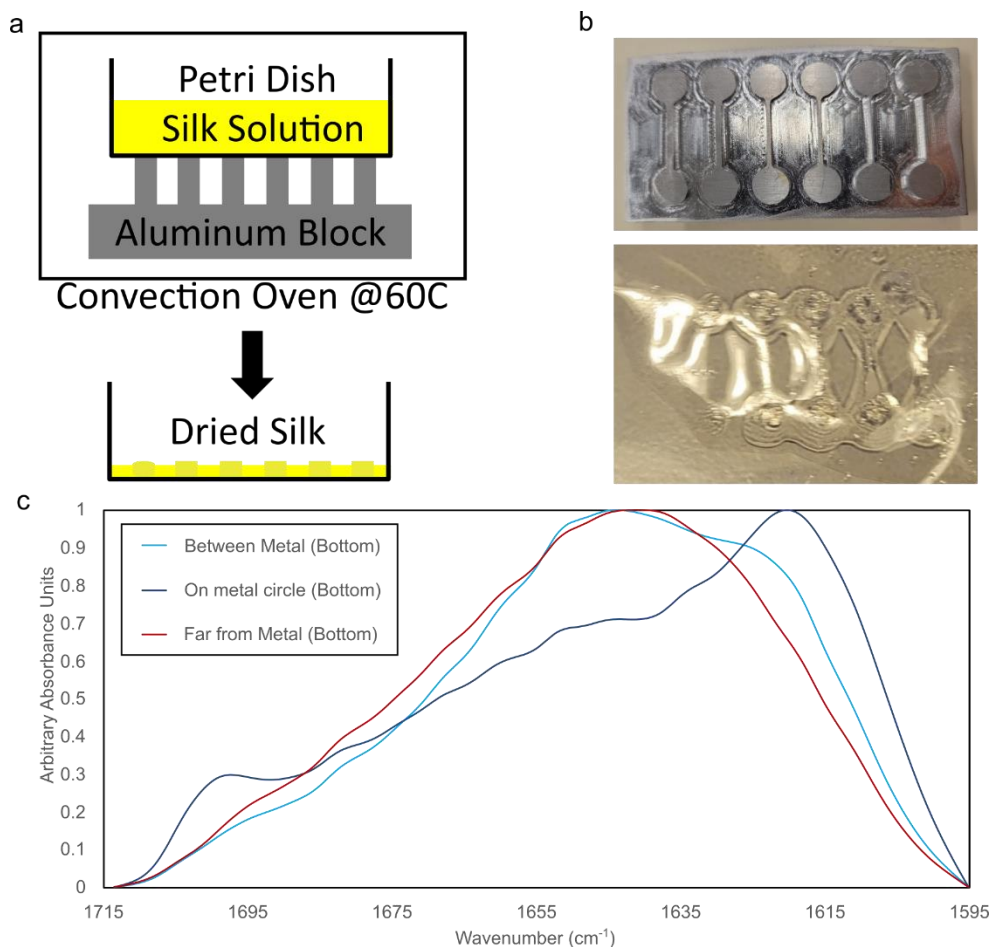


Figure 3.26 Thermal control over secondary structure and topology of silk fibroin cast as a solution and dried on shaped aluminum. (a) Schematic of process. (b) Aluminum block and dried film. (c) FT-IR spectra of various locations on dried film.

Similarly, the patterned aluminum block was used to thermally emboss a silk film, but the resulting construct exhibited increased crystallinity in areas not directly in contact or with the highest conduction,

but rather in between these regions. This is potentially due to the trapping of moisture in the enclosed pockets, where the heated water vapor could induce crystallization as is routinely employed for silk fibroin¹³². It is possible that other approaches for local heating of silk fibroin films, such as a photothermal-based process could sufficiently change the local environment to be favorable for increasing crystallinity. Further experiments are needed to understand these phenomena.

Use of mask-based approaches can be applied with humidity and solvent vapor to modulate secondary structure in a manner aligned with negative-tone resist processes¹³³. One route for this process is to use water impermeable shadow masks such as silicone, plastic, or metal closely mated to the surface of a silk fibroin film. This masked film can then be placed in a high humidity chamber with variable temperature, and the exposed portions of the shadow mask will experience an increase in humidity, and the area covered by the mask will have a reduced effect, if any, depending on diffusion during the humidity exposure (Figure 3.27). While this approach can provide spatial control over secondary structure, the minimum feature resolution that can be achieved was observed to be larger than with direct inkjet printing, likely due to diffusion under the masked areas through the unmasked portions of the film. Furthermore, the technique is prone to condensation when larger masks and higher temperatures are used, though this can potentially be mitigated by equilibrating the sample and mask to the appropriate temperature before placing into the humid environment.

