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Silk Cryogels for Microfluidics

by

Christopher David Hinojosa

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
in
Mechanical Engineering

Thesis Committee:
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Portland State University
2012

Abstract

Silk fibroin from silkworm cocoons is found in numerous applications ranging from textiles to medical implants. Its recent adoption as a biomaterial is due to the material's strength, biocompatibility, self-assembling behavior, programmable degradability, optical clarity, and its ability to be functionalized with antibodies and proteins. In the field of bioengineering it has been utilized as a tissue scaffolding, drug delivery system, biosensor, and implantable electrode. This work suggests a new application for porous silk in a microscale chromatography column. We demonstrate *in situ* cryotropic polymerization of highly porous structures in microscale geometries by freezing aqueous silk with a solvent. The resulting cryogels are experimentally characterized using flow parameters common in chromatography design; tortuosity, global pressure drop, pore diameter, and porosity. These empirical parameters are put into porous flow models to calculate an order-of-magnitude increase in functional surface area over the blank capillaries and packed-sphere columns used in traditional designs. Additionally, the pressure requirements to produce relevant flow rates in these structures are found not to threaten the integrity of microfluidic seals or connectors.

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1. Introduction

*"With time and patience the mulberry leaf becomes a silk gown"(Chinese Proverb),
"...or a cell-affinity chromatography column" (Chris Hinojosa).*

Humans have been utilizing silk threads for thousands of years due to their incredible luster and superior mechanical properties. Silk polymers are produced by the epithelial cells of silk glands in numerous species of mites, butterflies, spiders, worms and moths (Kaplan 1998), but this relatively small natural supply has never satisfied human demand. In recent years, scientists have genetically modified both plants and animals to produce silk in an attempt to increase the supply of this versatile polymer with numerous modern applications (Menassa 2004 and Karatzas 1999). The uses span time and place, and can be found in tools as diverse as ancient samurai shields (Ancient Discoveries 2009) and biologically-integrated electronics (Kim 2010).

Numerous new applications for the material, which is FDA-approved for human use, have been discovered in the last decade. Cell scaffolding (Kim 2005), food biosensors (Tao 2012), microfluidics (Bettinger 2007), biofunctionalized optical gratings (Lawrence 2008), artificial ligaments (Altman 2002), cornea replacements (Harkin 2011), and drug delivery systems (Hofmann 2006) are just a few examples of recent research with silk materials. This thesis characterizes new silk polymerization methods for novel

microscale applications in chromatography science and tissue engineering, and also gives results for both local and global flow characteristics in porous silk structures.

1.1 Silk producing species

The recent human adaptations of silk are numerous, yet spiders have been utilizing and evolving silk properties for at least 380 million years (Hayashi 2010)! A single garden spider (*Araneus diadematus*) produces seven genetically unique forms of silk for safety drag lines, wrapping eggs for reproduction, protective retreats, webs and glue for catching prey, and cement for attaching different silk types. The webs alone contain three separate forms of silk (Hayashi 2010), each containing their own specialized mechanical properties. The non-linear material response of silk threads to stress includes softening at the yield point and substantial stiffening at large strain until failure. This effectively localizes load-induced deformation, which results in robust webs with large load capacity (Figure 1.1). One study found that the typically golden orb web could sustain category IV hurricane winds before failure (Cranford 2012). Unfortunately, the independent nature of spiders, their relatively small lifetime silk production and sometimes deadly venom have made spider silk ranching unpopular and explains its underutilization in modern engineering.

Figure 1.1: A golden-orb spider captures a finch using its incredibly strong web (Malkin 2008).

On the other hand, the domesticated silkworm (*B. mori*) is so soft and benign that it is commonly raised in elementary classrooms studying life-cycles and in large-scale factories. The commercial farming of silkworms, or sericulture, has been practiced in parts of China since 2700 BC (Barber 1992). The silk moth lays eggs that hatch into millimeter long worms that survive solely on Mulberry leaves, which, after a month of continuous feeding, increases their weight 10,000 times (Range 2008). Their final act as a larva is to spin a silk cocoon around their body using a spinneret located near the mouth.

A moth allowed to reach full maturity will free itself by burning a hole in its surrounding cocoon with an acid it produces in its stomach. Usually the cocoons are thrown into an oven to kill the moth and prevent the destruction of the 1200 meter long

continuous fiber that makes up the cocoon. Typically, a few moths are allowed to reach full maturity and emerge from the cocoon, but thousands of years of domestication have created creatures that are unable to fly, eat, or live longer than needed to carry out their reproductive cycle. The worldwide sericulture industry is capable of producing 400,000 tons of dry cocoons per year using these ancient methods (Zhang 2002), yet scientists are now looking to genetic engineering for cheaper ways to produce large quantities of silk for the evolving market.

The first attempts at creating transgenic organisms that could produce silk utilized yeast and bacteria in culture (Prince 1995 and Fahnestock 1997). This approach was successful at producing silk, but had limited production capability and was not cost effective. The next successful attempts used transgenic goats, a common animal model for genetic modification, to produce dragline silk in their milk (Karatzas 1999). The most recent work to produce spider silk proteins has utilized transgenic plants, due to their large-scale production potential. Tobacco leaves have been reported as being good silk producers (Menassa 2004), but the low yield (69 grams/ hectare) and federal regulations against transgenic products still prohibit wide scale adoption.

1.2 Silk Applications

1.2.1 Textiles

Figure 1.2: A dress woven entirely from the silk of 1.2 million golden orb spiders (Jones 2012).

Humans identified the superior properties of silk threads thousands of years ago and began utilizing them in woven textiles. Woven silk absorbs moisture and keeps the wearer cool in the summer, and the material's low conductance helps to hold warm air near the skin for winter insulation. Throughout history, woven silk has been used for men's ties, gowns (Figure 1.2), underwear, scarves, parachutes, an ancient Roman currency equivalent to the value of gold, ancient noble attire, thrones, banners, art, and space insulators (Schoeser 2007). Ancient samurai horseman wore 'horos'- silk capes that ballooned behind their rider and protected them from arrows fired at their backs. This technique was recently shown to be completely effective at protecting a retreating rider from an expert archer (Ancient Discoveries 2009). The properties of silk make it so

sought after that dispute over its trade has even sparked wars (Schoeser 2007). The most recent unconventional use of silk thread was in the creation of violin strings with a "soft and profound timbre" (Osaki 2012).

1.2.2 Biosensors

Silk has many properties that make it a good candidate as a biomaterial. Purified silk shows excellent biocompatibility *in vivo* and *in vitro* (Vepari 2007). Purification, storage, and manufacturing of silk protein is done with aqueous methods that do not require expensive equipment, exotic chemicals, or extensive technical prowess. The protein exhibits self-assembly around nanoscale features, and the regenerated films are optically clear in the visible light range (Lawrence 2008).

All of these properties are advantageous for microfluidic devices that have integrated biosensors. Microfluidic devices of this type typically utilize injection-molded polystyrene or elastic silicon polymers molded with soft lithography. In a soft lithography process, a hard silicon substrate is patterned as a male mold using traditional photolithography methods. A rubbery silicon polymer, typically PDMS, is poured onto this mold and polymerized. The PDMS is then removed from the mold and bonded to a glass substrate to create fluid channels in the mold imprints (Xia 1998).

Using soft lithography, silk has been demonstrated as a material for microfluidic devices (Bettinger 2007). Silk surfaces promote cell adhesion without the protein

coatings that are required in PDMS devices (Bettinger 2007), and make it a viable alternative material in micro- cell culture devices for studying disease models or personalized drug toxicity (Huh 2010). It is also possible to integrate proteins and antibodies onto the channel surfaces of silk materials. In one example of this, a liquid silk solution is mixed with hemoglobin, a protein utilized by red blood cells to capture dissolved oxygen. An optical grating mold is made with this doped silk solution using a soft lithography procedure, and the resulting biofunctionalized grating shows a unique absorbance spectrum that is a function of the amount oxygen interacting with the grating (Lawrence 2008). This novel oxygen sensor could be integrated into a stem cell culture device in which measuring cellular respiration and maintaining oxygen levels is imperative to proper cell proliferation (Csete 2005). This technique might have advantages to current silicon oxygen sensors, which give inaccurate readings in human stem cell culture (private communications, Human Spare Parts, Tampere, Finland).

