POLYMER MICRONEEDLES FOR TRANSDERMAL DELIVERY OF BIOPHARMACEUTICALS

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POLYMER MICRONEEDLES FOR TRANSDERMAL DELIVERY OF BIOPHARMACEUTICALS

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To Sarah and the Cecil Sullivans,

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LIST OF SYMBOLS AND ABBREVIATIONS

AIBN	azobisisobutyronitrile
AICHI	subtype of H3N2 influenza virus
Al_2SO_4	aluminum sulfate
ANOVA	analysis of variance
BCA	bicinchoninic acid
BPO	benzoyl peroxide
BSA	Bovine Serum Albumin
CO_2	carbon dioxide
CPE	chemical permeation enhancer
DI	deionized
DMF	N, N Dimethylformamide
ELISA	Enzyme-Linked ImmunoSorbent Assay
H	hemagglutinin protein
HA	hemagglutination
HAI	hemagglutination inhibition
HAU	hemagglutination activity units
H+E	hematoxilyn and eosin
HRP	horseradish peroxidase
H1N1	subtype of influenza A virus
H3N2	subtype of influenza A virus
H_2O_2	hydrogen peroxide

H_3PO_4	phosphoric acid
IACUC	Institutional Animal Care and Use Committee
IgA	Immunoglobulin A
IgG	Immunoglobulin G
LD_{50}	lethal dose for 50% to die
M	matrix protein
MA	micro BCA reagent A
MAA	methacrylic acid
MB	micro BCA reagent B
MC	micro BCA reagent C
MDCK	madin-darby canine kidney
MN	microneedle
MWCO	molecular weight cut off
N	neuraminidase protein
Na_2CO_3	sodium bicarbonate
NP	nucleoprotein
OPD	o-Phenylenediamine dihydrochloride
P	p statistical value for t-test
PBS-T	PBS + 0.05% Tween
PDI	poly dispersity index
PDMS	polydimethylsiloxane
PFU	plaque forming unit
PGA	polyglycolic acid

PLA	polylactic acid
PLGA	poly(lactic-co-glycolic acid)
PMAA	polymethacrylic acid
PR8	strain of H1N1 influenza virus
PVP	polyvinylpyrrolidone
PVP-MAA	poly(vinyl pyrrolidone-co-methacrylic acid)
RBC	red blood cell
RDE	receptor destroying enzyme
SC	stratum corneum
SU8	polymeric photoresist epoxy
VP	vinylpyrrolidone
X-gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside
X31	strain of H3N2 influenza virus

SUMMARY

Biopharmaceuticals, including peptides, proteins, DNA and vaccines, are one of the fastest growing segments of the overall pharmaceutical market. While the current delivery device for these molecules, the hypodermic needle, is effective, it also has limitations, including low patient compliance, need for medically trained personnel and biohazardous sharps after delivery. As a result, there is a need for an alternative delivery system for biomolecules that is effective and has a higher patient compliance than the hypodermic injection. One possible solution is dissolving polymer microneedles, which are microscopic needles large and strong enough to insert into the skin and deliver the encapsulated drug effectively while also being small enough to not interact with the pain causing nerve fibers deep in the skin. One additional benefit of this system is that the needles completely dissolve in the skin, leaving behind no biohazardous sharps. The overall goal of this study was to develop a dissolving polymer microneedle system as an effective and patient compliant delivery method for biopharmaceuticals.

The first part of this study focused on developing a new fabrication process for dissolving polymer microneedles, which is gentle and allows for the encapsulation of active biomolecules. The new process focused on UV initiated free radical polymerization of a liquid monomer solution within a microneedle mold. This process produced sharp microneedles with the identical geometry of the original master structure. The polymer polyvinylpyrrolidone (PVP) was chosen as the main structural material for the polymer microneedles for the following reasons: high water solubility for fast dissolution within the skin after insertion, high mechanical strength due to a ring structure

in the chemical backbone, ability to be formed using radical polymerization, and a history of use in clinical settings. PVP polymer microneedles were shown to successfully insert into pig skin in vitro and deliver the encapsulated molecules. In addition, proteins retained full activity after encapsulation and delivery via the new polymer microneedles.

The remainder of this study focused on the use of microneedles for the delivery of the influenza vaccine. First, it was important to determine if the influenza vaccine retained antigenicity and immunogenicity after reformulation within PVP polymer microneedles. Since this fabrication process involves polymerization of a solution of drug and liquid monomer, the drug to be encapsulated must be in solid form. It was shown that lyophilization of the H1N1 or H3N2 influenza viruses had no significant impact on the activity of the viruses. This included full protection against lethal challenge in mice vaccinated with the lyophilized virus. Immunizations with the the influenza vaccine that was reformulated within PVP polymer microneedles resulted in a lower immune response than the response for the lyophilized or unprocessed virus. There was still a protective immune response against a lethal challenge, but it was not as strong as the response for the control intramuscular injection. Interestingly, a similar lower immune response was also seen in immunizations with the unprocessed virus that was in solution with the PVP polymer. It was believed that the interactions of virus and PVP polymer in solution alone decreased the activity of the virus, and that the encapsulation process had little to no deleterious effect on the virus.

Next, it was important to determine if a proper immune response could be induced via microneedle-based delivery of the influenza vaccine. Here, we used coated metal microneedles, which have been shown to be successful in numerous applications in the

past. The process of coating and drying the influenza virus onto metal microneedles resulted in a decrease in the in vitro antigenicity of the virus. In vivo immunizations resulted in a lower immune response for the metal microneedles compared to hypodermic injection control. However, this immune response was still strong and mice immunized with the influenza vaccine using the metal microneedles had full protection and survival against lethal challenge. This proved that microneedles could be used for successful immunizations of the influenza vaccine.

The final part of this study was to determine if dissolving polymer microneedles, created via the new fabrication process, could be used to induce a protective immune response for the influenza vaccine in mice. It was confirmed that PVP polymer microneedles produced similar antibody levels to the IM injection and resulted in full protection against lethal challenge after one immunization with the H1N1 influenza virus. These results were also confirmed with an H3N2 strain of the influenza virus. Finally, after challenge, the mice immunized using the PVP microneedles had a more efficient clearance of the virus from their lungs than the group immunized via the intramuscular injection.

The work done in this study showed that polymer microneedles can be created using a gentile fabrication process that allows for the retention of activity of encapsulated biomolecules. In addition, polymer microneedle based delivery produced an equivalent immune response for the influenza vaccine compared to the intramuscular injection. Furthermore, this delivery device offers multiple logistical advantages to the hypodermic needle, including higher patient compliance, possible self-administration and results in no biohazardous sharps waste. Looking forward, the microneedles produced in this study

offer an exciting alternate delivery device for multiple classes of biomolecules in the future.

CHAPTER 1

INTRODUCTION

Biopharmaceuticals, which include proteins, DNA, vaccines and other biologically related molecules, make up one of the fastest growing segments of the overall pharmaceutical market[6]. However, there are significant delivery limitations. Specifically, oral delivery is difficult due to poor absorption and degradation that occurs in the GI tract and liver[7, 8]. Transdermal drug delivery allows for direct access to the systemic blood supply, bypassing these degradation and absorption issues. However, the stratum corneum, the outer layer of the skin, is an effective barrier to the transport of biomolecules into the skin[9]. Currently, the most common delivery vehicle for these molecules is the hypodermic needle, which is effective, but also has limitations, including painful delivery, the need for trained medical personnel, the disposal of biohazardous sharps waste after injections, and problems in a mass immunization scenario. There is a distinct need in the market for a more patient compliant delivery method for biopharmaceuticals that is self-administered, lacks biohazardous sharps waste and could be used easily in a wide spread immunization effort. Polymer microneedles offer a delivery option that can meet all of the above goals.

Microneedles are microscopic needles that are large and strong enough to insert into the skin and deliver drugs into the skin, but short enough that they do not reach the deeper layers of the skin to stimulate nerves[10]. Dissolving polymer microneedles add an additional benefit of a lack of biohazardous sharps waste after delivery. The mode of delivery for these microneedles is by the degradation or dissolution of the polymer in the

skin after insertion, resulting in delivery of the encapsulated molecule and no needles left afterwards. This could be extremely beneficial for places where biohazardous sharps are a problem, including home use and developing countries. One current limitation to the use of polymer microneedles is the fabrication process, which should be at room temperature, to allow for retention of activity of biomolecules during encapsulation.

Microneedles offer an attractive delivery option for a number of classes of biomolecules, and are particularly appealing for the delivery of vaccines to the skin. Research has shown that the skin offers an appealing target for vaccine delivery due to the large number of immune cells present in the epidermis and dermis[11]. However, skin vaccination via an intradermal injection is a difficult process that requires highly trained personnel and can be time consuming. Specifically, the influenza vaccine has been shown to possibly allow for dose sparing in skin delivery versus the current intramuscular injection[12]. Microneedles offer an efficient method of delivering the antigen to the skin in a self-administered manner. Dissolving polymer microneedles in particular would allow for vaccination against the influenza virus via a self-administered microneedle patch that results in no biohazardous sharps waste.