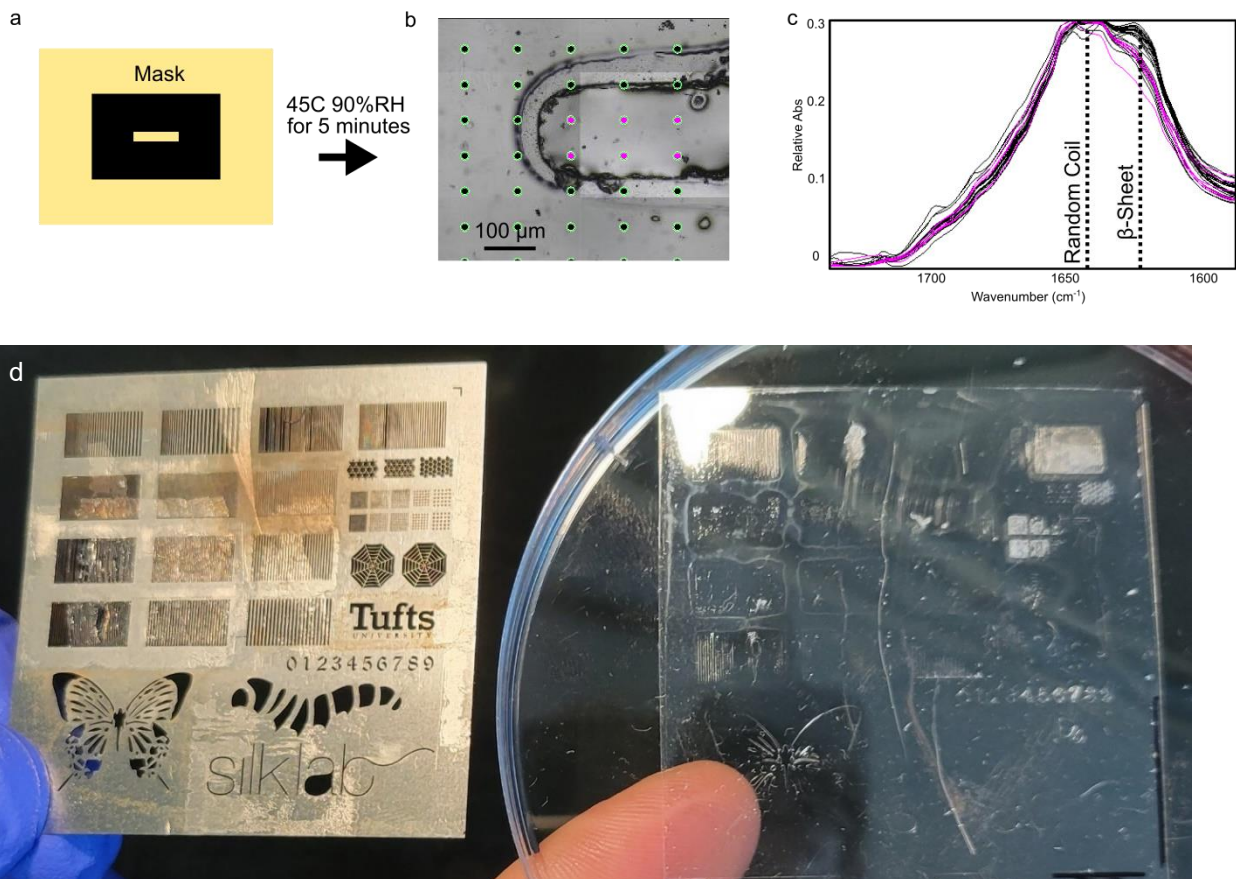


Figure 3.27 Resolution Test of Shadow-Masking during Water-Vapor Treatment. (a) Illustration of mask on amorphous film. (b) FT-IR Microscopy of exposed (pink) and covered (black) areas of film. (c) FT-IR analysis of treated film. (d) Finer resolution shadow mask example

Experiments were also conducted using directed steam rather than placing the sample into a humidity chamber (Figure A.6). This technique benefits from not being constrained by oven size, and the secondary structure was produced quickly after the start of application of steam, but the high flowrates caused condensation and reflow during initial moments of steam application. As an alternative, this approach was applied to silk-coated non-woven textiles and reflow was drastically reduced. Further work is needed to optimize the approach. As is the case for all mask-based approaches, these approaches require a unique mask be produced for each pattern, which both increases complexity and cost for each experiment, though has the potential to decrease variability between samples compared with direct patterning.

3.5 Future Directions

One area of the direct patterning technique that requires further investigation is in the understanding of the depth of the impact of the printed inks. There appears to be a trend for increased depth with increased layers of printing based on optical observation, and large volumes deposited at a single time, such as tens of microliters from a micropipette, can cause increase of crystallinity on the untreated side of a 20 μm amorphous silk film. While printed silk droplets spread to diameters of 30 μm or larger, no increase of crystallinity on the opposing film surface was observed using FT-IR spectroscopy. This could be due to the increased crystallinity on the printed side of the film reducing diffusion of the inks, or simply due to the relationship between evaporation and time needed for a sufficient ink quantity to diffuse beyond the crystalline surface layer. Generally, measuring the depth of secondary structure with the techniques readily available is not trivial. FT-IR and Raman spectroscopy coupled with optical microscopy both failed to produce resolution in the depth direction and require careful sample preparation to view a cross section without disturbing the printed structures on films of thicknesses produced in this work. Optical microscopy two-photon fluorescence microscopy or other techniques might provide valuable insight into the depth component based on previous work, and could be a solution to determining the depth component without requiring cross-sectioning^{134,135}. A better understanding of the mechanism and control over the depth of the effect of this technique could assist in the efficient design of structures and features using 3D-modeling and finite element analysis. Additionally, the ability to concisely measure swelling of various levels of crystallinity of silk fibroin would be quite important to building such models for composite actuators. Such a measurement was conducted with mechanical profilometry which suggest that films crystallized with water vapor annealing expand similarly to amorphous silk fibroin films, at least until reaching the relative humidity where the amorphous silk begins to dissolve due to moisture (Figure A.7). However, these results might be inaccurate at high humidities given the softening of silk fibroin at such conditions, as well as imprecise control of humidity in the profilometer enclosure and the effect of humidity on the instrument's readings.