Traditional silicon sensors have inherent issues with *in vivo* applications as well. They have dominated *in vitro* sensor applications, but these materials are too stiff to make good contact with the soft tissues. Silk, on the other hand, can be doped with functional electric circuits that conform to the curvilinear surfaces typically found in the human body (Kim 2010). Additionally, the silk can be programmed to dissolve after the electronics have integrated with the soft tissues. The neural signals of a living feline brain have been successfully monitored using these methods (Kim 2010). The same researchers have printed radio frequency identification coils on silk films that can

conform to fresh food surfaces (Figure 1.3) and exhibit different resonant responses during spoilage (Vickmark 2011).

Figure 1.3: An edible silk radio-frequency identifier (RFID) that adheres to fresh food and changes its resonant response during spoilage (Vickmark 2011).

1.2.3 Cell scaffolding and bandaging

Multiple silk polymerization methods result in highly porous structures (Figure 1.4) that closely mimic human tissue scaffolding (Nazarov 2004, Li 2001, Tamada 2005). One of the most studied applications of silk in biotechnology is the use of these scaffolds as artificial tissue for severe bone trauma repair. This is due to silk's large mechanical modulus and toughness, ability to be functionalized with growth factors, FDA approval for human use, and degradation that can be programmed to match the rate of natural tissue re-growth.

Figure 1.4: SEM image of porous silk scaffolding.

The mechanical modulus and ultimate tensile strength of natural silk polymers is superior to other common biodegradable polymeric materials. Silk's mechanical modulus is one order of magnitude larger than polylactic acid, and four orders of magnitude larger than collagen (Vepari 2007). Silk as a reconstituted porous scaffold has a compressive modulus at least the same order of magnitude as other common polymeric porous scaffolds, except Poly(propylene fumarate) (Vepari 2007).

Functionalization of silk scaffolds by covalent attachment of cell adhesion and signaling factors known to induce bone matrices has been demonstrated in the literature. Sofia *et al.* (2001) showed that silk scaffolding decorated with integrin recognition sequences (RGD) promote osteoblast-like cell adhesion, increase osteocalcin message levels, and ultimately stimulate osteoblast-based mineralization *in vitro*. A different study looked at the effect of RGD-modified silk fibers on the growth of anterior

cruciate ligament fibroblast (ACLFs), and found that ACLFs grown on modified silk fibers enhanced cell attachment and spreading (Chen 2003).

Novel mats for wound bandaging have also utilized silk fibers. These mats are manufactured using a traditional electrospinning process, and functionalized to improve cell adhesion or signaling. Epidermal growth factor (EGF) was incorporated into silk mats to study the effect of its controlled release on the healing process of wounded human skin-equivalents. The functionalized mats significantly decreased the time required for full wound closure when compared to a unmodified silk mat (Schneider 2009).

1.2.4 Drug elution

Many of the aforementioned silk attributes make it an excellent vessel for controlled drug delivery. Dextran and different proteins have been physically entrapped into silk fibroin's crystalline structure and then programmed to release over a certain time by controlling the hydrophobic properties of the structure (Hofmann 2006). The entrapped drugs are able to retain their functional properties through the manufacturing processes, storage, and even after controlled release from the silk matrix. One application of this research is implantable silk microspheres that are directed to areas of injury, where they can release chemical cues to cells to differentiate in a therapeutically beneficial manner (Hofmann 2006). In another application,

researchers (Omenetto 2011) entrapped penicillin in silk films and found that the drug does not lose its efficacy over long periods in desert conditions, which is quite remarkable considering the drug's traditional form requires sub-zero temperature storage. This has important implications for the storage and shipping of drugs to remote villages and outposts in the developing world.

1.2.5 Chromatography

There is an entirely undiscovered application of silk materials that has yet to be identified in patents or studied in the literature. It combines the porous morphology, ability for surface functionalization, and self-assembling nature of natural silk polymers into a monolithic porous material. Monolithic porous materials have generated interest in separation science as an alternative to packed columns that are used for cell separations, concentrators for proteins and antibodies, enzymatic flow-through reactors, and protein separators (Svec 2006).

Monoliths show promise over traditional packed columns because column permeability and mass transfer are independently optimized, which allows for highly efficient separations with low fluid pressure requirements (Svec 2006). Early attempts at creating this class of structures utilized soft materials that collapsed under pressure (Svec 2006). The current state-of-the-art overcomes these issues with silica, zirconia, or hafnia material that are photopolymerized to create porous monoliths (Hoth 2005). Silk

may soon be added to this list due to its high mechanical strength, optical clarity, ability for surface functionalization, and its ability to be polymerized *in situ*. This *in situ* polymerization is done by simply freezing the silk solution with a solvent. This method is known as cryogel processing, and has been previously demonstrated with acrylamide derivatives (Kumar 2010) for cell separation applications.

Cell separation techniques typically use physiochemical properties such as size (exclusion filters, density gradient centrifugation), light scattering properties (cell sorter), or electrical impedance (electrophoresis). However, these methods typically have low specificity and are difficult to process on a large scale (Kumar 2010). Cell-affinity methods use interactions between cell surface markers and specific molecules to tag and identify specific cells. Common applications of cell-affinity methods use fluorescent molecules to tag and optically sort cells (fluorescent-activated flow cytometry), or magnetic tags and a magnetic field to separate specific cells (magnetic bead separation technology). Both methods are accurate, but are very expensive and time consuming (Kumar 2010).

A new method for cell-affinity separations uses monolithic porous columns whose surfaces have been coated with specific molecules for cell capture. In this method a cell is specifically captured with an immobilized ligand (typically an antibody) on the porous surface, which can attach to specific antigens on the surface of a cell. The cells are separated from the bulk fluid and later released with shear stress or by washing with a powerful eluent (Dainiak 2007). Monolithic cell affinity chromatography

columns have the potential to have high-throughput (Kumar 2010), high specificity, and a significantly lower equipment cost than traditional flow cytometry methods.

A commercial version of this tool, Ceperate SC (CellPro), is available for separating hematopoietic stem cells from peripheral blood. The captured cells are removed from the column by mechanical agitation, which is reported to result in a 53% yield with 62% purity (Dainiak 2007). Many such cell-affinity columns have suffered from low yield due to the necessity of removing adhered cells in a manner that effects cell viability and function (Bell 1978). Very recently, cryogel cell separation columns were shown to release bound cells by simple elastic compression of the monolithic structure. Using this technique, human acute myeloid leukemia cells were separated from a bulk fluid with up to 85% viability and 80-85% recovery (Dainiak 2006). Particles have been shown to detach from cryogels using elastic deformation of the porous structure for a wide variety of bound biological particles with differing sizes and surface natures (Srivastava 2007).

Silk could have a significant benefit over current poly(acrylamide) cryogel cell separation columns due to its large compressive modulus (Table 2.1) and its numerous paths to antibody decoration. The applications for such a technology are numerous: specific isolation and characterization of intact fetal cells in maternal blood for non-invasive prenatal diagnosis, stem cell purification for cell-based therapy, separation of immunocompetent from non-immunocompetent cells, and separation of malignant from non-malignant cells.

Another particularly large market for this technology is T cell separation and counting, for determining the progression rate of human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS). Cell-affinity chromatography using optically clear capture matrices allow for optical cell counts using bright field microscopy. One study achieved yield and purity results comparable to flow cytometers, the gold standard of the field, by combining microfluidic channels and functionalized surfaces to capture and optically count helper T cells (Cheng 2007). Potential advantages to this method may be achieved by integrating functionalized porous silk structures in microfluidic channels. A few advantages are the significant increase in functionalized surface area, the increased variation in antibody orientation, the increased likelihood of cell-wall interactions, all of which can be done without obstructing optical access to the channel.

