



Silk Fibroin Optimization for Vaccine Delivery via Microneedle Patch

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Silk Fibroin Optimization for Vaccine Delivery via Microneedle Patch

A senior design project report submitted in partial fulfillment of the
requirements for the degree of Bachelor of Science in Engineering Sciences
at Harvard University

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Abstract

Vaccine-loaded microneedle patches have the potential to improve vaccine efficacy and access. Increasing body of evidence suggests that microneedle vaccine delivery increases the strength and breadth of immune response due to the sustained nature of vaccine antigen release. Vaccine-loaded microneedle patches also improve vaccine thermal stability, eliminating the need for expensive cold chain technologies that limit vaccination rates. Furthermore, microneedle patches allow for painless, self-administration of vaccines, eliminating sharps waste and the need to visit healthcare clinics. Vaxess's microneedle patches consists of a solid vaccine-loaded silk fibroin tip that prevents vaccine aggregation and improves thermal stability. Although commercial and academic sources of silk fibroin exist, creating a pure, shelf-stable, consistent silk fibroin material that does not introduce limitations in microneedle patch performance is a challenge. There is a need to systematically improve silk fibroin parameters, particularly those that have not yet been explored, to create stable silk fibroin that can be used in microneedle patches. The goals of the thesis are 1) to produce consistent and pure silk fibroin that is shelf stable even when subject to time and temperature stressors, and 2) to optimize the performance of dried silk fibroin microneedle patches for vaccine delivery. The improved silk fibroin material will prove critical in supporting the transition from bench scale silk fibroin and microneedle patch production to clinical manufacturing at Vaxess Technologies. Taken together, this thesis presents the first systematic enhancement of silk fibroin, both as a shelf-stable intermediate product and ultimately for optimized microneedle patch applications.

Chapter 1

Introduction

1.1 Background and Motivation

Despite recent rapid medical advancement, infectious diseases such as measles and influenza still pose a significant threat to society. For instance, the most common infectious disease—influenza—creates an economic burden of around \$11.2 billion per year in the United States, with 9.3 to 45 million cases per year, depending on the season [1] [2]. Vaccines have become an important tool in combating infectious disease, particularly in light of the recent pandemic, yet immunity does not cover entire populations because of two primary factors: vaccine efficacy and rate.

Vaccine effectiveness, while influenced by demographic and host factors, is primarily dependent on the degree of antigenic match between the virus in the vaccine and the circulating virus strain (**Figure 1.1**) [3]. Specifically, over the course of the flu season, accumulated influenza virus mutations minimize the effectiveness of strain-specific hemagglutinin (HA)—the most abundant glycoprotein on the influenza virus surface—antibodies, which no longer become effective in preventing infection [4] [5]. Possible solutions to improving strain match include production of immunogens based on the conserved region of the influenza virus, minimizing strain mismatch, and delivering the vaccine via the skin or nasal tract; however, research into these alternatives are still in early development [6] [7] [8]. In order to more immediately improve vaccines, the humoral and cellular immune responses of current influenza vaccines can be strengthened. While the immune response can be enhanced through increased vaccine dose, addition of adjuvants, or use of recombinant or cell-based products,

antigen kinetics—the temporal pattern of antigen uptake, processing, and presentation—have been shown to play a key role [9] [10] [11] [12]. Typically in a natural infection, the virus is presented for 1-2 weeks, leading to increased antibody generation, memory T-cell response, and heterosubtypic protection [13]. In contrast, traditional bolus vaccines result in antigen clearance within a day, minimizing antigen presentation to the lymph nodes. Therefore, mimicking natural infection by presenting the vaccine antigen for a longer period of time could not only enhance immune response to the included strain, but also against drifted strains [14].

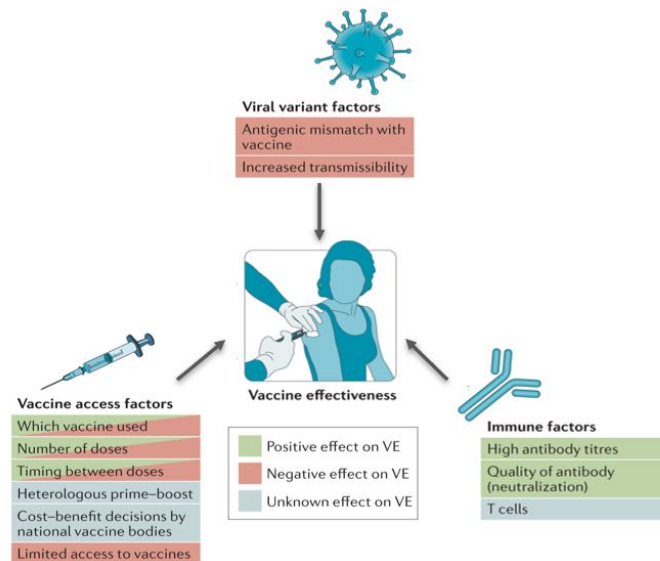


Figure 1.1: **Key factors related to vaccine effectiveness.** While demographic and host factors such as age and pre-existing conditions impact vaccine effectiveness, vaccine antigen match, vaccine access, and immune response play the largest roles in vaccine efficacy [15].

Meanwhile, vaccination rates are driven by vaccine access and willingness to vaccinate. Currently, vaccines are administered by healthcare professionals in clinics due to the stringent cold-chain storage conditions required to maintain antigen stability [16]. Not only do vaccine storage requirements limit vaccination in remote populations, cold-chain technology is expensive and results in wasted supply from temperature-control related issues (**Figure 1.2**) [17]. Willingness to vaccinate is influenced by a combination of public perception of vaccine effectiveness and needle phobias, prevalent in over 20% of the population [18].

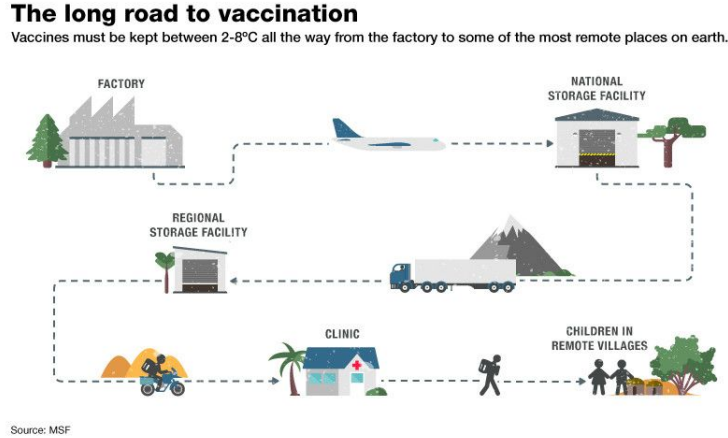


Figure 1.2: **Storage and shipment logistics related to vaccine distribution.** The World Health Organisation estimates that more than 50% of vaccines are wasted globally every year because of temperature control, logistics, and shipment-related issues arising from cold-chain storage requirements [17].

Microneedles have the potential to improve upon both vaccine efficacy and rate. Intradermal delivery via vaccine-loaded microneedles allows for direct targeting of professional antigen presenting cells in the immunocompetent skin tissue [19]. Furthermore, vaccine release kinetics from microneedle patches can be tuned depending on the encapsulating material to enable sustained release [5]. Biomaterials such as silk fibroin can extend antigen persistence in the skin for over 10 days, imitating natural infection and enhancing the magnitude, duration, and breadth of humoral immune response. Overall, vaccine efficacy is increased via microneedle delivery due to enhanced immunogenicity at lower dose levels and increased cross-reactivity of antibodies against drift strains. Use of biomaterials such as silk fibroin, which do not generate inflammatory byproducts upon degradation, can also prevent vaccine aggregation and improve thermostability, reducing the need for cold-chain storage and expanding vaccine access [20]. Furthermore, the needle-free technology enables painless, self-administration, potentially increasing vaccine acceptability [21].

Vaxess’s microneedle patch, MIMIX, consists of a solid vaccine loaded-silk fibroin “tip” and sugar-polymer “base” that dissolves upon application to deploy silk tip depots that remain in the skin over a period of days to weeks [22]. Vaxess Technologies is currently isolating silk fibroin from raw silk and using this liquid fibroin to construct microneedle

patches. Silk fibroin was selected for microneedle patch applications due to its biocompatibility, affordability, tunable degradation via crystallinity content, thermostabilization properties, and mechanical robustness required for skin penetration.

Silk Fibroin

Unlike synthetic polymers such as Polylactic acid (PLA) and Poly Lactic-co-Glycolic Acid (PLGA) used in drug delivery systems, biomaterials (i.e. silk fibroin) used in microneedles allow for microneedle processing under all-aqueous conditions without the need for ultraviolet cross-linking, elevated curing temperature, or high molding pressure, all of which impact vaccine antigen stability [23]. Furthermore, biomaterials result in noninflammatory degradation products. Compared to other biopolymers used in microneedle applications, silk fibroin's organized crystalline domains of β -sheets provide enhanced mechanical properties, particularly the necessary strength, stiffness, and toughness required for microneedle applications [24]. Furthermore, silk fibroin has been proven to protect biomacromolecules at elevated temperatures, providing a shelf-stable vaccine delivery system that does not require refrigeration [20] [25]. Finally, the sustained, programmable delivery of cargo from the silk fibroin matrix has been shown to elicit a more effective, broader, longer-lasting immune response [5] [11].

Given that silk fibroin is responsible for the distinguishing properties of Vaxess microneedle patches, a consistent, tunable source of silk fibroin is required to create effective, vaccine-stabilizing microneedle patches. Commercial silk fibroin is not suitable for generating microneedles due to previously documented inconsistent solution properties and the addition of excipients that result in solutions with high pH, around 10 [26]. Although academic groups have also produced silk fibroin, these solutions have been reported to remain stable for as short as ten days at 4°C, while manufacturing criteria dictate required stability of at least three weeks [27]. Since Vaxess commercial use will require silk fibroin production in advance of final microneedle manufacturing, the silk fibroin solution will be subject to

not only time but also elevated temperature stress, further shortening the shelf life. Thus, the stability of silk fibroin solutions, particularly subject to processing condition stress, represents a major limitation to utilizing silk fibroin as a biomaterial for microneedle patches and widespread technological applications [28]. Given the shortcomings of existing silk fibroin sources, the goal of this thesis is to create a consistent, pure, shelf-stable silk fibroin solution that enables microneedle patch applications. Furthermore, existing studies have not explored the impact of silk fibroin formulation process on ultimate microneedle patch performance.

1.2 Project Goals

The objective of this design project is to engineer pure, consistent silk fibroin that is shelf-stable and applicable for the manufacturing of vaccine-loaded microneedle patches (**Figure 1.3**). This will be accomplished by:

1. Designing reliable analytical methods used to assess the consistency of silk fibroin production, specifically to quantify the aggregation of silk fibroin solutions (Appendix A.1).
2. Varying the extraction and processing conditions of silk fibroin, measuring the impact of varied fill finish procedure, pH, conductivity, and molecular weight on silk fibroin characteristics and shelf life.
3. Printing vaccine-loaded silk fibroin microneedle patches with varied pH, conductivity, and molecular weight silk fibroin.
4. Evaluating vaccine release, vaccine stability, and mechanical outputs of microneedle patches printed with varied pH, conductivity, and molecular weight silk fibroin.
5. Determining improved silk processing conditions based on holistic evaluation of silk fibroin shelf life and vaccine-loaded microneedle patch performance

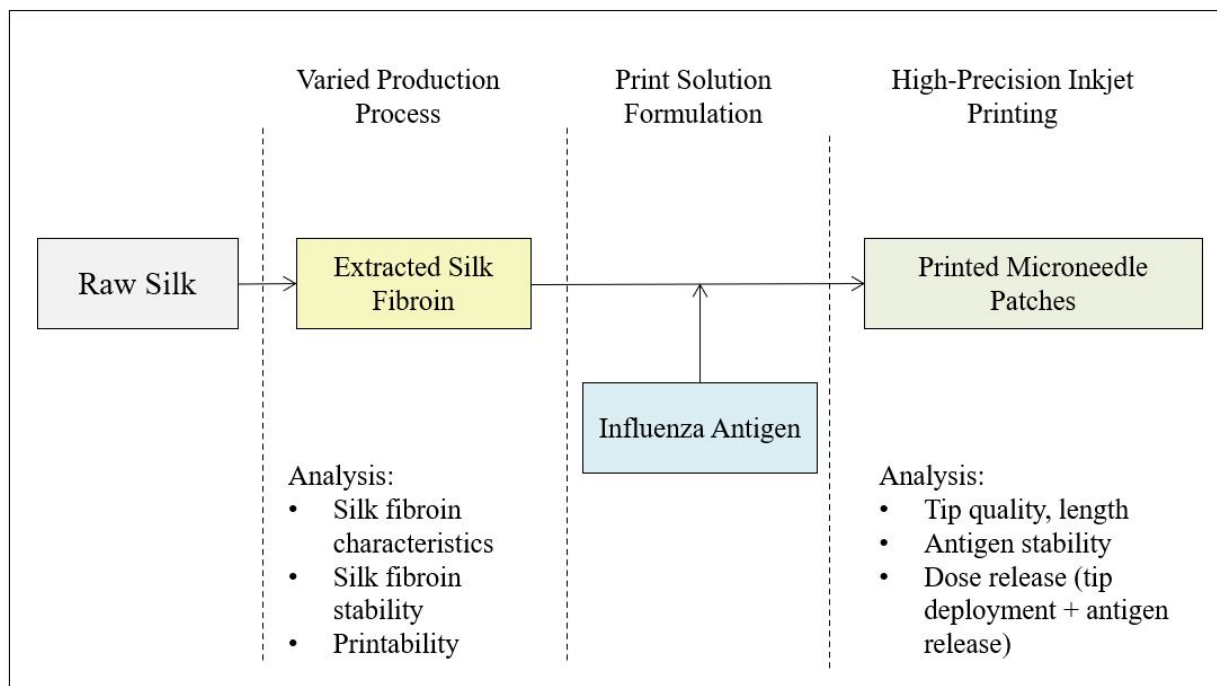


Figure 1.3: **Project workflow overview.** Key steps in project workflow, indicating material inputs, varied processes, and high-level analysis.

1.3 Potential Users and Stakeholders

The targeted end user of the engineered silk fibroin is Vaxess Technologies and relevant CMOs. The well-characterized, stable silk fibroin will be predominantly used for the manufacturing of microneedle patches ultimately used for vaccine delivery.

The stakeholders (**Figure 1.4**) for the developed silk fibroin solution can be broadly divided into two categories: Vaxess Technologies and academia or industry partners. The stakeholders for Vaxess’s microneedle patch technology include patients and healthcare professionals. Patients and families benefit from the self-administered, painless, needle-free technology that results in an enhanced immune response. Microneedle vaccine patches may eliminate the need for healthcare staff in vaccination, limiting patient cross-infection and allowing health professionals to dedicate their time to other causes. Even if microneedle patches are administered by healthcare staff, microneedle administration would reduce the cost, time, and sharps waste compared to current needle injections. Apart from Vaxess

Technologies, the silk fibroin solution can be distributed and used by Vaxess partners within academia and industry. The favorable qualities of silk fibroin can be capitalized on in other manufacturing and biotechnological applications. For instance, silk fibroin microneedle patches can be used for broader transdermal drug delivery in the areas of cancer, contraception, and diabetes among other applications [21].

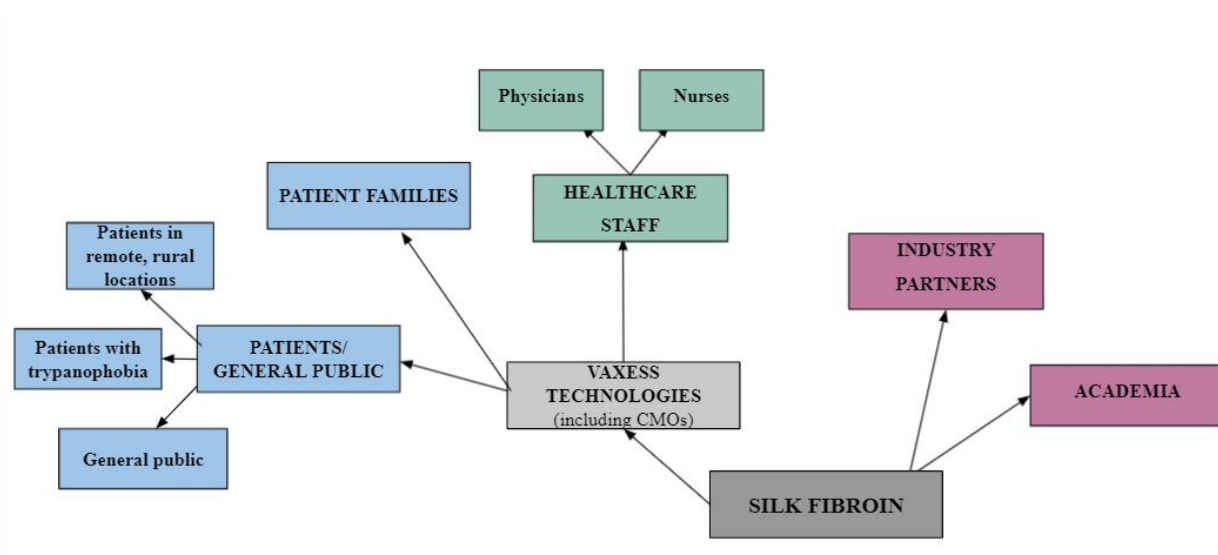


Figure 1.4: **Stakeholder map.** Stakeholders of the silk fibroin solution fall under Vaxess Technologies (green and blue boxes) or Vaxess Technologies’ partners (pink boxes).

