Silk solution processing automation

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Abstract

Natural silk produced by silkworms and spiders exhibits many unique properties, such as high tensile strength and extensibility, controllable degradation, biological compatibility, as well as a structure that can be manipulated chemically for particular applications. Silk has been used as a biomaterial in biomedical applications for years, most notably for sutures and tissue engineering scaffolds. For many biomedical applications, silk cocoons are transformed into a silk solution as the first step. The existing silk solution processing used by biomedical researchers at Tufts University involves degumming, drying, dissolving and dialysis, which require significant hands-on human involvement. In this thesis, a refined silk solution processing approach is proposed with a focus on a new automated boiling system for degumming silk cocoons. Using National Instruments' LabVIEW software for control and customized water sensors for feedback, the automated boiling system was demonstrated to perform correctly. Ongoing efforts to make monitoring and control of the process will be discussed as well.

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Introduction

Silk is a natural material with amazing mechanical properties as well as biocompatibility, and has been used as a biomaterial in biomedical applications for many years. Tufts University has a large research effort in tissue engineering, with a focus on the use of silk materials in various forms. In one form, silk solution is created from silkworm cocoons. The existing silk solution protocol process can be divided into four steps: degumming, drying, dissolving and dialysis. Degumming is the first step of silk solution processing, in which sericin, a protective glue-like coating is removed from cocoon silk. Drying is the second step, in which water is removed from degummed silk. Dissolving is the third step, in which degummed and dry cocoon silk is mixed with lithium bromide solution and placed in an oven at the temperature of 60 ℃ for 4~6 hours. Cocoon silk is transformed from fiber form to viscous liquid during the dissolving process. Dialysis is the fourth step. The lithium bromide used to dissolve the silk is exchanged with pure water by using osmotic pressure gradient across a dialysis membrane.

The current silk solution processing is a hands-on process which requires significant human involvement. In addition, the process is insufficiently automated and controlled, lacking *in situ* data collection and feedback. Furthermore, it is hard to get repeatable results and consistent, high quality silk solution for the current silk solution processing. There is an urgent need for automated silk solution processing.

In order to design an automated silk processing system, two parallel design tracks have been followed. Track one involves taking the existing process and current technology, and simply improving the processing steps with limited automation. Track two is a more fully automated approach that explores the use of different technology to achieve automation, while producing high quality as well as consistent silk solution. For track one, a silk automated boiling system, automated water change system and SnakeSkin dialysis approach with automated water change system were designed and manufactured. The designs in track one have sensors and a control strategy which is an improvement compared with the current silk solution processing steps. For track two, autoclave degumming and a SnakeSkin TFF dialysis system were designed and tested by using new technology but further research and testing is needed to determine its viability and the quality of silk solution generated.

This thesis is organized into 5 sections, starting with this introduction. The background section covers background information on different silks, silk properties and silk applications. In a separate silk processing section, the current silk solution process is described in detail. The third section introduces the track one refinement of the existing silk solution processing, including the design and manufacture of a prototype silk automated boiling system, exploration of G2 cassettes and a SnakeSkin dialysis approach. In addition, the track two approach is discussed, with focus on a prototype SnakeSkin TFF

continuous dialysis system. The fourth section is the conclusion section and the last section is future work.

Chapter 1 Background

1.1 Silk background

Silk, a natural protein fiber, well known in the textile industry for its impressive mechanical properties, such as high tensile strength and extensibility and luster, as well as its biological compatibility, is obtained from the cocoons of the larvae of mulberry silkworm *Bombyx mori* reared in captivity. The shimmering appearance of silk is due to the triangular prism-like structure of the silk fiber, which allows silk cloth to refract incoming light at different angles, thus producing different colors. Silk proteins are usually produced in epithelial cells by specialized glands after biosynthesis, followed by secretion into the lumen of these glands where the proteins are stored prior to spinning into fibers [1,2]. Silk fibroin comprises repetitive protein sequences which provide a structural function for nest building, web formation, cocoon formation, traps, egg protection and safety lines. Basically, silks are composed of beta-sheet structures due to the dominance of hydrophobic domains consisting of short side chain amino acids in the primary sequence. These structures provide a tight packing of stacked sheets of hydrogen bonded anti-parallel chains of the protein [1,3,4]. Information about the numerous but small beta-sheet crystals in the fibers is provided by the research of spider dragline silk proteins. Meanwhile, there is a high level of organization of the protein even in the less crystalline domains [25].

The difference between silks and collagens is that they have a highly repetitive primary sequence that leads to crucial homogeneity in secondary structure, for example, triple helices in the case of collagens and beta-sheets in the case of many silks. In contrast to the catalytic and molecular recognition functions of globular proteins, these types of proteins usually show impressive mechanical properties. This family of proteins provides an important set of material options in the fields of controlled drug release, biomaterials and scaffolds for tissue engineering because of these impressive mechanical properties. In comparison to globular proteins, the relative environmental stability of these families of proteins in combination with their biocompatibility, unique mechanical properties, and options for genetic control to tailor sequence provides a significant foundation to exploit these natural proteins for biomedical applications [2].

The most frequently characterized silks are from the domesticated silkworm, *Bombyx mori*, and from spiders such as *Nephila clavipes* and *Araneus diadematus*. There are many more evolutionarily advanced spiders producing different types of silks. The amino acid composition and mechanical properties for each of these silks are different depending on different specific functions, for example reproduction as cocoon capsular structures, lines for prey capture, lifeline support (dragline), adhesion and web construction [2]. In addition to biocompatibility, environmental stability, the ability for amino acid side charge modification to immobilize growth factors, controlled proteolytic

biodegradability and morphologic flexibility, silks from silk worms and spiders have amazing mechanical properties [1,2,6,8-18]. Table 1 shows the mechanical properties of biodegradable polymeric materials.

Table 1. Mechanical properties of biodegradable polymeric materials (modified from [2]).

Source of biomaterial	Modulus (GPa)	UTS (MPa)	Strain (%) at break	References
B. mori silk (with sericin)	5-12	500	19	[38]
B. mori silk (without sericin)	15-17	610-690	4-16	[38]
B. mori silk	10	740	20	[39]
N. clavipes silk	11-13	875-972	17-18	[39]
Collagen	0.0018-0.046	0.9-7.4	24-68	[40]
Cross-linked collagen	0.4-0.8	47-72	12-16	[40]
Polylactic acid	1.2-3.0	28-50	2-6	[41]

1.2 Silkworm silks

Silkworm silk was been used in commercial textile production and as biomedical sutures for many years [2]. The most important reason people prefer to use silkworm silks among the natural sources is that, among all of the animals that can produce natural silk, mulberry silkworms, also known as *Bombyx mori*, have great economic importance. The silkworms are fed with mulberry leaves until they are mature enough for metamorphosis into moths. At this point, the moth requires a protective shelter which is the silk cocoon.

After the completion of the silk cocoon, the silkworms are killed and the silk can be harvested. It is relatively easy to raise them in captivity compared to spiders. Another reason silkworm silks were been used widely is that it is pretty easy to increase silk production by maintaining a high density of larvae [2,18].

The structure of *Bombyx mori* silk fiber is shown in Figure 1. *Bombyx mori* silk fiber has a glue-like sericin coating outside two protein-monofilaments. The sericin coating glues two fibroin fibers together. There is a similar structure in other silkworm silks too [2,5,32]. The protein-monofilaments are named brins and the brin nanofibrils are composed of fibroin filaments. The diameter of brins is about 5 nm and a bundle of brins are about 100 nm. The nanofibrils have interaction between each other and they are parallel to the axis of the fiber [32]. The weight of sericin coating in *Bombyx mori* silk is around 25-30% of the total fiber volume [33,34].

There are at least two major fibroin proteins in *Bombyx mori* cocoon silk. These two fibroin proteins have different chains weights; one is a light chain, about 26kDa, the other is a heavy chain, about 360kDa [1,19]. The ratio of light chain and heavy chain inside the *Bombyx mori* silk is 1:1 [2].

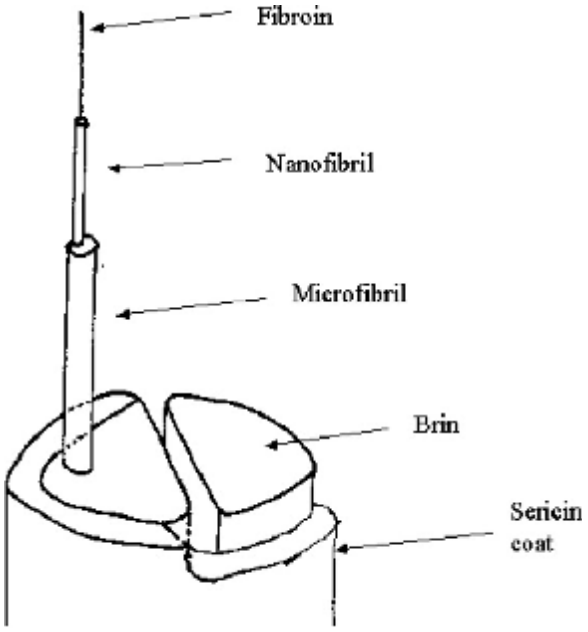


Figure 1. Structure of *Bombyx mori* silk thread [5].