The overall goal of this project was to develop a polymer microneedle system capable of delivering an active biopharmaceutical in vivo, producing the desired physiological response. The influenza vaccine was chosen as model biopharmaceutical to be used. This goal was investigated via the following four specific aims:

(1) Development of a new fabrication process for polymer microneedles for the delivery of biomolecules.

(2) In vitro and in vivo analysis of the reformulated influenza vaccine for delivery via PVP polymer microneedles

(3) Delivery of the influenza vaccine via coated metal microneedles to induce a protective immune response against lethal challenge in vivo

(4) Delivery of the influenza vaccine via dissolving polymer microneedles to induce a protective immune response against lethal challenge in vivo

CHAPTER 2

BACKGROUND

2.1 DRUG DELIVERY

In 2010, the global drug delivery market is forecast to have a value of 543.8 billion dollars[13]. The two most common methods of drug delivery are injections and oral formulations and each of these methods has advantages and disadvantages. Injections allow for a large bolus release of drugs directly in the bloodstream, but they also involve a painful delivery, can cause infection, and often require trained medical personnel [14, 15]. In addition, improper use of hypodermic needles can lead to needle-stick injuries and the possibility of blood-borne pathogen transmission through previously used needles[16, 17]. Since oral drug delivery usually only involves swallowing a pill, it has a higher patient compliance than injections. However, this form of drug delivery often suffers from low bioavailability due to enzymatic degradation and poor absorption in the GI tract and the first pass metabolism effect of the liver. This is especially significant with biopharmaceuticals, including proteins, DNA and vaccines which lose most of their activity if delivered via the oral route[8]. Thus, there is a need for a minimally invasive method for delivering biopharmaceuticals that is patient compliant and effective.

2.1.1 Skin Anatomy

One delivery route for biopharmaceuticals that can be effective and minimally invasive is across the skin, or transdermal drug delivery. The skin is a promising pathway for the delivery of drugs since it allows for delivery directly into the systemic

circulation without the degradation issues that occur in GI tract and liver in oral delivery. Another benefit is the ability to have continuous delivery, and to avoid systemic toxicity based on peaks and valleys of drug concentration [18]. The skin is made up of three distinct layers: the stratum corneum, the viable epidermis and the dermis. The stratum corneum is 10 – 20 μm thick and is primarily responsible as a barrier to the absorption of external compounds and to water loss[9]. Under the stratum corneum is the viable epidermis (50 – 100 μm thick), which is stratified squamous epithelium made up of keratinocytes, that vary in properties as they differentiate upward from the basal layer[19]. The epidermis is a self-renewing tissue that constantly balances the loss of the skin cells from the stratum corneum with the formation of new skin cells from the lower epidermis[9]. Below the epidermis is the dermis (1 – 2 mm thick), which provides mechanical support and is the location of the major blood vessels and nerves in the skin. A diagram of the skin can be found below in Figure 2.1.

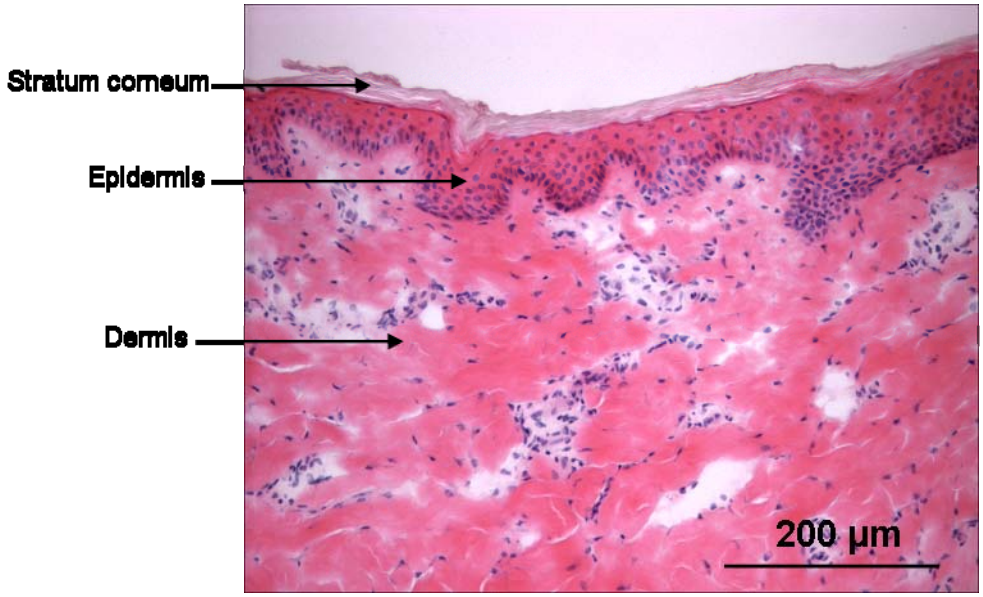

Figure 2.1: Skin anatomy. Histological image of in vitro porcine skin, stained with Hemotoxilyn and Eosin, showing the stratum corneum, epidermis, and dermis.

The stratum corneum (SC) is made up of differentiated keratinocytes from the epidermis. The properties of these cells are drastically different than in the lower layers of the epidermis. Here, the keratinoctytes have transformed into corneocytes, flat anucleated squamous cells that are packed mainly with keratin. The intact SC is sometimes described as a 'brick and mortar' structure[20], with the corneocytes making up the bricks and the lipid bilayers of fatty acids, ceramides, cholesterol and cholesterol esters, making up the mortar[21]. The stratum corneum makes up the main barrier to transdermal drug delivery. Due to the interconnected network, the primary route of compounds through the skin is intercellular, within the lipid bilayers. However, this is a limited pathway, and only molecules that have a low molecular weight (MW < 500) and are relatively lipophilic (1<log P<5) can be delivered passively across the skin [22, 23]. For the delivery of all other molecules, some perturbation of the skin is required.

2.1.2 Transdermal Drug Delivery

There are two main requirements for a transdermal delivery system that would replace the hypodermic needle. First, it must safely and effectively deliver the drug across the skin and into the systemic circulation. Second, it should be more patient compliant than the hypodermic needle, while maintaining a low cost of fabrication. This involves being possibly self-administered, less painful, having little to no chance of infection, and produces no biohazardous sharps waste. One promising option is the transdermal patch, which allows for the painless delivery of drugs across the skin and can be applied without medical supervision[24]. This system can also provide a continuous delivery of drugs, instead of one large bolus delivery offered by needle injections. However, this patch system is limited in the molecules that can be delivered. The patch works by providing a bath of solution to the surface of the skin. Then, the drug passively diffuses into the skin for delivery to the systemic circulation. As mentioned above, only a limited number of drugs have the correct properties to pass through the stratum corneum passively. This includes nicotine, nitroglycerin, clonidine, fentanyl, lidocaine, oestradiol, scopolamine and testosterone[25]. While there has been great success in using the patch for the delivery of these compounds, larger molecules, including biopharmaceuticals cannot be delivered via the conventional transdermal patch system.

In order to deliver larger molecules, the skin and specifically the stratum corneum must be disrupted by chemical or physical methods. This is the basis of most advanced transdermal drug delivery systems. In each of these categories, it is important that the disruption of the skin is transient, and that the skin returns to its protective nature in a short period of time. The main chemical method of disrupting the stratum corneum is by

using chemical permeation enhancers (CPEs), which include water, sulfoxides, azones, pyrrolidones, fatty acids, alcohols, and surfactants, amongst others[26]. The mechanism by which these chemicals disrupt the stratum corneum varies, including hydration, alteration of the cohesion of the skin corneocytes, modification of the lipid bilayer, and modification of the partitioning of the drug into the skin[26]. By whatever means, chemical permeation enhancers have been shown to increase skin permeability and thus increase the rate of drug delivery of large molecules across the skin[25]. However, these chemicals can cause irritation to the skin, and thus would limit their safety and marketability[27]. Also, there are some limitations on the class of molecules which can be delivered. More work is required to optimize the use of CPEs for transdermal delivery of biopharmaceuticals.