1.4 Design Criteria

The needs of Vaxess Technologies are prioritized in designing silk fibroin for vaccine-loaded microneedle patch applications. Understanding the impact of silk fibroin processing conditions on silk fibroin shelf life will extend the usability of individual silk fibroin batches, simplifying experimental procedures. Furthermore, engineering a silk fibroin solution that simultaneously optimizes silk fibroin shelf life and microneedle patch performance will hone production parameters, reducing inter-lot silk fibroin and microneedle variability. Successfully engineered silk fibroin solutions should have the following criteria:

- **Consistent and pure silk fibroin that is shelf stable even when subject to time and temperature stresses:** Silk fibroin production parameters (i.e. fill finish,

pH, conductivity, molecular weight) are suspected to impact solution stability. These parameters, as well as overall silk fibroin shelf life, will be quantified.

- **Easily printable silk fibroin solution:** Viscosity and surface tension impacts the ease with which solutions are printed using high-precision printing.
- **Results in effective microneedle patches:** Prototype microneedle patches will be printed using the variable silk fibroin solutions. Microneedle biomechanical outputs such as dose release (comprised of vaccine in vitro release and tip deployment), vaccine stability, tip length, and mechanical robustness will be evaluated and used to rank-order silk fibroin tip formulations.

This work is innovative in that it improves the understanding of process factors affecting silk fibroin shelf life, printability, and microneedle patch applications.

Chapter 2

Background Research

Silk fibers are natural, biocompatible proteins produced by both insects and spiders. Silkworm silks, such as silk from *Bombyx mori*, have traditionally been used in textile production and biomedical sutures [29]. Apart from the excellent material properties of silk, the biopolymer has also gained attention due to its affordability and availability. Silk consists of two parallel, semi-crystalline fibroin strands encompassed in amorphous sericin protein. Fibroin accounts for approximately 70-75% of silk fibers by weight, while water-soluble sericin makes up 25-30% of the total weight [24].

Silk fibroin, an amphiphilic copolymer consisting of hydrophobic blocks linked together by hydrophilic segments, is the usable component of silk fibers and the material of interest for this project (**Figure 2.1**). The hydrophobic domains align to form crystalline antiparallel β -sheet structures stabilized by hydrogen-bonded amides. An amorphous, random coil network of the hydrophilic domain surrounds the β -sheets [24]. The structure of silk fibroin allows silk to maintain both impressive flexibility and strength, while conserving water molecules within a dried matrix. Protein antigens can also become entrapped in the silk fibroin matrix during drying. The water-containing rigid, yet porous structure, is theorized to thermally stabilize antigen molecules while simultaneously enabling antigen release, a property useful for microneedle applications [22]. The rate of antigen release from microneedles is multi-modal, consisting of an initial burst release, a second phase of slower antigen diffusion, and a final phase of silk matrix degradation resulting in delayed antigen presentation to the immune system [30] [31].

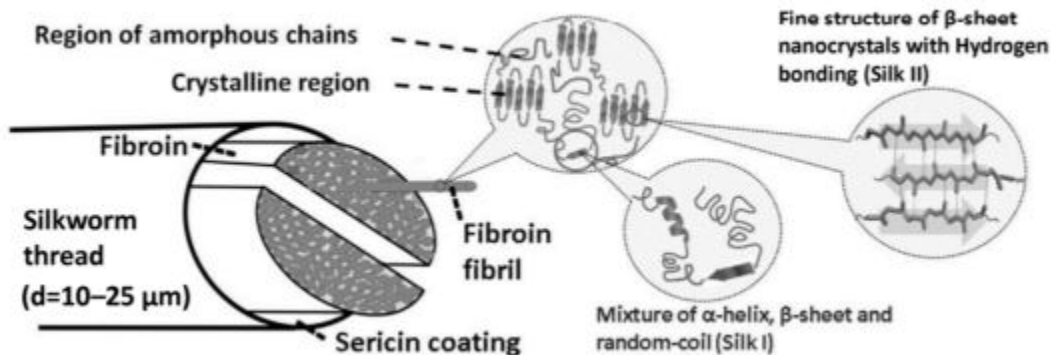


Figure 2.1: **Silk fibroin structure.** Silk fibroin is a block co-polymer containing large hydrophobic domains and small hydrophilic regions. Upon folding, silk fibroin forms organized crystalline domains (β -sheets) which provide insolubility in water and impressive mechanical strength [24].

2.1 Production Process

Silk fibroin is extracted from silkworm cocoons by exposing the fibers to heat and alkaline conditions to soften and hydrolyze the glue-like sericin coating, a process called degumming [27]. Fibroin is then cleaved into a desired molecular weight, referred to as denaturing. The final steps of silk fibroin processing include dissolution of the protein using lithium bromide, desalting via dialysis, and fill finish. The processing conditions of each step in silk fibroin extraction impact the ultimate properties of the silk fibroin solution [27]. The final silk fibroin product is a slightly viscous liquid with minimal aggregation.

2.2 Enhancing Stability: Silk Fibroin Processing

Available sources of silk fibroin do not result in a consistent, shelf-stable material suitable for microneedle applications. A number of studies have been conducted on influencing the silk fibroin shelf life by varying the processing conditions. One study varied the silk fibroin denaturing time from ten to sixty minutes and found that lower molecular weight silk fibroin results in a slower sol-gel transition and prolonged shelf life [32]. Decreasing the storage concentration of the silk fibroin solution can also extend shelf life [27]. How-

ever, exploratory studies conducted by Vaxess have demonstrated that silk fibroin properties and concentration must satisfy specific criteria for successful printing and performance of microneedle patches. Existing published studies did not examine the impact of varied silk fibroin on microneedle applications, which are included in the current thesis goals.

Although commercial silk fibroin solutions are stable for up to several months at 4°C, aggregates are visible in thawed solutions. Furthermore, enhanced stability can be explained by varied dissolution procedures, which significantly increase solution pH. Commercial silk fibroin sources, such as Advanced Biomatrix Silk Fibroin, have an 8.0-10.0 pH compared to a 5.2-6.9 pH for Vaxess silk fibroin solutions [26]. Alkaline silk fibroin pH levels interfere with antigen stability and potency, requiring time- and energy-consuming desalting procedures prior to use.

Taken together, although work has been conducted to prolong silk fibroin shelf life, existing studies do not explore the full impact of processing conditions on silk fibroin and its suitability for microneedle patch applications (**Figure 2.2**).

Silk Fibroin Properties	Commercial Sources	Research Labs
Pure, consistent silk fibroin material		
Silk fibroin shelf life >3 weeks		
Resistance to temperature stress		
Compatible with microneedle applications		

- Does not meet specified criteria
 - Occasionally satisfies specified criteria
 - Consistently satisfies specified criteria

Figure 2.2: **Existing sources of silk fibroin.** Commercial and academic sources of silk fibroin do not result in pure, consistent, and shelf stable silk fibroin that is compatible with microneedle patch applications.

Moreover, only a subset of silk fibroin attributes—molecular weight, concentration, pH—have been explored in literature [27][32][33][34]. Based on a comprehensive literature search, neither conductivity—influenced by residual lithium bromide from the desalting procedure—nor aggregation stemming from the fill finish procedure have been investigated as factors related to silk fibroin stability and ultimately microneedle patch performance (**Figure 2.3**).

Source	Degumming	Denaturing	Desalting	Fill Finish	Pitfalls
Kluge et. al. (2016) [19]		Increasing denaturing time from 10 to 60 minutes increased shelf-life at 4C up to 3 months (40mg/ml)		Lyophilization increases silk fibroin shelf-life up to 1 year but increases aggregate formation post reconstitution	Storage concentration lower than Vaxess requirements; Aggregate formation post reconstitution of lyophilized product; Narrow range of molecular weights explored
Matsumoto et. al. (2006) [25]	Increasing pH from 2 to 12 increased shelf life from <1 day to 20 days, respectively			Decreasing storage concentration from 7wt% to 1wt% increased silk fibroin shelf life up to 20 days	High pH interferes with antigen potency/stability; Storage concentration lower than Vaxess requirements
Wray et. al. (2011) [24]	Decreasing degumming time increases thermal stability of silk fibroin				Decreasing degumming time increases sericin content, leading to increased inflammatory response
Wang et. al. (2005) [26]				Decreased storage temperature increases silk fibroin solution stability	Limited temperature ranges explored in study

Figure 2.3: **Summary of previous approaches to prolonging silk fibroin aqueous solution shelf life.** Apart from failure to meet Vaxess technical requirements, existing studies on silk fibroin shelf life have not explored residual lithium bromide content from desalting and have only explored narrow molecular weight ranges and fill finish procedures.

2.3 Enhancing Stability: Alternative Techniques

An alternative to prolonging silk fibroin solution shelf life via parameter optimization is by converting the silk fibroin solution to a solid-state material for storage and reconstituting prior to use. Biologics such as silk fibroin are typically more stable in the solid state due to the reduced mobility of particles and elimination of degradation pathways such as hydrolysis [35]. A variety of techniques can be used to produce solid materials from a solution, including air or vacuum drying, spray drying, and lyophilization [27]. Previous research has utilized air, vacuum, and spray drying to produce solid-state silk fibroin. However, these drying techniques resulted in partially insoluble materials post reconstitution, inhibiting printing of microneedle patches [27] [26]. While producing solid-state silk fibroin improves the stability of the silk fibroin, these procedures present challenges for applications in microneedle patches due to large aggregates in solution. Furthermore, these drying techniques are both time and energy intensive. Thus, this project will not explore solid-state silk fibroin and will instead

focus on influencing previously unexplored processing conditions of silk fibroin to optimize shelf-life and microneedle patch performance.

2.4 Significance of Proposed Project

Existing studies demonstrate the feasibility of influencing silk fibroin shelf life without converting to a solid-state material by varying the silk fibroin processing conditions [27]. Yet the impact of varied silk fibroin processing conditions on microneedle patch performance has not been previously determined. This novel design project aims to produce a silk fibroin solution that satisfies both prolonged silk fibroin shelf life and vaccine-loaded microneedle patch applications. Furthermore, previously unexplored silk fibroin solution parameters will be optimized for this purpose, addressing gaps in current research. Specifically, this work will vary silk fibroin design parameters (fill finish, pH, conductivity, molecular weight) and evaluate success both as production of a pure, shelf-stable silk fibroin solution and in the overarching context of manufacturing microneedle patches.

2.5 Prior Lab Work

Prior self-directed work will serve as a foundation for evaluation of design specifications and criteria. Specifically, work conducted by the author on the development of the Thioflavin T assay will be used in the quantification of silk fibroin aggregation and ultimately silk fibroin shelf life (Appendix A.1). In addition, previous insights into the silk fibroin manufacturing process and areas for variability during silk fibroin processing will provide background knowledge. While previous work focused on silk fibroin characterization method development, the aim of the current project is to utilize developed methods to evaluate and optimize silk fibroin microneedle patch applications.

Chapter 3

Design Approach for Building

Variations in the silk fibroin production process impact the final silk fibroin characteristics, the stability of the silk fibroin solution, and microneedle patch performance. Specifically, the molecular weight, conductivity, pH, and aggregation from the fill finish process are all impacted by variations in the silk fibroin manufacturing process (**Figure 3.1**). Each of these silk fibroin components were iterated on to determine the set of silk fibroin characteristics that prolong silk fibroin shelf life and optimize microneedle patch performance. In the end, the optimized silk fibroin processing conditions were used in the final design iteration of producing silk fibroin used in in H1N1-loaded microneedle patches.

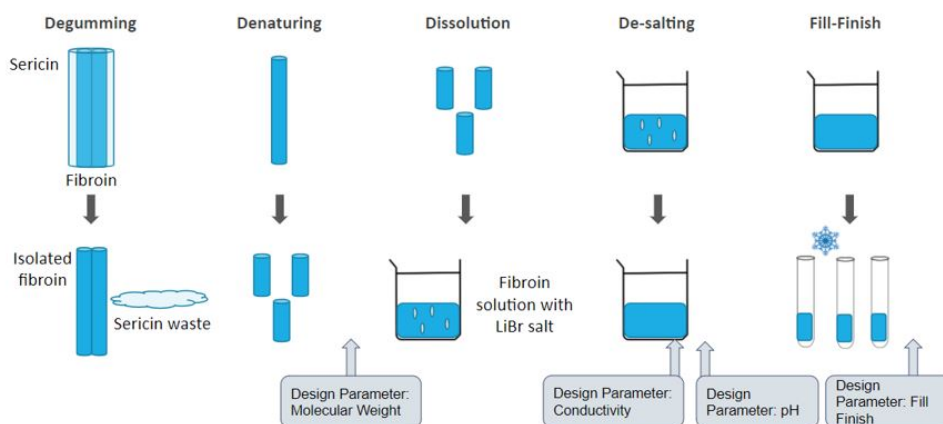


Figure 3.1: **Silk fibroin production process overview with design components of interest indicated.** Design components (molecular weight, conductivity, pH, fill finish) will be modified during the denaturing, desalting, and fill finish steps.

3.1 Design Components

3.1.1 Silk Fibroin Molecular Weight

Silk fibroin molecular weight is most directly influenced by the denaturing time during the extraction process. Denaturing the silk fibroin for a shorter period of time leads to larger silk fibroin polymers and vice versa.

It is hypothesized that lower molecular weight silk fibroin solutions prolong shelf life. Gelation results from formation of inter- and intramolecular interactions among the protein chains, including hydrophobic interactions and hydrogen bonds [12]. Smaller silk fibroin particles decrease intramolecular interactions, increasing the time required for the sol-gel transition and ultimately increasing silk fibroin shelf life.

Meanwhile reduced molecular mobility, caused by higher molecular weight fibroin, plays a role in the stabilizing effect of silk fibroin. It is hypothesized that in the dried state, silk fibroin stabilizes vaccines and other biomolecules both via the “water-replacement hypothesis” (thermodynamic stabilizing effect) and the “vitrification or glass dynamics hypothesis” (kinetics stabilizing effect) [20].

Varying the denaturing time of silk fibroin will provide insight into the effect of silk fibroin molecular weight on solution stability and microneedle patch performance. The range of explored denaturing times in this project is much wider than any existing studies.

3.1.2 Conductivity

Lithium bromide is used in the dissolution step of silk fibroin purification. Sufficient removal during the de-salting step is required for regulatory mandates [36]. Salt inclusion (i.e. sulfate salts) in silk fibroin aqueous solutions can increase fibroin cross-linking, accelerating sol-gel transition and reducing shelf life [37]. With regards to microneedle patch performance, salts have been shown to stabilize proteins and macromolecules [38], theoretically translating

to enhanced antigen stability. The impact of salt content on antigen release from the silk fibroin network has been contested, though it is generally believed lower salt content enhances release [39]. A better understanding of the impact of lithium bromide on silk fibroin shelf life, antigen stability, and antigen release from microneedle patches can be gained by exploring varied levels of lithium bromide in silk fibroin post de-salting.

Since the true lithium bromide content of silk fibroin solutions can only be determined by inductively coupled plasma mass spectrometry (ICP-MS), conductivity will be used as a proxy. Conductivity is determined by the number of charge carriers, how fast they move, and how much charge each one carries. In the case of silk fibroin solutions, differences in conductivity can be attributed to the amount of residual lithium bromide in the solution after the de-salting procedure. During the study, it was confirmed that true lithium content determined by ICP-MS is linearly correlated to conductivity, with an R-squared of 0.998, justifying the use of conductivity as a proxy for lithium bromide content (**Figure A.4**). Silk fibroin solutions have historically had a conductivity of 300-700 μ S/cm, but the study will explore 1000 μ S/cm and 2000 μ S/cm.

3.1.3 pH

The final pH of a silk fibroin solution is influenced by a culmination of the initial silk fibroin processing conditions, namely degumming, denaturing, dissolution, and desalting. Currently, the pH of pure silk fibroin solutions varies between 5.2 and 6.9, averaging around 6.0. Not only does a variable pH introduce inconsistencies in batch-to-batch performance of microneedle patches, the pH may have unexplored impacts on silk fibroin shelf life and antigen potency [40].