1.3 Spider silks

Silks produced by spiders differ in many aspects from those produced by mulberry silkworms. Spider silk is a protein fiber spun by spiders. Spiders use their silk to make astonishing orb webs for prey capturing, cocoons for offspring protection and other structures which function as adhesives used to anchor web or as leads for flying prey capturing [5]. The reason silkworm silk has been more exploited is that 600 to 900 m of fiber can be yielded from one silkworm cocoon but only ~137 m of fiber can be generated from spider ampullate gland and in a spider orb web, only ~12 m of silk can be found [5,7,20]. Table 2 shows a summary of the main differences between spider and mulberry silkworm silks.

Table 2. Comparison of key properties between spider and silkworm silk [5].

Properties	Spider silk	Mullberry silk
Number of silk types per species	Up to eight silk types	One type of silk
Functions	Making a web to catch prey, safety line, support for the web, wrapping prey and egg cocoon formation	Cocoon formation
Mechanical properties		
Strength σ_{max}	1.1(*Araneus* MA silk)	0.6(B.mori)
Extansibility ε_{max}	0.27(*Araneus* MA silk)	0.18(B.mori)
Coationg of thread	Glycoprotein mixture, not fully characterised	Sericins, antigenic proteins
Major structural protein	Spidroin	Fibroin
Behaviour in water	Supercontraction of dragline	Little change in silk shape/length
Mass production	Not yet achieved, small quantities extracted by pulling silk from spiders or collecting egg cocoons	B.mori, the domesticated silkworm, has been grown for mass production for millennia

Among the entire insect silks up to now, spider silks have the best mechanical properties. The silk produced by domesticated silkworms is rigid, but brittle on some level. It has been prove that under controlled conditions, the artificially reeled silk from a silkworm is much better than the natural silk from a cocoon [5,21-23]. Furthermore, there is a big difference between the spider silk obtained by forced silking and the silk obtained from a spider which can ambulate unrestrained. The possible explanation for this unexpected difference is that when the spider was forced into silking, there was an internal friction brake generated by the spider which acts as a resisting

force, and this resisting force has a large effect on the mechanical properties of spider silk [24].

The molecular weights of spider silk proteins are different for different sources. Normally, depending on the analysis method, the molecular weight range of spider silk is from 70kDa to 700kDa [2]. In order to get large quantities and more controllable spider silk, genetic engineering is expending great effort try to overcome the limits of using native organisms of spider. This approach improved the studies of silk protein structure and material processing [2,25,26]. Compared to the silkworm silk, the mechanical properties of spider silk is much better in some aspects and that is the reason why people show more enthusiasm on the research of spider silk.

1.4 Properties of silk

There is a distinct difference in mechanical properties between silk and all other natural materials. Some spider silks have more than 200% elongation and tensile strengths. These mechanical properties are almost the same as high performance fibers [31]. From Table 3 we can see that the toughness of some silk species is much better than Kevlar fiber [5]. The molecular structure, the thread substructure and the formation process are the main reasons that silk has this mechanical advantage. [27-30]. Silk is stable at both high temperature and low temperature. In the solid state, silk is hydrophobic and the mechanical properties of silk can be greatly influenced by water. With respect to energy

absorption, spider silk is unbeatable compared with natural fibers. These mechanical properties are much higher, especially for spider dragline silk from *Nephila clavipes*, than silkworm cocoon silks.

Table 3. Tensile properties of silks and other materials [5].

Silk type	Species	Extension (%)	Tensile strength (GPa)	Toughness (MJm^{-3})
Major ampullate	Araenus	27	1.1	160
Viscid	Araenus	270	0.5	150
Cocoon	Bombyx	28	0.6	70
Elastin	-	25	0.002	2
Kevlar 49 fiber	-	2.7	3.6	50

Silks are insoluble in water, dilute acid, dilute alkali and other solvents. Liquid crystalline phases and conformational polymorphism contribute to the architectural features within the fibers in the biological processing. Silks have different mechanical properties when produced under different conditions which including climate, the silkworm/spider's nutrition and the speed of reeling. Silks can exhibit different mechanical properties even for the same thread from the same source [5].

1.5 Silk applications

Silks are very useful and well suited for biomedical applications. Different silks have different mechanical properties and therefore a cautious selection of the advantages, disadvantages and limitations of different silks should be considered. Silk can be used as a substrate for collagen for the raising of animal cells and it is almost as good as collagen [5,35]. Table 4 shows applications of silk fibroin scaffolds in cell and tissue engineering.

Table 4. Cell and tissue applications of silk fibroin scaffolds [1].

Application	Morphologic form	References
Wound dressings	Film	[42]
	Sponge	[43]
Bone tissue engineering	Sponge	[44-50]
	Film	[12,51,52]
	Hydrogel	[16,53]
	Non-woven	[14,54]
Cartilage tissue engineering	Porous sponge	[50,55-58]
	Hydrogel	[59]
Ligament tissue engineering	Fiber	[60-62]
Tendon tissue engineering	Fiber	[63]
Hepatic tissue engineering	Film	[11]
Connective tissue	Non-woven	[64]
Endothelial and blood vessel	Non-woven mats	[9,65]
Antithrombogenesis	Film	[66]

In tissue engineering, using silk to make tissue scaffolds has been explored for years because it is biocompatible. Silk fibers also can be used as non-load

bearing spacers for tissue transplant due to their good mechanical properties [18,36]. In drug delivery, reliability and control is very crucial, especially in situations where the drugs may cause side effects. Silk proteins can work as drug carriers in drug delivery because of their biocompatibility and morphology [5].

Chapter 2 Current Silk Solution Processing

The current silk solution processing can be divided into four steps: degumming, drying, dissolving and dialysis. A lot of human involvement is required for each of these steps and the entire process takes a considerable time to complete. While a protocol is typically followed, it is quite hard to control the quality of silk solution generated in the current silk solution processing. The following sub-sections provide details of the major processing steps.

2.1 Degumming

Bombyx mori silk fiber is composed by two protein-monofilaments embedded in a glue-like sericin coating. It is reported that 25–30% of the weight of *Bombyx mori*'s silk fiber is the sericin coating [2,5,32]. Foreign body response (FBR) is a concern when using silk as the implantation material. The sericin coatings on silkworm fibers are implicated for foreign body response [1]. Degumming is a process that removes the sericin coating from silk. In simple terms, silkworm fibers are degummed by boiling silkworm fibers in distilled water with sodium carbonate (Sigma-Aldrich). Depending on the application for the silk solution, silk degumming time can be different. Figure 2 shows the current steps in the degumming process.

(a) (b) (c)

(d) (e) (f)

(a) Uncut cocoons.
(b) Cut cocoons into small pieces and remove dead silkworm.
(c) Weight 4.24g Na_2CO_3.
(d) Put Na_2CO_3 into a 2L beaker and heat with heating plate.
(e) Put cocoon pieces into boiled aqueous solution of Na_2CO_3.
(f) Rinse with pure water.

Figure 2. Cocoon degumming process.

The first step of the current degumming process is cutting cocoons. Cocoons are cut into small pieces with titanium scissors (Figure 2b) and the dead silkworms are removed by hand. Then 4.24g of sodium carbonate is weighed (Figure 2c) and a 2L beaker is filled with distilled water. The sodium carbonate is added to a beaker and heated up to about 90℃(Figure 2d and 2e). When the aqueous solution of sodium carbonate starts boiling, the cocoon pieces are added to the boil water. After boiling the cocoon pieces for 30 to 90 minutes, depending on the application of silk solution, the silk is taken out of the beaker and put it into a plastic beaker filled with distilled water for rinsing (Figure 2f). The silk is placed into fresh milli-Q water and rinsed 30 minutes

for 3 total washing periods. A stir bar is used when rinsing silk with pure water.

There are several challenges in transforming this process to be automated. First of all, it is difficult and takes a lot of time to cut cocoons into pieces and remove the dead silkworm, depending on the particular cocoon source. Chinese cocoons were pre-cut, so it was not necessary to cut them. For Japanese cocoons, they came whole, with the dead silkworm inside. Second, when boiling the cocoon pieces with an aqueous solution of sodium carbonate, overflow is a problem. The boiling water can overflow the beaker often, so that constant monitoring is required. Currently, process users will be on stand-by to mix the silk and adjust heating plate temperatures to increase or reduce the rate of boiling.

2.2 Drying

Drying is a process which removes water from silk. In the current process, a silk rinsing process is required before drying. This process is performed 3 times to ensure remaining sodium carbonate is removed. After the third (3 times of each 30 minutes) rinsing cycle, the silk is removed from a plastic beaker and squeezed to remove much of the remaining water. The silk is stretched by hand and placed in a fume hood (Figure 3) to air dry until it becomes fully dried.

Figure 3. Silk laid out in fume hood to dry.

2.3 Dissolving

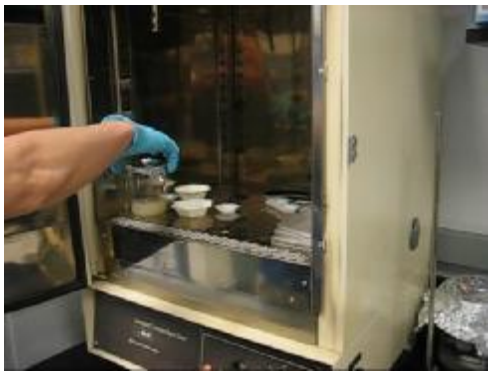
Dissolving is a process of breaking down a solid into solution through the use of a solvent. In this process, the silk crystal lattice is broken down into molecules, ions or atoms, which can transport into the solvent.