While this work focused on the modulation of mechanical properties of these monomaterial constructs, the same process also modulates the solubility and permeability of silk fibroin. For example,

printed portions become water insoluble compared to the soluble amorphous material (Figure 3.28).

Highly crystalline regions generally resist enzymatic degradation more than less crystalline regions^{28,67,114,136}. This opens the technique for use in applications where controlled degradation is useful, such as in implantable devices, as well as in the controlled release of molecules from a silk film or matrix for drug delivery, or chemical reactions. Crystallinity can also influence the release of molecules contained inside a silk matrix^{28,136–139}. In parallel, pattern designs can be used to change exposed surface area of different crystallinities of silk fibroin (Figure 3.29).

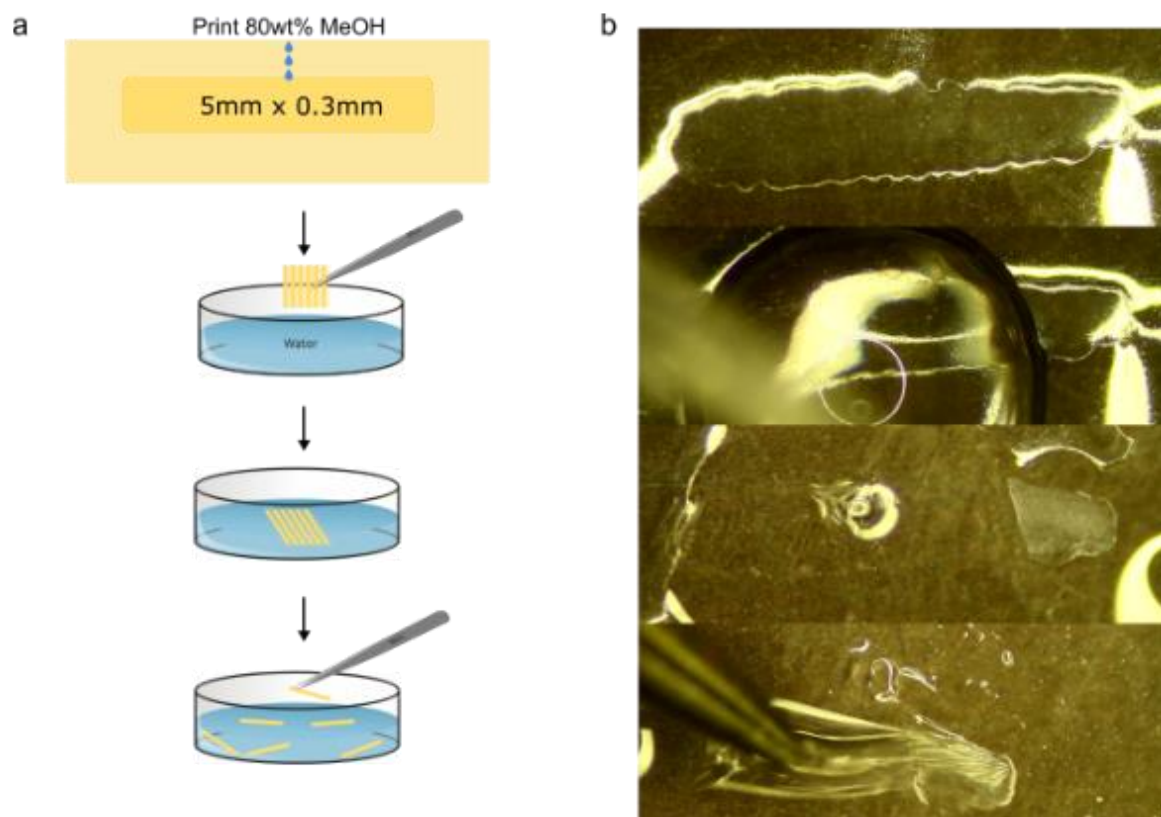


Figure 3.28 Example of extraction of crystalline printed pattern after immersion in water. (a) Schematic of process. (b) Time lapse images of dissolution and extraction (immersion time of 4 minutes).

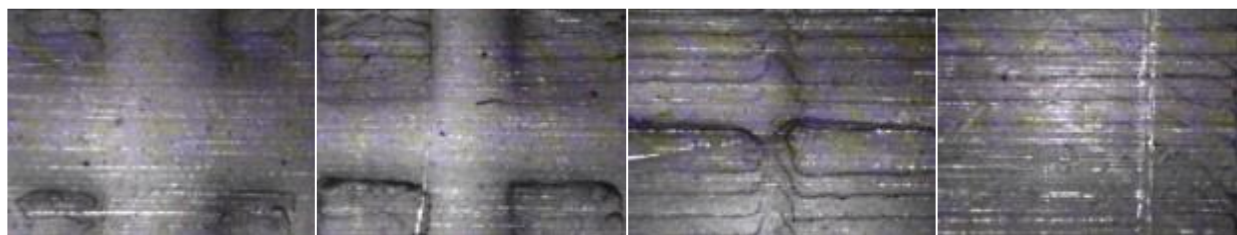


Figure 3.29 Infill patterns of (from left to right) 12.5%, 25%, 50%, and 100% using squares, 80vol% methanol, 1 layer print.