Previous research has demonstrated that increasing the silk fibroin pH to over 8.0 increases silk fibroin shelf life [27]. However, bulk vaccine antigen solutions typically have a pH in the 7.2 to 7.4 range. Thus, although alkaline pH (> 7.0) may improve silk fibroin stability, basic pH values may have unexplored negative effects on antigen stability and po-

tency [27]. Two pH values, 6.0 and around 7.6 representing the natural silk fibroin solution pH and bulk vaccine pH values respectively, were selected for the optimization experiment.

3.1.4 Fill Finish Process

The fill finish process is the final step in extracting silk fibroin and involves aliquoting and freezing the aqueous solutions. The frozen silk fibroin is then thawed and filtered prior to use as a stabilizing excipient in microneedle patches. The freezing process can be prolonged or accelerated depending on the fridge temperature and the use of hyper-cooled ethanol. It is hypothesized that freeze-thaw cycling during the fill finish process or due to imperfect freezing conditions (i.e. slow freezing) may introduce aggregates into the solution. Larger, aggregated silk fibroin particles in solution may increase the total inter- and intramolecular interactions, increasing the rate of sol-gel transition and reduced shelf life post-thaw [12].

Meanwhile, increased aggregation introduced by the fill finish process may result in decreased solution printability, inconsistent tip consolidation, inhibition of vaccine release, and inaccurate vaccine dosage levels. The optimal fill-finish formula will be engineered in order to limit silk fibroin aggregation, which negatively impacts silk fibroin solution stability, printability, and vaccine release efficacy. Freezing temperature, storage temperature, and use of ethanol as an accelerating freezing compound will be analyzed to ultimately minimize silk fibroin aggregation during fill finish.

3.2 Prototype

Given these design choices, varied silk fibroin solutions will be produced and subsequently used to create H1N1-loaded microneedle patches via high-precision printing (**Figure 3.2**).

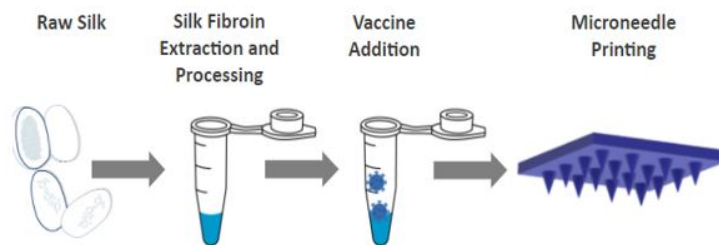


Figure 3.2: **Overview of microneedle patch prototype generation using silk fibroin.** For each described design component, silk fibroin batches was extracted, formulated with the H1N1 antigen, and printed into microneedle patches.

Each microneedle patch consists of an 11x11 array of conical shaped microneedles. The slowly dissolving tip is comprised of H1N1 antigen and the silk fibroin solution of interest, with a rapidly dissolving polymer base on top (total length of tip silk fibroin tip plus polymer base is less than 1mm). The adhesive backing, attached during the demolding process, acts as a carrier for the microneedles and enables adherence to the skin. In practice, the adhesive backing remains on the skin for five minutes, after which the microneedles detach from the adhesive and stay embedded in the skin, enabling sustained release of the antigen.

3.3 Experimental Design

Experiments are divided into three installments: optimization of fill finish (**Table 3.1**), optimization of pH and conductivity (**Table 3.2**), and optimization of molecular weight (**Table 3.3**), performed in that order. The list of silk fibroin parameters used for each experiment are indicated in the tables below, with blue cells indicating standard Vaxess parameters or parameters that were deemed to be optimal based on the conducted optimization experiments. The project was concluded with a final iteration—involving silk fibroin extraction, microneedle patch production, and analysis—based on the optimized silk fibroin parameters determined through each experiment.

Molecular Weight Denaturing Time	Conductivity	pH	Fill Finish
180 minutes	300 μ S/cm	6	Freezing in -80°C
			Freeze in EtOH(5 mins), store -80°C
			Freezing in -80°C, store -20°C
			Freeze in EtOH(5 mins), store -20°C
			Freezing in -20°C

Table 3.1: Silk fibroin parameters for fill finish optimization experiment.

Molecular Weight Denaturing Time	Conductivity, pH	Optimal Fill Finish
180 minutes	300 μ S/cm, pH 6	Freeze in EtOH(5 mins), store -80°C
	300 μ S/cm, pH 7.6	
	1000 μ S/cm, pH 6	
	1000 μ S/cm, pH 7.6	
	2000 μ S/cm, pH 6	

Table 3.2: Silk fibroin parameters for conductivity and molecular weight optimization experiment.

Molecular Weight Denaturing Time	Optimal Conductivity	Optimal pH	Optimal Fill Finish
60 minutes	300 μ S/cm	6	Freeze in EtOH(5 mins), store -80°C
120 minutes			
180 minutes			
240 minutes			

Table 3.3: Silk fibroin parameters for molecular weight optimization experiment.

Chapter 4

Technical Specifications

The design specifications for the project were selected based on prior literature or data to holistically evaluate the success of engineered silk fibroin solutions and microneedle patches.

4.1 Silk Fibroin Solution

Silk fibroin solution specifications have a broad range to accommodate initial optimization experiments, but were refined with completion of each optimization experiment.

Technical Specification	Numerical Value & Measurement	Justification
pH	5-8, Mettler Toledo SevenCompact pH meter S220	Shelf stable silk fibroin has a pH in this range based on historical analysis [33]
Conductivity	100–1000 $\mu\text{S}/\text{cm}$, Mettler Toledo SevenCompact Conductivity Meter S230	Based on historical analysis, silk fibroin solutions require a conductivity in this range to maintain shelf stability. Additionally, the ICH Permitted Daily Exposure is 100 μg of lithium (5% silk patch at 10,000 $\mu\text{S}/\text{cm}$ is 16 μg) [36].

Table 4.1: Specifications for silk fibroin solution.

Technical Specification	Numerical Value & Measurement	Justification
Shelf life of >3 weeks at 4°C	No gelation; <0.1 absorbance (OD Assay); <400,000 fluorescence (ThT Assay) (Figure A.2), BioTek Synergy H1 multi-mode microplate reader	Silk fibroin solution should remain un-gelled for at least three weeks, the longest expected period of time between silk fibroin production, transportation to facility, and expected use in microneedle patch production according to Vaxess specifications.
Shelf life under temperature stress (25°C) of >2 days	No gelation; <0.1 absorbance (OD Assay); <400,000 fluorescence (ThT Assay) (Figure A.2), BioTek Synergy H1 multi-mode microplate reader	Stress stability studies are typically conducted over 24-48 hours [41]. Furthermore, silk fibroin may be left in ambient conditions or subject to higher temperatures for up to 2 days during the transportation process.

Table 4.2: **Specifications for silk fibroin shelf life.**

Technical Specification	Numerical Value & Measurement	Justification
Viscosity	$5 \pm 3\text{cP}$ at 25°C , Brookfield DV2T Viscometer	Ink-jet printing requires a higher ink viscosity (2-5 cP) for proper printing [42].
Surface tension	$<75 \text{ mN/m}$, Pen- dant Drop Method	Surface tension plays a critical role in drop formation for high-precision printing with an operating range of 20–70 mN/m and optimal range at 30–50 mN/m [43].

Table 4.3: Specifications for silk fibroin printability.

4.2 Microneedle Patches

Technical Specification	Numerical Value & Measurement	Justification
Stable H1N1 antigen for >3 weeks at 25°C	$>90\%$ recovered potent antigen, VaxArray assay	The vaccine should be stable and immunogenic at room temperature for 20 days, the average time period required to transport, distribute, and administer vaccines [44].

Table 4.4: Specifications for vaccine stability.

Technical Specification	Numerical Value & Measurement	Justification
Vaccine release	>80%, Fluorescent in vitro release and VaxArray assay	Alternative vaccine delivery systems release 60-80% of their load over the course of 50 days [45]. The selected silk fibroin parameters should allow for delivery of 80% of the appropriate vaccine dose over the course of a month.
Tip deployment	>95%, deployment into ex-vivo skin models	Current Vaxess standards label >95% deployment of demolded tips as successful. Specified tip deployment, combined with vaccine release criteria, allows for dose release comparable with alternative systems.

Table 4.5: **Specifications for antigen dose release.**

Technical Specification	Numerical Value & Measurement	Justification
Tip length	<1mm total tip length, ImageJ analysis of dissolved patches	Tip length in this range has been found to maximize tip retention in skin. Microneedles must be long enough to penetrate the 10-15 μ m thick stratum corneum which consists of dead cells, but sufficiently short enough to allow the stratum corneum to close over and prevent expulsion from the skin. Furthermore, less pain is felt at <1 mm under the skin [46] [47].
Critical load factor	≥ 1 , COMSOL Solid Mechanics	Qualifies the microneedle tips as a mechanically safe and stable structure [21]. The critical load factor is the ratio of the failure force to the applied load [48].

Table 4.6: **Specifications for microneedle physical characteristics.**

Technical Specification	Numerical Value & Measurement	Justification
Cost	<\$23.96, quantitative cost analysis	The lower bound of influenza vaccines are \$12.45/dose, including the \$0.75 Federal Excise Tax [49]. The total variable cost per injection averages around \$11.51 [50]. Combined, current material plus administration costs are \$23.96.

Table 4.7: **Specifications for cost.**

Chapter 5

Materials and Methods for Fabrication and Measurements

The design components for the silk fibroin solution were used to produce variable silk fibroin solutions, each of which was subsequently prototyped into microneedle patches and evaluated to determine whether technical specifications were met.

5.1 Methods of Fabrication

5.1.1 Silk Fibroin Production

Silk fibroin is extracted from silkworm cocoons through four distinct phases: degumming and denaturing, dissolution, de-salting, and fill finish. Silk fibers are obtained and boiled with gentle stirring in 0.02M sodium carbonate for one to four hours, depending on the desired molecular weight. This first step removes adhesive sericin and other impurities, as well as denatures and hydrolyses the silk fibroin to the desired molecular weight. The purified fibroin is then rinsed and soaked in water, followed by air drying. Hydrolyzed denatured fibroin is subsequently dissolved in a 9.3M lithium bromide solution for four hours at 60°C during the dissolution step. The dissolved silk fibroin is de-salted using a 3,500 MWCO dialysis cassette for 48 hours, with an average of three water exchanges every 24 hours. After de-salting, the silk fibroin is filtered with a 5 μ m filter. The concentration of the silk fibroin solution is determined via dry-weight analysis and absorbance at 280nm in order to dilute the solution to a 6% concentration. The silk fibroin is aliquoted, frozen, and thawed

via the appropriate fill finish procedure. The pH, conductivity, molecular weight, viscosity, and aggregation levels are determined post thaw.

5.1.2 Print Solution Formulation

Frozen silk fibroin solutions are thawed in a 37°C water bath for approximately five minutes, or until completely liquid. Thawed silk fibroin is formulated with Alexa Fluor 647 (AF647)-labeled or unlabeled H1N1 antigen, a wetting agent, and Mili-Q water to reach a final solution consisting of 1% silk fibroin, wetting agent, and 6.2% AF647-labeled or unlabeled H1N1 antigen (equivalent to a standard vaccine dose). The surface tension of the formulated print solution is measured prior to printing microneedle patches. If necessary, formulated print solutions are stored in 4°C.

5.1.3 Microneedle Patch Printing

Formulated print solutions are loaded into the PicoPulse nozzle and valve and machine settings are adjusted to a drop volume of 10nL (**Figure 5.1**). For each formulated print solution, fifteen patches are printed. Before loading the next solution, the PicoPulse nozzle is rinsed and machine settings are re-calibrated as necessary. Printed patches are dried overnight. The next day, the remainder of the microneedle patch molds are filled with the polymer base solution and once again dried for at least twenty-four hours. Once the sugar-polymer base is completely dried, the microneedle patches are demolded using the adhesive backing. Demolded patches are either transferred to microtubes for tip length determination and in vitro release or loaded into the microneedle patch applicator for tip deployment tests.

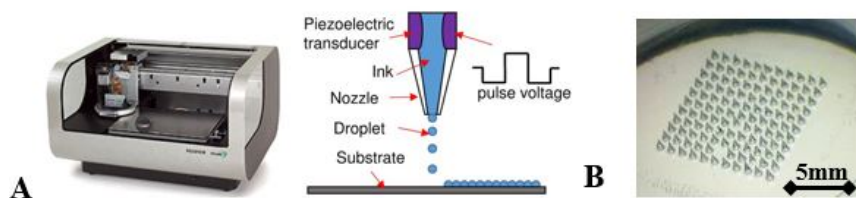


Figure 5.1: **Printing of silk fibroin patches.** (A) High-precision printing to deliver repeatable microdeposits as microneedle tips [51]. (B) Image of printed microneedle tips.

5.2 Methods and Analysis of Silk Fibroin Solution

5.2.1 Solution Characteristics (pH, Conductivity, Molecular weight)

pH of the silk fibroin solution equilibrated to 25°C is measured using a pH meter (Mettler Toledo SevenCompact pH meter S220). The pH meter is calibrated with pH 4,7, and 10 standard solutions prior to taking the pH values. Measurements are conducted in triplicate and averaged for the final value. Similarly, conductivity of the silk fibroin solution equilibrated to 25°C is measured using a conductivity probe (Mettler Toledo SevenCompact Conductivity meter S230). The probe is calibrated using a 500 μ S/cm standard, and measurements are conducted in triplicate and averaged for a final value. Molecular weight of the silk fibroin solution is determined via size-exclusion chromatography (SEC-HPLC). A 50mM Phosphate Buffer, pH 6.8 is prepared as the mobile phase. Prior to standard and sample preparation, the system with no column is run for 10 minutes at 0.25mL/min with the mobile phase to flush any leftover solution. Prior to beginning analysis, the system is initialized and calibrated for thirty minutes. The appropriate analysis method is selected and run. After analysis completion, the retention times of standards are compared to historical data. If the retention times are within 5% of prior analysis, the data is accepted and analyzed using the Cirrus GPC software to determine the Mp, Mn, Mw, PDI of all silk fibroin samples.

5.2.2 Silk Fibroin Shelf Life

Silk fibroin solutions are held at 4°C for at least three weeks, with an added temperature stress of two days at 25°C on the second week, in order to evaluate shelf life under manufacturing-mimicking conditions. Silk fibroin solutions are designated as “stable” if solutions do not undergo sol-gel transition and exhibit minimal aggregation over the time-course study. Sol-gel transition and aggregation are evaluated both qualitatively and quantitatively. Qualitative evaluation involves visual observation every two to three days. Aggregation is quantified via two assays, the thioflavin T (ThT) assay and absorbance at 550nm (OD), every six to seven days (unless visual observation of gelation is detected, which warrants daily aggregation quantification). For the ThT assay—a measure of β -sheet fibril aggregation—1% silk fibroin solutions with 88uM thioflavin T are incubated for twenty minutes and fluorescence is evaluated using a microplate reader (BioTek Synergy H1 multi-mode microplate reader) with 430nm excitation and 490nm emission wavelengths [52]. Both normalized fluorescence based on the original value and absolute fluorescence are recorded for each silk fibroin sample to account for excipient interference (Appendix A.1). A fluorescence of >400,000 a.u. is indicative of sol-gel transition. OD—a quantitative measure of non-specific aggregation including micelles, fibrils, and larger aggregates—is determined by absorbance at 550nm of a 1% silk fibroin solution using a microplate reader (BioTek Synergy H1 multi-mode microplate reader). As with the ThT assay, normalized absorbance (to account for excipient interference) and absolute absorbance, with a cutoff at 0.1 a.u., are recorded and compared for each silk fibroin sample. The ThT and OD assays were previously verified as indicative of silk fibroin aggregation by comparison to silk fibroin particle size diameters obtained from dynamic light scattering. These results, as well as additional method suitability studies conducted on the assays are described in the Appendix A.1.

5.2.3 Printability of Silk Fibroin Solution

Viscosity and surface tension for each silk fibroin solution are assessed to determine printability of the silk fibroin solution. Viscosity of the silk fibroin solution is measured by pipetting 250 μ L of 6% silk fibroin into the viscometer chamber (Brookfield DV2T Viscometer). Viscosity is measured for one minute at 25 rpm, 25°C, and with a CP-40 spindle in triplicate and averaged.

The pendant drop method is used to determine surface tension (**Figure 5.2**) [53]. A custom 3D printed syringe holder is used to hold a syringe filled with the silk fibroin of interest on the base plate of the Instron. A DinoLite camera is set up to image the drop formation. The syringe is pushed down to form a drop and an image is captured using the DinoLite camera once the drop is static and at equilibrium. Images continue to be captured as the syringe is pushed down until the drop eventually falls. The image of the drop at its largest size is saved and used for subsequent analysis in ImageJ. The Pendant Drop analysis feature on ImageJ is used to calculate surface tension based on the empirically-determined silk fibroin densities [54]. For each silk fibroin solution, surface tension measurements are performed in triplicate and averaged. The surface tension is related to drop shape by the following equation: $\gamma = \frac{\Delta\rho g R_0}{\beta}$ where γ is the surface tension, $\Delta\rho$ is the density difference between fluids, R_0 is the drop radius of curvature at the apex, and β is the shape factor. β can be defined through the Young-Laplace equation expressed as dimensionless first-order equations: $\frac{dx}{ds} = \cos\Phi$, $\frac{dz}{ds} = \sin\Phi$, and $\frac{d\Phi}{ds} = 2 + \beta z - \frac{\sin\Phi}{x}$.