For the current silk dissolving process, the first step is weighting 14g of dried cocoon silk. Then 9.3M Sigma-Aldrich lithium bromide (LiBr) solution is prepared by mixing 45.24g LiBr powder with pure water. The final volume of 9.3M LiBr solution is 56ml. After that, the dry cocoon silk is dissolved in the 9.3M LiBr solution, then placed into an oven and heated 4~6 hours at 60°C. Figure 4 shows the steps of the current dissolving process.

(a) Weighting of LiBr.

(b) Making 9.3M LiBr solution.

(c) Silk mixing with 9.3M LiBr solution.



(d) Heating in oven for 4-6 hours.

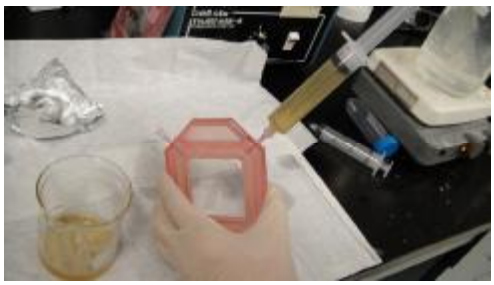
(e) Dissolved silk

Figure 4. Dissolving silk with LiBr solution.

2.4 Dialysis

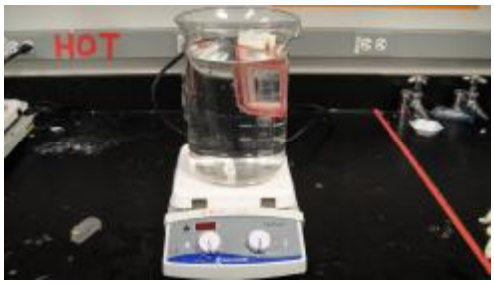
Dialysis is a process in which molecules in solution are separated by the difference of their diffusion rates through a semi-permeable membrane. Dialysis is a very common technique both in a laboratory, such as for removing salt from a protein solution, and in medical applications, such as for providing an artificial replacement for lost kidney function in people with renal failure. Normally a solution is placed into a sealed semi-permeable dialysis bag. The dialysis bag is placed into a container which holds a pure water or different solution. The small molecules (often water, salts and other small molecules) will move to the lower concentration side and the larger molecules (often proteins, DNA, or other larger molecules) will be kept inside the dialysis bag because their sizes are larger than the membrane pore size [71].

Thermo Scientific 3.5K MWCO Slide-A-Lyzer Dialysis Cassettes are used in the current silk dialysis process. Dissolved silk solution is injected into a 30ml cassette by a syringe (Figure 5a), which is placed into a 2L beaker of milli-Q water. A foam float is attached on the top of the cassette in order to make sure the cassette will not sink to the bottom of the beaker (Figure 5b). The water in the beaker is changed 6 times over a 2-day dialysis period. It is very obvious that the current silk solution dialysis process needs a lot of human involvement, for injecting dissolved silk solution, setting up beakers and cassettes, and performing water change.



(a)

(b)



(c)

(a) Dissolved silk solution is injected into 30ml 3.5K MWCO Dialysis Cassettes.
(b) A foam float is attached on the top of cassette.
(c) Dialysis cassettes are placed into a 2L beaker which is filled with milli-Q (pure) water.

Figure 5. Silk solution dialysis process using beakers in the current process.

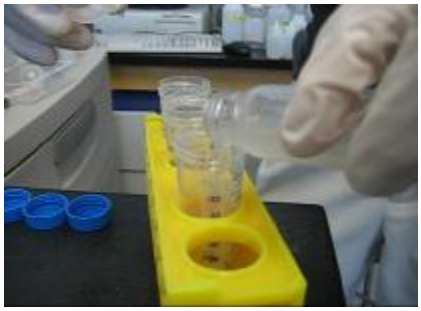
2.5 Silk solution post-processing

There are two important post-processing steps in the current silk solution processing, concentrating and centrifuging. Because there are still some impurities inside the silk solution after dialysis, centrifuging is needed to remove most of the impurities. Figure 6 shows the procedure for centrifuging silk solution. First, 4 centrifuge tubes are cleaned. Silk solution is injected into a centrifuge tubes and placed in centrifuge. The centrifuge is run for 20 min at 11000 RPM. When centrifuging is done, silk solution is removed, transferred into BD Falcon Tube, and stored in refrigerator. The concentration of final silk

solution product is about 7%-8% w/v (weight per volume).

(a)

(b)



(c)

(a) 4 centrifuge tubes are prepared.
(b) Silk solution is put into centrifuge tubes and placed into centrifuge.
(c) Silk solution is stored in BD Falcon Tube.

Figure 6. Centrifuging steps in current silk solution processing.

Chapter 3 Refined Silk Processing

The focus of this thesis was the development of a more automated process for transforming native cocoon silk to a soluble form for tissue engineering applications. It is very challenging to develop a fully automated system for the entire silk solution process, a dual-track design approach has been initiated. The first track is focused on the design and manufacture of a semi-automated system for each step of the silk solution processing. The second track is focused on fundamentally new technology that is higher-risk and higher-cost, but shows promise for providing a more fully automated system.

3.1 Refined degumming

The current silk solution processing approach needs a lot of human interaction and takes a really long time to get the final silk solution product from cocoon. Meanwhile, the current silk solution processing lacks data produced during processing and no quality control. In order to reduce the time and labor cost during silk solution processing and improve the final silk solution quanlity, refinement of silk solution processing was investigated.

3.1.1 New Silk Automated Boiling System

In order to produce more stable and controllable silk solution, an automated silk boiling system was designed (Figure 7).

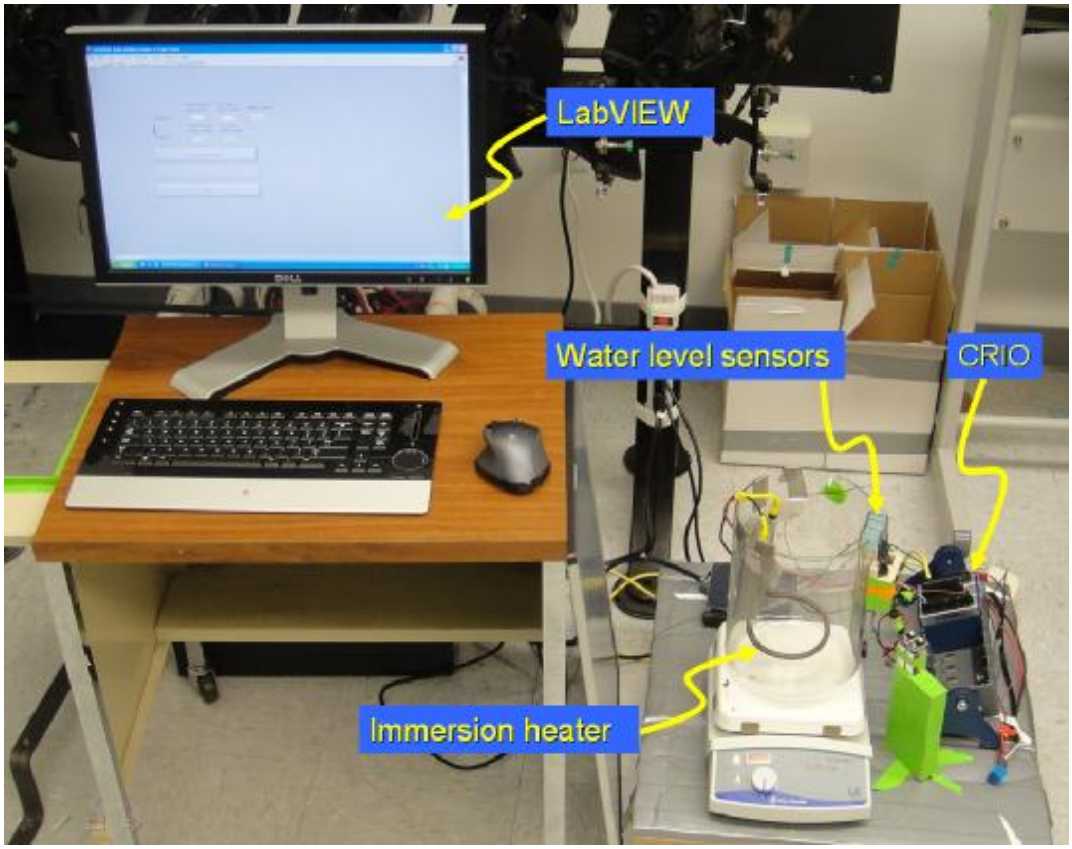
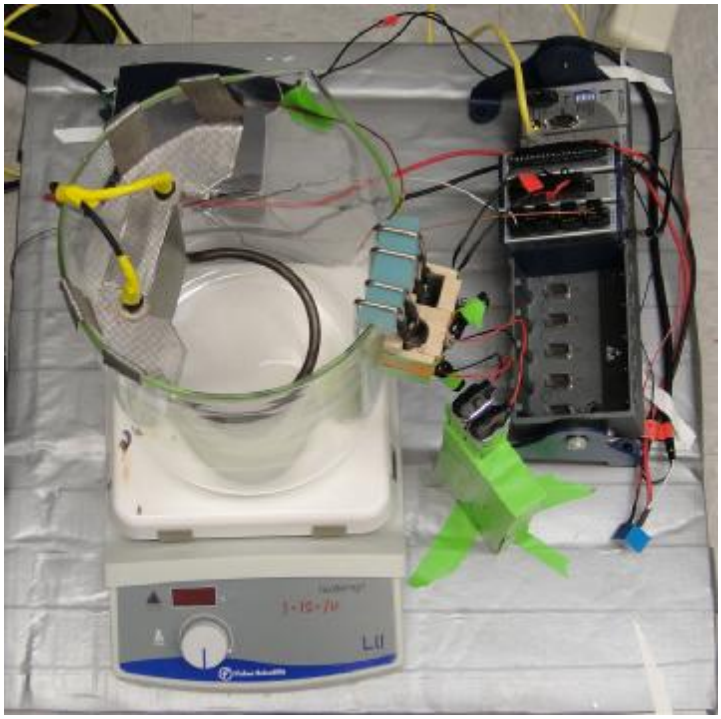


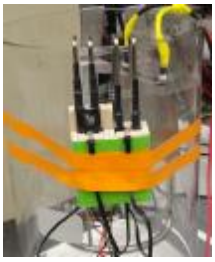
Figure 7. Silk automated boiling system.