1.3 Characterization gaps

Utilization of porous silk structures in the commercial market may begin shortly as researchers quantify their performance and discover new applications. Design and production of these structures on a large scale will require fundamental flow characterizations and processing specifications. The current silk literature has almost completely overlooked two crucial characterizations: fluid flow through porous silk, and

how the rate of heat transfer in cryogel silk processing affects pore morphology. The goal of this work is to characterize these two unstudied aspects of silk materials.

1.3.1 Fluid and diffusion characteristics for design

It is imperative to know the flow characteristics within porous silk structures. The pressure drop across porous structures is necessary for the design of fluid channels, pumps, vessel seals, and fluid connections required for integrating porous silk structures in microfluidic devices and chromatography tools. Additionally, multiphysics modeling of the performance of these devices will require knowledge of certain parameters common in porous media equations. These parameters include tortuosity, pore size, and porosity.

Tortuosity, a measure of the twisted path a particle must take within a porous media, is important for understanding residence time within the structure. It is also a typical parameter found in flow models for porous media.

Pore diameter is a length scale used to estimate pressure drop across the structure, it determines if cells or secondary phases will get physically stuck as they flow through the structure, and it sets an effective diffusion length scale for characterizing diffusion time scales.

Porosity, the volume of the empty voids in the solid porous structure, is an important parameter in flow models for porous media, and gives a measure of the volume of coating chemicals that will be needed to functionalize surfaces.

This thesis presents experimentally determined values for all of these parameters of porous silk structures made with cryogel methods. Additionally, the experimental methods developed will serve as a guide for other engineers characterizing similar structures for new applications.

In addition to guiding design engineers, knowledge of these parameters is also important for tissue engineers. It can help in the development of more controlled ways to flow cells into tissue scaffolds, which is typically done by pouring a cell solution over the scaffolding. These parameters are also a key component to accurate computational modeling and design of nutrient and oxygen mass transfer in complex tissue scaffolding (Yu 2009). The development of experimental methods to determine model parameters is critical as engineers attempt to design and quantify artificially vascularized, or highly specialized, tissue scaffolds.

1.3.2 Pore morphology for processing conditions

Systematic knowledge of how processing methods define pore morphology is imperative for commercial-scale manufacturing of porous structures for chromatography and tissue scaffolding. In some methods for making porous silk, such

as those utilizing porogens (Kim 2005) or freeze-drying (Nazarov 2004), the relationship between pore size and processing conditions is relatively well-defined. For other pore-forming methods more conducive to *in situ* manufacturing, such as cryogel processing (Tamada 2005), the morphological effect of the rate of heat transfer in freezing step has never been studied. This work examines how the rate of heat transfer affects pore size, and also looks at how the confining dimensions of the porous mold affects morphology.

2. Background

2.1 Silk

The following is a review of the material properties of silk and of the techniques researchers have developed to produce different types of structures. The numerous methods are relevant for the application of silk as a material in chromatography columns, tissue engineering, biosensors, and microfluidics.

2.1.1 Chemistry

Domesticated silkworm (*B. mori*) silk fibroin is a high-molecular weight organic polymer consisting of light (~26 kDa) and heavy chain (~390 kDa) proteins present in a 1:1 ratio (Vepari 2007). These proteins are linked by a disulfide bond and coated with a

glue-like hydrophilic protein (20-310 kDa) known as secerin (Vepari 2007). These proteins are characterized by highly-repetitive hydrophobic and hydrophilic peptide sequences that naturally assemble into regular structures analogous to synthetic block copolymers (Hofmann 2006). The surfaces of the assembled structure reveal a large presence of short-side chain amino acids glycine, serine, and alanine (Hofmann 2006). The silk surfaces can be decorated using these modifiable amino acid side-chain groups, 3.3% of which contain the carboxyl side groups (Vepari 2007) that are most commonly utilized for functionalization (Wu 2011, Wang 2011, Sofia 2001, Chen 2003, Schneider 2009).

2.1.2 Crystalline structure

X-ray diffraction has been used to identify three organizational states of assembled silk proteins. The first is the random-coil state that occurs prior to crystallization (Kaplan 1998). The second, Silk I is the water soluble state that is found in an organism's silk gland prior to spinning. The last structure is silk II, or β -sheet, which is a crystalline state induced by shear-stress during the spinning process. The basis for silk's strength and toughness are the β -sheet layers that form into thermodynamically stable crystalline structures using strong hydrogen bonds and van der Waals forces (Vepari 2007, Hofmann 2006). These sheets exclude water molecules and are insoluble

in many solvents including mild acid, alkaline solutions, and several chaotropes (Vepari 2007).

It is possible to alter the crystalline structure of silk, and therefore its solubility, *in vitro*. If a solution of purified silk protein and water is allowed to evaporate, the proteins self-assemble into a combination state of silk I and random-coil structure. When the resulting film is less than ~70 μm it will rapidly solubilize in water, but if it is greater than 100 μm thick it forms a highly elastic hydrogel that swells in water (Jin 2005). Conversion of silk I to silk II structures *in vitro* is done by treating these films and hydrogels with a methanol or potassium chloride treatment (Huemmerich 2006), and results in long-term water stable structures. In addition to chemical treatments, physical stretching or shearing of the films induces a change from amorphous silk I structure to crystalline silk II (Jin 2005).

2.1.3 Purification

Purifying silk fibroin from silkworm cocoons is done with all aqueous processing, at atmospheric pressure, and with easily accessible and affordable tools. Cocoons from *B. mori* silkworms are obtained by raising the silkworms from egg to cocoon (Figure 2.1 left), raiding a local preschool studying life-cycles using the silkworm, buying locally from your city's strange worm-lady, or buying online from the numerous dealers selling cocoons for textiles or beauty care (some beauty salons use the cocoons as a facial

exfoliate). The cocoons are cut in half, and any remaining parts of the moth are removed. They are then boiled in an alkaline solution and rinsed to remove the sericin glue-like proteins. The extracted silk is dissolved in a lithium bromide solution, and then dialyzed against water until all the salt has been removed. The result is a clear solution of diluted silk protein in water (Figure 2.1 right).

Figure 2.1: Cocoons with moths removed (left), silk fibers after sericin is rinsed away (middle), and purified silk protein in water (right).

2.1.4 Manufacturing

2.1.4.1 Thin films

Thin silk films are produced by casting an aqueous silk solution onto a mold (polystyrene, a silicon wafer, PDMS) and allowing the water to evaporate. The silk proteins self-assemble during this evaporation process and conform to the surface geometries of the casting surface. By slowing the rate of evaporation, it is possible to create films with a higher content of silk I structure and a smaller percentage of random-coil structures (Jin 2005). It is possible to use soft-lithography methods to

pattern silk films using a procedure similar to the one used for PDMS. High fidelity features down to 400 nm are produced with silk protein self-assembly (Bettinger 2007). Layered thin sheets of silk produced in this manner form a mechanically robust and stable structure (Jiang 2007). Researchers have created optical gratings (Lawrence 2008), holographic devices (Lawrence 2008), lenses (Figure 2.2, Lawrence 2008), and microfluidic channels (Bettinger 2007) with thin films produced on molds.

Figure 2.2: Silk holographic films demonstrating different optical patterns (Tobin 2008).

2.1.4.2 Porous structures

Porous structures are created by silk protein assembly around a porogen such as salt or ice crystals. The process of using ice as a porogen is known as cryotropic polymerization, and the resulting structure is known as a cryogel. In this method, a

solvent and polymer solution are placed in a freezer, causing most of the solvent to freeze, and concentrating the dissolved solute into a non-frozen liquid microphase in which the polymerization proceeds (Kumar 2010). When the sample is thawed, the solvent crystals melt and leave behind a system of continuously interconnected polymer pores that act as capillary channels. In the specific case of silk, methanol is typically added to the silk solution before the freezing step. The methanol precipitates silk aggregates (Nazarov 2004) that serve as nucleation sites for silk assembly during the freezing step.