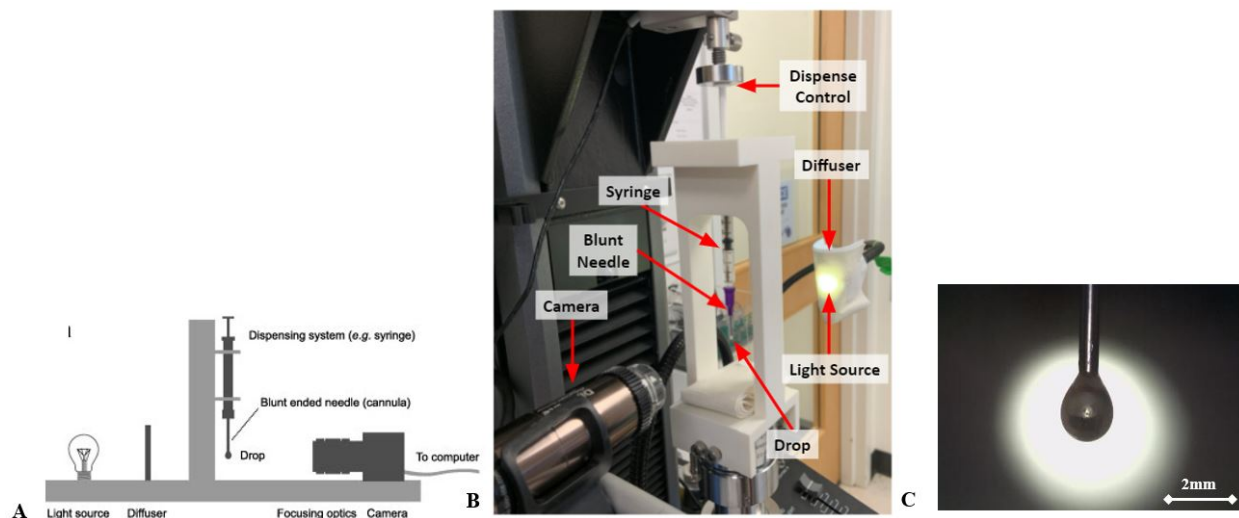


Figure 5.2: **Set-up for measurement of surface tension.** (A) Diagram of surface tension measurement set-up. (B) Configuration of Instron, syringe, and DinoLite to measure surface tension. (C) Example of captured drop at its largest size, ultimately used in ImageJ analysis for surface tension determination.

5.3 Methods and Analysis of Microneedle Patches

5.3.1 Stability of H1N1 Antigen

Immunogenicity of the antigen recovered from microneedle patches after storage at room temperature is evaluated using the VaxArray Platform and compared to neat antigen storage at room temperature. Briefly, the VaxArray Platform involves binding of target antigens to monoclonal antibodies spotted on the array surface. Detection antibodies conjugated with a fluorescent label are added to the arrays, bound to corresponding target antigens, and wells are imaged. Printed microneedle patches are dissolved in PBS and supernatant is sampled. The removed supernatant is diluted in Protein Blocking Buffer 2.0 with detergent so the estimated concentration is in the middle of the calibration curve. Standards are prepared by serial dilution in the provided strip tubes. $50\mu\text{L}$ of each standard and sample are added to the wells and incubated for 60 minutes at room temperature. During the incubation period, label solutions are prepared, added to each well, and incubated for 30 minutes at room temperature. Wells are washed with two wash buffers and subsequently air-dried. The slide

is imaged using an excitation range of 510-540nm and emission collected from 570-600nm. The collected data is processed by the VaxArray Imaging and Analysis software. For each group of microneedle patches, immunogenicity of five microneedle patches is evaluated at designated time points of 1 hour, 1 day, and 3 days post dissolution.

5.3.2 Dose Release

Dose release from printed microneedle patches is influenced by antigen release from microneedle tips as well as tip deployment efficacy in skin.

Antigen release from microneedle patches is evaluated using the VaxArray assay (described above) or via the fluorescent in vitro release assay. For the fluorescent in vitro release assay, microneedle patches printed with fluorescently-labeled H1N1 antigen are placed in Eppendorf tubes and dissolved in PBS. Similar to the VaxArray procedure, samples are placed in black box on a 37°C shaker at 100rpm. Samples are then diluted and plated onto black 96-well plates along with AF647-H1N1 standards. Fluorescence is recorded using a BioTek Synergy H1 with excitation at 640nm and emission at 681nm. Mass and percent release of the antigen is calculated based on the prepared standard curve and fluorescent intensity. Percent release is calculated as: $percent\ release = \frac{cumulative\ antigen\ release\ over\ time}{total\ antigen\ in\ microneedle\ patch}$. For each group of microneedle patches, antigen release from five patches is evaluated at 1 hour, 1 day, 2 days, 7 days, 14 days, 21 days, and 28 days post dissolution.

Tip deployment efficacy is evaluated in ex-vivo pig skin models. Pig skin is thawed as necessary, shaved, and wiped down to remove excess hair and dirt. Prior to deploying, microneedle patches are examined and imaged using a stereo and confocal fluorescent microscope to determine tip morphology. Microneedle patches are loaded into the applicator and deployed on the pig skin in the designated area. After leaving the patches on the skin for five minutes, patches are removed by pulling straight up. Pig skin deployed with printed with AF647-labeled antigen is imaged using the Dino-Lite, and microneedle patches are imaged using the Stereo microscope to determine the number of tips deployed (**Figure 5.3A**).

Microneedle patches printed with unlabeled H1N1 antigen are stained with Methylene Blue for 30 minutes, wiped down, and imaged using a stereo microscope(**Figure 5.3B**). Tip deployments are performed in triplicate and averaged to determine final deployment efficiency.

Deployment efficiency was calculated as $tip\ deployment = \frac{total\ tips\ deployed}{total\ tips\ demolded\ per\ patch}$.

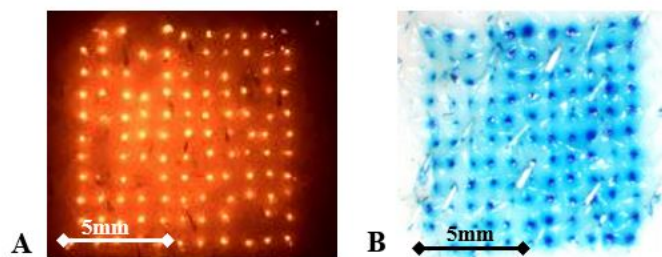


Figure 5.3: H1N1-loaded microneedle tips embedded in ex-vivo pig skin. (A) Representative example of AF647-labeled antigen microneedle tips deployed in ex-vivo skin models imaged using Dino-Lite. (B) Representative example of unlabeled H1N1 antigen microneedle tips deployed in ex-vivo skin models, stained with Methylene Blue, and imaged using the stereo microscope.

Diffusion Modeling

Upon drying of microneedles, antigen proteins become entrapped in the silk fibroin matrix with antigen release dictated by silk matrix swelling, drug diffusion, and silk matrix degradation. Silk matrix swelling results in an immediate burst release of vaccine antigen. Drug diffusion comprises the second stage of drug release. Silk fibroin degradation, the last phase of drug release, is influenced by diffusivity of the matrix and the surface area relative to the volume of the tip. Vaccine diffusion, the second phase of dose release, is the focus of the COMSOL model.

In order to model diffusion on COMSOL, the diffusion coefficient of the silk and antigen formulation are required. Two 10mL syringes are filled with water and a colored silk fibroin plus antigen formulation, respectively. Tubing and metal inserts are used to connect the syringes and outflow tubing to a microfluidic device. The microfluidic device is positioned under a DM-143-FBLED-A5 Motic Digital Stereo Microscope, and the Motic software is opened on a laptop (**Figure 5.4A**). The flow rate on the syringe is set to 0.700 mL/min prior to pressing run. Images of the flow are taken at select locations around the microfluidic device

(**Figure 5.4C**). At each of these locations, ImageJ is used to measure the concentration profile by plotting the intensity profile. The concentration profile is then exported from ImageJ into Matlab, used to fit an error function (**Figure 5.4D**). Nine different locations along the device are translated into time and used to calculate the diffusion coefficient. The final diffusion coefficient value is an average of all nine values.

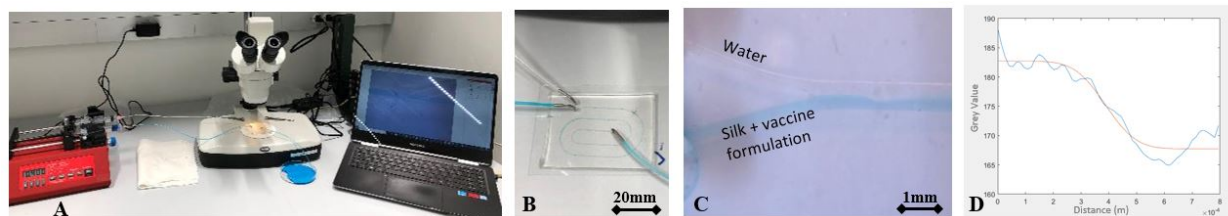


Figure 5.4: Set-up for diffusion coefficient determination. (A) Syringe pump, syringes, microfluidic channel, Stereo microscope, and laptop set-up for data capture. (B) Microfluidic channel with two inlets, for water and the colored print formulation, and one outlet. (C) Enlarged image of flow between water and the colored print formulation. (D) Fitted error function from Matlab script on top of the ImageJ exported concentration profile.

COMSOL is used to model the combination of silk fibroin and vaccine diffusion. The microneedle tip is modeled as a 3D cone shape with a density of $1,800,000 \text{ g/m}^3$, Poisson's ratio of 0.45, and Young's Modulus of 2 GPa, based on literature and historical values. An empirically diffusion coefficient of $1.58\text{E-}08 \text{ m}^2/\text{s}$ is used for the silk fibroin and vaccine antigen formulation, while $1.49\text{E-}9 \text{ m}^2/\text{s}$ is used as the water diffusion coefficient, representing the bodily fluids surrounding the injected microneedle. The formulation concentration is set to 0.105 mol/m^3 , and the concentration of the surrounding fluid is set to 0 mol/m^3 . Diffusion is modeling using a parametric sweep from 0 to 5000 seconds at 100 second intervals.

5.3.3 Tip Length

Tip length is assessed by dissolving microneedle patches in PBS and imaging the tips in solution using a stereo-microscope. Tip morphology is visually observed, while the tip length is determined from captured images using ImageJ. For each image, five tips are selected, analyzed, and averaged to determine tip length.

5.3.4 Physical Robustness

The physical robustness of the microneedle tips is modeled using the Structural Mechanics module in COMSOL [55] [56]. The microneedle tip is modeled as a 3D cone shape with a density of $1,800,000 \text{ g/m}^3$, Poisson's ratio of 0.45, and Young's Modulus of 2 GPa, based on literature and historical values. The Solid Mechanics interface is used to apply an axial pressure of 3.18MPa—enough to pierce the skin—at the microneedle base. Using linear buckling analysis, the critical load factor and maximum force is determined and recorded. Critical load factor is defined as: $\text{critical load factor} = \frac{\text{failure force}}{\text{applied load}}$.

5.3.5 Cost

The cost of microneedle patch production and administration is calculated by totaling the cost of administration, price of bulk vaccine, and microneedle patch material costs. The microneedle patch costs are further divided into the silk fibroin, mold, polymer base, backing, and packaging costs. The final cost of microneedle patch production and administration is compared to industry standards.

5.3.6 Statistical Analysis

All data shown are mean $\pm$ standard error of the mean. Statistical significance is determined using one- or two-way ANOVA as appropriate ($p < 0.05$).

Chapter 6

Results

6.1 Fill Finish Optimization

Since fill finish is the final step of the silk fibroin production process, the fill finish procedure was optimized prior to subsequent silk fibroin production parameter optimization. A batch of silk fibroin was split into five 5mL vials, each of which was frozen with one of the fill finish procedures as described in Section 3.3. Silk fibroin vials were held frozen for 28 days, representative of the longest time fibroin is frozen before microneedle printing, and thawed in a 37°C water bath for five minutes. After thawing, the silk fibroin characteristics (pH, conductivity, molecular weight), silk fibroin shelf life (visual sol-gel transition, fluorescence and absorbance values over time from the ThT and OD assays), and printability (viscosity) were assessed. The silk fibroin samples were stored at 4°C post thaw.

Original experimentation determined that the fill finish procedure does not have a significant impact on silk fibroin characteristics (pH, conductivity, molecular weight) or printability (viscosity) of the post-thaw solution. Varied fill finish procedures resulted in silk fibroin solutions that met the predetermined technical specifications. Specifically, thawed silk fibroin solutions recorded a pH between 6.1-6.3, conductivity of 349-370 $\mu\text{S}/\text{cm}$, and viscosity of 2.18-2.34 for all solutions (**Table A.1**).

Silk fibroin shelf life post-thaw was evaluated over 56 days at 4°C. None of the silk fibroin solutions displayed visible signs of aggregation or sol-gel transition post storage for >56 days, including after a 48 hour temperature stress of 25°C. Aggregation assay results are displayed below as normalized pre-freeze fluorescence or absorbance values, for the ThT

and OD assays respectively (**Figure 6.1**).

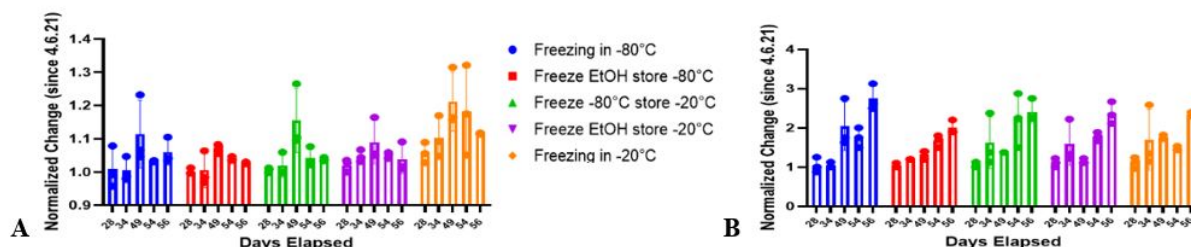


Figure 6.1: **Quantified aggregation of silk fibroin solutions with varied fill finish procedures.** (A) Normalized fluorescence values based on the pre-freeze values determined from the ThT assay. (B) Normalized absorbance values based on the pre-freeze values determined from the OD assay. Data show as mean $\pm$ standard deviation, $n=3$.

The silk fibroin solutions frozen in chilled ethanol or in -80°C directly resulted in the smallest increase in fluorescence (ThT Assay) over the examination period. Fill finish procedure had a statistically significant impact on normalized fluorescence values based on a two-way ANOVA with a $p=0.0442$. Tukey's multiple comparison, used to identify means that differ from a set of means, revealed that by the end of the examination period, the silk fibroin solution frozen in ethanol, stored in -80°C and the solution frozen in -80°C , stored in -20°C had statistically significant lower aggregation levels as measured by the ThT assay compared to the solution frozen in -20°C . The silk fibroin solution frozen in ethanol and stored in -80°C consistently had the lowest increase in absorbance (OD assay) over the examination period. However, fill finish procedure did not have a statistically significant impact on normalized absorbance values based on the conducted two-way ANOVA.

Post aggregation quantification, the silk fibroin samples were qualitatively examined for 186 days (6 months) for sol-gel transition at 4°C . During the examination period, the silk fibroin samples frozen without chilled ethanol underwent sol-gel transition. The performed extended time-course study confirmed the hypothesis that rapid silk fibroin freezing minimizes aggregation and prolongs shelf life.

6.2 Conductivity and pH Optimization

The conductivity of the silk fibroin solution is predominantly influenced by the residual lithium bromide content, which among other silk fibroin processing conditions also impacts the pH. Altering the conductivity of the solution can also impact pH; therefore, an original factorial design experiment was conducted to determine the impact of conductivity and pH on the silk fibroin solution and microneedle patch performance, as outlined in Section 3.3.