In this automated boiling system, a Tayama soy milk maker immersion heater (Figure 8c), which is controlled by a National Instrument CompactRIO-9074 was used to boil the pure water. Immersion heaters are designed for direct contact heating of water, oils, viscous materials, solvents, process solutions and gases for many industrial heating applications. Since all heat is generated inside the liquid, virtually 100% thermal energy efficiency is achieved. The immersion heater's working voltage and current is 110V and

5.6A respectively. The power of the immersion heater is, therefore around 616 watts. Two relays were used to control the on/off of immersion heater. One relay is Tyco Electronics OUDH-SS112D mechanical relay and the other is National Instruments NI9485 8-channel solid state relay (Figure 8d). OUDH-SS112D is a Tyco Electronics general purpose relay that has 9VDC (Max) control on voltage and 1.2VDC (Min) control off voltage. National Instruments NI9485 solid state relay has a 1.2A/channel switching current for up to 4 channels. The principle is using NI9485 relay to control the on/off of the OUDH-SS112D relay and immersion heater was controlled by these two relays.



(a)



(b)

(c)

(d)

(a) Silk auto boiling system.
(b) Water level sensor.
(c) Immersion heater.
(d) National Instruments NI9485 8-channel solid state relay.

Figure 8. Prototyped Silk automated boiling system details.

The silk automated boiling system includes sensor-based system that can automatically prevent the boiling water from overflowing. There are two water level sensors (Figure 8b), a top water level sensor and a bottom water level sensor. The mechanism of the top water level sensor is that when water reaches the sensor probe, the immersion heater will be turned off automatically. The bottom water level sensor is used to prevent the container from going dry, which is a hazard for the immersion heater and can cause damage and/or a fire. The automated boiling system will not start working unless there is sufficient water in the beaker. The water level sensors are two leads attached to RAM 45 boat saver pumps. When water reaches the leads, a voltage output signal will be sent to CompactRIO and the CompactRIO will turn off the immersion heater. The sensors are powered by a 9V battery and sensor probes are stainless steel, which prevents corrosion. The heater is turned off as the water level approaches the top water level sensor, and then turned back on as the water level returns to an acceptable level. This bang-bang open loop control strategy is repeated over the duration of the cooking period.

Compared to the current degumming process, the automated boiling system has many advantages. First, the high power of the immersion heater immersing in the water produces boiling temperature in about 30 minutes, which saves time. For the current degumming process that uses a heating plate, it takes at least 1 hour to heat the pure water to boiling temperature. Second,

since the silk automated boiling system is controlled by a CompactRIO, it is very easy and convenient to control the degumming process with a LabVIEW program. Meanwhile, because there are water level sensors in the automated boiling system, no human interactions are needed during the boiling period.

There is a stainless steel mesh outside the immersion heater to help preventing silk from directly contacting the immersion heater. After several test runs, it was determined that the immersion heater would not burn the silk, even if there is direct contact between the immersion heater and silk. The only problem is in some cases silk will wrap around on the immersion heater, which hard to remove silk from heater.

Figure 9 shows the LabVIEW control panel of the automated boiling system. The bottom and top water level sensor trigger level can be set on the panel. When the system is running, the control panel can display the bottom and top water level sensor output voltage in real time.

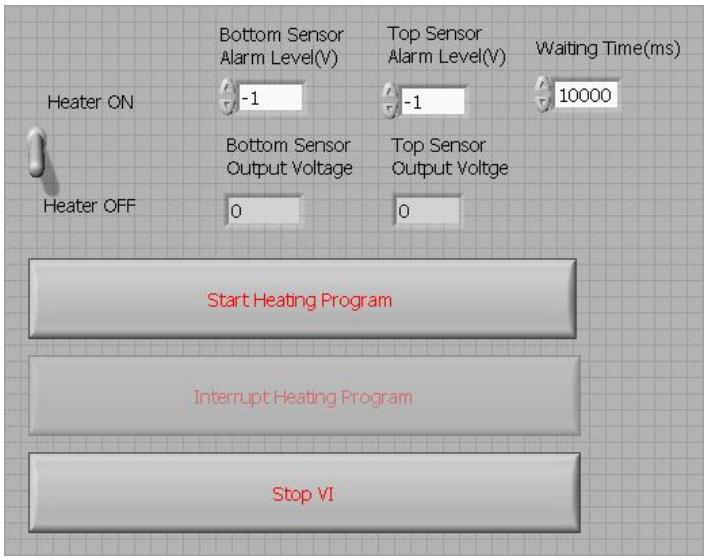
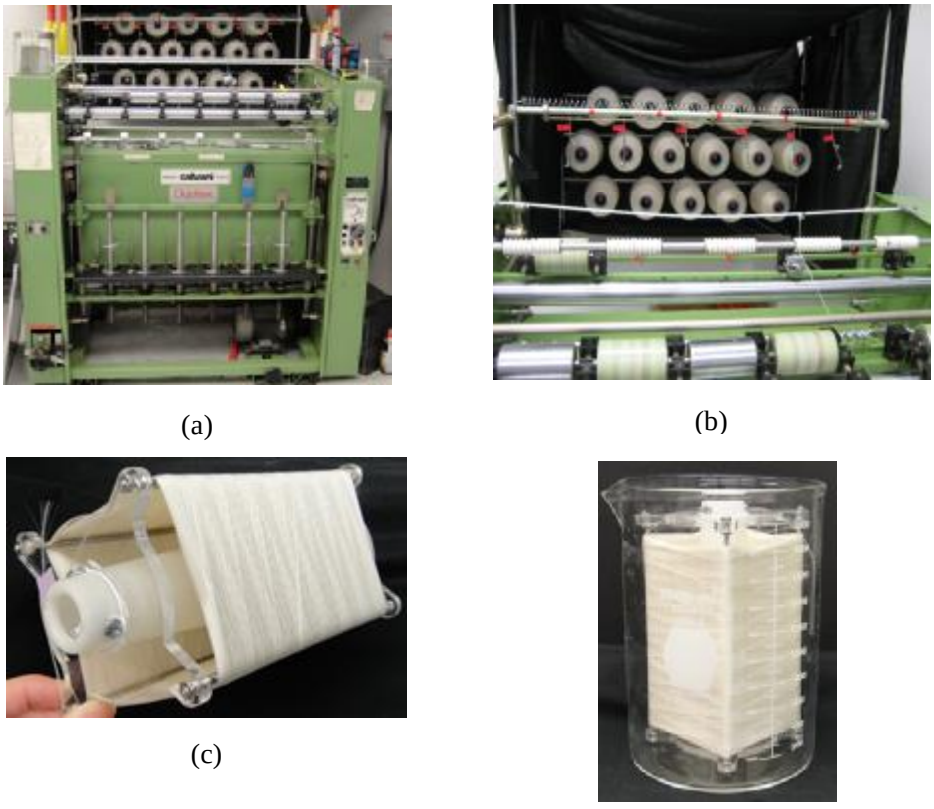


Figure 9. LabVIEW control panel for automated boiling system.

3.1.2 Customized bave silk yarn degumming

Figure 10 shows an alternative degumming process being explored, which uses silk yarns as the source. Silk yarns manufactured from bave silk also can be used to make silk solution. In order to get silk yarn, the very first step is winding bave silk using a Calvani Fancy Jet Ring Twister 6/SP. Unlike silkworm cocoons, there is a wax coating on bave silk which means not only sodium carbonate but also soap needs to be used in the degumming process for wax removal.



(a) Calvani Fancy Jet Ring Twister 6/SP.
(b) Brazil bave silk.
(c) Silk yarn.
(d) Degumming silk yarn in a beaker.

Figure 10. Brazil bave silk and silk yarn.

3.1.3 Silk autoclave degumming

An autoclave is a device used to sterilize equipment and supplies by subjecting them to high pressure saturated steam at 121 °C or higher, typically for 15-20 minutes depending on the size of the load and the contents [37]. It is proposed that a degumming procedure in an autoclave could provide the same degumming outcome as the current degumming process. First, a 5L beaker is prepared by filling it with 4L pure water. 8.48g of Na_2CO_3 is put into a beaker, then fully mixing with water. After that, 14g of dry silk fiber is put into the beaker. The beaker is covered with aluminum foil, and placed into a Consolidated Mark II Controlled Sterilizer (Autoclave) (Figure 11). The degumming process is finished under high temperature and high pressure in the autoclave. There are two problems in this process. First, it is a challenge to run degumming automatically using the autoclave because a lot manual interaction is involved. Second, it is still unknown how effective the approach is for removing sericin, and if damage could occur from the autoclave.

Figure 11. Consolidated Mark II Controlled Sterilizer (Autoclave) use in a proposed degumming process.