Another way of altering the stratum corneum is by physical methods. Some of the most prevalent current methods include iontophoresis, electroporation, thermal ablation, ultrasound, and microneedles. Iontophoresis involves increasing the permeation of a topical drug by applying a low level of electrical current to the skin[19]. Usually, transdermal iontophoresis involves the delivery of a charged drug solution, however neutral drugs can also be delivered by means of electroosmosis[28]. Electroporation increases skin permeability to a topical drug by the application of short high voltage pulses, which are thought to create transient pores for delivery[29]. This method of delivery has been shown to be effective for the delivery of a number of molecules, including large biopharmaceuticals[30]. Thermal ablation increases the permeability of a topical drug by using a short burst of high temperatures to also create transient pores for delivery. This method has been shown to work for the delivery of vaccines and other

biopharmaceuticals[31]. Ultrasound increases topical transdermal delivery by disrupting the stratum corneum after treatment of low frequencies (<100 kHz) of ultrasonic energy for a short period of time. This method has been shown to be effective in the delivery of many large molecules, including insulin[32] and tetanus toxoid[33].

While all of the devices mentioned above are capable of delivering drugs to the skin, they also have some limitations. Most of these methods require external equipment to aid delivery, which can be cumbersome and expensive to produce. Also, in terms of iontophoresis and electroporation, skin irritation can occur during the electrically driven delivery process[34]. Finally, there are some restrictions on the number and class of molecules that can be delivered by these delivery methods. An alternative to these delivery methods is microneedles. Microscopically piercing the skin with micron-scale needles offers an effective and convenient alternative for the delivery of biomolecules, due to the efficient delivery [35, 36], lack of pain [10, 37], ease of use and the expected low cost of fabrication.

2.2 MICRONEEDLES

Microneedles are typically prepared as an array of microscopic needles that are sufficiently large to deliver drugs effectively to the body across the stratum corneum, and also small enough to avoid the nerve fibers in the deeper tissue that elicit a pain response[37]. The length of the needles can range from 100-1000 microns (μm). These dimensions are sufficient to transverse the stratum corneum, which is 10-20 μm[38]. The microneedles effectively deliver the drug into the epidermis or upper layers of the dermis, where it can diffuse into the dermal capillaries and enter the systemic blood supply. In

addition, local delivery and uptake by cells in the skin can also occur. This could be beneficial for vaccination due to the large population of immune cells present in the skin.

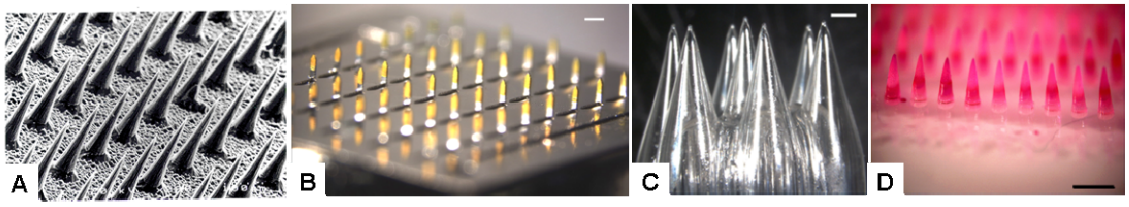

Figure 2.2: Microneedle images. 2.2A: Silicon microneedles, 100 µm in height [1]. 2.2B: Coated Metal Microneedles, 750 µm in height [2]. 2.2C: Glass Hollow Microneedles [3]. 2.2D: Dissolvable Polymer microneedles, 750 µm in height [4].

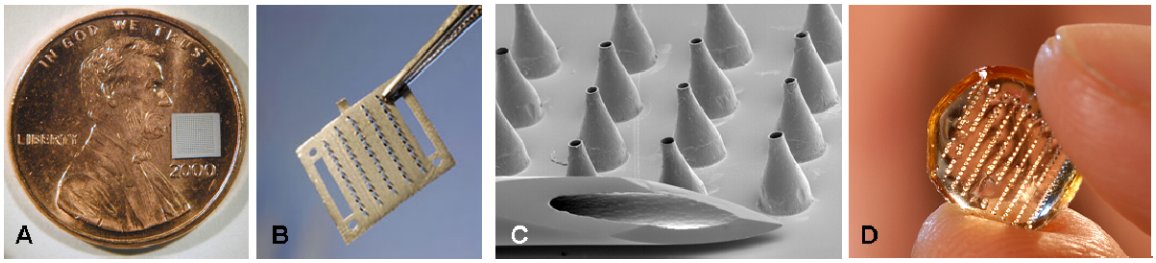

Figure 2.3: Relative sizes of microneedle arrays. 2.3A: Array of 400 silicon microneedles on top of a penny. 2.3B: Array of 50 metal microneedles held with a tweezer. 2.3C: Array of 16 hollow metal microneedles next to a hypodermic needle [5]. 2.3D: Array of 225 polymer microneedles held between two fingers.

There are four main methods of delivery using microneedles for transdermal drug delivery. Figures 2.2 and 2.3 above illustrate the four different types of microneedles. First, solid microneedles can be applied to the skin to create microchannels to increase skin permeability and allow for the delivery of a topical drug solution. Second, solid microneedles can be coated with a drug on the surface of the microneedles. Here, the microneedles are inserted into the skin, and the coating dissolves off of the needles and

into the skin. A third delivery option is hollow microneedles, in which the microneedles are inserted into the skin and a liquid drug solution is delivered via pressure driven convection through the microneedle into the skin. A final option is dissolvable polymer microneedles, in which the drug is encapsulated within the polymer material. After insertion of the microneedle array, the polymer degrades or dissolves, releasing the encapsulated drug. All of these microneedle delivery options have been used to successfully deliver biomolecules to the skin, in vitro and/or in vivo.

2.2.1 Evolution of microneedles

Creating needles of micron size typically requires the use of microfabrication technologies[39]. For proper delivery, the microneedles must have extremely sharp tips[40], be made of a mechanically strong material, and the fabrication process cannot degrade any encapsulated or coated drug. Some of the various techniques that have been used to produce microneedles include lithography and wet or dry etching of silicon, laser cutting, molding and other fabrication methods [1, 36, 41-43]. The original work concerning microneedles involved the use of solid microneedles made of silicon or metal, where the needles pierce the skin to increase permeability or the needles are coated with drug for delivery [1, 42]. Silicon was used as the first material due to its extensive use in the microelectronics industry, and thus much research has been done involving the fabrication of silicon microdevices[39]. However, silicon has some drawbacks. It is more expensive than many metals or polymers, is rather brittle, and is not a material with an FDA history. Thus, metals are often preferred. They come at a cheaper cost, greater strength, and have an FDA track record of safety[44]. Using this system, a number of

biomolecules have been delivered effectively in vivo to animals, including insulin[45], DNA[46] and human growth hormone[47].

Another microneedle delivery method involves using coated metal microneedles. As mentioned above, stainless steel microneedles are extremely strong and microneedles with extremely sharp tips have been created using an IR laser. An important aspect of coated metal microneedles is to have a coating that is strongly adhered to the microneedle, can be used on a wide variety of compounds and is dissolved off of the needle and into the skin quickly after insertion[2]. In addition, the coating process should be optimized to preferentially coat the microneedle shaft and not the base of a microneedle array[2]. Coated metal microneedles have been used in vivo in humans to deliver a number of biomolecules, including ovalbumin[48], desmopressin[49], and the influenza vaccine[50, 51]. In fact, there are companies who are currently evaluating the use of coated metal microneedles in clinical trials.

Hollow microneedles offer another well-researched microneedle delivery method which involves the delivery of a drug solution to skin. Unlike the coated metal microneedles above, little to no reformulation of a drug solution is required for hollow microneedle-based delivery. Both metal and glass hollow microneedles have been fabricated, with each material being strong enough to insert into the skin. Hollow microneedles have been used for the delivery of insulin[5], and the influenza vaccine[52] amongst other biomolecules to animals. This work has focused on the use of single hollow microneedles with pressure driven flow. Future work may focus on the use of hollow microneedle arrays, which may allow for higher flow rates of delivery to the skin.

Polymer microneedles offer another delivery option. While the glass and metal microneedles described above are capable of delivering drugs to the skin, there are, however, safety concerns if microneedles made of these materials were to break off in the skin, or if they were accidentally or intentionally reused. In contrast, the use of water soluble or biodegradable polymers could eliminate these concerns, because the needles completely and safely dissolve or degrade within the skin, and the needle-free patch backing could be safely discarded, leaving no biohazardous sharps waste.

2.2.2 Polymer microneedles

While polymer microneedles offer an exciting and possibly effective transdermal delivery option, there are significant material challenges that must be met for this system to be successful. The ideal polymer material needs to be strong enough to penetrate the skin, degrade or dissolve rapidly once in the skin, and be safely excreted by the body. Also, the fabrication process for these microneedles should produce microneedles with sharp tips, take place at ambient temperatures, without organic solvents, and avoid damaging fragile biomolecules during encapsulation. Another benefit of polymer microneedles is the possibility of a controlled-release delivery system. The delivery kinetics of this system is based on the rate of degradation, or dissolution of the chosen polymer. Thus, different polymers could be used for the delivery of different molecules, depending on the desired rate of drug delivery.