Additionally, carefully designed patterns may impart additional functionality not deeply explored in this work, such as those which impart multi-directional stability to amorphous films, such as grid or hexagonal patterns, or those which cause more complex three-dimensional shapes to form, such as rounded curvature, or even meta-material which can provide flexibility through structure, such as the Miura Ori and its negative Poisson's ratio (Figure 3.30). These might be especially helpful in the case of silk fibroin, which is brittle without the inclusion of a plasticizer, and becomes even more brittle with increasing crystallinity. Combining these pattern motifs by optimizing printing parameters to create areas of resistance to bending and areas of specific bending patterns could potentially enhance toughness at ambient humidity.

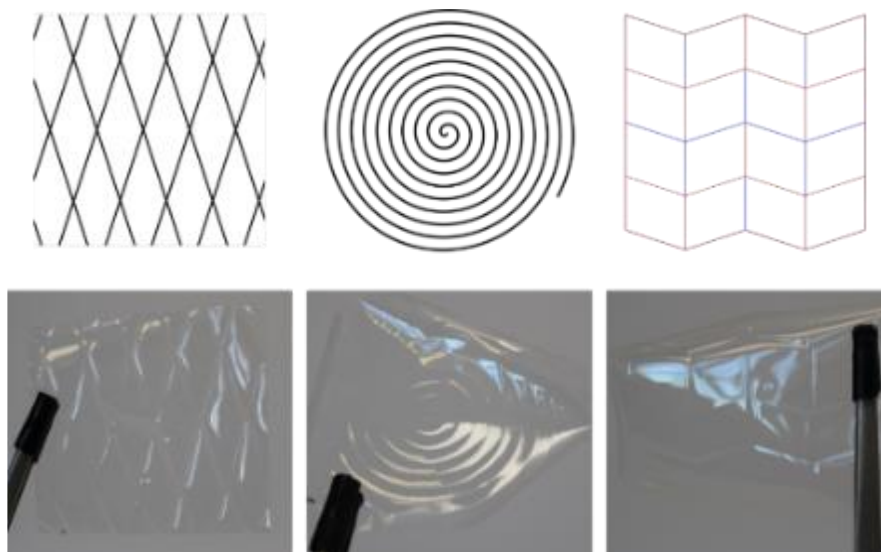


Figure 3.30 Several patterns printed to explore pattern influence on final film shape.

Chapter 4: Concluding Remarks

In Chapter 2, coating was successfully implemented at the lab scale through a deeper understanding of the influence of surface tension, substrate choice, and concentration. Lab-based experiments were also carried out to explore drying temperature, orientation, and coating thickness. The understanding gained from lab scale experiments was used to produce films using a pilot-sized slot-die coating system. Learnings from this pilot trial were then applied to a larger, industrial scale knife-blade production line. Several functional films were produced using techniques compatible with R2R production and demonstrate that RSF films can be printed, patterned, crystallized, and made water soluble.

In Chapter 3, the technique of applying inkjet printing technology to spatially control the secondary structure of otherwise homogeneous, amorphous silk fibroin films was developed. By assessing characteristics of several short-chained alcohol aqueous solutions and their impact on secondary structure, inks which can be printed on demand and that are able to induce crystallinity in silk fibroin films were developed. By modulating printing parameters, the printing process was refined for several of these alcohol-based inks, and the technique's effect on secondary structure was assessed via FT-IR microscopy. This technique was employed to precisely modulate material properties, such as mechanical robustness, by dictating the arrangement of molecular structures within the film. Furthermore, the ability to manipulate these properties at specific locations of a silk fibroin film enables the creation of actuatable materials that can respond dynamically to environmental stimuli, such as humidity gradients. Despite the vast library of potential applications where silk fibroin has already demonstrated promise, this technique introduces a new dimension to the functionality of further transforming it into a smart material suited for advanced applications in biomedicine, textile engineering, and environmental sensing.

While silk fibroin is an impressive material with growing scientific interest, a lack of scalable processes for converting the regenerated silk into functional forms limits its utility despite the availability of the raw material due to the well-established sericulture industry. In this work, roll-to-roll production and inkjet printing methods were investigated as scalable means of filling this technological gap. These techniques demonstrated the ability to maintain and even control the delicate process by which

regenerated silk fibroin proteins transition from a solution to the solid state in film formation. This work expands upon prior work in silk fibroin processing and devices manufacture, providing a foundation for future work in scalable production and functional tunability. Moreover, these developments hold promise for transforming the use of natural polymers such as silk fibroin, offering efficient, controllable, and versatile solutions that could meet the demands of various applications, from food coatings to transient electronics. The implications of this research extend beyond academic inquiry confined to the laboratory, providing pathways for industrial adoption and commercial exploitation of silk fibroin as a high-performance, programmable material.