There is an intriguing effect on dissolving time when using autoclave as degumming equipment. Unlike normal degummed silk, autoclave degummed silk reduces dissolving time significantly. Silk fibers transform to a liquid state as soon as contact is made with LiBr solution. This interesting phenomenon may influence dissolving time greatly.

3.2 Refined dialysis processes

Because the current silk dialysis process requires significant human interaction and it is not very easy to handle, a new dialysis approach is desired. Dialysis cassettes and SnakeSkin tubes were considered for pursue in an automated silk solution process.

3.2.1 Automated water change dialysis system

In a prototyped automated water change system designed by Tim Lo, silk solution dissolved by LiBr is inserted into 30ml 3.5K MWCO Thermo dialysis cassettes. The cassettes are placed into an automated water change system. The automated water change system has a Vigoro 196-036 waiting timer which can control the customized water valve in the system to change water. The automated water change system changes pure water automatically every 6 hours for two days. When the water change is completed, the silk solution was extracted from the Dialysis cassettes using a syringe. Compared to the current dialysis process using a beaker, the automated water change system saves time and reduces a lot of human interaction. Figure 12 shows the dialysis process using the automated water change system.

(a)

(b)

(c)

(a) Insert dissolved silk solution into dialysis cassettes.
(b) Using automation system to change water every 6 hours for two days.
(c) Take out silk solution using a syringe after two days.

Figure 12. Dialysis process using automation water change system.

3.2.2 Automation dialysis system using SnakeSkin Dialysis Tubing

Thermo Scientific SnakeSkin Dialysis Tubing is an easy and ready to use form of traditional dialysis membrane tubing that allows desalting and buffer exchange for 15-100 ml samples. SnakeSkin Dialysis Tubing is regenerated-cellulose dialysis tubing that is supplied as an open, pleated (telescoped) tube. It is supplied in eight-inch (20-cm) sticks containing 35 feet

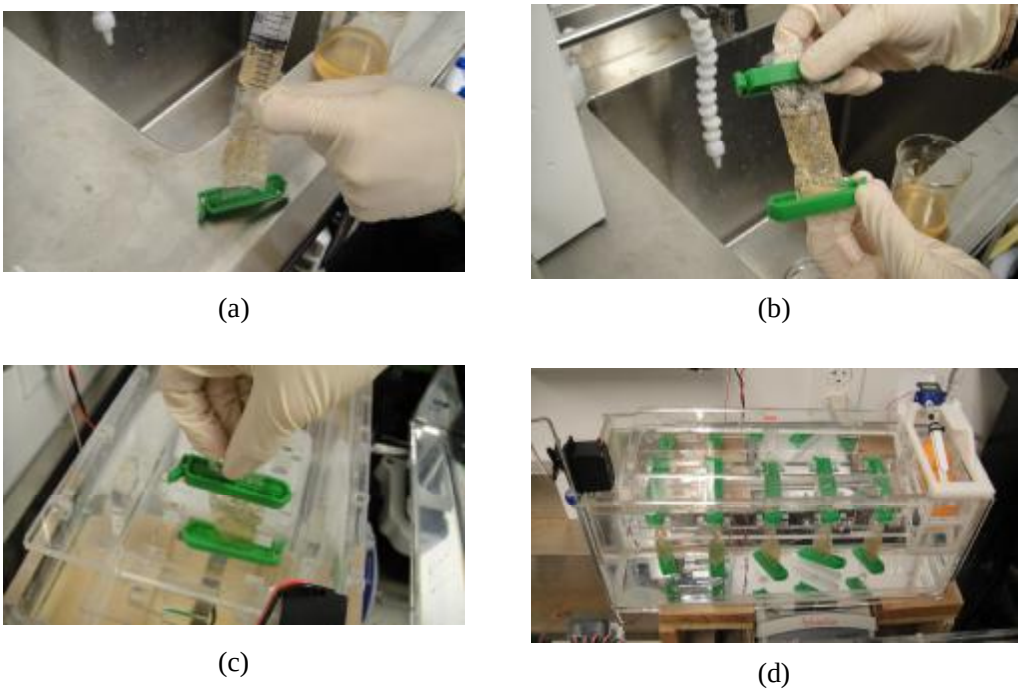
of dialysis tubing having a 22mm circular internal diameter. Equivalent in capacity to 10.5 meters of 34mm dry flat width tubing, hydrated SnakeSkin Tubing holds ~3.7 ml of sample per centimeter of length.

Traditional dialysis tubing supplied in a dry, flattened roll is difficult to open and often requires extensive soaking in water or buffer before it can be used. SnakeSkin Dialysis Tubing was developed by Thermo Scientific to simplify large sample dialysis. The pleated format of SnakeSkin Dialysis Tubing makes it easy to open and ready to use, streamlining dialysis preparation. The tube is used by cutting it to lengths, folding over one end and applying a clip to close it. Then solution is added in the open end, and a second clip is used to close the remaining end.

The pleating process does not change the tubing's MWCO. Also, any low MW contaminants present are removed during the dialysis process. Because SnakeSkin Dialysis Tubing is made from the same type of regenerated cellulose as flat tubing, its dialysis performance matches that of conventional tubing.

Figure 13 shows SnakeSkin Dialysis Tubing to be used in dialysis process. The SnakeSkin Dialysis Tubing is cut into 5 pieces approximately 6cm long each. The one open end of SnakeSkin Dialysis Tubing is closed by clip. After that, a syringe is used to put dissolved silk solution into SnakeSkin (Figure 13a) and the other open end is sealed by a clip (Figure 13b). All of the SnakeSkin Dialysis Tubings are put into automated water change system for

two days (Figure 13c and 13d). When the dialysis process is done, either side of the tube is opened, and silk solution can be easily removed.



(a) (b)

(c) (d)

(a) Silk solution is injected into SnakeSkin by a syringe.
(b) SnakeSkin is sealed by a clip.
(c) Sealed SnakeSkin is put into the automated water change system.
(d) Water changes are made for two days in the automated water change system.

Figure 13. SnakeSkin dialysis automation using the automated water change system.

There are several advantages of using SnakeSkin Dialysis Tubing in an automation system. Compared to the current dialysis process using Slide-A-Lyzer Dialysis Cassettes, SnakeSkin Dialysis Tubing saves a lot of time and reduces manual manipulation. It is very easy to put dissolved silk solution into SnakeSkin, but complicated if using dialysis cassettes. When using dialysis cassettes for the dialysis process, cassettes must be soaked in water for several minutes before using them. It is not that easy to inject dissolved silk solution into dialysis cassettes by a syringe with a needle

because the pressure inside a dialysis cassette is increasing while silk solution is injected into the cassette.

3.2.3 G2 cassette tests with manometer

The Thermo Scientific Slide-A-Lyzer G2 Dialysis Cassette is an easy to use cassette. It has a pipette-accessible port for easy sample loading and retrieval which saves time. Also, there is a chamber on the top of G2 cassettes that provides buoyancy and vertical orientation which is good for doing dialysis in a beaker of water.

The most unique aspect of this Slide-A-Lyzer G2 Dialysis Cassette is the pipette-accessible port. The cassette cap is twisted 30 degrees for removal. The silk solution can be easily injected into the cassette through the large hole in the cassette by a pipette or syringe. Also, the shearing on the silk is reduced because of the large hole.

A G2 cassette pressure test set up can be seen in Figure 14. A Sper Scienctific 5 PSI manometer was attached to a cassette to record the pressure change inside the cassette. There was an aluminum tube passing though the G2 cassette cap and connecting to the manometer by rubber tubing. The manometer was connected to a laptop through a USB port. A LabVIEW program was generated in order to record the pressure data. The pressure inside the G2 cassette was measured every 10ms and the data automatically saved into a txt file.

Figure 14. G2 dialysis cassette pressure test set up.

Figure 15 shows the LabVIEW graphic interface which can provide real time pressure data. On the front panel, the pressure inside the G2 cassette is shown in real time. Also, there is a graphic shows the pressure change.