Polymer microneedles have been created that effectively deliver molecules to the skin in vitro [53, 54]. The fabrication process for these polymer microneedles involved the melting of solid polymer pellets into a microneedle mold. A polydimethylsiloxane (PDMS) mold is made as the inverse template of an SU8 masterstructure, which is

created via a lens based microelectronics process[53]. This process produced microneedles made of the biodegradable materials, PLGA, PLA and PGA, which properly insert into the skin in vitro and are capable of delivering the encapsulated cargo, including albumin and microspheres[54]. One drawback of this process is the high temperatures ($T > 140\ ^{o}C$) that is required to melt the polymer pellets to form the microneedle structures. This high temperature could result in the degradation of fragile biomolecules during the fabrication and encapsulation process.

Another fabrication process for polymer needles that has been developed uses a dextrin and drug mixture that is spread onto polypropylene tips to form needle structures. This process takes place at room temperature and has produced needles that allow for the successful delivery of a number of biomolecules, including insulin[55], heparin[56] and erythropoietin[57] to animals in vivo. However, the length of these needles is greater than 3 mm, a length which could cause similar pain of a hypodermic needle. Also, the fabrication process forms needles on a singular basis and is not conducive for mass fabrication. While polymer microneedles offer a promising method of transdermal drug delivery, none of the current fabrication processes allows for the mass production of biodegradable or dissolvable microneedles of micron dimensions or occur at gentle conditions allowing for the delivery of fragile biomolecules. An alternative fabrication method could use free-radical polymerization of a liquid monomer solution to create polymer microneedles. This type of polymerization can occur at room temperature by UV curing and has been used previously in situ in the presence of cells for tissue engineered transplants[58], and biomolecules for implanted drug delivery devices[59].

2.3 POLYMERIZATION FOR POLYMER MICRONEEDLES

2.3.1 Free-radical polymerization

Free radical polymerization is a common method of creating polymers from vinyl monomers, those which have carbon-carbon double bonds. Many polymers can be made using this method, including polystyrene, poly(methyl-methacrylate) polyvinylpyrrolidone, poly(acrylic acid), and branched polyethylene, among others. There are two main components needed for this process to occur, a vinyl monomer, typically without an inhibitor present, and a free radical initiator. The most commonly used initiators for free radical polymerization are azo compounds (azobisisobutyronitrile, AIBN) and peroxy compounds (benzoyl peroxide, BPO)[60]. Free radical polymerization has three main steps: initiation, propagation, and termination. Initiation is the creation of a free radical species, which begins the process. This occurs when the free radical initiator is converted into a free radical species. For example, in the case of AIBN, the free radical is created after the two C-N bonds are broken, releasing N_2 gas and two identical free radical molecules[60]. This bond in the AIBN molecule can be broken by a number of methods including increased temperature (>50 ^{o}C), and ultraviolet light[61]. The next step is propagation, which is the formation of the polymer backbone. Here, the free radical formed in the initiation step reacts with the double bond of the vinyl monomer creating a free radical vinyl monomer species. This process continues and a vinyl chain is formed. The amount of free radical initiator that is used is typically in the range of 0.1 – 3 mol% of the monomer solution[62]. Finally, the process is finished with

the termination step, which can occur by coupling of two radical species or disproportionation.

Some of the benefits of the free radical polymerization process include that it is a well-established simple process, can be performed in a number of different environments and can be used to make a number of polymers and copolymers. However, there are some drawbacks, including the lack of control of the molecular weight of the polymer, which can lead to polymers of high molecular weight[63]. In terms of biocompatibility, the general molecular weight cutoff for polymers is 42,500 – 50,000 Da for glomerular excretion by the kidneys[64]. However, the upper limit for excretion could be as low as 25,000 depending on the molecular shape and polarity of the polymer. Polymers that are larger may be retained in the body which could lead to future health issues. Thus it is important to determine both the average molecular weight and poly dispersity index (PDI) of the polymer prior to clinical use to verify that it has the correct size for proper clearance.

Free radical polymerization offers a new option in the fabrication process of polymer microneedles. Using UV-initiated photopolymerization, the conversion of the liquid monomer and drug solution into the drug encapsulated polymer microneedles can occur at room temperature, thus avoiding high temperature degradation effects that could occur in previous polymer microneedle fabrication processes. Also, this polymerization process could be scaled for mass fabrication as photopolymerization is currently used in a number of industrial settings[62, 65].

2.3.2 Photopolymerization

Photopolymer technology encompasses chemical and physical reactions of organic-based materials that are initiated by the application of electromagnetic radiation, including ultraviolet (UV) and infrared (IR) light[65]. One of the most common commercial applications of photopolymer technology is photopolymerization. This involves the photoinitiated polymerization of monomers and oligomers using a radical-generating photoinitiator to form cross-linked polymers[66]. Concerning photoinitiated free radical polymerization, the process is initiated when the free radical initiator absorbs a photon from the UV light, resulting in a hemolytic bond rupture of the initiator converting it into a free radical species[67]. Photopolymerization has been used commercially in a number of applications, including coatings, adhesives, sealants, and microelectronic resists[65]. It is also popular for medical applications in vivo, including implantation of drug delivery devices in the skin[59], insertion of dental resins for oral surgery[68], and the formation of hydrogel coatings on arterial surfaces to prevent restenosis after angioplasty[69], amongst other applications[59]. Typically, the use of polymerization in vivo is rare due to issues with high temperatures needed for initiation, and long times for the reaction to occur. Photopolymerization is a viable option since it can be initiated at low temperatures and is typically rapid[59]. Photoinitiated free radical polymerization is an exciting option for the fabrication of polymer microneedles since it is fast acting and can occur at room temperature, possibly preventing the damage of any encapsulated biomolecules. Also, it can be used for the synthesis of a number of polymer and copolymer systems, thus allowing for the tuning of polymer microneedles with specific properties.

2.3.3 PVP and PVP-MAA

In this project, the free radical polymerization process could be used to produce a number of polymers as the structural basis of the polymer microneedle system. Specifically, two polymers were used in this project, polyvinylpyrrolidone (PVP), and poly(vinyl pyrrolidone co-methacrylic acid) (PVP-MAA). The chemical structures of the monomers vinyl pyrrolidone (VP) and methacrylic acid (MAA) are shown below in Figure 2.4.

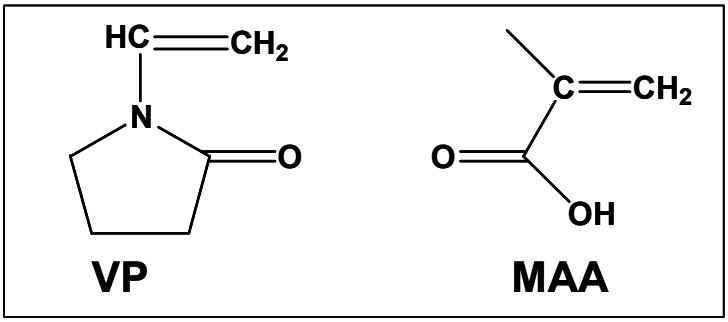

Figure 2.4: Chemical structures of monomers vinyl pyrrolidone (VP) and methacrylic acid (MAA) used for polymer microneedles.

Polyvinylpyrrolidone is a homopolymer formed by the monomer N-vinyl-2-pyrrolidone. It is a water soluble polymer and has been used in a number of industries, including pharmaceuticals, food, beverage, cosmetic, toiletry, and photography[70]. Its wide use can be attributed to a number of properties, including biological compatibility, low toxicity, film-forming and adhesion, complexing ability, inert behavior towards salts and acids and resistance to thermal degradation in solution[71]. In the pharmaceutical industry, the most common use of PVP is as a tablet binder for oral delivery. The addition of PVP has been found to enhance the solubility and biocompatibility of poorly-

water soluble drugs. The first medical use of PVP was as a plasma expander during World War II[72]. It was a popular alternative due to its nonantigenic properties, requiring no cross-matching, and avoiding the dangers of infectious diseases inherent to blood[71]. PVP has been shown to be effective in large volumes for the treatment of shock to correct for low blood volume. Other medical applications of PVP include the addition to many drugs to increase their pharmacological effect[73] and as a component of implanted medical devices[74] and transdermal wound healing products[75].