Appendix A:

Table A-1 Slot-Die Roll-to-Roll Trial at University of Massachusetts

4/25/2023 R2R Trial - Slot Die			Oven Speeds				Substrate Tension		Length per condition (m)	
Gap to substrate	Shim Size	Line Speed	Vacuum	Zone 1 Supply (Hz)	Zone 2&3 Supply (Hz)	Zone 1,2&3 Exhaust (Hz)	Dryer Substrate Tension (kg)	Unwind Tension (kg)		
0.432mm	125um	15mm/s	On	40	55	60	2.3	2.3		
Solution	Substrate	Pump Speed (ml/MIN)	Infrared (%)	Corona Treatment	Web Temperature (°C), Zone:			Temperature (°C) Setpoint	3.5m	
					1	2	3	Zone 1,2,3	Notes:	
20wt% SF	Melinex ST505 (125µm PET)	53							At this pump speed, silk fibron solution floods the die head	
		30							Reduced pump rate for improved coating uniformity	
		20				22	49	22	60	Measurements were taken at different pump speed to test dry thickness.
		25				34	52	21	60	
		25				36	53	33	60	
		27.5				23	53	22	60	Dry coating is clear and can be peeled away from the substrate
		27.5		60 Watts	26	53	33	60		
20wt% SF	FLX002698/Outside	27.5				40	53	21	60	Flexcon substrates curl severely with Infrared drying, likely due to thinness
		27.5				37	52	16	60	
		27.5	25	25	60 Watts	44	51	22	60	
			20			21	53	21	60	
20wt% SF	Melinex ST505	20			24	37	22	80	Increase temperature to control better drying	
		25			24	38	22	80		
20wt% SF	Melinex ST505	20			26	69	22	80	Lacquer puddling at the die continue throughout each setting.	
5wt% Glycerol		20			24	37	23	80	Coating thickness was less than 20 um at each conditions. Samples provided for further evaluation	

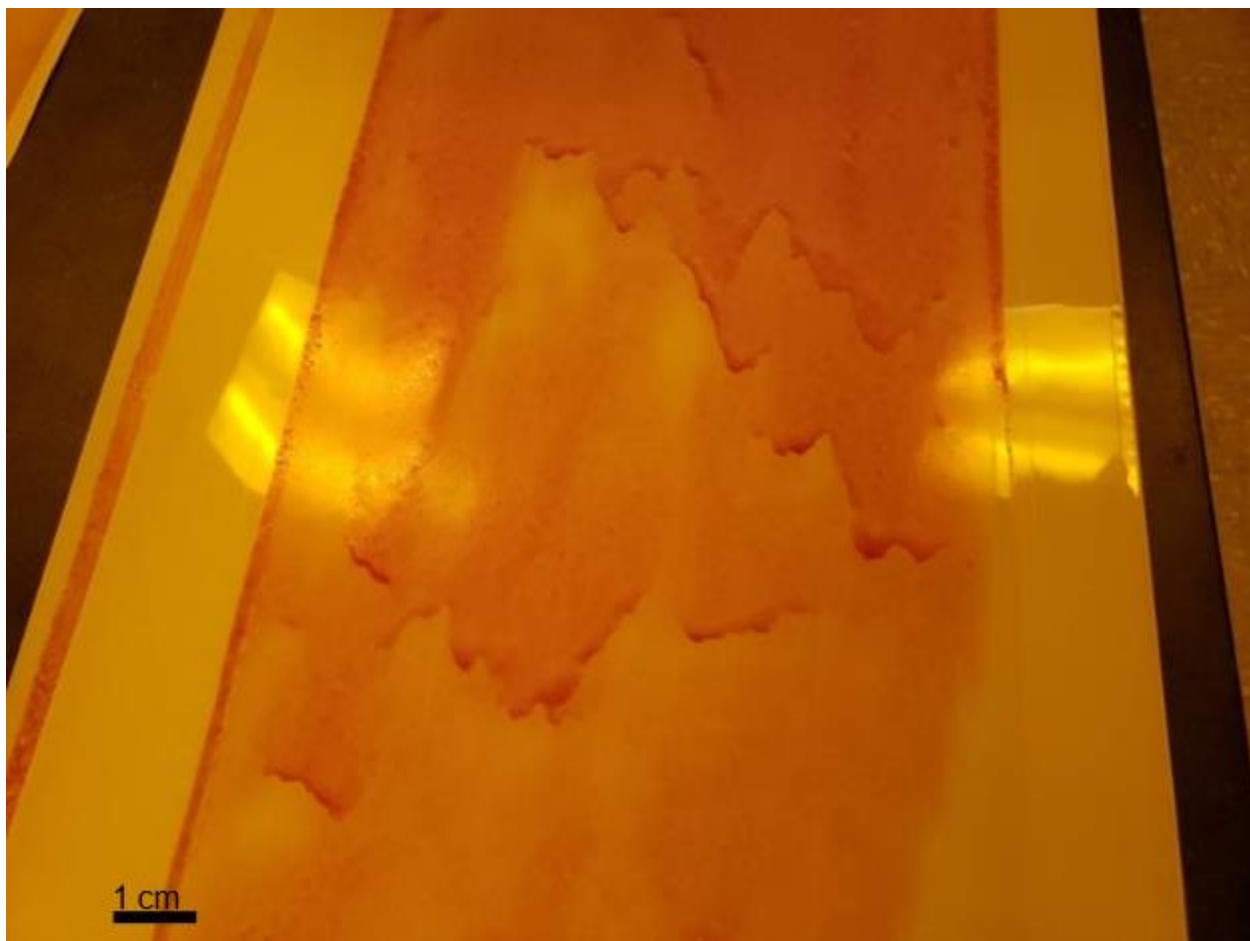


Figure A.1 Example of defects from RSF ink with phenol red that was dried in a vertical orientation