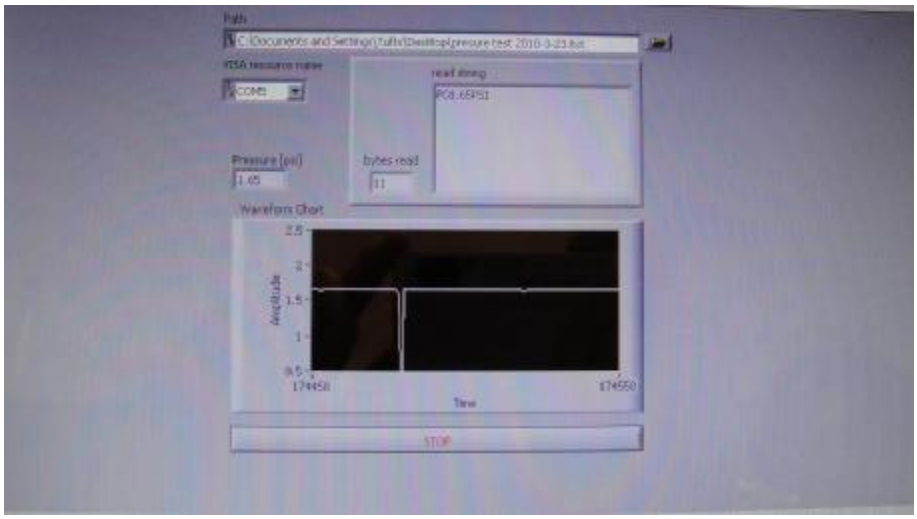
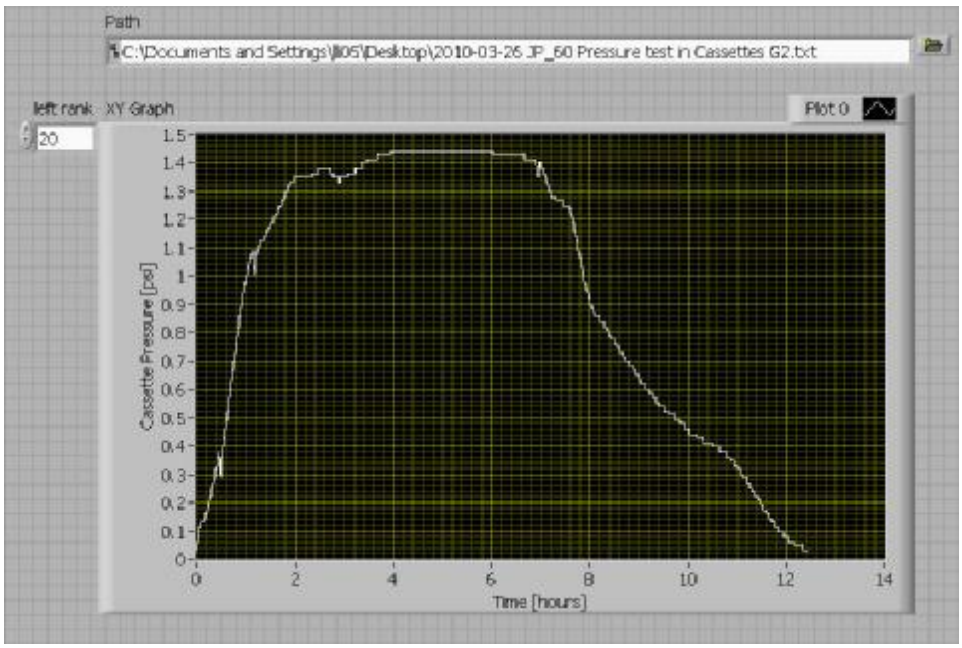
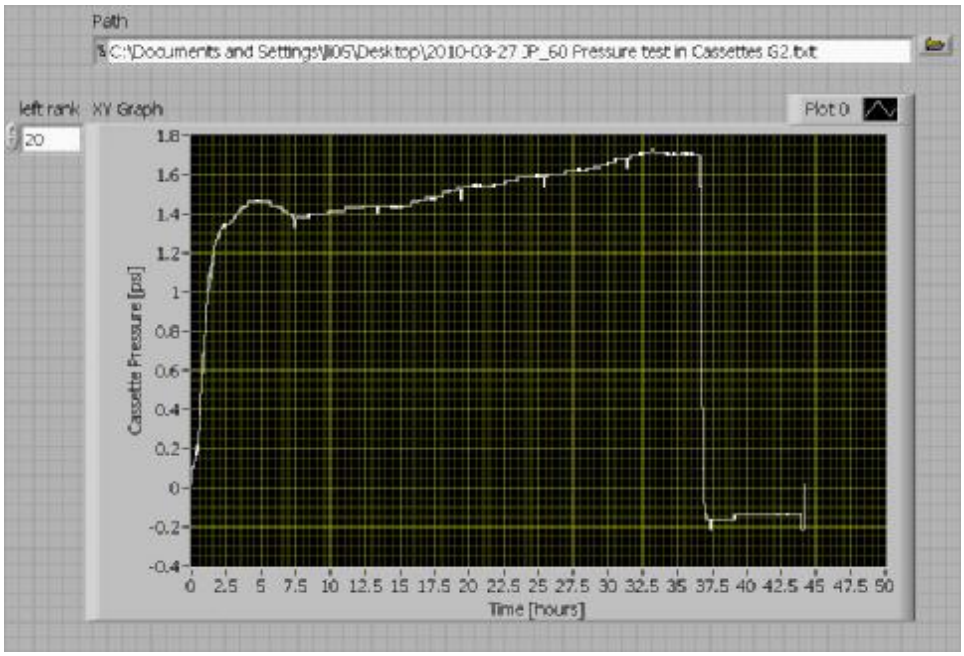


Figure 15. LabVIEW interface for real-time pressure data display.

Two successful G2 Dialysis Cassette tests were conducted. A third test was inconclusive due to software failure. The first test used 60 minute degummed Japanese cocoon silk solution. All three of the cassette membranes were broken after about 8.5 hours (Figure 16a). The first test showed maximum pressure inside the G2 cassette before breaking was 1.44psi. The second test used 60 minutes degummed Japanese cocoon silk solution and the membrane of the cassette failed after about 37.5 hours (Figure 16b). The maximum pressure inside the G2 cassette before failure was 1.73psi. The G2 cassette membrane area is about 22.96 cm^2, so that the force applied on membrane can be calculated. The force applied on the membrane is approximately 22.79 N for the first test and 27.36 N for the second test.



(a)

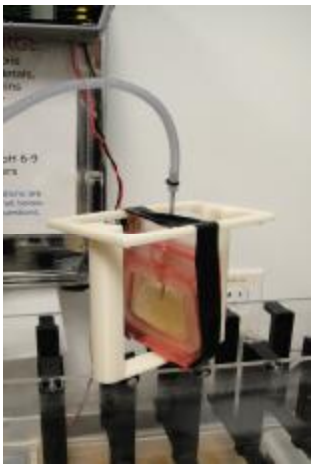


(b)

(a) The first JP 60 G2 Dialysis Cassette pressure test data.
(b) The second JP 60 G2 Dialysis Cassette pressure test data.

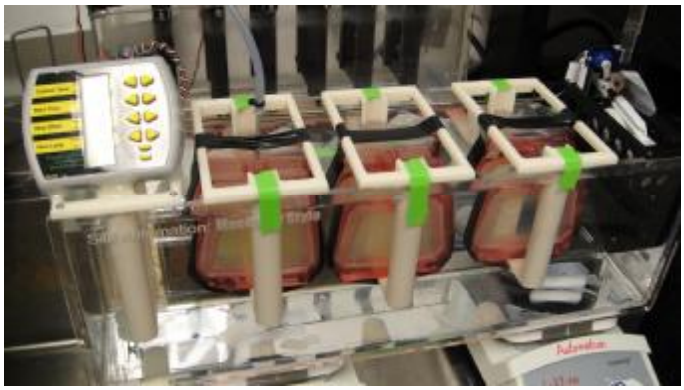
Figure 16. JP 60 G2 Dialysis Cassette pressure test data.

A new test experiment was designed (Figure 17a). 60 minutes degummed Japanese silk solution was prepared and injected into three Thermo 30ml 3.5K MWCO G2 cassettes. The silk solution volume added to each cassette varied; 30 ml silk solution was added to the first cassette, while 20ml and 10ml to the remaining cassettes. The manometer (Figure 17b) was turned on and the LabVIEW program used to record pressure data. Through observation, the pressure starts to increase as long as the cassette is placed into automation water change system.



(a)

(b)

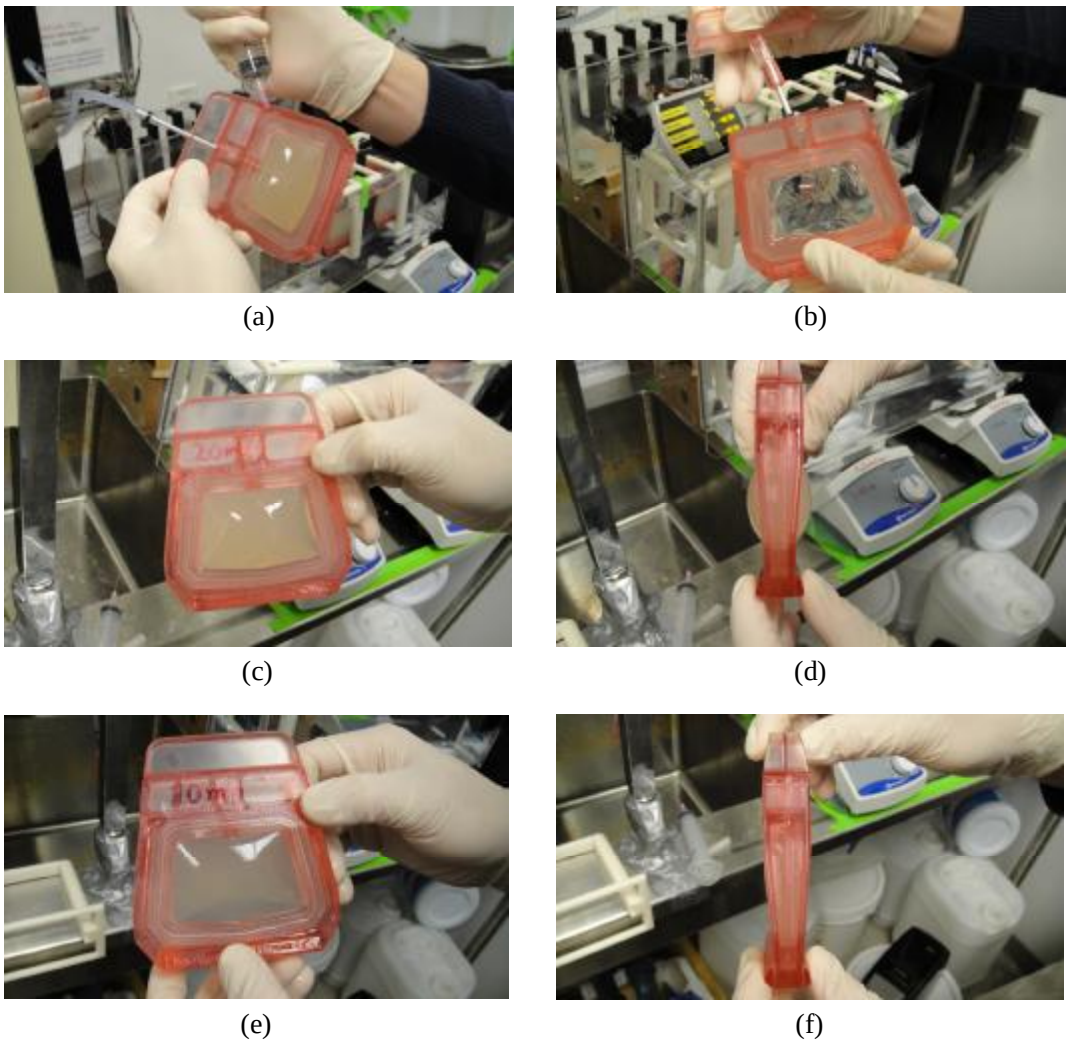


(c)

(a) G2 cassette was connected to manometer by tube.
(b) Sper Scientific 5 PSI manometer.
(c) Three different silk solution volume (from left to right: 30ml, 20ml, 10ml) 30ml G2 cassettes in automation system.