Recently, there has been some concern of the long term effects of the injection of PVP. Some patients who have been injected with large amounts (>200 g) of high molecular weight (>100,000 Da) PVP have incurred a storage disease, labeled PVP thesaurosis or PVP storage disease[76]. Specifically, PVP polymers that are less than 20,000 MW can be easily excreted by the kidneys. However, PVP polymers with larger molecular weights can be partially retained in the body, phagocytosed and stored in the reticular endothelial system[77]. The polymer is not metabolized in the body and is considered biologically inert. While there has been no evidence to specific clinical symptoms related to the storage of this polymer, the evidence of the retention of the polymer has led to prohibition of use of the higher molecular weight PVP for systemic administration[78]. In the use of lower molecular weight PVP (MW<20,000), less than 1% is left at the injection site 24 hours after subcutaneous injection of a large amount of PVP ([71]. The majority of the PVP polymer (>90%) is excreted in the urine and feces within 48 hours[71].

One of the enticing properties of using PVP as the structural material for polymer microneedles is the ability for rapid dissolution within the skin due to the high water

solubility. However, it may be desirable to use a polymer with a slower dissolution rate. One possible choice is to make copolymers of polyvinylpyrrolidone with other polymers, including polymethacrylic acid (PMAA). Methacrylic acid is also a vinyl monomer and can be formed into the polymer PMAA by free radical polymerization. The liquid MAA is quite miscible with VP, which allows for a homogenous solution. PMAA has been used in the past for drug delivery purposes and has a high mechanical strength due to the rigidity of its chemical backbone [79]. In addition, a copolymer of P(VP-MAA) could have additional mechanical strength due to hydrogen bonding between the side chains of the VP and MAA monomeric units of the polymer [80]. The copolymer of polyvinylpyrrolidone-co-methacrylic acid P(VP-MAA) has been used in the past for ophthalmic inserts[80].

There are many properties of polyvinylpyrrolidone that are enticing as the structural material for polymer microneedles. This includes the ability to form the polymer from monomer at room temperature, high water solubility, being biologically inert and biocompatible at low molecular weights. It is important to restrict the molecular weight of this polymer to below 20,000 Da for any clinical use. The copolymer PVP-MAA could also be used as the structural basis of microneedles, as it is also biologically inert. However, further research is required to verify the biocompatibility of this copolymer before use clinically.

2.4 BIOPHARMACEUTICALS

2.4.1 Molecules

Biopharmaceuticals have been defined as pharmaceuticals that are inherently biological in nature and manufactured using biotechnology[81]. This includes a large

group of molecules, including DNA, proteins, peptides and vaccines, which are increasingly becoming more popular in the overall pharmaceutical market. In fact, the global biopharmaceutical market exceeded 80 billion dollars in 2007 and is growing at an annual rate of 17%, a higher growth rate than the overall pharmaceutical industry[6].

2.4.2 Delivery methods for biopharmaceuticals

As mentioned previously, oral delivery of biopharmaceuticals is difficult due to poor absorption and degradation in the GI tract and the first pass metabolism effect of the liver[8]. For biomolecules to be delivered orally in pill form, significant reformulation would be required. Unfortunately, the formulations that are traditionally used for small molecule oral drugs do not protect biomolecules for oral delivery. Instead, the biomolecules incur poor permeability, lumen and cellular enzymatic degradation, rapid clearance and poor stability and conformation[7]. Currently, the two main advances in improving the oral delivery of biomolecules are the addition of absorption enhancers and enzyme inhibitors to the formulation[82]. While there have been some success in this work, it appears that the formulation will be highly compound specific, especially with the oral delivery of peptides and proteins[83].

Alternate delivery routes for the delivery of biomolecules include nasal, buccal, pulmonary and transdermal. All of these routes avoid the GI tract and first pass effect of the liver and thus the associated absorption and degradation issues. Nasal delivery offers a highly permeable and vascularized region for delivery. While many biomolecules have been delivered via this route, there are some limitations, including limited volume of delivery, molecular weight limitations, and possible irritation of nasal mucosa[84]. Buccal delivery has similar promise to nasal delivery with a highly vascularized region of

the mouth and quick onset of delivery. The main issues with this delivery is absorption and retention time, as keeping a delivery system in the mouth for an extended period of time can be cumbersome to the patient. Another option is pulmonary delivery which offers a route of delivery to the lungs with a extremely large and absorptive surface area of 35 – 140 m^2[85], a thin diffusion path and high blood flow[86]. For delivery by inhalation, the drug must be formulated into aerosol form and must be in a specific size range. A number of biomolecules have been delivered via this method, including insulin, erythropoietin, and growth hormone. However, there are some limitations to this route of delivery, including rapid clearance of drug, protein instability, and the difficulty of precise dosing[86]. Finally, transdermal delivery also offers a promising route of delivery. The major options for transdermal delivery that were described previously are all relevant for the delivery of biomolecules. One important issue for all of these delivery systems is formulation and retention of activity of the biomolecule in the delivery system and during storage and delivery. All of the microneedles systems, including coated, hollow and dissolving polymer, have gentle reformulation methods and could be used for the delivery of biomolecules.

2.4.3 Transcutaneous Immunization

Recent work has shown that the skin, especially the epidermis, provides a rich environment for vaccine delivery [11, 87-89]. In fact, studies have shown that vaccine delivery to the skin may require a smaller antigen payload than the dose required for an intramuscular vaccine injection[12]. This may be due to the large number of immune cells that move in and out of the epidermis, allowing for an increase in antigen presentation to the immune system. The primary immune cells in the epidermis are the

Langerhans cells, which are dendritic cells that move between the bone marrow and the skin and are involved in antigen presentation and immune surveillance[90]. In fact, these Langerhans cells cover 20% of the skin's surface area along its basal surface[91]. On the other hand, intramuscular injections deliver the antigens into a region with few to no immunogenic cells. The process of inducing immunization through the skin begins with the introduction of a foreign antigen into the epidermis. Here, the large amount of Langerhans cells will interact and phagocytose the antigen. The Langerhans cells then migrate to the draining lymph node, where they interact with T-cells and the immune response is inducted[87]. Transcutaneous immunization has been shown to be capable of inducing strong mucosal (IgG) and secretory (IgA) antibody responses, and has offered protective immunity against a lethal mucosal challenge [88, 89]. This is important because many viruses, including influenza, enter through the mucosal surfaces.

Even though the skin appears to be a promising target for vaccines, there is still a problem concerning delivery to this region. As mentioned previously, vaccines are large molecules and thus unable to passively transverse the stratum corneum and enter the epidermis[38]. Using a needle for injection is difficult due to the small thickness of the epidermis and dermis, approximately 1-2 mm. Gas powered injections has been used for vaccination, but these devices are painful and can be difficult to control the location of delivery, which is quite important in this form of delivery [92, 93]. Microneedles offer an exciting opportunity that allows for delivery of the vaccine specifically to the epidermis and the upper layers of the dermis while also being more patient compliant than the current forms of injection.

2.5 LYOPHILIZATION OF BIOPHARMACEUTICALS

Lyophilization, or freeze drying, has become a widely used process in the biopharmaceutical industry. The main use of this process is to increase the stability and long-term lifespan of biologically related molecules, especially proteins by converting a liquid solution into a solid powder[94, 95]. As the number of biomolecules used has risen over the years, there has been a greater need to increase the stability of these molecules. Biomolecules and specifically proteins suffer from limited stability in solution due to physical and chemical degradation. In fact, the typical shelf life of a protein drug is just 18 – 24 months[96]. Since most of these molecules are currently delivered via injection, the biomolecules are stored for some period of time in liquid form, which often requires some form of the cold chain for retention of activity. Storing the molecules at low temperatures can be expensive and cumbersome, especially for use in areas that lack adequate refrigeration. Also, storage of biomolecules in liquid form can increase the extent and speed of degradation, due to a number of chemical and physical processes, including deamination, oxidation, aggregation, unfolding, and adsorption to surfaces[97-99]. It has been shown that storing biomolecules in solid form allows for better stability and easier handling during shipping and storage. Lyophilization is one of the most widely used methods of converting liquid solutions of biomolecules into solid form.

2.5.1 Lyophilization processes

The goal of the lyophilization process is to remove all solvent from a liquid drug solution, resulting in a solid powder of drug and possibly an excipient. The lyophilization process is made of three main steps: freezing, primary drying and

secondary drying. In addition, it is important what type of solvent the biomolecule is found in and what the storage conditions are after the lyophilization is complete. Usually, the lyophilization conditions need to be optimized for each of the biomolecules used. This includes the solvent used; the length and time of freezing; the temperature, pressure and time of the drying steps; and the temperature and relative humidity for the storage conditions. All of the steps are important to verify that the biomolecule retains activity during and after this process.