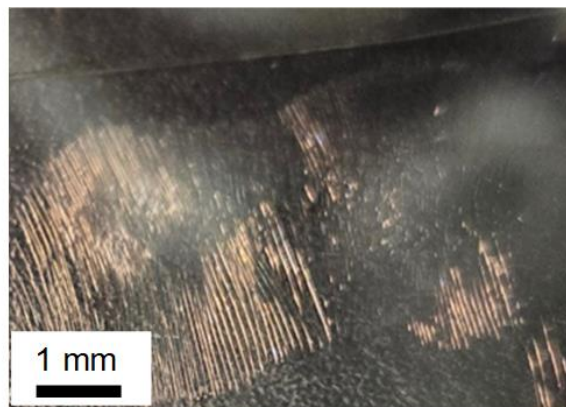
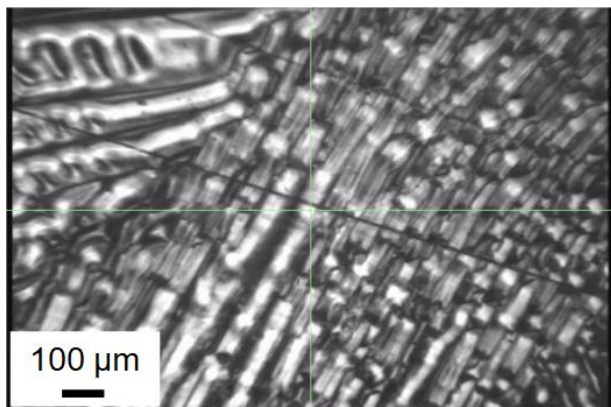


Figure A.2 Cracking of 90vol% Methanol printed pattern viewed with optical microscope (left) and macroscopic camera (right)

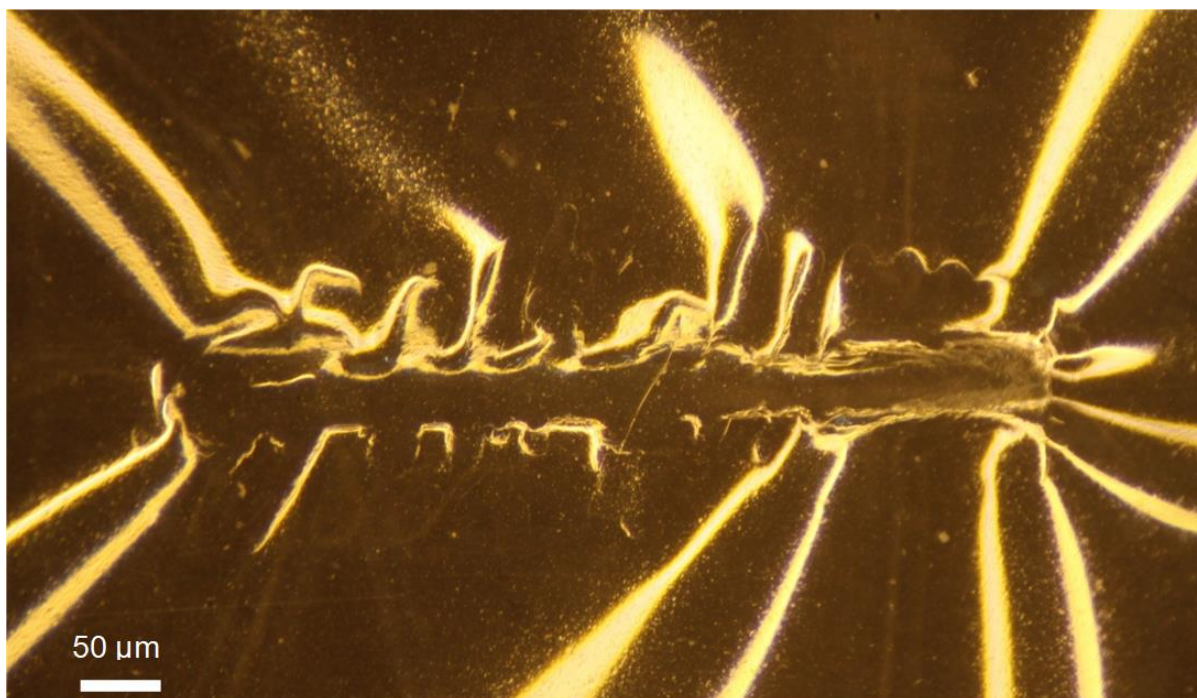


Figure A.3 Contraction observed when applying 80vol% Methanol ink onto amorphous silk fibroin film (20μm thick)