In practice, conductivity and residual lithium bromide content are directly related to the extent of desalting. Meanwhile, pH is influenced by a combination of degumming, denaturing, dissolution, and desalting conditions. Precisely controlling the pH or conductivity by influencing the silk fibroin processing conditions is difficult. Instead, to achieve solutions with desired pH and conductivity, silk fibroin solutions were produced using the same upstream processing conditions and modified prior to the final fill finish step. Dilute lithium bromide or sodium-potassium phosphate based buffers were added to the silk fibroin solutions to alter the conductivity or pH, respectively. Unmodified silk fibroin solutions had a pH of around 6 and conductivity of $300\mu\text{S}/\text{cm}$. Adding $4\mu\text{L}$ or $10\mu\text{L}$ of 9.3M lithium bromide solution to 4mL of 6% silk fibroin resulted in conductivities of $1000\mu\text{S}/\text{cm}$ or $2000\mu\text{S}/\text{cm}$, respectively. Meanwhile, $375\mu\text{L}$ of sodium-potassium phosphate buffer was added to 4mL of a 6% silk fibroin solution to achieve a pH of 7.6. Lithium bromide and buffer were added as appropriate to produce five solutions of variable pH and conductivity ¹. Silk fibroin solutions were subsequently frozen in ethanol and stored in -80°C based on the previously determined optimal fill finish procedure.

¹Samples are referred to by the pre-buffered conductivity with post-buffered pH and conductivity described in Table A.2

Silk Fibroin Solution Analysis

All five post-thaw silk fibroin solutions of varied pH and conductivity met the pH, conductivity, and molecular weight technical specifications, although each solution expectedly differed in the measured pH and conductivity. All silk fibroin solutions also met the printability specification, with viscosity of 2.05-2.1 (**Table A.2**).

Silk fibroin shelf life post-thaw was evaluated over 42 days. None of the silk fibroin solutions displayed visible aggregation or sol-gel transition post storage in 4°C for >42 days, including after a 48 hour temperature stress at 25°C. Aggregation assay results are displayed below as normalized pre-freeze fluorescence or absorbance values, for the ThT and OD assays respectively (**Figure 6.2**).

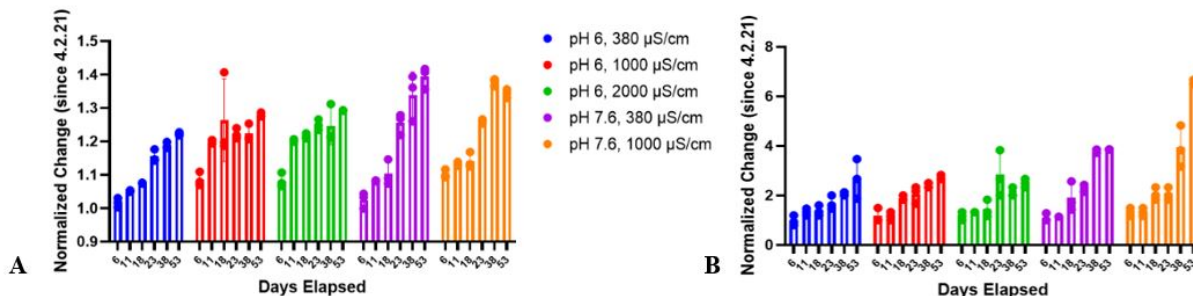


Figure 6.2: **Quantified aggregation of silk fibroin solutions with varied pH and conductivity.** (A) Normalized fluorescence values based on the pre-freeze values determined from the ThT assay. (B) Normalized absorbance values based on the pre-freeze values determined from the OD assay. Data show as mean $\pm$ standard deviation, n=3.

Modifying the pH and conductivity of the silk fibroin solutions had a statistically significant impact on the change in normalized fluorescence and absorbance values based on the conducted two-way ANOVA, with $p < 0.0001$. The pH 6, 380 μ S/cm silk fibroin solution had the most stable absorbance and fluorescence values over the course of measurement, confirmed by Tukey's multiple comparison. Meanwhile, the high pH (7.6) silk fibroin solutions had the greatest increase in fluorescence and absorbance over the time-course study, indicating higher pH leads to increased aggregation and shortened shelf life.

Post aggregation quantification, the silk fibroin samples were qualitatively examined for

186 days (6 months) for sol-gel transition at 4°C. During the examination period, the silk fibroin solutions with a 7.6 pH underwent sol-gel transition. Gelation of the high pH (7.6) silk fibroin validated the quantitatively observed trend that buffered silk fibroin accelerated silk fibroin aggregation and reduced shelf life.

Microneedle Patch Analysis

The five silk fibroin solutions of varied pH and conductivity were used to print microneedle patches with fluorescently labeled H1N1 antigen (**Figure 6.3**). Fifteen patches were printed per silk fibroin solution, resulting in a total of 75 patches. Dose release, comprised of antigen release from microneedle tips and tip deployment, and tip length were analyzed.

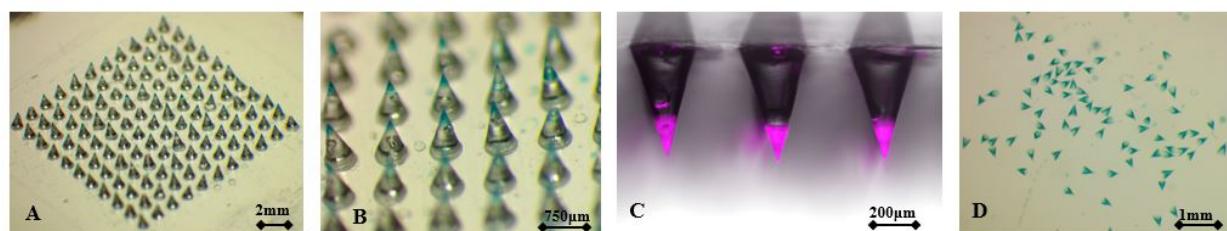


Figure 6.3: **Representative images of fabricated H1N1-loaded microneedle patches.** (A) Image of entire 11x11 array of microneedle patches. (B) Image demonstrating tip morphology with fluorescently labeled H1N1 antigen. (C) Fluorescent image demonstrating antigen localization in tips. (D) Patches post dissolution of base used to calculate tip length.

H1N1 antigen release was evaluated over a 28-day period for microneedle patches using the fluorescent in vitro release assay. Total vaccine release ranged between 42% with the pH 6, conductivity 1000µS/cm silk patches to 54% with the pH 6, conductivity 380µS/cm silk patches (**Figure 6.4**). While none of microneedle patches achieved the desired release of 80% over the measurement period, the microneedle patches printed with the pH 6, 380µS/cm silk fibroin solution were the closest to this specification. Overall, the microneedle patches printed with low conductivity silk fibroin solution—regardless of the pH level—had slightly improved vaccine release, although this trend was statistically insignificant. In the future, assay media that better mimics in vivo conditions can be used to enhance vaccine release.

Tip deployment efficiency ranged between 74% for the pH 6, conductivity 380 μ S/cm group to 95% for the pH 6, conductivity 1000 μ S/cm group (**Figure 6.4**). Besides the pH 6, conductivity 380 μ S/cm group, the remaining patches had deployment efficiencies above 80%, meeting the technical specification. The pH 7.6, conductivity 1000 μ S/cm group had the highest tip deployment efficiency, though the improvement was not statistically significant based on a one-way ANOVA.

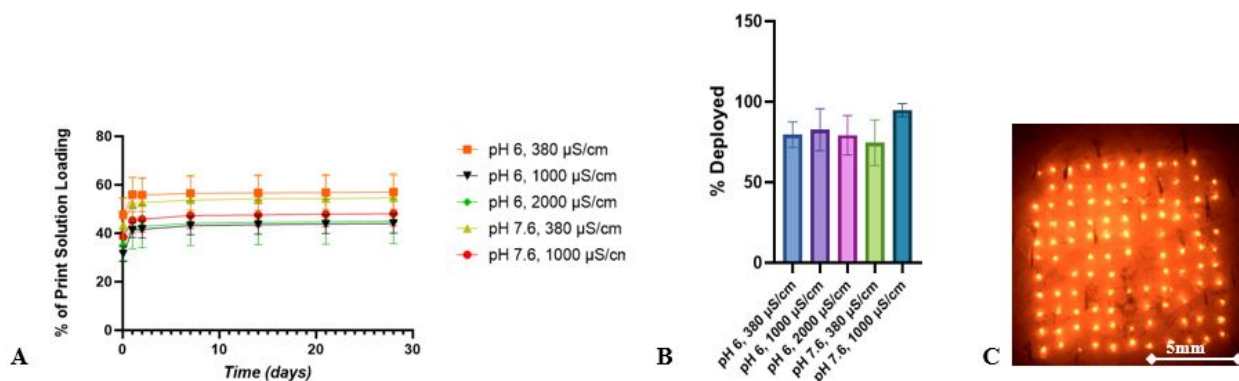


Figure 6.4: Dose release from microneedle patches printed with variable silk fibroin conductivity and pH. (A) Percent vaccine release of total loaded H1N1 antigen for all five microneedle groups. (B) Percent tips deployed in ex-vivo pig skin models. (C) Representative image of tip deployment in ex-vivo skin model imaged using DinoLite. Data show as mean $\pm$ standard deviation, n=5.

Measured microneedle tip lengths ranged between 210 μ m for the pH 7.6, conductivity 1000 μ S/cm group to 250 μ m for the pH 6, conductivity 380 μ S/cm group (**Figure 6.5A**). All the printed patches met the technical specification for tip length. Shorter tip length was correlated with enhanced tip deployment efficacy (**Figure 6.5B**).

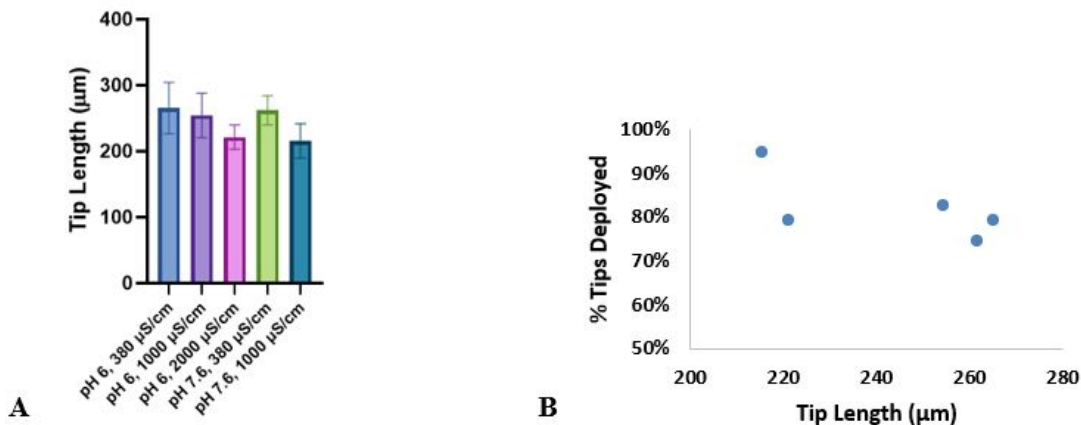


Figure 6.5: **Calculated tip length and correlation to tip deployment efficiency for microneedle patches printed with variable silk fibroin conductivity and pH.** (A) All printed microneedle patches met the technical specification for tip length. (B) Observed inverse relationship between tip length and tip deployment. Data show as mean $\pm$ standard deviation, $n=5$.

6.3 Molecular Weight Optimization

Silk fibroin molecular weight is governed by denaturing time. Explored denaturing times of 60, 120, 180, and 240 minutes resulted in molecular weights of 109, 96, 88, and 80 kDa, a much wider range of analyzed molecular weights compared to literature. Each batch of silk fibroin was then separately dissolved in lithium bromide, desalted, frozen in ethanol, and stored in -80°C .

Silk Fibroin Solution Analysis

All four silk fibroin solutions of varied molecular weight met the empirically determined target pH and conductivity technical specifications, with pH of 5.6-6.8 and conductivity of 250-353 $\mu\text{S}/\text{cm}$. All silk fibroin solutions met the molecular weight, viscosity (1.78-3.4 CPa), and surface tension (57-72 mN/m) technical specifications (**Table A.3**).

Silk fibroin shelf life post-thaw was evaluated over 176 days (6 months). None of the silk fibroin solutions displayed visible aggregation and sol-gel transition post storage in 4°C for >176 days, including after a 48 hour temperature stress at 25°C . Silk fibroin molecular

weight had a statistically significant impact on the normalized fluorescence and absorbance values based on the conducted two-way ANOVA, with $p < 0.001$. Higher molecular weight silk fibroin consistently had significantly higher normalized fluorescence values compared to lower molecular weight silk fibroin solutions based on Tukey's multiple comparison. All four silk fibroin solutions also had statistically significant differences in normalized absorbance values, with the exception of the two lowest molecular weight silk fibroin solutions. Over the extended aggregation time-course study, the higher molecular weight silk fibroin had a greater increase in normalized absorbance values, indicative of increased aggregation and reduced shelf life.

Microneedle Patch Analysis

The four silk fibroin solutions of varied molecular weight were used to print microneedle patches with fluorescently labeled H1N1 antigen. Fifteen patches were printed per silk fibroin solution, printing 60 patches total. Dose release, comprised of antigen release from microneedle tips and tip deployment, and tip length were analyzed.

Over the course of the 28 day measurement period, total vaccine release ranged from 46% with the lowest molecular weight silk fibroin patches to 52% with the highest molecular weight silk fibroin patches based on the fluorescent in vitro release assay, with statistically significant differences. While none of the printed microneedle patches achieved desired release, the highest molecular weight silk fibroin groups were the closest in meeting the technical specification.

Tip deployment efficiency ranged from 54% for the highest molecular weight silk fibroin patches to significantly higher 79% for the lowest molecular weight silk fibroin patches ($p=0.001$). The lowest molecular weight silk fibroin patches nearly met the technical specification, though none of the microneedle groups achieved desired deployment efficiency. Tip deployment efficiency was comparatively lower for this optimization iteration, likely due to the use of aged pig skin which negatively impacted deployment efficacy.

Measured microneedle tip lengths ranged from $176\mu\text{m}$ - $247\mu\text{m}$ for the range of molecular weight silk fibroin patches explored. All the printed patches met the technical specification for tip length.

6.4 Final Design

The optimal silk fibroin production parameters result from a 180 minute denaturing time, low pH, low conductivity, and a fill finish procedure of freezing in ethanol and storing in -80°C . In addition to producing silk fibroin based on the determined optimal parameters, the final design iteration encompassed an optimization experiment intended to achieve more consistent, precise silk fibroin conductivity. Typically, silk fibroin is desalted via cassette dialysis against Mili-Q water, which results in somewhat variable end-point conductivity. For the final design iteration, dialysis was performed against dilute solutions of lithium bromide set at the target silk fibroin end-point conductivity. Lithium bromide solutions at conductivities of $300\ \mu\text{S}/\text{cm}$ and $500\ \mu\text{S}/\text{cm}$ were used to further refine optimal silk fibroin conductivity and evaluate the method's precision. The selected conductivity of $300\ \mu\text{S}/\text{cm}$ was based on the empirically determined optimal conductivity (Section 6.2), while $500\ \mu\text{S}/\text{cm}$ fell in between the high and low conductivity values explored in Section 6.2.

After degumming, denaturing, and dissolving the silk fibroin in lithium bromide, half of the silk fibroin batch was dialyzed against the $300\ \mu\text{S}/\text{cm}$ lithium bromide solution and the other half of the silk fibroin solution was dialyzed against the $500\ \mu\text{S}/\text{cm}$ lithium bromide solution. After the standard forty-eight hours of dialysis, the silk fibroin solutions were frozen in ethanol, stored in -80°C , thawed for analysis, and then kept at 4°C .

Silk Fibroin Solution Analysis

Both silk fibroin solutions of varied dialysis procedures met the pH and conductivity technical specifications, with the silk fibroin conductivity falling within $60\ \mu\text{S}/\text{cm}$ of the

lithium bromide solution used for dialysis. Dialysis against the lower conductivity lithium bromide solution led to silk fibroin with conductivity of 273 $\mu\text{S}/\text{cm}$ and pH of 6.2, while dialysis against the 500 $\mu\text{S}/\text{cm}$ lithium bromide led to silk fibroin with conductivity 444 $\mu\text{S}/\text{cm}$ and a pH of 6.3. Both silk fibroin solutions met the remaining silk fibroin technical specifications of viscosity (2.1-2.18 CPa) and surface tension (62-68 mN/m) (**Table A.4**).

Silk fibroin shelf life post-thaw was evaluated over a 35 day period. By day 21, the silk fibroin solution dialyzed against the 500 $\mu\text{S}/\text{cm}$ lithium bromide solution gelled, so absorbance and fluorescence values were no longer collected. The silk fibroin solution dialyzed against the 300 $\mu\text{S}/\text{cm}$ did not display any visible signs of aggregation or sol-gel transition post storage in 4°C for >35 days, even after a 48 hour temperature stress of 25°C. Aggregation assay results are displayed below as normalized pre-freeze fluorescence or absorbance values, for the ThT and OD assays respectively (**Figure 6.6**).