In the freeze-drying method (Nazarov 2004), an aqueous silk solution (5.8 wt %) is mixed with 25 vol% of methanol, such that some silk precipitates from the solution and creates a gel. This gel mixture is then placed in a Teflon mold and frozen in dry ice (-80 °C) for 2 hours, and then placed in another container that controls the freezing rate at -1 °C/min. The container is then placed in a -20 or -80 °C freezer for two hours. The frozen composite is lyophilized to create the final porous structure. Interconnected pores with an average size of 50 +/-20 µm are formed with a -20 °C freezing temperature. Pore diameter is decreased to 15 +/- 7 µm when the freezing temperature is reduced to -80 °C.

When the freezing temperature of the silk solution is above the glass transition zone of the aqueous solution (-20 to -34 °C, Li 2001), the ice crystals will nucleate and grow. In this temperature regime, the ice crystal size and resulting pore diameter is dependent on freezing temperature, the duration of the freeze, temperature gradients,

and confining dimensions. When the freezing temperature is decreased below the glass transition temperature of the solution, ice forms rapidly and does not have time to form a coherent crystalline structure. The duration of the freeze has no impact on crystal growth in this regime.

One method of producing porous silk structures is given by Yasushi Tamada (2005). In this method, a diluted organic solvent solution is added to an aqueous silk solution while stirring. The mixed solution is placed into a mold and frozen for at least eight hours. The mold is then allowed to thaw and the resulting silk sponge is placed in a water bath to remove the solvent. This method produces sponges with a typical pore size of 80-150 μm , and is best suited for concentrations of silk fibroin ranging from 1-5% (w/v%). Pore size is a weak function of both silk fibroin concentration and freezing duration, and a strong function of solvent concentration. No sponges are formed for solvent concentrations over 1% by volume (Tamada 2005).

Salt-leaching methods are also utilized to form porous silk structures. These methods are similar to those employed to create porous scaffolds using other polymers. In one technique (Kim 2005), granular NaCl (particle size; 850-1000 μm) is added to the aqueous silk fibroin solution (4-10%, w/v%) and allowed to cure for 24 hours. The surface of the salt is dissolved in the solution, while most of the salt stays as solid particulate due to the saturation of the solution. The salt particulates create the pores, which are then removed by immersing the polymerized silk in water for 2 days. Kim *et al.* found that the pore size of the derived scaffolds was typically about 90% of the

porogen diameter, and pore size was not highly dependent on silk protein concentration in the solution.

The gas-foaming method (Nazarov 2004) for producing porous scaffolds is similar to the salt-leaching method, except that ammonium bicarbonate is used (150-250 μm) in place of sodium chloride. The porogen is added to a silk/HFIP solution in a 20:1 weight ratio, and the solution is allowed to evaporate at room temperature. The scaffolds are then immersed in 95 °C water for 10 minutes to induce gas-foaming . The resulting scaffolds are placed in a water bath for 24 hours to remove the salt, and then allowed to air dry. This method produces highly-interconnected open-pore morphology with an average pore size of 155 $\pm$ 114 μm .

The different methods of creating porous silk structures result in different morphologies and mechanical properties. Table 2.1 gives a summary of the compressive strength and modulus of porous structures created using the above manufacturing methods. This is compared with three types of porous structures typically found in biological systems. While silk does not have the strength of human bone, it is still stronger than other commonly utilized tissue alternatives such as collagen or poly(acrylamide).

Material	Compressive Strength (kPa)	Compression Modulus (kPa)	Source
HFIP/aqueous silk derived with gas foaming	280 +/-4	900 +/-94	Nazarov 2004
Aqueous derived silk sponge using salt porogen	320	3330	Kim 2005
Aqueous silk using freezing-drying	80 +/- 1	170 +/- 7	Nazarov 2004
Cortical Bone	131,000-224,000	17-20e6	Yaszemski 1996, Cullinane 2001
Cancellous Bone	5000-10,000	50,000-100,000	Yaszemski 1996, Cullinane 2001
Collagen (freeze dried)	~15	~150	Kim 2001
Poly(acrylamide)	n/a	42-86	Srivastava 2007

Table 2.1: Mechanical properties of porous biomaterials.

2.1.5 Functionalizing surfaces

2.1.5.1 Avidin-biotin interactions (Wang 2011)

Avidin and its commercial derivatives (Streptavidin and Neutravidin) are homotetramers with four biotin binding sites. Avidin can be covalently bonded to the surface of silk through carboxyl groups on its surface. The covalently trapped avidin has a high affinity for biotin ($K_d=10^{-15}$ M), which allows for biotinylated molecules to quickly link to the material surface under mild conditions (neutral pH and at room temperature). This coupling strategy is very effective for the biofunctionalization of silk materials due to the large number of biotinylated molecules available on the commercial market. Incubating Neutravidin-functionalized surfaces in a protein solution

such as bovine serum albumin significantly reduces the amount of non-specific binding that occurs on the silk surface, and may be a useful strategy in situations where highly-specific biosensors are required. A detailed procedure for functionalizing porous silk in capillaries with Neutravidin and biotinylated anti-CD4 is given in Appendix A.

2.1.5.2 Non-covalent bonding

It is also possible to attach antibodies to silk surfaces without the use of intermediate reactions. This is done by utilizing the numerous carboxyl groups on the surface of silk films (Vepari 2007). In one method, antibodies are mixed directly into the liquid silk solution, which then assemble into the silk structure during solvent evaporation. Antibodies attached to silk surfaces using these methods appear to retain the functionality, but the level of non-specific binding to the surfaces has yet to be studied. If further research into this method reveals high levels of non-specific binding, then it will not be useful in producing highly selective biosensors.

Another possible issue with this method is that antibodies are distributed throughout the silk matrix, which means larger amounts of antibodies are needed to coat the active surfaces. To get around this problem, researchers have added the antibodies to virgin silk films in their last stages of drying. This has the effect of immobilizing the antibody closer to the active surface, and thereby reducing the volume of antibodies needed for surface functionalization (Lu 2011). This additional step may

not be required for functionalization of porous materials since the pore walls are usually ~200 nm, and the ratio of surface area to volume is large.

2.2 Models of fluid flow in porous media

A model of the fluid flow through porous media is important for the design of any porous fluid device. First, the pressure drop across such structures is an important numeric that will provide pumping requirements. Second, the fluid velocity within these structures will be used in the equations describing chemical reactions and in the determination of fluid shear stress on cells. Third, these models will allow for the calculation of the functional surface area of a reactor, which will be important in design and in comparing the performance of novel porous devices to more traditional packed-bed reactors.

There are numerous equations to describe the pressure drop and velocity relationship through a porous media. A common equation is Darcy's law, which can be stated as (Richardson 2002),

$$u_c = -B \frac{\Delta P}{\mu l}$$

Equation 2.1

where u_c is the average velocity of flow of the fluid, also defined as $(1/A)(dV/dt)$, where A is the total cross sectional area of the bed, V is the volume of fluid flowing in time (t),

B is permeability coefficient of the bed, μ is viscosity of the fluid, l is the length of the porous structure, and ΔP is the pressure drop across the bed.

An expanded model uses a general relation between pressure drop and mean velocity in terms of porosity and specific surface area of the bed. This model builds on Darcy's model by assuming that the void space within a pore can be modeled as a bundle of parallel capillaries. The equation for laminar flow through a single round tube is

$$u = \frac{d_t^2}{32\mu} \frac{-\Delta P}{l_t}$$

Equation 2.2

where u is the mean velocity of the fluid, d_t is the diameter of the tube, and l_t is the length of the tube. A resistance factor, K , is added to Equation 2.2 to account for the additional resistance of numerous bundled capillaries that are analogous to a porous bed

$$u_e = K \frac{d_t^2}{32\mu} \frac{-\Delta P}{l_t}$$

Equation 2.3

where u_e is an equivalent average velocity corresponding to that increased resistance.

The K in Equation 2.3 is a factor that includes such geometric considerations as tortuosity, porosity, and surface area. It can be expanded to include those specific

terms by assuming the porous media consists of a series of tortuous cylinders. Equation 2.3 is then rewritten as

$$u_1 = \frac{d_m'^2}{K' \mu} \frac{(-\Delta P)}{l'}$$

Equation 2.4

where $d_m'^2$ is some equivalent diameter of the pore channels, K' is a dimensionless constant that depends on the structure of the bed, l' is the length of the channel, and u_1 is the average velocity through the pore channels.