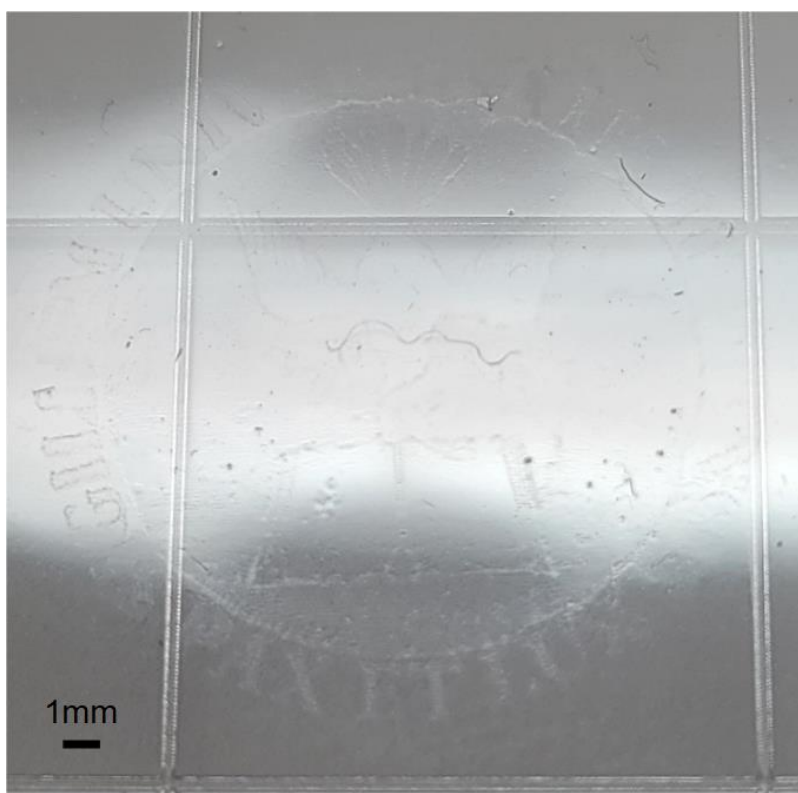


Figure A.4 Printed Tufts Seal, 25 micron drop spacing, 80vol% Methanol.

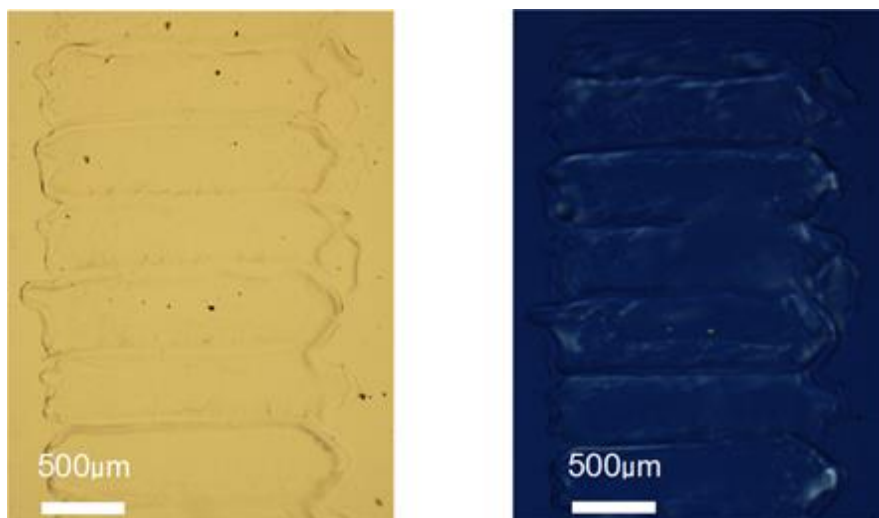


Figure A.5 Optical microscopy of printed silk fibroin film in brightfield transmission without (left) & with cross polarization (right) (16 layer, 80vol%Methanol).