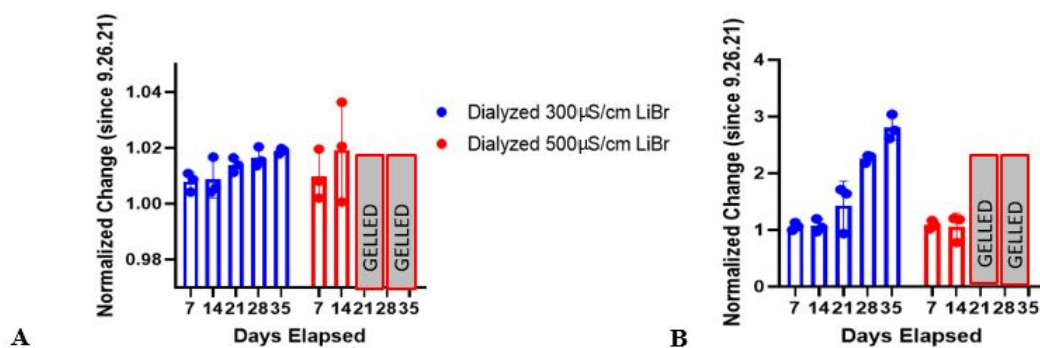


Figure 6.6: **Quantified aggregation of silk fibroin solutions from the final design iteration.** (A) Normalized fluorescence values based on the pre-freeze values determined from the ThT assay. (B) Normalized absorbance values based on the pre-freeze values determined from the OD assay. Data show as mean $\pm$ standard deviation, n=3.

Microneedle Patch Analysis

Both silk fibroin solutions of varied dialysis procedures were used to print microneedle patches, with 15 patches printed per group. For this final iteration, the VaxArray assay—rather than the fluorescent in vitro release assay—was used to assess potency and antigen in vitro release from printed microneedle patches held at room temperature for 5

days. While the previously used fluorescent in vitro release assay quantified total antigen release—regardless of potency—the VaxArray assay quantified only stable, potent vaccine release. Over the course of the 3 day measurement period, total potent vaccine release averaged around 100% (**Figure 6.7A**), meeting the vaccine release technical specification. No significant difference in the release profiles between the two groups of microneedle patches was observed. Additionally, the VaxArray assay confirmed that the H1N1 antigen remains potent in the silk fibroin matrix of the microneedles when held at room temperature for several days. Although the full three week time period of antigen stability was not explored due to time constraints, preliminary thermostabilization effects of silk fibroin on vaccines were observed.

Out of successfully demolded microneedle tips, deployment efficiency ranged from 98% for the group dialyzed against 300 μ S/cm lithium bromide to 99% for the group dialyzed against 500 μ S/cm lithium bromide, meeting the defined technical specifications (**Figure 6.7C**). No significant difference in tip deployment efficiency was observed between the two groups.

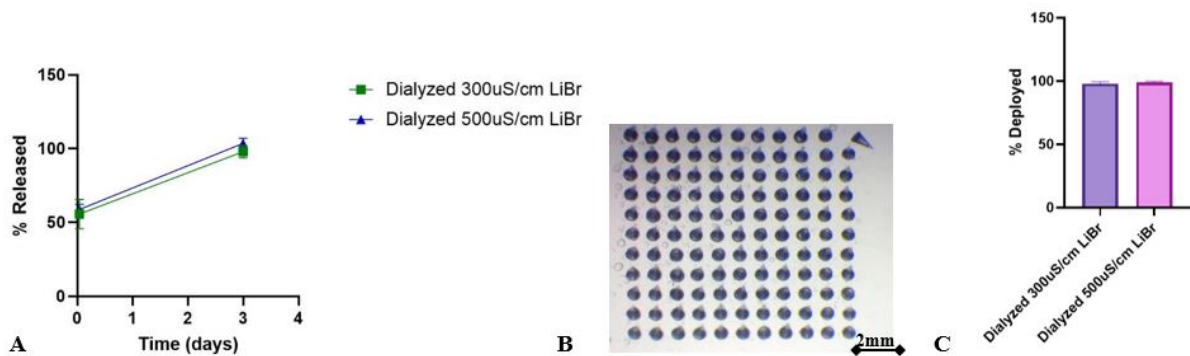


Figure 6.7: **Dose release from microneedle patches printed with the final iteration silk fibroin.** (A) Percent release of potent antigen based on total loaded H1N1 antigen. (B) Representative image of printed microneedle patch observed using a stereo microscope. (C) Percent tips deployed in ex-vivo pig skin models. Data show as mean $\pm$ standard deviation, $n=5$.

Microneedle tip length ranged from $252\mu\text{m}$ - $253\mu\text{m}$ (**Figure 6.8A**), meeting the technical specification. Dialysis against the $300\mu\text{S}/\text{cm}$ lithium bromide solution resulted in the longer tips, while dialysis against the $500\mu\text{S}/\text{cm}$ lithium bromide solution resulted in the shorter tips ($p<0.001$). Overall, lower conductivity silk fibroin results in longer tips, a trend also observed in the conductivity optimization experiment.

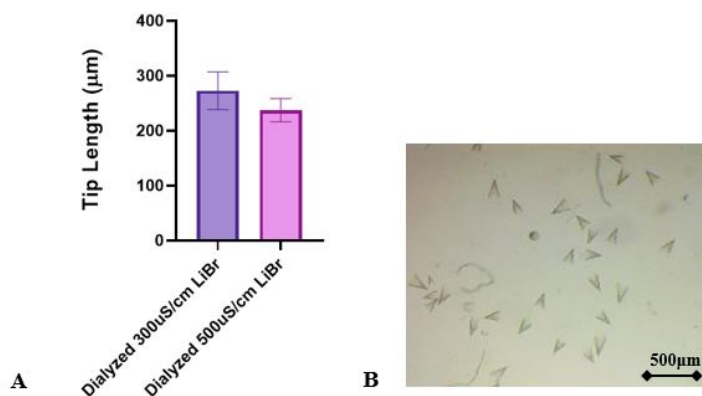


Figure 6.8: **Tip length of final iteration microneedle patches.** (A) All printed microneedle patches met the technical specification for tip length. (B) Representative image of dissolved polymer microneedle base used to calculate tip length. Data show as mean $\pm$ standard deviation, $n=5$.

6.5 Mechanical Structure

Applying a pressure of 3.18 MPa—enough to pierce the skin—in the axial direction resulted in a critical load factor >1 during linear buckling analysis, indicating the microneedles

should not buckle upon pressure application during skin insertion (**Figure 6.9A**) [55]. The determined critical load factor was 12.125, translating to a maximum load of 4.84N given the microneedle tip dimensions. The microneedles met the mechanical strength technical specification based on the COMSOL model, but physical experiments should be conducted on varied silk fibroin microneedles to empirically confirm these results.

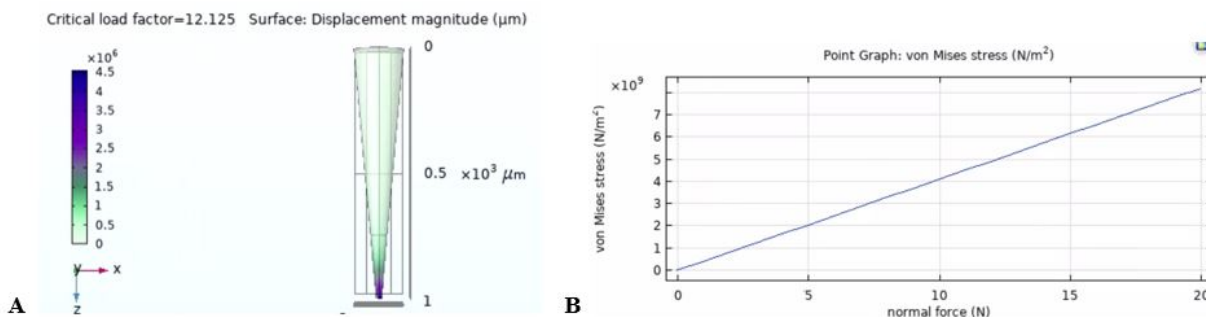


Figure 6.9: **Calculated critical safety for microneedle tips.** (A) COMSOL linear buckling analysis was used to demonstrate that microneedles should not buckle upon pressure application. (B) Linear elastic properties of silk fibroin and polymer base in microneedle tips.

In addition to the conducted linear buckling analysis, a parametric sweep of axial loading from 0N to 20N with 1N increments was performed (**Figure 6.9B**). The von Misses stress versus the applied force was plotted, and a linear relationship was observed, confirming expectations of the microneedle materials' linear elastic properties.

6.6 Vaccine Release: Diffusion

Antigen entrapped in the silk fibroin matrix exhibits multi-modal release beginning with silk matrix swelling, followed by vaccine diffusion, and concluding with silk matrix degradation. Vaccine diffusion from the silk fibroin matrix was modeled using COMSOL convection-diffusion, given a vaccine concentration of $0.103 \text{ mol}/\text{m}^3$ and empirically derived diffusion coefficient of $1.58\text{E-}8$.

Concentration of the silk fibroin and vaccine formulation was modeled for up to 5000 seconds at two points: at the microneedle tip and 0.05cm above the tip. At the microneedle

tip, the formulation concentration increased to a maximum at 200 seconds then gradually declined until ultimately leveling off by 1000 seconds (**Figure 6.10 A,B**). Meanwhile, at 0.05cm above the microneedle tip, the formulation concentration increased rapidly for 500 seconds, followed by a more gradual increase, and ultimate leveling off by 1500 seconds (**Figure 6.10 C,D**). Based on this COMSOL model, around 25 minutes is required for complete diffusion of the formulation to occur from the microneedles.

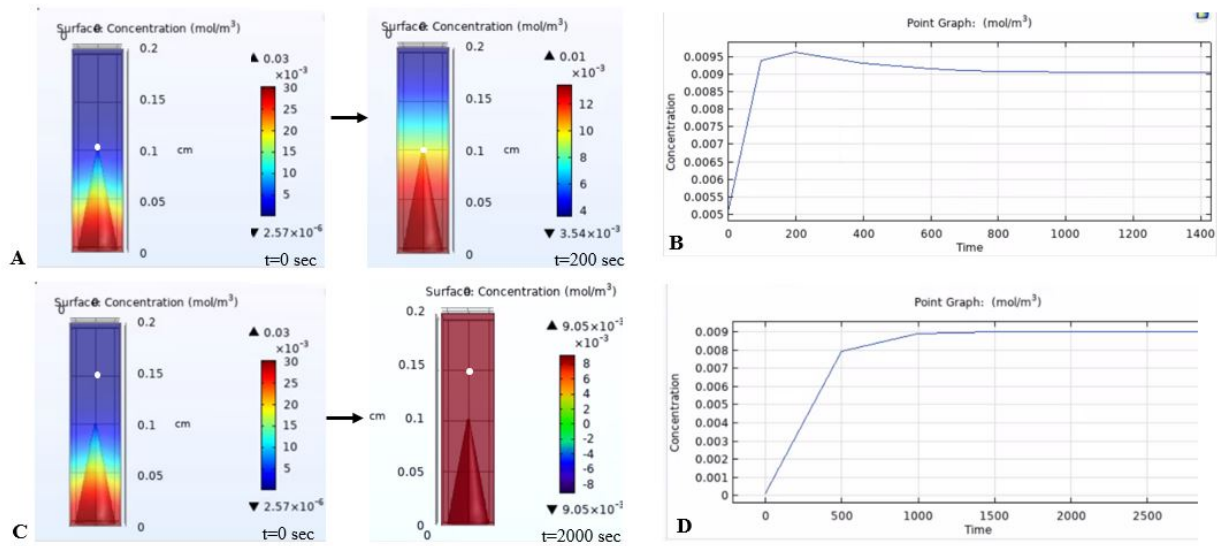


Figure 6.10: **COMSOL modeled vaccine diffusion.** (A,B) Vaccine diffusion analyzed at the microneedle tip. (C,D) Vaccine diffusion analyzed 0.05 cm above the microneedle tip.

6.7 Cost

The cost of microneedle patch production and administration was calculated by totaling the cost of administration, price of bulk vaccine, and microneedle patch material costs, which was further divided into the silk fibroin, mold, polymer base, backing, and packaging costs. Altogether, the cost of microneedle patch production is estimated at \$6.67 (**Figure 6.11**). Although additional costs (sterilization, quality control, labor, CAPEX, fixed costs, equipment) need to be considered, the cost of microneedle production is below current influenza vaccine prices (\$12.45/dose) [49]. The primary cost advantage of microneedle patches can be attributed to the lack of expensive cold chain technology typically required

to maintain vaccine stability. Furthermore, the dominant cost in the microneedle—antigen cost—would decrease even further with bulk orders and large scale production. The total cost of microneedle production and administration is also close to \$6.67 due to near zero administration costs, which is well below current standards (\$23.96) [50].

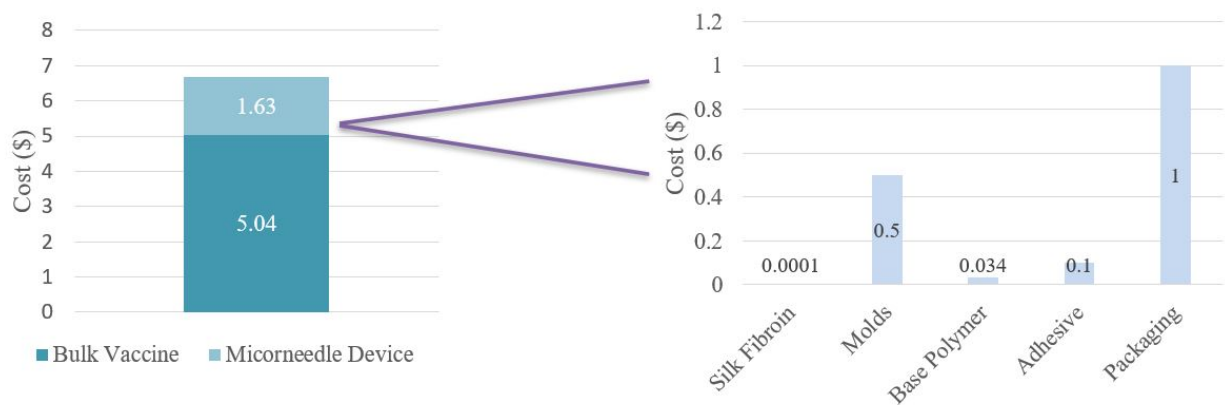


Figure 6.11: **Calculated cost of microneedle production and administration.** Microneedle patches will be self-administered, setting the administration costs close to zero. Material costs are the primary cost in microneedle production and administration.

Chapter 7

Discussion of Results

7.1 Fill Finish

All five fill finish procedures (Section 6.1) resulted in solutions that met the silk fibroin solution and printability technical specifications. Over the entire 186 day (6 month) time-course stability study, silk fibroin samples frozen without use of chilled ethanol underwent sol-gel transition. The freezing curves provide an explanation for this phenomenon (**Figure 7.1**) [57]. An ideal fill finish procedure minimizes time in the critical zone between 0°C to -10°C, the temperature range in which silk fibroin maintains mobility and the possibility of aggregation. Apart from minimizing overall freezing time to avoid silk-lithium bromide separation and silk fibroin gelation, hyper-cooling should also be avoided.

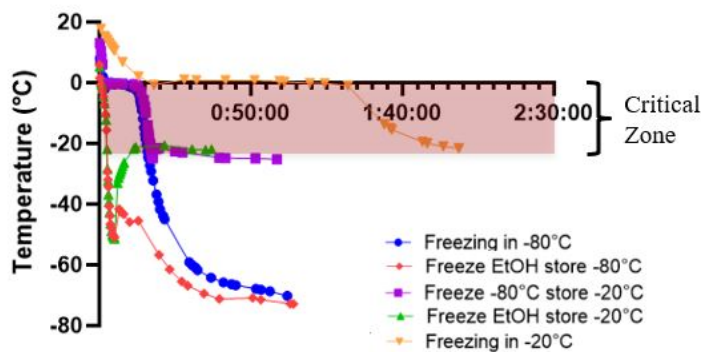


Figure 7.1: **Experimental freezing curves.** Freezing in ethanol and transferring to -80°C represents the most ideal freezing progression, in which time in the critical zone is minimized and hyper-cooling is avoided.

Freezing in -20°C left the silk in the mobile, “freezing” period for over an hour—much longer than any of the other fill finish procedures—increasing the likelihood of aggregate formation. Freezing in -20°C did in fact increase the quantified aggregation compared to

other silk fibroin solutions based on the increase in fluorescence (ThT Assay). Freezing directly in -80°C (freezing in -80°C or freezing in -80°C , storing -20°C) resulted in silk above -10°C for a longer period of time compared to freezing in ethanol. Overall, freezing without the use of chilled ethanol increased time in the critical zone, increasing the likelihood of aggregation and decreasing overall shelf life. Comparing the two samples frozen in ethanol, the sample frozen in ethanol and transferred to -20°C experienced an undesirable, hypercooling spike during transfer to -20°C . Meanwhile, freezing in ethanol and transferring to -80°C reduced the solid cooling phase, minimized the freezing time, and avoided hypercooling the solution. Combined with the time-course aggregation results, the freezing curves justified the selection of freezing in ethanol and transferring to -80°C as the final fill finish procedure.