Note that u_1 and l' in Equation 2.3 represent conditions in the pore and are not the same as u_c and l in Equation 2.1. However, Dupuit (1863, translated from French by Richardson) thought it reasonable that l' is proportional to l , and u_c proportional to u_1 through the following argument: in a porous cube with side length ' X ' and porosity ' e ', the volume of void space is given as eX^3 . The average cross-sectional area of this cube is then the volume of the void space divided by the projected area, eX^2 . If the volumetric flow rate through this cube in terms of Darcy's average velocity is $u_c X^2$, then the average pore velocity is given by

$$u_1 = \frac{u_c X^2}{eX^2} = \frac{u_c}{e}$$

Equation 2.5

To make Equation 2.5 generally useful, it is necessary to find an expression for $d_m'^2$. This was done by Kozeny (1927, again translated from German by Richardson) as

$$d'_m = \frac{e}{S_B} = \frac{e}{S(1-e)}$$

Equation 2.6

where S_B is the wetted surface of the structure and S is the surface area of the structure divided by the volume. Combining Equation 2.4, Equation 2.5, and Equation 2.6, and knowing $l' \propto l$, we get

$$u_c = \frac{1}{K''} \frac{e^3}{S^2(1-e)^2} \frac{1}{\mu} \frac{(-\Delta P)}{l}$$

Equation 2.7

where K'' is the Kozeny constant. Carman (1937) showed the dependence of K'' on tortuosity and cross-sectional geometry with this relationship

$$K'' = (\tau)^2 + K_o$$

Equation 2.8

where τ is the tortuosity of the porous structure, and can be simply defined as the ratio of the fluid path length in a structure to the overall structure length, and K_o is a factor that depends on the shape of the porous cross-sectional area. The value of the K'' has been experimentally found for a number of packed-bed geometries. A bed of silk fibers with different porosities is shown in Table 2.2: Experimental values for K'' for silk fiber packed columns of different porosities (Lord 1951).Table 2.2.

Experimental Constants For Silk Fibers	
Porosity, e	K''
0.8	5.35
0.9	6.8
0.95	9.2
0.98	15.3

Table 2.2: Experimental values for K'' for silk fiber packed columns of different porosities (Lord 1951).

This entire derivation is based on the assumption that the fluid flow in the porous structure is entirely laminar. A modified Reynold's number for flow in porous media (Richardson 2002) provides this confirmation in the following experiments. This modification multiplies the characteristic length, the average velocity, and fluid density (ρ) and divides by viscosity to get

$$Re_p = \frac{u_1 d'_m \rho}{\mu} = \frac{u_c}{e} \frac{e}{S(1-e)} \frac{\rho}{\mu} = \frac{u_c \rho}{S(1-e)\mu}$$

Equation 2.9

3. Methods

3.1 Silk Purification

Thirty silk cocoons are cut into fourths and boiled for 1 hour in 1 liter of 0.02 M Na_2CO_3 solution, and then rinsed 3 times with distilled water to remove the sericin proteins. After drying, the extracted silk is dissolved in 100 mL of LiBr solution (Tokyo Chemical Industry) (10 M) at 60 °C for 3 hours. The solution is added to 8 cm long dialysis tubes (SnakeSkin, 3,500 MWCO, Thermo Scientific) that are each dialyzed against 1 liter of distilled water and replaced every 24 hours for 3 days. After dialysis, the solution is centrifuged twice for 15 minutes to remove any silk aggregates that formed during the process. The result is an optically clear solution that is stored at 4 °C until use. The final silk concentrations are typically 2-4 % (w/v), as determined by weighing a silk film resulting from the evaporation of 1 mL of solution.

3.2 Polymerization of porous structures

Porous structures are created using a method similar to that of Tamada (2005). A silk solution (2.9-3.6% w/v) is thoroughly mixed with 1-25% Methanol (v/v) by agitation in a plastic syringe. One end of a capillary is placed in a small droplet (25-500

μL) of the silk solution, and filled by capillary wicking. The capillary tubes have round and square cross-sectional areas with length scales ranging from 50 μm to 3 mm. Both ends of the capillary are sealed with PTFE thread sealant tape.

In one experimental case, the prepared capillaries are placed in foam stands designed to hold them in air during the freezing process and ensure axially-symmetric heat transfer (Figure 3.1). The stands are placed in a freezer ($-15\text{ }^{\circ}\text{C}$ or $-20\text{ }^{\circ}\text{C}$) for 12-24 hours, and then removed and thawed at room temperature. At this stage, the silk has formed a porous structure, with melted ice filling the porous voids. The silicon tape is removed from the ends and the remaining water is evaporated at room temperature ($25\text{ }^{\circ}\text{C}$) or in an oven (IsoTemp 285A, Fischer Scientific) at $60\text{ }^{\circ}\text{C}$ until all the liquid is gone.

For additional variation in freezing temperature, capillaries are prepared as above, but then placed between dry ice layers ($-80\text{ }^{\circ}\text{C}$) or in a saltwater-ice bath ($-8\text{ }^{\circ}\text{C}$) for 8 hours. One capillary sample is removed every hour, allowed to thaw at room temperature, and checked for porous structure formation under a microscope. This process is repeated for an entire 8 hour experiment.

Figure 3.1: Foam holders to keep capillary tubes held in the air during freezing process.

Capillaries with different thicknesses of insulation layers are created to vary the rate of heat transfer from the structure while keeping the confining capillary length constant. This is done by fixing a sealed glass capillary ($\varnothing 1.4 \text{ mm} \times 100 \text{ mm}$ long) in the center of a plastic mold containing uncured PDMS. This mold is then cured in an oven, which polymerizes the liquid PDMS into an elastic rubber around the capillary (Figure 3.2). Silk solution is then injected into the glass capillary and the end is re-sealed. PDMS caps are added to each end of the insulation layer to reduce heat transfer end effects. Devices with different insulation thicknesses (0 mm, 3 mm, 5 mm, 10 mm) are placed on foam stands in a freezer at -20°C for 24 hours.

Figure 3.2: PDMS insulation around a $\phi 1.4$ mm glass capillary not showing end caps.

3.3 SEM Imaging

Capillaries containing porous structures are fractured along their cross-section using a razor blade. Four cross-sections for each sample (1.4 mm, 550 μm , and 50 μm capillaries) are mounted upright on a microscope slide using epoxy. These samples are sputter coated using a modified tabletop turbo sputter coater (Plasma Prep II, SPI) with nitrogen gas and a silver electrode. The samples are then placed in a modified scanning electron microscope (SEM) (XL-40, FEI) and imaged with a 20 kV incident beam. Images are captured with an Oxford Link ISIS microanalysis system.

3.4 Tortuosity using particle tracing

A 2D approximation of tortuosity is determined using optical tracking of microspheres caught in the flow. Porous silk structures are polymerized in square borosilicate glass capillaries (Friedrich & Dimmock Inc., 400 μm inner diameter, 100 μm wall) mounted to a glass microscope slide. The capillary is attached to a syringe pump

(Popper & Sons 5cc glass syringe, Havard Apparatus PHD2000 syringe pump) using Tygon Tubing and epoxy glue. The prepared slide is clamped to the stage of an inverted microscope (Nikon TE-2000), and focused 10 μm from the inner capillary wall using a 20X objective lens (Nikon Plan Flour, air immersion, NA=0.45, DOF=5.8 μm). A mixture of 4 μm polystyrene spheres and distilled water (0.1 w/v%) are loaded into the syringe and pumped through the capillary at flow rates between 1 $\mu\text{L}/\text{min}$ -4 $\mu\text{L}/\text{min}$. Videos of the flowing particles are recorded (~30 second lengths) at multiple locations along the length of the structure using a CCD camera (GeviCAM 3360, 656x494 pixel array) that captures 30 frames per second (Coyote Application, Pleora Technologies). The resulting videos are analyzed frame-by-frame with ImageJ (Wayne Rasband, National Institute of Mental Health) by manually tracking an in-focus particle moving through the camera's field-of-view. This raw coordinate data is imported into Excel (Microsoft Office 2012) for further analysis and presentation purposes.