The freezing stage is the first step of the lyophilization process. Here, most of the water is separated from the drug, and the system is separated in multiple phases, with an ice phase and a drug phase[100]. It is important to determine the proper cooling rate, including the freezing temperature and the length of cooling time. For many biomolecules, the cooling rate should be fast, which can be accomplished using liquid nitrogen. Slow freezing can increase the amount of protein degradation due to an increase in the amount of time of phase separation and protein unfolding. For the influenza vaccine, a fast cooling rate has been shown to be optimal to reduce the level of degradation [101]. The freezing process imparts a number of destabilizing stresses on protein drugs. The two main stresses are pH shifts and the introduction of the ice-aqueous interface. To reduce the effects of the pH effect, the amount of salts should be minimized and chosen carefully or water should be used as the primary solvent[102]. As for the issue of the presence of an interface, the protein concentration can be increased or surfactants could be used to eliminate this problem [103].

The next step of the lyophilization process is primary drying, which is the longest step and is where the unbound water is removed by sublimation. Prior to the start of

primary drying, the system must be completely frozen. Here it is important to control the shelf temperature, condenser temperature and chamber pressure. The chamber pressure is reduced and the shelf temperature is raised to supply the heat removed by ice sublimation[100]. During this process, the chamber pressure needs to be well below the vapor pressure of ice and the ice is transferred by sublimation from the product to the condenser. This process needs to be optimized to remove the largest amount of unbound water, and also in a slow enough manner not to damage the physical structure of the system. If the process of sublimation occurs too rapidly, the different phases of the solid could collapse, damaging the frozen protein concentrate. At the end of the primary drying process, there is typically only 5% of the moisture present in the system[104].

The final step of the lyophilization process is secondary drying, where the bound water is removed from the system. This water needs to be removed since even with as low as 5% water in the system, chemical and physical degradation processes can still occur. Here, the water is removed by desorption and requires more energy than sublimation. The shelf temperature of the system is slowly raised to reduce the chance of collapse of the product. It is better to set the temperature used for secondary drying high for a short amount of time, instead of lower for a longer amount of time in order to maximize the amount of water removed during this step[105]. The desired final moisture amount in the system is less than 1%[100]. After the secondary process is finished, it is important to store the lyophilized powder at the correct temperature and relative humidity. While the product should be more stable in solid form than liquid form, there could be some degradation and absorption of water, so the optimal conditions need to be determined.

2.5.2 Addition of excipients for cryoprotection and lyoprotection

The lyophilization process involves a number of stresses imparted on the biomolecule drug, and it may be necessary to add excipients to the original solution to minimize the degradation. The presence of the excipient is important during all three steps of the lyophilization process. Usually, in order for excipients to protect the biomolecule during lyophilization, the ratio of excipient/protein is quite large, around 50-100:1. However, this amount needs to be optimized for each biomolecule. If there is too little excipient, the protein could be damaged during the freeze drying process, and if there is too much of the excipient the physical stability of the solid protein could be decreased leading to physical collapse during storage[106]. The main excipients that are used during the freeze drying process are sugars, including trehalose, inulin, sucrose, glucose and maltose among many others[107].

During lyophilization, protection against degradation is required for the protein solution during the freezing process (cryoprotection) and the drying process (lyoprotection). During the freezing step, it is accepted that sugars stabilize proteins through the preferential exclusion mechanism, by which the excipient solute is excluded from the protein and helps keep it in the native folded state [108] During the drying process, water is being removed from the system, which can lead to degradation of the protein. This could include physical unfolding and denaturation or chemical degradation. There are two separate hypotheses in the literature that account for the protection offered by excipients during the drying steps. The first theory is kinetic in nature and is labeled the ‘glass dynamics hypothesis’[109, 110]. Here the large amount of frozen sugar forms a rigid, inert matrix in which the protein is widely dispersed. The sugar is in such high

amounts (50-100:1) that the protein molecules have little to no contact with one another. The limited mobility of the protein minimizes unfolding or chemical degradations, which typically require some mobility to occur. Thus, the degradation steps are slowed to a point where little to no effect occurs. The second hypothesis is thermodynamic in nature and is labeled the 'water replacement hypothesis'[111, 112]. Here, the hypothesis poses that the removal of the water causes thermodynamic instability of the protein, leading to unfolding. The presence of a large amount of sugar replaces the water and forms hydrogen bonds with the protein, making the native state thermodynamically more stable and the unfolded state.

Lyophilization is an important process that is vital to the long-term storage and use of biopharmaceuticals. It is important to determine the specific conditions needed to preserve activity of the molecule of interest. This may include the inclusion of excipients in the drug formulation to prevent degradation during the freezing and drying steps. In terms of polymer microneedles, lyophilization may be used to convert a drug solution into a solid powder, which can then be easily encapsulated within the polymer microneedle system. Here, the drug will be stored in solid state, which may lead to better stability and not require the use of the cold chain for storage.

2.6 INFLUENZA VACCINE

2.6.1 Influenza virus

Influenza infections are responsible for a large number of deaths and illnesses each year[113]. During a normal year, 36,000 people die from influenza in the United States. This number nearly triples if deaths caused by influenza-related respiratory

diseases including pneumonia are included. In addition, 5-20% of the population is infected with the flu each year, with over 200,000 Americans hospitalized[114]. During a pandemic threat, the number of deaths has risen to 500,000 Americans and over 20 million worldwide[113]. Due to the broad reach of this virus, 113 million doses of the influenza vaccine were distributed during the 2007-2008 season and a record 145 million doses of the influenza vaccine were produced for the 2008-2009 flu season[115].

The influenza virus falls under the family of orthomyxoviridae viruses[116], and can be further subdivided into three groups, influenza virus A, influenza virus B and influenza virus C. The differences between these groups is based on antigenic differences between the two major structural proteins: the matrix protein (M) and the nucleoprotein (NP)[117]. The influenza A virus is the most common form that infects humans[118]. This virus is divided into subtypes based on two of the viral surface proteins, hemagglutinin (H) and neuraminidase (N). There are a large number of subtypes of the influenza A virus, with sixteen H subtypes and nine N subtypes. The influenza virus is identified by the following information, type of isolate, the geographic location where the isolate was found, the year of isolation, a laboratory identification number and a subtype of the H and N surface proteins. One example is H3N2/A/Panama/2007/99.

The influenza viruses are enveloped and contain segmented RNA-negative sense genomes[117]. The structure of the virus is shown in Figure 2.5 below. The virus structure resembles spherical 80-120 nm particles with a surface layer of spike-like projections (10-14 nm) of the H and N surface proteins. The H protein is the main antigen that the body's antibody immune response is directed. This protein is responsible

for the attachment of the virus to the host cell surface during the first parts of infection[117]. Antibodies against the specific H protein are believed to prevent the onset of influenza infection. The N protein is responsible for the release of the mature virus from the infected host cell. Antibodies against the N protein are thought to limit the spread and severity of the influenza infection. The basis for the current vaccines is immunoprotection specific to these two surface proteins.

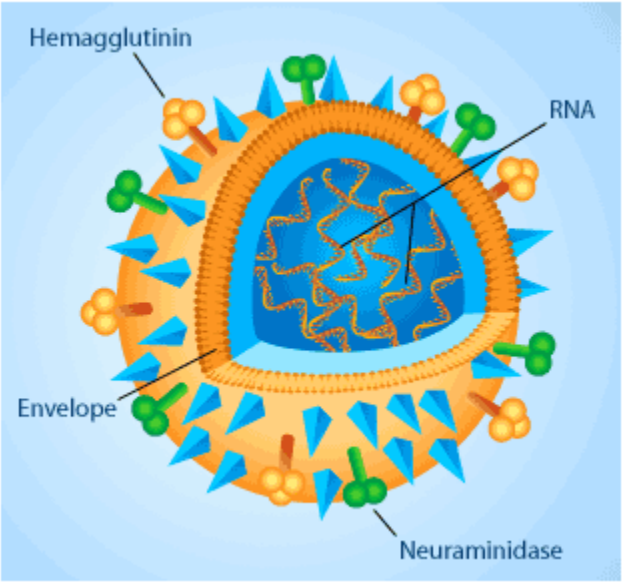

Figure 2.5: Influenza virus structure.
http://www.abc.net.au/health/library/img/flu_virus_diag.gif

Currently, it is suggested that the population should be immunized each year against the most current common form of the influenza virus. This is due to the antigenic drift of the virus[119]. The different subtypes of the H and N proteins are serologically different, and while some cross reactivity is observed between the subtypes, the extent of cross-protection is not fully understood[117]. Further verification into the extent of cross protection could lead to a decrease in the need for a yearly vaccination.

The two most common subtypes of the influenza A virus are H1N1 and H3N2, and these are included in the yearly seasonal vaccine. Recently, a large amount of discussion has centered on the H5N1 influenza virus, which is also known as the 'bird flu'. This virus occurs primarily in birds, is highly contagious and can be deadly. Recently, this virus was shown to be able to be transmitted from birds to humans and can lead to severe illness, including death [120]. It is believed that most if not all of the human cases of the H5N1 virus have been caused by direct contact with an infected poultry. Currently, it is believed that this virus strain is not capable of being transmitted by human to human contact. If this does occur in the future, there is a worry of a large pandemic since little to no protection currently exists against the H5N1 virus amongst humans. Because of this, there is a large amount of work currently being done to produce a vaccine against this virus and for medications to reduce the severity of the infection.