Figure 17. 30ml G2 Dialysis Cassettes test with manometer.

The membrane of the G2 cassette filled with 30ml silk solution was broken after one day, while the membrane of 20ml (Figure 18c and 18d) and 10ml (Figure 18e and 18f) silk solution cassette survived at the end of test after two days. The concentration of silk solution retrieved from the 20ml and 10ml silk solution cassettes is about 4%-5% w/v, lower than the normal silk solution which is around 7%-8% w/v.



(a) (b) 30ml JP60 silk solution after dialysis using 30ml G2 cassette (failed).
(c) (d) 20ml JP60 silk solution after dialysis using 30ml G2 cassette.
(e) (f) 10ml JP60 silk solution after dialysis using 30ml G2 cassette.

Figure 18. G2 Dialysis Cassette testing in automation water change system.

Because almost all the 30ml G2 cassettes failed, the 15ml G2 cassettes dialysis test was investigated. Figure 19 shows the experimental test setup of 15ml G2 cassette. In this test, Mettler Toledo InLab®731 Conductivity probe and Mettler Toledo InLab®Expert Pt1000 pH probe was used. Table 5 shows that conductivity increases dramatically as long as the cassettes were preset in

the automation water change system, but there is almost no change in pH.

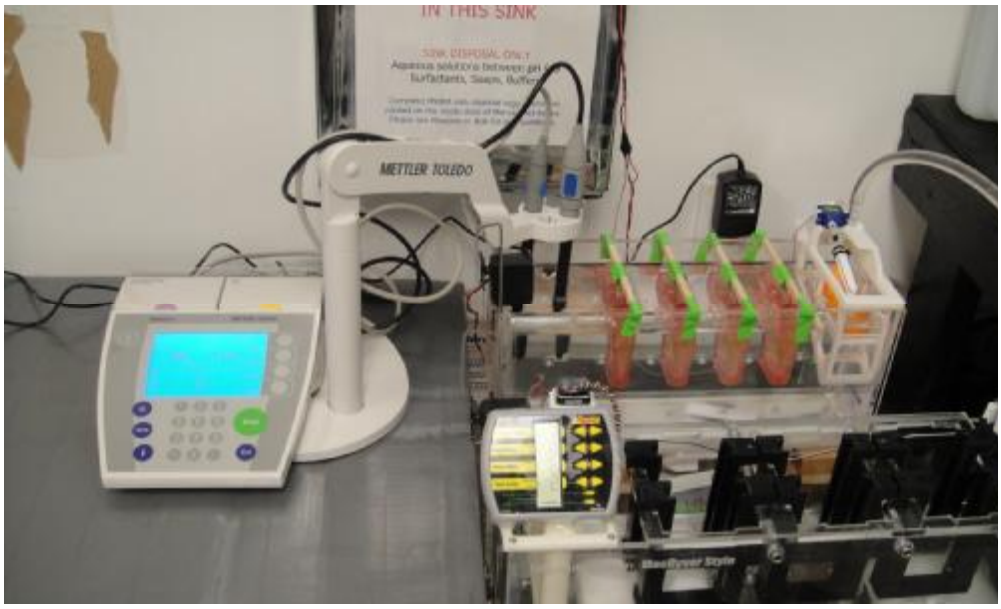


Figure 19. JP30 silk solution 15 ml G2 cassette test with conductivity sensor.

All the cassette membranes were broken after one day. The possible reason for all the failures are that compared to the Slide-A-Lyzer Cassette, the bottom of G2 cassettes is narrower than the top. When the pressure increases, the non-flat cross section of G2 cassette causes non-uniform pressure contribution along the membrane edge.

Table 5. Conductivity and pH change for 15ml G2 Dialysis Cassettes test.

Test	Conductivity	pH
1	727μs/cm	6.740
2	950μs/cm	6.732
3	1804μs/cm	6.725
4	8.00ms/cm	6.728

3.3 Silk solution post processing

3.3.1 Filter

For the current silk solution processing, a centrifuge is used to remove the impurities inside the silk solution. However, it takes really long time and much human interaction to use a centrifuge. Instead of centrifuging, a proposed idea of using a filter to remove impurities has been studied.

Figure 20 shows a Nalgene 167 Filter Unit being used to filter the silk solution. Silk solution is poured into the upper cup of the filter. The filter is connected to a vacuum by a plastic tube. Silk solution will be extracted into the lower cup by vacuum pressure. There are several advantages using filter to remove impurities. First, it saves time and reduces the need for human interaction compared with centrifuge. Second, the silk solution is sterilized after filtering, which can be good for biomedical applications. The potential issue is that there might be shear on the silk solution because the pore size of the filter is very small, around 0.45μm.

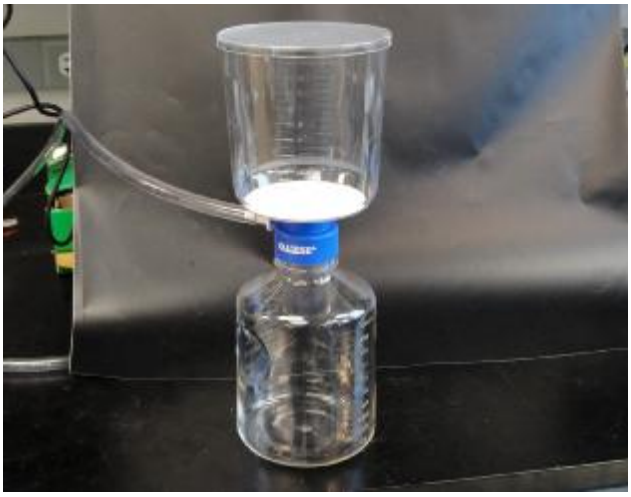


Figure 20. NALGENE 167 Filter Unit.

3.3.2 Lyophilizer

Lyophilization (freeze-drying) is a dehydration process that is used for perishable material storage and transport. The perishable material is frozen and the pressure around the material is reduced. Then enough heat is added in order to let the frozen water sublime from a solid phase to a gaseous phase. Figure 21 shows a VirTis Genesis 25L Lyophilizer. Self-assembly is an issue when using refrigerator to store silk solution. In order to keep the silk solution longer enough, silk solution is freeze-dried by a lyophilizer and stored. When silk solution is needed, mixing lyophilized silk solution with pure water then silk solution will be ready to use.

Figure 21. VirTis Genesis 25L Lyophilizer.

Chapter 4 Conclusions

The focus of the research reported in this thesis was the development of a more automated process for converting native cocoon silk to a soluble form for tissue engineering applications. Since it is very challenging to develop a fully automated system for the entire silk solution process, a dual-track design approach has been initiated. The first track has focused on the design and manufacture of a semi-automated system for each step of silk solution processing. The second track has focused on fundamentally new technology that is higher-risk (no guarantee at this early stage to perform well), but shows promise for providing a more fully automated system. It has been the intention in both tracks to provide a computer- and sensor-based interface to enable system control and monitoring, crucial steps in implementing *in situ* quality control.

As part of the first track, an automated boiling system was prototyped. The working prototype was faithful to the current boiling process used to degum cocoon silk; boiling water with sodium carbonate would be used to remove the sericin from the silk fiber. Initial indications are that the automated system, which utilizes an immersion heater, custom water level sensors, and a computer-based control and feedback system, works as well as the current manual process. Track two proposed designs include an autoclave-based degumming, a combined machine to perform degumming, washing, and drying, and a more elegant circulating bath and immersion circulator approach

to degumming. Each of these designs has its advantages and disadvantages. Further research and testing is needed.

Dialysis is another step that requires significant human interaction and would greatly benefit by automation. In a track one design approach, a previously designed automated water change system was utilized with a newly introduced SnakeSkin dialysis membrane. In addition, a unique G2 dialysis cassette design was studied for possible usage in dialysis. The water change system has previously been shown to greatly reduce human intervention in the dialysis processing and has been proved to be reliable. Using a watering system microcontroller, the system can easily be implemented using a computer- and sensor-based automation system. The G2 dialysis cassette has accessibility for pipettes, which can greatly reduce concern over solution shearing when standard syringes are used. However, a series of experiments demonstrated that the membranes on such cassettes are not reliable enough for the application. The SnakeSkin automation dialysis design has been tested and demonstrated to work fine. Further research needs to be pursued, including measurement of residual Lithium and Bromine to determine if the solvent material has been removed from the solubilized silk.