3.5 Porosity with volume void method

A porous silk structure is polymerized in part of a capillary tube (Fischer Scientific, $\varnothing$ 1.4 mm x 100 mm long). Isopropanol alcohol is introduced to one side of the capillary at a known volume using a micropipette (FinnPipette II, Fisher Scientific) without wetting the porous structure. A camera (iPhone 4s, Apple) is fixed above the capillary to obtain top view images of the capillary, and a ruler is placed next to the

capillary to provide the scale. An image is recorded before the alcohol slug wets the porous structure. The slug is then allowed to fully wet the porous structure and another image is recorded. The length of the slug before ($L_{unwetted}$) and after wetting (L_{wetted}) the structure is obtained using Spotlight image analysis software (Robert Klimek and Ted Wright, NASA Glenn Research Center), as well as the length of the porous structure within the capillary ($L_{occupied}$). With these lengths known, it is possible to calculate the volume of the alcohol before and after wetting the silk structure, the volume of the silk structure itself, and the porosity of the structure using the equation derived below,

$$V_{silk} = V_{wetted} - V_{unwetted}$$

Equation 3.1

where V_{silk} is the volume of the silk structure, $V_{unwetted}$ is the volume of the alcohol before wetting the silk structure, and V_{wetted} is the volume of the alcohol and silk structure together. It is possible to calculate the volume of the void within the porous structure (V_{void}) with the ratio of the volume of the silk structure to the volume of the region in the capillary that the porous structure occupies ($V_{occupied}$).

$$V_{void}(\%) = \frac{V_{silk}}{V_{occupied}} \times 100$$

Equation 3.2

Substituting Equation 3.1 into Equation 3.2,

$$V_{void}(\%) = \left(\frac{V_{wetted} - V_{unwetted}}{V_{occupied}} \right) \times 100$$

Equation 3.3

By plugging in the volume equations for a round capillary tube, Equation 3.3 becomes

$$\begin{aligned} V_{void}(\%) &= \left(\frac{\pi r^2 L_{wetted} - \pi r^2 L_{unwetted}}{\pi r^2 L_{occupied}} \right) \times 100 \\ &= \left(\frac{L_{wetted} - L_{unwetted}}{L_{occupied}} \right) \times 100 \end{aligned}$$

Equation 3.4

The porosity can then be calculated as

$$e(\%) = 100 - V_{void} = 100 - \left(\frac{L_{wetted} - L_{unwetted}}{L_{occupied}} \right) \times 100$$

Equation 3.5

3.6 Pressure drop

The pressure drop for water flowing across the porous structures at room temperature is measured using a simple manometer. Porous slugs of different lengths are polymerized as described above using $\varnothing 1.4$ mm x 100 mm long glass capillaries (Fisher Scientific). The capillary is connected to a syringe (Popper & Sons 5cc) fixed in a syringe pump (Havard Apparatus PHD2000) using Tygon tubing. A T-junction is inserted at the entrance to the capillary tube and a glass tube manometer (4 mm x 25 cm) is attached perpendicularly to the flow direction and held vertically by a fixed level/ruler. The exit end of the capillary is inserted into a hole in a reservoir that utilizes a weir to maintain a constant pressure head during the length of the experiment (Figure 3.3). A flow rate relevant to the design of a microfluidic cell-affinity chromatography device (Cheng 2007) is generated by the syringe pump (5-500 μL), which corresponds to a Reynolds number (ratio of inertial forces to viscous forces, Equation 2.9) in the porous structure of 2×10^{-4} to 8×10^{-4} . A pressure develops in front of the capillary due to the viscous losses in the porous structure, and causes fluid to rise in the manometer. For every flow rate, the height of fluid rise is recorded with a camera (iPhone 4s, Apple) fixed parallel to the manometer and ruler/level. The pressure head (Pa) required to move water through the structure for a given flow rate is determined from hydrostatics as,

$$\text{Pressure Head} = \rho g(h_m - h_r)$$

Equation 3.6

where ρ is the density of water, g is gravitational acceleration, h_m is the height of the fluid column in the manometer, and h_r is the height of the fluid in the reservoir.

Figure 3.3: Diagram of pressure drop measurement experiment. A set flow rate is generated with the syringe pump, pressure measurements are recorded from the manometer with a camera, and excess fluid is sent over a weir built into the constant height reservoir.

4. Results and Discussion

4.1 Morphology for different freezing temperatures

Previous research in cryotropic polymerization of silk only studied the freezing temperature and freezing duration variable. A more thorough study of freezing methods is challenging because freezing variables are numerous and difficult to control. Some of the known factors that affect ice formation are temperature and temperature gradients, the direction of heat transfer, proteins in solution, and nucleation sites in container walls (Patapoff 2002). Despite these variables, other studies showed freezing regimes in which macroscale molds would freeze and create porous structures (Nazarov 2004, Li 2001, Tamada 2005). The main goal of this work was to determine if these processing conditions would produce porous structures on smaller scales inside capillaries.

All silk samples in capillaries placed between dry ice layers (-80 °C) froze within a few seconds. However, no capillaries frozen at this temperature over any duration produced porous structures (Table 4.1). This is consistent with the results of Tamada (2005) under similar processing conditions, and is due to the freezing temperature being below the glass transition temperature of the solution. Below this temperature, the solution freezes into an amorphous state, which prohibits the formation of the ice crystal porogens.

The silk solutions frozen at -8°C and -20°C did not produce consistent porous structures throughout any of the capillaries. In some cases there was limited porous formation on one side of the capillary, and it was hypothesized that maybe the silk protein was settling before being frozen. To test this hypothesis, concurrent samples were frozen vertically and horizontally, but showed no difference in porous structure formation. These results are not consistent with those of Tamada (2005), who found good porous formation at this temperature. This may be attributable to their utilization of freezing molds that were an order of magnitude larger in diameter or their control of boundary conditions, both of which could affect both the rate of heat transfer and the temperature gradients in the mold during freezing.

Table 4.1 shows that every size capillary frozen at -15°C produced porous structures. The pore size for each of these different structures was measured from SEM images (Figure 4.1 & Figure 4.2), and the results are shown in Table 4.1. As the confining length scale of the capillary is reduced, so is the size of the pores. This may be due to the physical limitations the capillary walls provide for ice crystal formation. It could also be due to the increased rate of heat transfer from the smaller capillaries, which results from insulative glass walls that are $1/3$ the thickness of the larger capillary. This increased rate of heat transfer has been shown to result in many smaller ice crystals (Nazarov 2004). Different thicknesses of insulation were added to one type of capillary before freezing to separate the variables of heat transfer and confining length scale. However, none of these insulation layers exceeded the critical insulation radius

needed to decrease heat transfer ($r_{cr}= 7\text{mm}$). Additionally, none of the insulated capillaries produced consistent porous structures after freezing for 24 hours.

Freeze Temp.	Confining Length Scale		
	50 μm	550 μm	1.4 mm
-8 °C	X	X	X
-15 °C	11$\pm$15	41$\pm$15	141$\pm$36
-20 °C	X	X	X
-80 °C	X	X	X

Table 4.1: Summary of pore size (μm) in different sized capillary for different freezing temperatures. ‘X’ indicates no consistent pore formation. All silk solutions were ~3% w/v with 1% methanol v/v and frozen for 24 hours.

The pore morphology also changes as the confining dimension of the capillary is reduced. The pores in the 1.4 mm capillary are well-distributed and interconnected, with a leaf-like structure (Figure 4.1A). This is qualitatively similar to what is seen in the SEM images of cryogel silk at 1% solvent by Tamada (2005). The pores in the 550 μm capillary are 70% smaller than in the 1.4 mm case, and the shape of the pore is qualitatively different. In the 550 μm capillary the pores are spindle-like, and are missing the flat walls characteristic of the 1.4 mm capillary (Figure 4.1A & Figure 4.1B). This spindle formation is even more apparent in the 50 μm capillary. At this scale the silk has not formed full pores, but instead, the structures look like neural axons that emanate from the channel walls.