7.2 pH and Conductivity

All five analyzed silk fibroin samples (Section 6.2) met the silk fibroin characteristic and printability technical specifications. Decreasing the pH and conductivity of the silk fibroin solutions decreased quantified aggregation, translating to an increased shelf life. The main effect of pH and conductivity on absorbance and fluorescence values for the OD and ThT assay, respectively, were calculated. Increasing the pH played a greater role in increased aggregation levels, for both the ThT and OD assays, compared to increasing the conductivity based on the main effect calculations (**Figure A.5, A.6**). Buffering the silk fibroin solution to increase the pH introduced potassium phosphate ions, which are more kosmotropic compared to lithium bromide. Kosmotropic ions have been found to increase formation and stabilization of silk fibroin micelles, contributing to the observed silk fibroin aggregation over time [33].

Decreasing the conductivity by reducing the lithium bromide content generally improved H1N1 release (**Figure A.9**). Based on the Hofmeister series, lithium bromide is a chaotropic

salt, which destabilizes silk fibroin micelles but increases protein-silk interactions [38]. Addition of lithium bromide increases interaction of silk fibroin with the H1N1 antigen, which hinders antigen release from the silk fibroin network.

High pH and conductivity decreased tip length, resulting in increased tip deployment. Increase in lithium bromide and potassium phosphate salts reduced repulsion of anionic silk fibroin during microneedle printing, improving tip consolidation and reducing tip length. Shorter microneedle tips experience decreased expulsion and increased retention in ex-vivo skin models.

The combination of pH and conductivity that increased silk fibroin shelf life and enhanced microneedle patch performance are summarized below (**Table 7.1**). Low conductivity ($380\mu\text{S}/\text{cm}$) and low pH (6)—selected for the final design—provided the optimal balance of maximized silk fibroin shelf life and antigen release.

Design Parameter	Solution Printability	Minimized Aggregation	Highest Tip Deployment	Elevated H1N1 Release
pH	All explored values	6	7.6	6
Conductivity	All explored values	$380\mu\text{S}/\text{cm}$	$1000\text{-}2000\mu\text{S}/\text{cm}$	$380\mu\text{S}/\text{cm}$

Table 7.1: **Optimal silk fibroin pH and conductivity for technical specifications of interest.**

7.3 Molecular Weight

All four silk fibroin samples (Section 6.3) with varied molecular weight met the silk fibroin solution (pH, conductivity, molecular weight) and printability (viscosity, surface tension) technical specifications. Higher molecular weight increased silk fibroin aggregation. Larger fibroin protein chains increase intramolecular interactions, decreasing the time required for sol-gel transition, increasing silk fibroin aggregation and reducing shelf life [12]. Denaturing silk fibroin for 180 minutes enhanced shelf stability and microneedle patch performance, justifying the selection of the 180-minute denaturing time.

7.4 Final Design

Both silk fibroin solutions (Section 6.4) produced via dialysis against dilute lithium bromide met the silk fibroin solution (pH, conductivity, molecular weight) and printability (viscosity, surface tension) technical specifications. Importantly, dialyzing against dilute lithium bromide led to a more consistent, accurate silk fibroin endpoint conductivity within $60\ \mu\text{S}/\text{cm}$ of the dialyzing solution.

The silk fibroin solution dialyzed against $500\mu\text{S}/\text{cm}$ lithium bromide underwent sol-gel transition by day 21 of shelf life analysis. Since the conductivity optimization experiment empirically determined that conductivity does not have a significant impact on aggregation quantified, the observed rapid gelation may be explained by contamination. Nonetheless, additional experiments need to be performed to confirm gelation is not a result of processing conditions.

After five days of storage at room temperature, both groups of microneedle patches released close to 100% of potent, viable antigen. Near complete antigen release importantly proves the dose delivery capabilities of the microneedle patches. Furthermore, release of potent antigen after microneedle storage at room temperature indicates the vaccine thermostabilization potential of the silk fibroin matrix.

Silk fibroin dialyzed against $500\mu\text{S}/\text{cm}$ lithium bromide reduced tip length. Increased lithium bromide reduced electrostatic repulsion of silk fibroin, improving tip consolidation and decreasing tip length. Tip deployment efficiency marginally increased with the higher conductivity silk fibroin group, but not by a statistically significant amount.

Based on all four conducted optimization experiments, the optimal silk fibroin production process that results in a consistent, shelf-stable silk fibroin solution and resulting effective vaccine-loaded microneedle patches consists of 180 minutes denaturing, desalting against $300\mu\text{S}/\text{cm}$, maintaining low pH, freezing using ethanol, and storing in -80°C .

Chapter 8

Conclusions

8.1 Achievement of Technical Specifications

For the final iteration of silk fibroin production, the pH, conductivity, and molecular weight of the extracted silk fibroin met the silk fibroin technical specifications (**Table 8.1**). For future manufacturing and analysis, insights gained from the optimization experiments can be used to set even narrower optimal silk fibroin parameters.

The silk fibroin dialyzed against the $300\mu\text{S}/\text{cm}$ lithium bromide solution remained unaggregated over the examination period, even post stress at 25°C . Ideally, the full shelf life would be evaluated by quantifying aggregation until gelation. However, there is reason to believe that shelf life at 4°C is around 6 months based on conducted optimization experiments. Silk fibroin shelf life under more strenuous temperature and shear stress should also be explored to more accurately mimic shelf life under processing and manufacturing conditions.

For the final design, VaxArray was utilized to simultaneously evaluate the potency and in vitro release of the H1N1 antigen. A total of 97-100% potent H1N1 antigen was released from the silk fibroin microneedle patches. Tip deployment efficiency was around 98% and tip length was around $250\mu\text{m}$. Taking into account the percent of tips correctly demolded, total vaccine release averaged around 91% for both microneedle patch groups in the final iteration. Overall, microneedles printed in the final design exceeded the technical specification of 80% vaccine release.

COMSOL was used to determine the critical load factor and maximum load. The critical

load factor was 12.125 upon application of a 3.18MPa pressure to the microneedle, resulting in a reasonable maximum load of 4.84N. In the future, the maximum load of microneedle tips printed with varied silk fibroin solutions should be experimentally confirmed via Instron measurements.

The diffusion component of vaccine release was modeled in COMSOL using an experimentally determined diffusion coefficient. While the COMSOL model did not represent silk matrix swelling nor silk fibroin matrix degradation—both of which contribute to vaccine release—the model demonstrated that antigen fully diffuses from the microneedle tips over the course of 25 minutes, if diffusion were only governed by concentration gradients and diffusion coefficients. A more realistic model would represent silk fibroin-antigen electrostatic and hydrophobic interactions, which tend to lengthen diffusion. Nonetheless, the developed COMSOL model provides a starting point for future analysis.

The optimized final design silk fibroin produced via dialysis against 300 μ S/cm lithium bromide met all the silk fibroin technical specifications. When successfully used to print and analyze H1N1-loaded microneedle patches, the printed patches also met all the microneedle technical specifications. Compared to existing studies on silk fibroin, this design project produced consistent, shelf-stable silk fibroin applicable for vaccine delivery via microneedle patches. This study also comprehensively explored and optimized a wide range of silk fibroin production parameters. The original project goals were accomplished, and attainment of design specifications satisfied all design criteria.

Design Aspect	Target Specification	Optimized Value
Silk Fibroin Solution	pH 5-8	pH 6.2-6.3
	100–1000 $\mu\text{S}/\text{cm}$ Conductivity	270-440 $\mu\text{S}/\text{cm}$
Silk Fibroin Shelf Life	Shelf life at 4°C >3 weeks (No gelation, <0.1 absorbance, <400,000 fluorescence)	<0.1 absorbance, <400,000 fluorescence for >5 weeks for the silk fibroin solution dialyzed against 300 $\mu\text{S}/\text{cm}$
	Shelf life under temperature stress (25°C) >2 days (No gelation, <0.1 absorbance, <400,000 fluorescence)	<0.1 absorbance, <400,000 fluorescence post temperature stress
Silk Fibroin Printability	5cP $\pm$ 3cP viscosity	2.1-2.2 cP
	75 mN/m Surface Tension	62-68 mN/m
Microneedle Performance	H1N1 antigen potent for >3 weeks at 25°C storage	97-100% released potent antigen post 5 day storage
	>80% vaccine release	97-100% released potent antigen
	>95% tip deployment in ex-vivo skin models	98-99% tip deployment
	200 $\pm$ 60 μm tip length	252-253 μm
	$\geq$ 1 safety factor	12.125
Cost	<\$23.96	\$6.67

Table 8.1: Achieved design specification of optimized silk fibroin.

8.2 Limitations and Future Work

This bioengineering design project, comprehensive in its engineering design and evaluation, will aid the translation of microneedle patches into the clinic. However, a number of additional studies could be performed to address limitations and enable clinical translation.

First, the full silk fibroin shelf life until aggregation should be evaluated, both at 4°C and subject to external stress (i.e. temperature, shear) in order to fully understand the usability of the silk fibroin solutions under processing conditions.

In addition, stability of the H1N1 antigen in the silk fibroin matrix was evaluated after only five days of storage at room temperature, due to time constraints. In order to fully prove that silk fibroin enhances thermostability of vaccines, microneedle patches should be subject to longer, elevated temperature stresses, with potent antigen release subsequently evaluated via the VaxArray assay. The antigen-silk fibroin interaction could be further explored by differential fluorescent tagging of the proteins, followed by qualitative and quantitative examination of colocalization of the H1N1 antigen with silk fibroin.

Furthermore, the COMSOL modeled maximum load should be confirmed experimentally, particularly exploring the impact of varied silk fibroin processing conditions on microneedle mechanical strength. Similarly, influencing the silk fibroin processing conditions and ultimate silk fibroin properties impact vaccine release from the silk fibroin matrix. A more elaborate COMSOL diffusion and vaccine release model considering hydrophobic, electrostatic, and physical interactions between the antigen and variably processed silk fibroin could be developed to explore these hypotheses. An enhanced diffusion model would also consider release into bodily fluid rather than water.

Finally, this design project did not explore the impact of varied silk fibroin solutions on the immunogenicity of microneedle patches. Although previous studies have demonstrated that delivery via Vaxess microneedle patches enhance the magnitude, duration, and breadth of humoral immune response, additional studies should be conducted to determine whether

the optimized silk fibroin impacts these results. Furthermore, additional studies should be conducted to confirm that silk fibroin subject to variable processing conditions does not generate inflammatory byproducts. Finally, the microneedle patches should be tested on several animal models to prove efficacy of the technology on several relevant models.

8.3 Impact

This design project resulted in the optimization of silk fibroin production parameters for microneedle patch applications, enabling more robust future manufacturing processes. Novel experimentation determined that low pH, low conductivity, lower molecular weight, and a fill finish procedure involving ethanol freezing and -80°C storage resulted in silk fibroin solutions with prolonged shelf life and well-performing H1N1-loaded microneedle patches.

Although this particular design project focused on optimization of silk fibroin microneedle patches for H1N1 antigen vaccines, the microneedle platform can be applied for delivery of a variety of therapeutic applications from other vaccines to insulin and anti-cancer agents. Therefore, the impact of this project is well beyond the immediate impact of this study and positively contributes to the emerging transdermal drug delivery field. Specifically, the slow-release tips uniquely made from silk fibroin mimic natural infections, allowing the body to mount a stronger, more durable response. Meanwhile, the microneedle patches do not require refrigeration and can be self-administered pain free. Altogether, this therapeutic delivery platform results in improved treatment efficacy and the possibility for significantly cheaper, more-widespread distribution.

Appendix A

Appendix

A.1 Assay Development

Optical density (OD) and the Thioflavin T (ThT) assay were identified as assays that could quantify silk fibroin aggregation without requiring extensive preparation and instrumentation access (compared to the use of SEC and DLS typically used to quantify particle size and aggregation). The following subsections describe the background method suitability tests conducted on both assays.

Repeatability of ThT stock solution preparation

A method to easily determine the concentration of freshly prepared ThT stock solution was established. Using existing ThT stock solutions, a series of serial dilutions from 100% to 0.35% were prepared. The absorbance of each solution at 412 nm was measured, recorded, and plotted. A linear regression was performed to determine the line of best fit, used to calculate the concentration of freshly prepared ThT stock solution based on a molar extinction coefficient of 36,000 M/cm (**Figure A.1**).

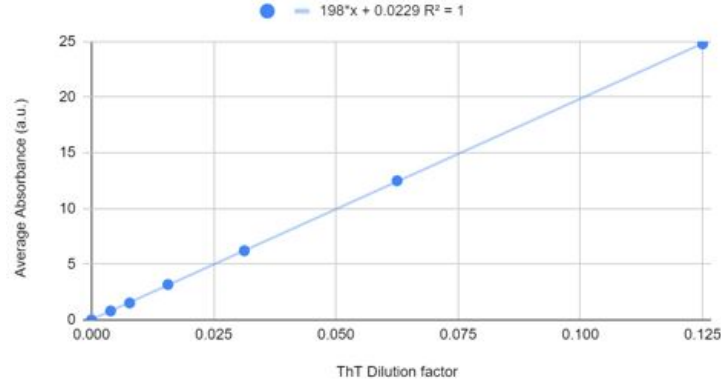


Figure A.1: **ThT stock concentration determination.** Linear regression of serial dilutions of ThT stock solution.

Range

Based on a comparison of silk samples stored for varied time periods, it was determined that freshly prepared, unfiltered silk fibroin has fluorescence (a.u.) values of around 140,000 based on the ThT assay and absorbance (a.u.) values of around 0.01-0.02 based on the OD assay (**Figure A.2**). Meanwhile, silk fibroin solutions that are near gelation have fluorescence (a.u.) values around 1,000,000 and absorbance (a.u.) values at 0.1-0.6. Both assays are sensitive to silk gelation. Un-aggregated silk fibroin solutions have fluorescence and absorbance values that are an order of magnitude smaller compared to aggregated silk fibroin solutions. The findings of this experiment were used to set the technical specification values of acceptable aggregation levels.

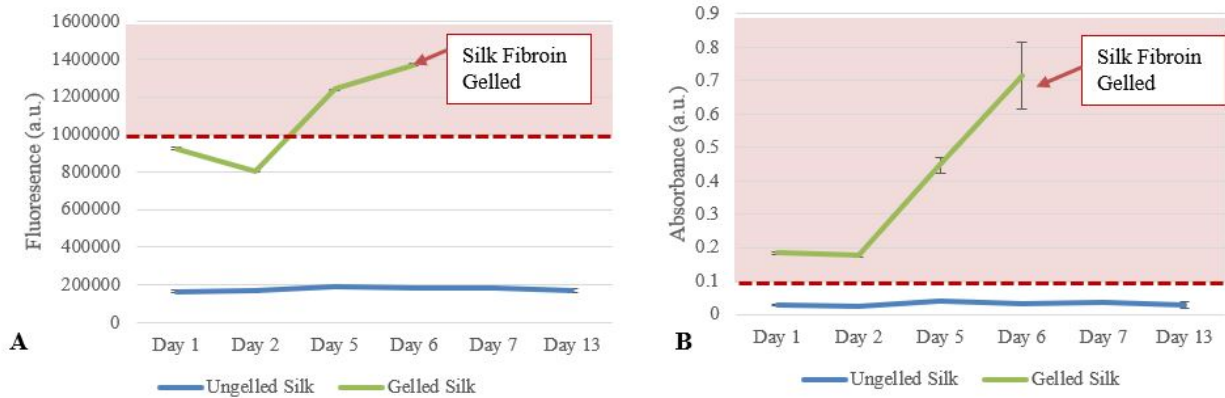


Figure A.2: **Aggregation assay values representative of gelation.** Absorbance and fluorescence values of silk fibroin at sol-gel transition.

Precision

Precision was quantified by the coefficient of variation between replicate preparation of the assays. The coefficient of variation for the ThT assay was 0.274%, while the coefficient of variation for the OD assay was 8.3%, meeting the accepted coefficient of variation of <10%.

Linearity

Blends of freshly prepared silk fibroin (representing un-aggregated silk) and aggregated, near gelation silk fibroin were prepared for assessment of linearity. The ratios of unaggregated to aggregated silk fibroin blends were: 100:0, 75:25, 50:50, 25:75, 0:100, prepared via direct dilution. The fluorescence (ThT assay) and absorbance (OD assay) were plotted, and linearity curves comparing the ratio of aggregated silk fibroin to fluorescence/absorbance were generated. Both assays had a correlation coefficient of >0.9.

Impact of Excess Lithium Bromide

In order to ensure that excess lithium bromide does not interfere with aggregation assay readout, 9 μ L of 0.93M lithium bromide solution was spiked into two separate silk fibroin solutions during ThT and OD assay preparation. Spiking in lithium bromide resulted in a 5% change in absorbance (OD assay) and 6.9-9.5% change in fluorescence (ThT assay). Due to these changes, normalized absorbance and fluorescence values were reported in the results.