2.6.2 Types of influenza vaccines

Currently, the two types of vaccines available are the inactivated influenza virus and the live attenuated virus. Each of these vaccines includes three virus subtypes, one H1N1/A, one H3N2/A, and one B virus. The total dose of the vaccine is 45 μg, which is made up of 15 μg of each of the three subtypes

The first step of making the influenza vaccine is to determine the most prevalent strain of the virus. This is carried out by the World Health Organization using an international surveillance to find the most prevalent form of H1N1/A, H3N2/A and B subtypes[121]. The seed strains are then prepared by genetic reassortment using the different strains determined by the WHO, and tested for the similarities to the wild type virus[122]. Once this is verified, the bulk production of the virus is performed in

embryonated eggs and the live virus is harvested. For the inactivated vaccine, the virus is inactivated using formalin or β-propriolactone and purified using ultra-centrifugation. The virus is then split, reformulated and tested for antigenicity and immunogenicity[123]. The virus is stored at 4 – 8 ^{o}C and may include a preservative to protect the antigenicity. Anyone over the age of six months can be immunized with the inactivated vaccine.

The other vaccine available is the live attenuated virus. After determining the specific strain, three strains are developed: host-range (hr) mutant virus, temperature sensitive (ts) and cold-adapted mutant strains[117]. The virus is propagated in specific pathogen-free (SPF) hen's eggs. After the virus is harvested from the allantoic fluid, it is centrifuged and stabilized with the addition of sucrose-phosphate-glutamate (SPG). The virus is loaded into an aerosol sprayer and 0.2 mL is delivered to the patient nasally.
The live attenuated influenza vaccine can be used by healthy patients between the ages of 2 and 49 years old[117].

2.6.3 Delivery methods for the influenza vaccine

According to the Centers for Disease Control and Prevention (CDC), currently there are two acceptable methods of delivery for the seasonal influenza vaccine[124]. A hypodermic needle injection is the most commonly used method and it is used for the delivery of the inactivated vaccine. The other method of delivery is the nasal spray by Flumist®, which is used for the delivery of the live attenuated influenza vaccine.

Research into alternate delivery methods for the influenza vaccine is currently underway, with some methods being tested in clinical trials. In theory, all of the delivery technologies described previously for use with biopharmaceuticals could also be used to deliver the influenza virus. Many of these methods deliver the vaccine to the skin to take

advantage of the robust number of immune cells present there. These new methods are desired for skin immunization since it is difficult to use a common hypodermic needle due to the thickness of the skin (1-2 mm). One system that has worked is an intradermal injection using a 1.5 mm microinjection system, which has been shown to induce a comparable immune response for the influenza vaccine to the IM injections with a lower antigen dose in humans [125]. Other transdermal delivery systems have been successfully proven in animals for influenza vaccination, including thermal ablation[126], tape stripping[127], and hollow microneedles[128] (1 mm). While more work needs to be done on these methods, the skin is a promising target for immunizations with the influenza vaccine.

Research has also been done using oral vaccines for the delivery of the influenza vaccine. Oral influenza vaccines have been shown to induce significant mucosal IgA antibody levels clinically but have had very low sera IgG levels [129]. It is unclear if this strategy alone would provide adequate protection for humans. One issue with oral vaccination is the delivery site, and the influence of absorption and enzymatic degradation. Recent work in mice showed that targeting vaccine delivery to the lower GI-tract (colon-rectum) could allow for a more effective oral vaccination strategy against influenza [130]. While oral influenza vaccination would have a high patient compliance, more work needs to be done to enable protective immunization in this manner.

As an alternative, dissolving polymer microneedles offer a potentially patient compliant and effective manner of delivering the influenza vaccine to the skin. As with the delivery of other biomolecules, some concerns that must be answered are the delivery

efficiency and the activity of the vaccine after any reformulation or processing steps required in microneedle based delivery.

CHAPTER 3

MATERIALS AND METHODS

3.1 POLYMER MICRONEEDLE FABRICATION AND DELIVERY ANALYSIS

3.1.1 Free radical polymerization process

A previous method for creating polymer microneedles was used as the framework for the new fabrication process[53]. This method involved the use of a microfabricated microneedle array as the master structure template for the process. The master structure was created via a lens-based fabrication process of SU8 photoresist. Next, a microneedle mold was produced by pouring polydimethylsiloxane (PDMS, Dow Corning, Midland, MI) over the master structure and allowing the mixture to cure overnight at 37 ^{o}C. Then, the cured PDMS mold was peeled off the microneedle master structure. This microneedle mold provides the template for the production of the subsequent polymer microneedles. In the previous process, polymer microneedles were created by melting polymer pellets onto the microneedle mold and applying a vacuum to allow the polymer melt to enter the microneedle cavities of the mold. Finally, the polymer microneedle system was removed, allowed to cool to room temperature and peeled out of the mold.

The goal of this project was to create polymer microneedles using a room temperature fabrication process. While the previous process created robust microneedles that inserted properly into the skin, the fabrication involved elevated temperatures (>180 ^{o}C) to melt the polymer pellets. These temperatures could lead to the degradation of many encapsulated biomolecules. The new fabrication process developed in this project involved the free radical polymerization of a liquid monomer solution that was injected

inside the microneedle mold. Most of the work done in this project used the liquid monomer, N-vinyl pyrrolidone (VP, 99%, Sigma-Aldrich, St Louis, MO). Here, 1.0 mole % of the free radical initiator azobisisobutyronitrile (AIBN, 99%, Sigma-Aldrich, St Louis, MO) was added to 1 mL of vinyl pyrollidone and approximately 75 μL of the solution was added to the surface of the microneedle mold, enough volume to cover the surface area of the base of the microneedles. Next, the system was placed inside a vacuum oven (VWR, Cornelius OR) at -30 in Hg for 1 minute to allow the monomer to fill the mold and remove any air bubbles. The microneedle cavities of the mold were considered to be completely filled when no bubbles remain on the liquid surface.

Polymerization of the monomer solution was induced via UV light. After the vacuum is applied to fill the microneedle mold with monomer, the microneedle/mold system was placed approximately six inches under a UV lamp (100 W, 300 nm, BLAK RAY, Upland CA). The system was left for approximately 30 minutes where the free radical polymerization occurred and resulted in sharp microneedles that retain the structure of the original microneedle master structure. A detailed diagram of this fabrication process can be found in Figure 4.1 in Chapter 4 of this thesis. The polyvinyl pyrrolidone (PVP) microneedles were then stored in a desiccator prior to use to prevent absorption of water. It is important to note that polyvinylpyrrolidone is hygroscopic, and can absorb water if left in a high humidity environment. When PVP microneedles were left out in a humid environment, the polymer absorbed water, becoming more ductile and resulted in more difficult insertions into skin.

3.1.2 Fabrication process for polymer microneedles

3.1.2.1 Distillation of liquid monomer prior to free radical polymerization

In order to use a liquid monomer to create polymer microneedles via free radical polymerization, the monomer must first be distilled to remove the inhibitor that prevents polymerization during storage. There were two monomers that were used during this project, N-vinylpyrrolidone(VP) and methacrylic acid (MAA, 99% Sigma-Aldrich, St Louis, MO). The boiling point of the inhibitor (260^{o}C for 0.01% sodium hydroxide) was much higher than the boiling points of the monomers (130 ^{o}C for VP and 140 ^{o}C for MAA) allowing for a relatively easy distillation. Briefly, 500 mL of the monomer was placed in a round bottom flask with a stir bar and attached to a distillation column (Distilling Head 14/20, 24/40, CHEMGLASS, Vineland, NJ) with an empty round bottom flask at the opposite end to collect the light component. The system was placed under a light vacuum (15 in Hg) and cold water was circulated at the top of the column to cool the monomer vapor. The flask containing the original monomer solution was placed in oil, which was slowly heated to above the boiling point of the pure monomer (130 ^{o}C for VP and 140 ^{o}C for MAA). The liquid that was removed included monomer with little to no inhibitor present. Finally, the monomer was stored in an amber bottle in a closed cabinet away from light or heat that could induce polymerization.

3.1.2.2 Polymer microneedles with encapsulated drug

The fabrication process for polymer microneedles described above detailed the creation of microneedles entirely of a polymer, with no drug encapsulated. To encapsulate a drug in the microneedles, the drug was dissolved or suspended in the liquid monomer solution prior to the formation of the microneedles. Because of this, the drug

must be in solid form to be encapsulated using this process. If the drug was found in liquid solution, subsequently it was lyophilized (freeze-dried) to convert the drug solution into solid form. The drug was then dissolved or suspended in the liquid monomer. If the lyophilized powder settled, either more monomer was added or the solution is pipetted up and down to better mix the solution. Next, the vacuum was applied and the system was placed under the UV light, where polymerization occurs. The resultant array included polymer microneedles with an encapsulated drug within the needles.