Figure 4.1: Cross-sectional SEM images of porous silk structures in 1.4 mm (A), 550 μm (B), and 50 μm capillaries (C).

The throats of the pores, the openings that form in between neighboring pores, serve as a qualitative indicator of possible inertial losses through the structures at large Reynold's numbers. In Figure 4.2, the throats of the pores, D_t , are only slightly smaller than the largest diameter of the pore itself, D_p . This suggests that the fluid may not

have significant contraction/expansion losses, and that the pressure drop should be mainly attributable to viscous wall drag.

Figure 4.2: Detail of pore structure in a 1.4 mm capillary. The white arrows give an example of how the pore diameter, D_p , and throat diameter, D_t , were identified.

These experiments also confirm that it is possible to create porous structures of varying lengths within a capillary. Structures ranging in length from 1-100 mm are consistently obtained after freezing at -15 °C with 1% Methanol. It is also possible to introduce multiple slugs of silk solution into a capillary, which results in distinct porous regions after freeze treatment. Each of these fluid slugs can include different capture antibodies, which suggests a new method to create total analysis devices in parallel or in series.

4.2 Porosity

The porosity of the structures in the 1.4 mm capillary is $89.4 \pm 1.7\%$ (Figure 4.1A). The evaporation of alcohol in the capillary was measured over the experiment length to ensure that evaporation was not contributing error to the measurement. Evaporation over 3 minutes was not measurable. The porosity of the 550 μm was found to be $82.6 \pm 2.0\%$ (Figure 4.1B).

In both measurements, there were a number of microbubbles trapped in the structure. Multiple techniques were attempted to remove the bubbles; placement of the capillary in a vacuum, creating an adverse pressure gradient to slow capillary wetting, and a number of different wetting fluids (0.65 cS Silicone oil, isopropanol, ethanol, water). None of these attempts were successful.

The bubbles effectively act the same as the stationary silk phase in the porous structure, which has the effect of making the structures less porous. However, extended flow and high fluid pressure are both effective at removing the bubbles from the structure. To determine the theoretical porosity of the structure if these bubbles were removed, the total volume of the bubbles was approximated. All of the microbubbles within a structure were imaged using the inverted microscope. The volume of each microbubble was determined by approximating them as perfect spheres, measuring their diameter in Spotlight, and plugging it into the equation for the volume of a sphere. These volumes were summed to find the total volume taken up by the bubbles. The approximate porosity of the 1.4 mm capillary with no bubbles is $91.3 \pm 1.7\%$ and for the 550 μm capillary it is $84.6 \pm 2.0\%$.

4.3 Tortuosity

The tortuosity of the porous structures in the 550 μm capillaries is very close to unity. The average tortuosity is 1.12 ± 0.05 , and a number of representative particle pathlines are shown in Figure 4.3. The small average tortuosity value indicates that particles travel through the structure in a relatively unimpeded straight path, but Figure 4.3 suggests differently. The path the particles take are often straight, but the paths themselves typically move at an angle deviating from the flow direction. The distance between consecutive points shows how the velocity of the particles changes as they

move through the structures. When the particles are flowing in the pores, the velocity is nearly constant throughout the frame, as indicated by consistent distances between points. When the particles in Figure 4.3B are forced through pore throats, indicated by bumps in pathlines, they are strongly accelerated.

For this measurement, the objective lens has a 5.8 μm depth of focus, which effectively confines the measurements to a psuedo-two-dimensional space. In reality, the particle could move $\pm 13 \mu\text{m}$ out of the focal plane and still appear in focus in the recorded images. An average pore velocity was calculated and compared to the average particle velocity in the video-recordings to determine how much this out-of-plane movement contributed to error in the tortuosity measurement. The average velocity is calculated from the induced flow rate and the effective flow area, which is determined from porosity data. The calculated average pore velocity was 116 $\mu\text{m/s}$ and the average velocity of the particles was 127 $\mu\text{m/s}$. An 8% error suggests that out-of-plane motion is not significantly contributing to an under prediction of tortuosity in the measurements.

During the experiment, it was noted that the 4 μm particles did not become permanently trapped in the silk pores. At a flow rate of 1 $\mu\text{L/min}$, some particles clumped in the smaller pore throats, which can be seen in Figure 4.3A & B, near the pore walls. However, these particles were washed away by increasing the flow rate to 4 $\mu\text{L/min}$. If the particles were deformable, as in the case of many bioparticles, it can be imagined that the cells would be even less likely to clump in pore throats.

Figure 4.3: Particle pathlines overlaid against backlit images of the porous structures they are flowing through at two different locations in the capillary (A & B).

4.4 Pressure drop

The graph in Figure 4.4 shows pressure losses across porous structures of different lengths, and includes a measurement in a capillary with no porous structure. The measurement in a blank capillary provides an experimental comparison to the analytical solution for pressure drop across a capillary tube (Equation 2.2), and quantifies the error the T-junction and corresponding expansion/contractions add to the measurement. At 250 $\mu\text{L}/\text{min}$, this minor loss contributes 16 Pa to the overall pressure loss, which is less than 2% of the measured pressure drop across the shortest porous structure. These pressure losses were calculated for each flow rate and subtracted from the data in Figure 4.4 and Figure 4.5.

Figure 4.4: Pressure loss across capillaries containing different lengths of porous inserts for a range of flow rates. Error bars, due to resolving the meniscus in the pressure tap and variability in the syringe pump, are hidden by the data points.

Equation 2.7 predicts that pressure drop is a linear function of porous structure length. Figure 4.5 shows the result of dividing pressure by structure length, and confirms the relationship with a 0.9889 R^2 -value for a linear curve fit to the data. The slope of this curve is compared to Equation 2.7 to get an estimate of the surface area of the porous structure. By choosing a K'' from the experimental literature for flow through silk fibers (Table 2.2), the calculated surface area of the porous structure is calculated as 7×10^5 (1/m). When compared to a specific surface area of 3×10^3 (1/m) for a blank

capillary, there is a two order of magnitude increase in functional surface area. A comparison of the slope of the curve-fit to the blank capillary and the porous structures in Figure 4.5 shows that this increased surface area comes at a cost of a three-order of magnitude increase in pressure loss. This is indicated by the slope of the curve fit which is $3e13$ for the porous media and $4e10$ for the blank capillary.

This data was also compared to the theoretical pressure drop in a packed-bed column formed from $100\text{ }\mu\text{m}$ spheres. In this packed-sphere case, the porosity is 0.4, K'' is 5, and the specific surface area is 6×10^4 (1/m) (Richardson 2002). Plugging these values into Equation 2.7 results in the following relationship between pressure and flow rate,

$$Pressure/Length \left(\frac{kg}{m^2 s^2} \right) = 1.43 \times 10^{12} * Q \left(\frac{m^3}{s} \right)$$

Recall that the silk porous structure has a porosity of 0.9 and a specific surface area of 7×10^5 (1/m). The silk structure has more than double the porosity, one-order of magnitude greater surface area, and it only costs a one-order of magnitude increase in pressure loss.

Figure 4.5: Pressure drop divided by structure length as a function of flow rate with a linear curve fit through the porous data. The curve appearing on the x-axis is the pressure drop for a capillary with no porous structure. Error bars are again hidden by the data points.

5. Conclusions and Future Work

This work demonstrates that porous silk structures can be polymerized *in situ* in confining dimensions as small as 50 μm , and provides the freezing regime in which this is possible. The morphology of these structures is qualitatively similar to those found in the literature for freeze-dried (Nazarov 2004) and cryotropically polymerized (Tamada 2005) silk scaffolds processed in larger molds. In future studies, it would be useful to study the effect of the rate and direction of heat transfer on porous morphology. Insulation layers with a radius larger than 7 mm should be added to the capillaries to

decrease the rate of heat transfer during freezing. Additionally, it might be possible to achieve anisotropic porous structures by controlling the direction of heat-transfer using similar methods to those used by the pharmaceutical industry (Patapoff 2002).