The initial ideas on how to implement process control and monitoring focused on PC-based LabVIEW control of National Instruments CompactDAQ hardware with appropriate data module interfaces. The initial prototype applied to the automated boiling system was promising. Using a

web-based approach, a LabVIEW-based web server was set up, in which a web-accessible control program could be either directly controlled or monitored from a computer not directly connected to the experiment. Overall, significant progress has been made in track one development of a working prototype for each stage of the silk solution process. Some clever ideas and proposed design solutions for the track two approach have been identified. These ideas will soon be ready to be tested by designing actual working prototypes.

Chapter 5 Future work

5.1 National Instrument CompactRIO silk solution process automation system

Figure 22 shows a proposed design that uses a National Instruments CompactRIO to control and monitor the whole silk solution process. Every individual step would be controlled by CompactRIO based on a LabVIEW control program. There are sensors proposed for every individual sub-system which can provide feedback to the CompactRIO. Meanwhile, remote control and monitoring can be accomplished by the CompactRIO based on internet. There is a feature in LabVIEW called “web server” which can allow users to generate a webpage and this webpage can be accessed anywhere as long as there is internet access. The advantage of this web server feature is that it can not only provide real-time monitoring of the silk solution processing system but also can be used as control interface to control the silk processing remotely. Limits of authority can be added to the silk processing control webpage. For the common user, it would only provide real-time data of the silk solution processing system. For the system manager, it would allow control inputs to the silk solution processing system.

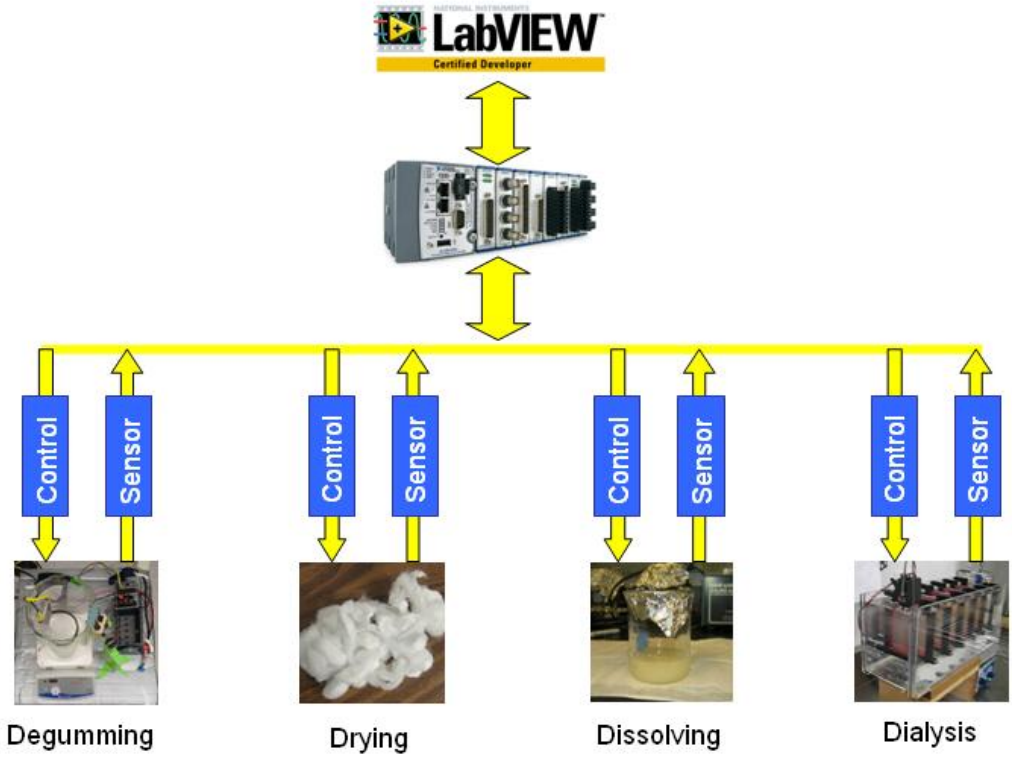


Figure 22. Silk solution process integrated by CompactRIO.

5.2 Combination machine for silk degumming and drying

Figure 23 shows a proposed design for a combination cooking and washing machine that performs both the degumming and drying processes in one machine. The most fascinating part of this design is that it can combine multiple processing steps which greatly reduce human involvement. Also, it is fairly easy to scale up, depending on the quantity of degumming silk needed.

The proposed cooking/washing/drying machine degumming process would start by providing pure water into the machine chamber. Sodium carbonate is added into the machine and the water is brought to a boil. After the solution is at boiling temperature, cocoons are placed into the machine. The machine would automatically stop after a pre-set time. Then, water inside the

cooking/washing machine would be released out and pure water provided for rinsing. After several rinsing cycles, all the water inside the machine would be removed from the machine. Then the inside chamber of the machine would rotate in order to remove any remaining water trapped inside the silk. At the same time, the machine chamber would be heated up, just like a drier and silk would then dry. The degummed silk could be removed from the machine after it is fully dry.

Figure 23. Proposed cooking/washing/drying machine for silk degumming.

5.3 Silk degumming using circulating bath and immersion circulators

Inspired by the automated boiling system, another degumming design is being pursued. Figure 24 shows a Thermo Scientific Haake Shaking Water Bath that can be used as a silk degumming system. It has an adjustable over temperature protection and PID control for accurate temperature and speed performance. Speed and temperature are set via a keypad and viewed on a digital display [67].

Figure 24. Circulating bath proposed for degumming.

Baths feature a high level of safety and can support continuous running. The heater and control sensor are located underneath the bath, making the bath easy to clean. An optional transparent plastic roof-shaped bath cover prevents water spillage due to fluid turbulence formation. Because it is observed that silk can wrap on the immersion heater of the silk automated boiling system, it is necessary to have some mechanism to stop it from happening. For the circulating bath, the heater and sensor are located underneath the bath which can avoid silk wrapping on the heater. Meanwhile, the water inside the bath will circulate, which can help in degumming the cocoons.

Another possible idea is using a PolyScience® Standard Immersion Circulator, 120 VAC (Figure 25) to perform the degumming process. The immersion circulator has a two speed pump, and an LED display that reads temperature in °C or °F. The set point is rapidly changed with a rotary encoder or with three user-selectable preset temperature set points. PID control ensures precise temperature under changing load conditions. The two-speed pump minimizes turbulence in small tanks, while providing greater uniformity in large tanks. A directable nozzle can set the direction of pump flow [67]. This design is similar as the circulating bath design. Basically, the immersion circulator can provide heat and circulation for silk degumming. It is easy to scale up depending on the needed volume of silk.

Figure 25. Immersion circulator degumming.

5.4 Bave silk yarn continuous degumming

Figure 26 shows a proposed design for using bave silk yarn in a fully automated degumming process. There are two baths for degumming and pure water rinsing.

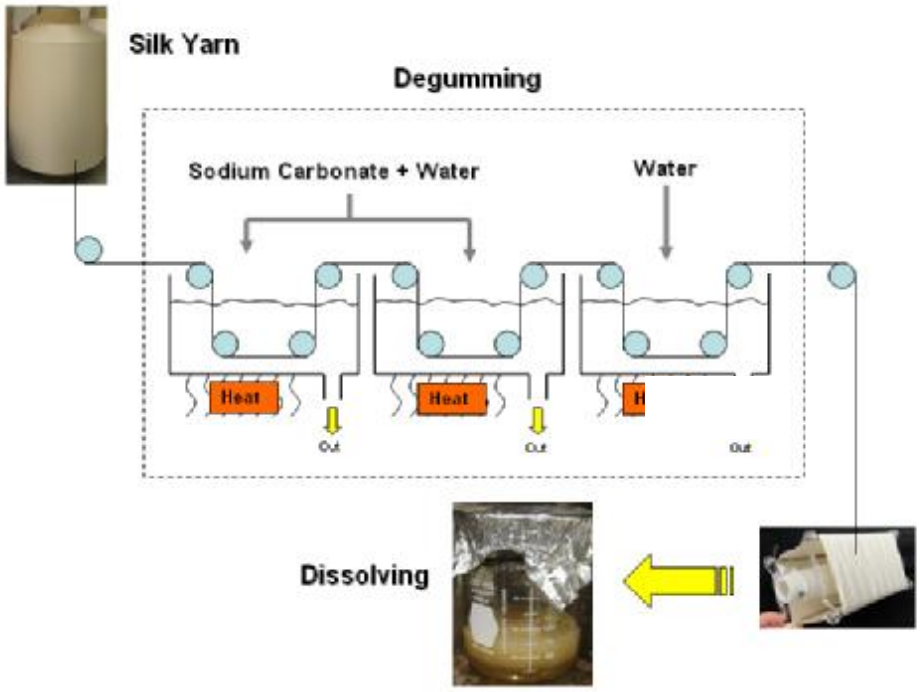


Figure 26. Bave silk yarn continuous degumming

The bave silk is pulled off of its spool and brought into two baths. Sodium carbonate solution is added into the two baths and the baths are heated to boiling temperature to degum silk. After a controlled period of time, all the sodium carbonate solution and water will be refreshed in order to maintain the silk degumming quality. This design can greatly reduce human involvement in the silk degumming process. As long as the system is set up, it can run the degumming and rising process continuously. Meanwhile, it is easy to scale up depending on the needed of silk which can improve the silk degumming process greatly.

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