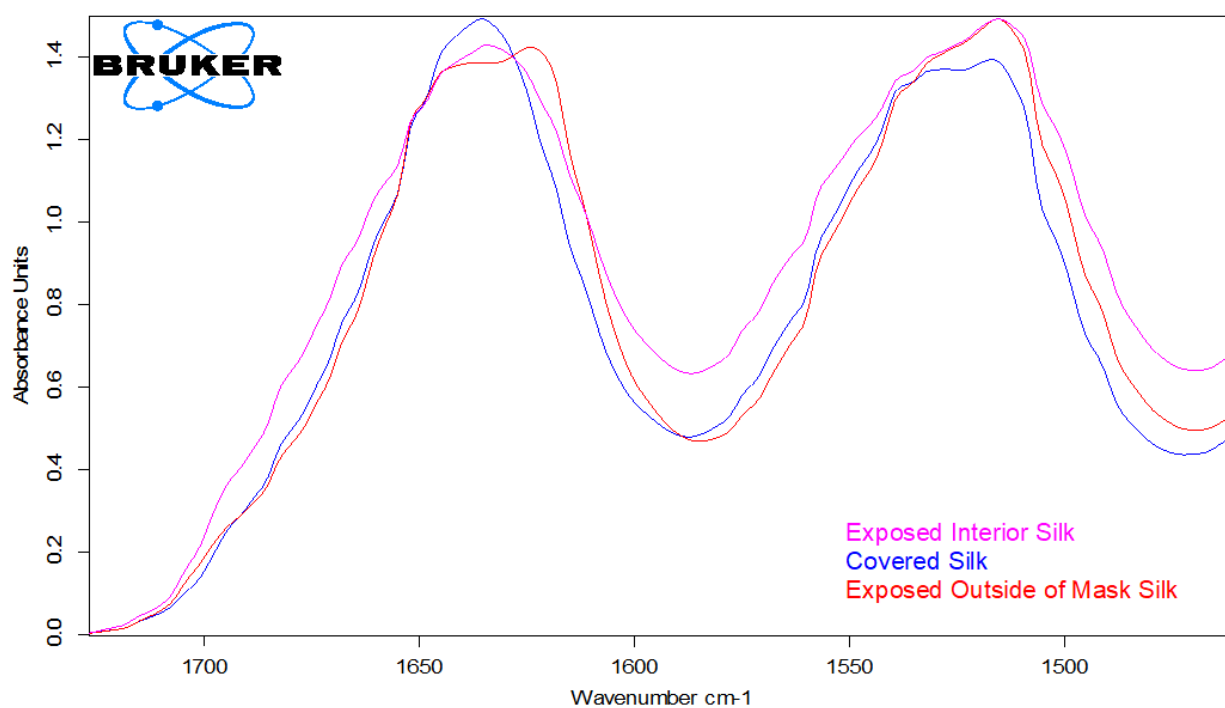


Figure A.6 FT-IR spectra of steam-treated silk-film using acrylic shadow mask showing increased crystallinity in the internal portion of the film exposed by the mask, but even higher crystallinity in completely exposed portions outside of masked region.

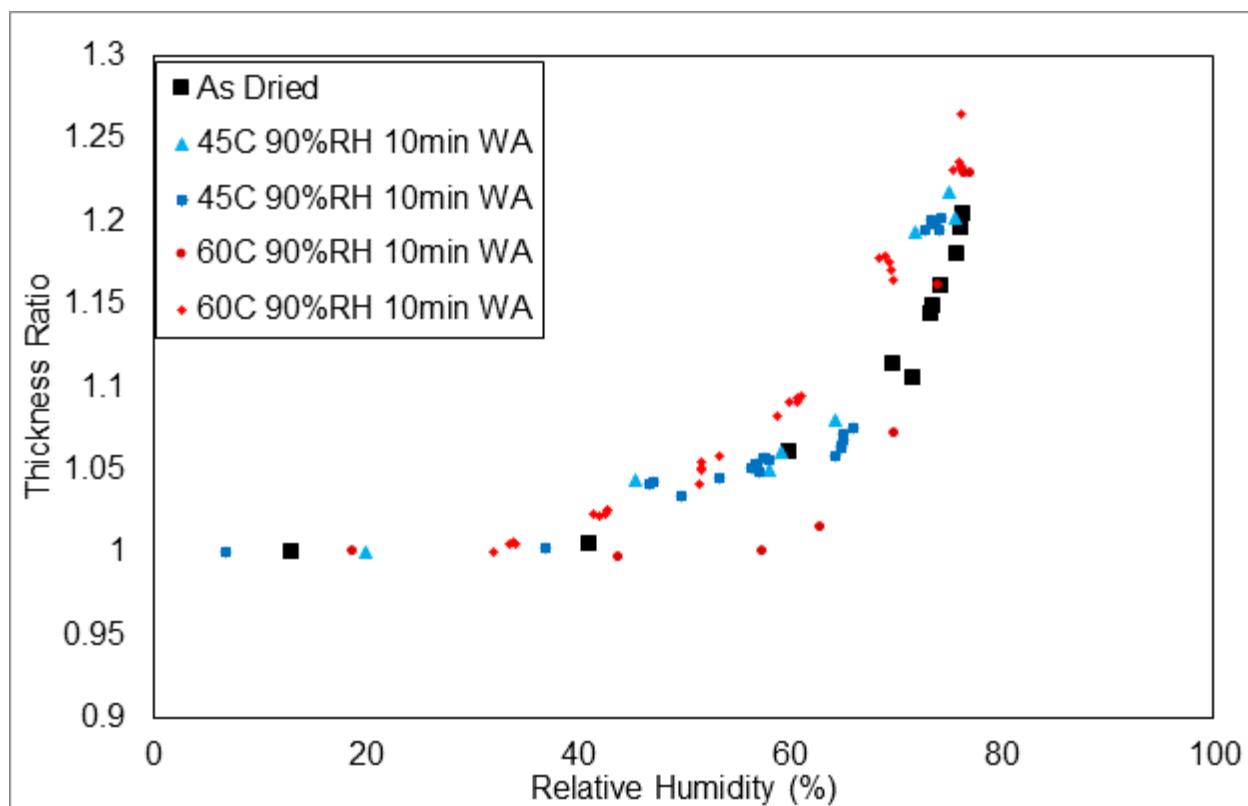


Figure A.7 Swelling behavior of 20-micron silk fibroin films under increasing humidity measured via contact profilometer.

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