If different pore morphologies are produced using these methods, then a systematic study of pressure drop for different pore morphology could be conducted. A scaling analysis of pressure drop in porous structures is given in Appendix B.

Investigating non-dimensional pressure as a function of pore size, porosity, pore throat diameter, or pore anisotropy may reveal important variables for pressure drop in porous media. This could be useful to chromatography science, engineers creating cell-separation tools, microfluidicists integrating porous cryogels into their devices, or civil engineers studying transport through porous media.

These experiments demonstrate that porous silk structures have a significant increase in functional surface area compared to blank capillaries and traditional packed-sphere chromatography columns. This is achieved without increasing the pressure drop across these structures to levels that will harm seals common to microfluidic devices.

This thesis also shows that multiple distinct porous structures can be created by introducing multiple fluid slugs into the capillary before freezing. This improves possibilities for devices that have multiple filters, in series or parallel, that have different functionalizations. This may be utilized in a negative-selection cell separator, a single device for counting multiple cell types, or a complex protein concentrator.

The pores that are created using the developed methods have pore diameters in a range that will not cause cells to be physically trapped during cell-affinity chromatography. This was confirmed in experiments using rigid microspheres. The compressive properties of silk make it an excellent candidate for a biocompatible material to be used in a similar way to poly(acrylamide) cryogels that exhibit bioparticle release from their functionalized surfaces under elastic compression. The literature shows that silk porous structures have a compression modulus that is two-orders of magnitude greater than that of poly(acrylamide) cryogels. This knowledge, along with silk's ability to be functionalized through many chemical paths, suggests an important new application for porous silk in cell separation science.

Most of the research on silk has focused on its potential for cell-scaffolding, but as of yet this field is still in its basic research stage. However, cell-separation techniques are widely used for diagnostic and therapeutic applications. Studies need to be done on the performance of silk structures for cell-affinity capture. The morphology of the silk pores is such that antibodies adhered on the surface will have a large diversity of orientations for interactions with surface markers on passing cells. This random orientation could significantly increase the efficiency of a porous silk device for cell-affinity separation. Additional studies should examine the effectiveness of silk-bound cell release during elastic compression of the silk structure. Finally, the performance of these devices should be compared to other cell-separation tools in terms of purity, yield, and cell viability. An excellent protocol for conducting such a study can be found

in the work of Kumar and Srivastava (2010), using the silk surface functionalization methods developed in Appendix A.

Another important market for porous silk structures may be in diagnostic tools for counting cells from whole blood or saliva. Such a device could have increased functionalized surface area, an increased variation in capture-antibody orientation, and a greater probability for cell-wall interactions. The methods of Cheng (2007) will serve as a state-of-the-art benchmark for measuring the performance of such a diagnostic device. Figure 5.1 gives an example of a simple PDMS device and protocol that could be used to study the efficiency of cell capture from complex fluids, and the efficiency of cell release during elastic compression of the silk structure.

Figure 5.1: Possible design and protocol for a microfluidic device capable of separating and counting T cells from whole blood.

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7. Appendix A: Functionalization using Neutravidin and Biotinylated anti-CD4

Stock Solutions

- Prepare PBS stock solution.
 - a. Empty one PBS foil pack into 500 mL of water and stir until dissolved.
 - b. Label this mixture PBS.
- Prepare EDC/NHS stock solution.
 - a. Use a syringe to remove 1 mL of PBS solution.
 - b. Add 8 mg of EDC and 22 mg of NHS and mix well
 - c. Label this syringe EDC/NHS
- Prepare Neutravidin stock solution.
 - a. Dissolve 3 mg Neutravidin in 3 μ L of DI water in a glass vial (Deltware No. 94399).
 - b. Use a pipette to add 0.3 mL PBS to the glass vial.
 - c. Label this vial Neutravidin.
- Prepare Hydroxylamine Hydrochloride (Hydroxylamine HCL) stock solution.
 - a. Dissolve 1 mg Hydroxylamine HCL into 1 mL of DI water in a syringe.
 - b. Label this syringe HCL.
- Prepare BSA stock solution.
 - a. Dissolve 10 mg of BSA powder into 1 mL of DI water in a syringe.
 - b. Label this syringe BSA.

Procedure

1. Prepare 3 mm long porous structures in square capillaries (550 μ m inner diameter, 20 mm long) using established methods.
2. Use micropipette to add 5 μ L of PBS to the porous structure and let sit for one hour.
3. Place KimWipe near one end of capillary and allow PBS to completely wick from structure.
4. Use micropipette to add 5 μ L of EDC/NHS solution to capillary and let sit at room temperature for 15 minutes.
5. Remove EDC/NHS solution using KimWipe, flush with clean PBS solution, and remove excess fluid with KimWipe.
6. Add 5 μ L BSA solution to capillary, place in a covered dish near a wet KimWipe to reduce evaporation, and let sit overnight at room temperature.
7. Remove BSA solution with a KimWipe.

8. Use micropipette to add 5 μL of Neutravidin solution to capillary and let sit in covered dish for 5 hours.
9. Flush capillary of Neutravidin solution twice using 10 μL of Hydroxylamine HCL solution.
10. Let fresh Hydroxylamine HCL sit in capillary for 10 minutes.
11. Flush with DI water three times.
12. Use micropipette to add 5 μL of anti-CD4 solution to capillary and let sit for 20 minutes.
13. Flush with PBS solution.
14. Add 5 μL of fresh PBS solution to capillary, seal ends with silicon tape, and store at 4 $^{\circ}\text{C}$ until use.

Storage

Store anti-CD4 in its original buffer solution at 4 $^{\circ}\text{C}$ (39.4 $^{\circ}\text{F}$) in refrigerator.

Store Neutravidin powder at room temperature under the fume hood to keep dry.

Seal stock solutions in vials and store in refrigerator. Prepare enough porous samples so that you can use all of the Neutravidin stock solution in one sitting.

8. Appendix B: Dimensionless Pressure

We begin with the z-component of the Navier-Stokes equation in cylindrical coordinates

$$\rho \left(\frac{\partial u_z}{\partial t} + u_r \frac{\partial u_z}{\partial r} + \frac{u_\phi}{r} \frac{\partial u_z}{\partial \phi} + u_z \frac{\partial u_z}{\partial z} \right) = -\frac{\partial P}{\partial z} + \mu \left(\frac{1}{r} \frac{\partial}{\partial r} \left(r \frac{\partial u_z}{\partial r} \right) + \frac{1}{r^2} \frac{\partial^2 u_z}{\partial \phi^2} + \frac{\partial^2 u_z}{\partial z^2} \right) + \rho g z$$

and remove extraneous terms.

$$\rho \left(\cancel{\frac{\partial u_z}{\partial t}} + u_r \cancel{\frac{\partial u_z}{\partial r}} + \frac{u_\phi}{r} \cancel{\frac{\partial u_z}{\partial \phi}} + u_z \cancel{\frac{\partial u_z}{\partial z}} \right) = -\frac{\partial P}{\partial z} + \mu \left(\frac{1}{r} \frac{\partial}{\partial r} \left(r \frac{\partial u_z}{\partial r} \right) + \cancel{\frac{1}{r^2} \frac{\partial^2 u_z}{\partial \phi^2}} + \cancel{\frac{\partial^2 u_z}{\partial z^2}} \right) + \cancel{\rho g z}$$

steady, fully-developed laminar flow

axis-symmetric, fully developed, no elevation

We are left with

$$\frac{\partial P}{\partial z} = \frac{\mu}{r} \frac{\partial}{\partial r} \left(r \frac{\partial u_z}{\partial r} \right)$$

and we identify the length scales of the flow

$$r \sim R_p \text{ and } z \sim L$$

where R_p is the radius of the pore and L is the length of the structure. Plugging these scales in

$$P \sim \frac{\mu u_z L}{R_p^2}$$

In terms of flowrate

$$u_z = Q / e\pi R_c^2$$

Where Q is flowrate, e is porosity, and R_c is the radius of the confining capillary. Putting velocity in terms of flowrate we get

$$P \sim \frac{\mu Q L}{e\pi R_c^2 R_p^2}$$

which is the pressure scale for flow in our porous media.