DLS Measurements

Dynamic light scattering (DLS) was performed to validate the ThT and OD assays. DLS particle diameter trends aligned more closely with the OD absorbance trend. While the OD assay and DLS may quantify non-specific fibrils, micelles and other aggregates, ThT may quantify only β -sheet fibrils, explaining the observed trends [52] (**Figure A.3**).

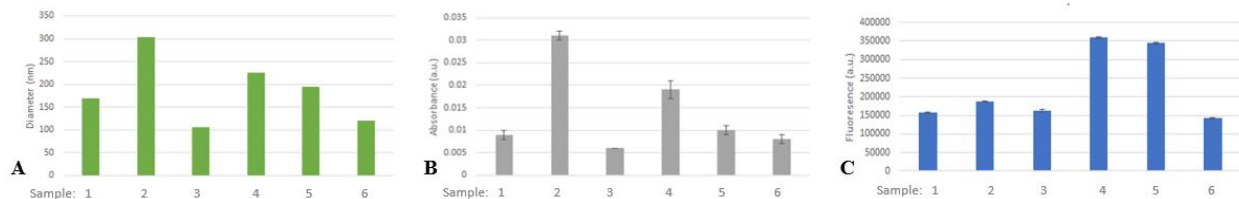


Figure A.3: **DLS validation of aggregation assays.** (A) Particle size trends as observed by DLS. (B) Absorbance trends as observed by the OD assay. (C) Fluorescence trends as observed by the ThT Assay.

A.2 Conductivity and Lithium Bromide Content

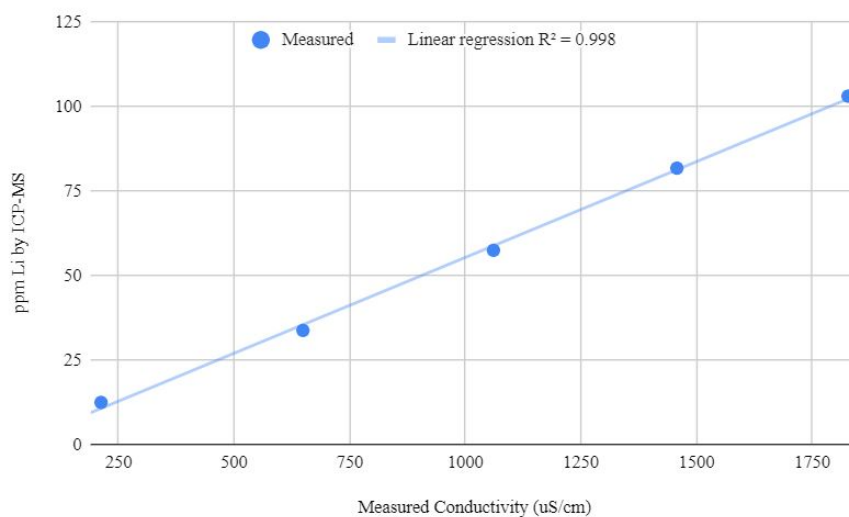


Figure A.4: **Relationship between lithium content and conductivity.** A linear relationship between lithium content as measured by ICP-MS and the conductivity is observed with a coefficient of determination of 0.998.

A.3 Silk Fibroin Solution Experiment Values

Fill Finish Procedure	pH 5-8	Conductivity 100-1000 $\mu\text{S}/\text{cm}$	Viscosity 5 (± 3) CPa
Freezing in -80°C	6.3	370	2.22
Freeze in EtOH (5 mins), store -80°C	6.2	366	2.18
Freezing in -80°C , store - 20°C	6.1	360	2.33
Freeze in EtOH (5 mins), store -20°C	6.1	366	2.19
Freezing in -20°C	6.1	349	2.34

Table A.1: **Silk fibroin solution values for fill finish optimization.** Values are reported post-thaw as an average of triplicate readings.

pH 5-8	Conductivity 100-1000 $\mu\text{S}/\text{cm}$	Viscosity 5(± 3) CPa
6.0	380	2.08
6.0	1045	2.06
6.1	2086	2.05
7.5	2363	2.1
7.5	3389	2.07

Table A.2: **Silk fibroin solution values for conductivity and pH optimization.** Values are reported post-thaw as an average of triplicate readings.

Denaturing time (min)	pH 5-8	Conductivity 100-1000 $\mu\text{S}/\text{cm}$	Viscosity 5 (± 3) CPa	Surface Tension <75 mN/m
60	6.4	284	3.4	57
120	6.8	353	2.75	69
180	6.3	319	2.24	71
240	5.6	250	1.78	72

Table A.3: **Silk fibroin solution values for molecular weight optimization.** Values are reported post-thaw as an average of triplicate readings.

Conductivity of LiBr Dialy- sis Solution	pH 5-8	Conductivity 100-1000 $\mu\text{S}/\text{cm}$	Viscosity 5 (± 3) CPa	Surface Tension <75 mN/m
300	6.2	273	2.1	62
500	6.3	443	2.18	68

Table A.4: **Silk fibroin solution values for final design iteration.** Values are reported post-thaw as an average of triplicate readings.

A.4 Main Effect Calculations of pH and Conductivity

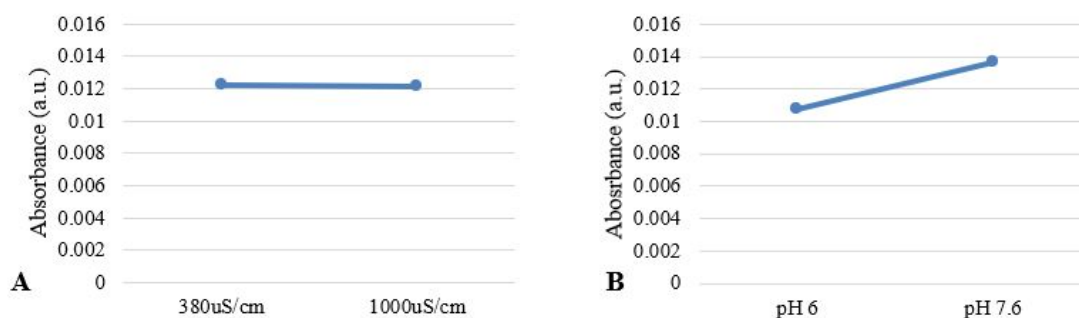


Figure A.5: **Main effect of conductivity and pH on aggregation as measured by the OD assay.** (A) Main effect of conductivity on measured absorbance. (B) Main effect of pH on measured absorbance.

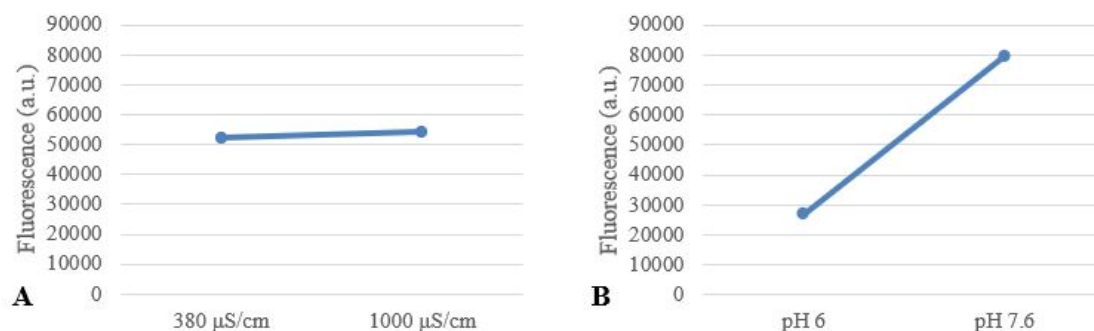


Figure A.6: **Main effect of conductivity and pH on aggregation as measured by the ThT assay.** (A) Main effect of conductivity on measured fluorescence. (B) Main effect of pH on measured fluorescence.

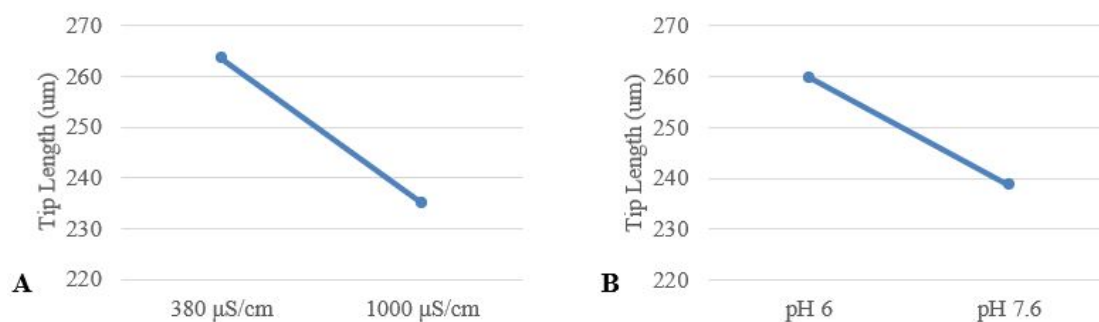


Figure A.7: **Main effect of conductivity and pH on tip length.** (A) Main effect of conductivity on tip length. (B) Main effect of pH on tip length.

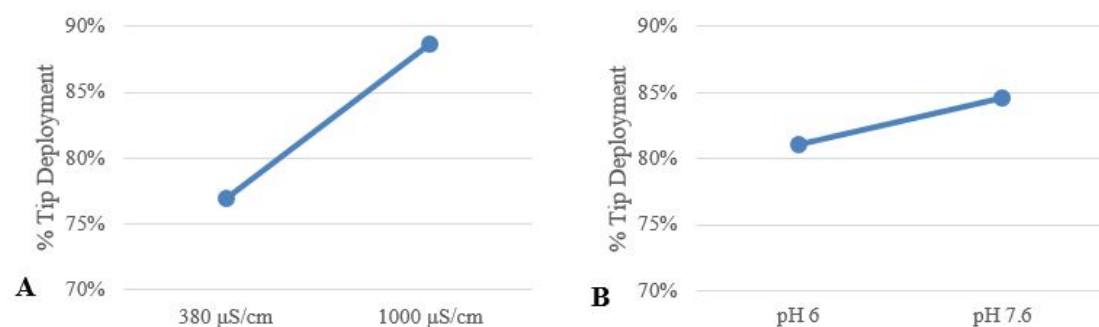


Figure A.8: **Main effect of conductivity and pH on tip deployment efficiency.** (A) Main effect of conductivity on tip deployment efficiency. (B) Main effect of pH on tip deployment efficiency.

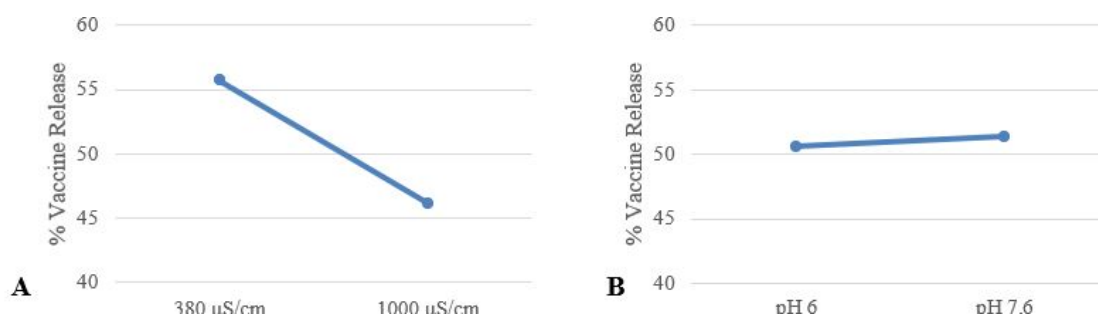


Figure A.9: **Main effect of conductivity and pH on percent vaccine antigen release.** (A) Main effect of conductivity on percent vaccine antigen release. (B) Main effect of pH on percent vaccine antigen release.

A.5 Budget

The tables below document the total cost of items used in the project as well as a detailed breakdown of the cost of a single microneedle device. Equipment, instrumentation, and other non-consumables are not included in the cost calculations for these tables. In Table A.5, these items are simply listed as "Accessed", either at Vaxess or through the Active Learning Labs (ALL).

Table A.6 outlines a more detailed cost breakdown of a microneedle patch. The cost of silk fibroin was calculated under the assumption of a 50kg batch size. The cost of injection molds, backing, and packaging are based on internal quotes. For this calculation, the cost of the H1N1-antigen was based on a quasi-bulk estimate. Sterilization, cleaning and gowning supplies, quality control, labor, CAPEX, fixed costs, and equipment costs are not included.

Item	Cost of Item	Cost to Me	Source
Raw silk (100g)	\$10.80	\$0	Vaxess
Sodium carbonate (25 g)	\$190.00	\$0	Vaxess
Lithium Bromide (100g)	\$67.70	\$0	Vaxess
H1N1 Antigen (10mg)	\$800.00 (bulk)	\$0	Vaxess
Methylene blue (25g)	\$84.30	\$0	Vaxess
Thioflavin T dye (100mg)	\$81.00	\$0	Vaxess
PBS (50mL)	\$49.82	\$0	Vaxess
BSA (10g)	\$76.60	\$0	Vaxess
Human Sera (20mL)	\$58.80	\$0	Vaxess
5% sodium azide (500mL)	\$125.60	\$0	Vaxess
Water-based dye set	\$4.19	\$0	Owned
Microfluidic channel	\$40	\$0	ALL
Skin models	\$205.00	\$0	Vaxess
Dialysis Cassettes, 3.5K MWCO (10)	\$178.75	\$0	Vaxess
Assorted pipettes, tubes, plates, beakers	\$300.00	\$0	Vaxess
SEC-HPLC	Accessed	\$0	Vaxess
PicoPulse	Accessed	\$0	Vaxess
Stereo/ Confocal Microscope	Accessed	\$0	Vaxess
Nanodrop	Accessed	\$0	Vaxess
Microplate reader	Accessed	\$0	Vaxess
VaxArray system, reagents	Accessed	\$0	Vaxess
pH, conductivity meter	Accessed	\$0	Vaxess
Microfluidics Double Syringe Pump	Accessed	\$0	ALL
Total (own expense)		\$0	

Table A.5: List of the project's required materials, equipment, and expenditures.

Item	Cost	Quantity Sold	Quantity Required	Cost per Device
Silk Fibroin	\$450	1kg	2mg	\$0.0001
Microneedle Molds	\$0.50	ea	1	\$0.50
Base polymer	\$0.54	1g	0.06g	\$0.034
Adhesive backing	\$0.10	ea	1	\$0.10
Packaging	\$1.00	ea	1	\$1.00
H1N1 antigen	\$84.00	1mg	0.06mg	\$5.04
Total				\$6.67

Table A.6: Total cost per microneedle device.

A.6 Matlab Code to Determine Diffusion Coefficient

```

1 %%Load the data
2 %data=intensityvalues4;
3 clc
4 clear
5 Num=input('How may files you want to run? ');
6 DCoef=zeros(Num,1);
7 for Counter=1:Num
8
9 [filename,path] = uigetfile('*.dat','*.txt','*.csv');
10 addpath(path);
11 import=importdata(filename);
12 im1=import.data;
13 YesNo=menu('Do you want to determine the position based on your
    file name?
14 (It has to be a single digit name)', 'Yes', 'No');
15 if (length(filename)>5) || isempty(str2num(filename(length(
    filename)-4))) || YesNo == 2 %#ok<*ST2NM>
16     Position=menu('Select Needed Position', '1','2','3','4','5','6
    ','7','8');
17 else
18     Position=str2num(filename(length(filename)-4));
19 end

```

```

20 % Specify distance
21 Distance = [10.77]*1e-3;
22 %%Break down the data into its X and Y vectors, the plot it
23 xdat=im1(:,1)*1e-3;
24 ydat=im1(:,2);
25 plot(xdat,ydat)
26 %%Creates a model function with open fitting parameters for the
    unknowns,
27 %%then fits based on the initial guesses
28 modelfun=@(b,x)b(1)+b(2)*(1-erf( (x+b(3)) / b(4) ));
29 beta0=[42 58 -1e-4 1e-4];      %Initial guesses
30 beta=nlinfit(xdat,ydat,modelfun,beta0);
31 %Fits and outputs best fits for each parameter
32
33 %%This plots the data and the fit
34 figure(1);
35 plot(xdat,ydat)
36 hold on;
37 plot(xdat,modelfun(beta,xdat))
38 hold on;
39
40 %%Takes parameters and calculates diffusion coefficient
41
42 coeff=beta(4);
43 disp(coeff);
44 L=Distance(1);    %%%Input the distance in the channel in meters
45 A=75*10^-6*1*1e-3;
46 Q=(0.1*10^-6)/60;
47 D=( (coeff/2)^2 / (L*A/Q) );
48
49 txt = [ 'D= ' num2str(D) ];
50 text(5e-5,30,txt)
51 hold off
52 DCoef(Counter)=D;
53 saveas(figure(1), fullfile(path,num2str(Position)),'png');
54 end

```

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