3.1.2.3 Polymer microneedles with drug encapsulated only within the microneedles

The fabrication process detailed above resulted in the drug encapsulated within the microneedle array and the microneedle base. However, the drug that was encapsulated within the base was unlikely to be delivered to the skin. Thus, an adaption was made to the process to preferentially encapsulate the drug within the microneedles and not the base. The first steps of the process remained the same, including the use of PDMS molds from an initial SU8 master structure. The drug was dissolved or suspended in a liquid monomer solution of vinyl pyrrolidone and approximately 75 μL was added to the surface of the microneedle mold. The vacuum was applied and the monomer/drug entered the microneedle mold. The next step involves the removal of the monomer/drug on the base using a pipette. The removed solution was stored for later use. After all of the monomer/drug solution was removed from the base, 100 μL of vinyl pyrrolidone with no drug dissolved was added to the surface of the microneedle mold. This system was then placed under the UV lamp for 30 minutes, where polymerization occured. The resultant polymer microneedles are shown in Figure 4.2 in Chapter 4 of this thesis.

In order to minimize the amount of biomolecule that was used in the encapsulation process, it was important to pipette all of the excess monomer off of the surface of the microneedle mold. This included pipetting the monomer that may seep into the sides of the mold. Also, the amount of monomer that was added to the surface was minimized to only cover the microneedle array surface. The amount of monomer used was as low as 35 μL. Also, during the fabrication of the polymer microneedle arrays, it was important to keep the microneedle mold flat and not angled. When the surface was angled, the monomer moved off of the surface of the mold, and it required more monomer (>100 μL) to cover the entire surface of the microneedle mold, which resulted in a higher percentage of wasted solution.

3.1.2.4 Polymer microneedles made of copolymer PVP-MAA

Most of the work done in this project involved the use of a single monomer, vinyl pyrrolidone, which was polymerized to form polyvinylpyrrolidone. However, there were times when one polymer did not have the desired properties for delivery, and a copolymer needed to be used as the structural basis of the polymer microneedles. Specifically, some of the work in this project used the copolymer PVP-MAA (polyvinylpyrrolidone-co-methacrylic acid). The fabrication process for this system was quite similar to the previous processes described above. The liquid monomers vinyl pyrrolidone and methacrylic acid were mixed in a set ratio. These monomers are quite miscible, which was important so that the polymer's structure was consistent throughout. The co-monomer solution was then added to the microneedle mold in the same manner as described above and placed under the UV light under the same conditions described above where polymerization occurs.

3.1.3 Verification of in vitro insertion of polymer microneedles

3.1.3.1 Skin dot insertion test

Due to the microscopic size of microneedles, it was difficult to visually determine if the needles properly insert into the skin. Previous work in this lab resulted in a protocol to determine the number of microneedles in an array that properly inserted into the skin[53]. This protocol involved the use of a tissue marking dye, trypan blue (Sigma-Aldrich, St Louis, MO), which preferentially stained the holes created by a microneedle array and not the skin surface. In this experiment, polymer microneedles were created out of PVP by the free radical polymerization process detailed previously. In vitro pig skin was used to test the microneedle insertion. The pig skin was thawed to room temperature, and shaved with a metal razor to remove all of the hair. Prior to insertion, the PVP polymer microneedles were taped to a metal applicator, which was made of a flat surface with an attached cylindrical pole. This applicator allowed for easier handling for delivery than simply using your thumb. The needles were inserted into the skin and removed immediately. The insertion was accomplished by grasping the cylindrical pole of the applicator and inserting the needles into the skin in a quick motion. 100 μL of a 50% trypan blue solution was then applied to the skin, covering the entire surface of the holes left by the microneedle array, and left for 5 minutes. The skin was then washed thoroughly using DI water and a towel to remove all excess dye from the surface. Finally, the skin was analyzed under a light stereomicroscope (Olympus SZX9, Japan). Each dot represented a hole created by microneedle insertion. Figure 4.3A in Chapter 4 of this thesis shows an image of a successful microneedle insertion.

3.1.3.2 Histological analysis to determine depth of microneedle insertion

The skin dot test described above allowed for an evaluation of the number of microneedles that inserted into the skin. However, it did not allow for a determination of the depth of delivery. A different protocol involving histology of skin was used to determine the depth of insertion of polymer microneedles. Here, polymer microneedles were made of polyvinylpyrrolidone via the new UV initiated polymerization method and pig skin was used for the insertion test. Sulforhodamine (0.1%, Invitrogen, Eugene, OR), a fluorescent molecule, was encapsulated within the microneedles to better visualize the microneedles within the skin. The PVP microneedles were then inserted into pig skin. The skin, with the microneedle array inserted within, was quickly placed in a cryomold, with the stratum corneum facing up, and a gel of optimal cutting temperature compound (OCT, Tissue-Tek: Sakura Finetek USA, Inc., Torrance, CA) was added to the cryomold, completely covering the skin. Next, the cryomold was flash frozen in liquid nitrogen, in preparation for histological sectioning.

The frozen skin sample was placed securely in an adaptor in the microcryostat (MICROM HM560, Waldorf Germany). The adaptor was needed to hold the frozen sample in place during the cutting process. Next, 10 μm histological sections were cut and fixed to glass slides. The sections were examined using a fluorescent microscope (Nikon E600W, Japan) to find the section with a microneedle embedded in the skin, and the depth of insertion was determined. In addition, this section was stained to better visualize the different layers of the skin. Here, the chosen histological sections were fixed using cold acetone for 15 minutes. Next, the fixed section was placed in a staining machine (LEICA AUTOSTAINER XL, Germany) and stained with Hematoxilyn and

Eosin (H+E). A cover slip was added to the stained section and left overnight. The sections were viewed using a light stereomicroscope to show the depth of insertion. Figures 4.4A and 4.4B in Chapter 4 of this thesis show the depth of the microneedle insertion, including a microneedle embedded in the skin.

3.1.4 Verification of in vitro drug delivery via polymer microneedles

To test the ability of this polymer microneedle system to deliver drugs to the skin, a fluorescent molecule was encapsulated within the microneedles. These molecules were easily visualized in frozen histological sections under a fluorescent microscope and thus allowed for an evaluation of the delivery profile in the skin.

3.1.4.1 Delivery of sulforhodamine via polymer microneedles

The first fluorescent molecule used was sulforhodamine (MW=558.66, Invitrogen, Eugene, OR). A liquid monomer solution was made of VP, AIBN (1.0 mol%) and sulforhodamine (10^{-6} M). 100 μL of this solution was added to a microneedle mold and a vacuum (2 in Hg) was applied to pull the solution into the mold. Next, the solution that remained on the surface of the mold was carefully pipetted off and returned to the bulk solution for future use. Then, a solution of vinyl pyrrolidone with no sulforhodamine was added to the mold to act as a base for the microneedle array. The system was placed under a UV lamp, where polymerization occurs.

The delivery of sulforhodamine was tested in pig skin in vitro. The skin was prepared as described previously. The polymer microneedles were applied to a metal applicator, as described previously. The needles were inserted into the skin with force being applied for 15 seconds, and the needles were left in the skin. Then, the needles

were removed from the skin after a set amount of time, ranging from 30 seconds to 15 minutes, and inspected under the microscope. The skin, containing the insertion site, was observed under a fluorescent microscope to visualize the delivery. Next, the section of skin was cut and placed in a cryomold and processed for histology as previously described. 10 μm sections were made of this skin sample and the sections were visualized using a fluorescent microscope to determine the holes created by microneedle insertion and the fluorescent delivery that emanated from these holes. The skin sections that had the most promising results were then stained with Hemotoxilyn + Eosin to better visualize the microneedle insertion site. Figures 7.2D and 7.2E in Chapter 7 of this thesis show images of skin sections after delivery of sulforhodamine.

3.1.4.2 Delivery of fluorescently labeled proteins via polymer microneedles

To further show the capabilities of PVP polymer microneedle delivery, a fluorescently tagged protein, Texas-red albumin (Invitrogen, Eugene, OR) was delivered to in vitro pig skin. This delivery was quite similar to the work described above involving sulforhodamine, with Texas-red albumin (10^{-6} M) suspended in the vinyl pyrrolidone monomer. Polymer microneedles were created with Texas-red albumin encapsulated within the polymer microneedles and a base of polyvinylpyrrolidone polymer. The microneedles were inserted into pig skin and left for 1 minute. The microneedles were subsequently inspected under a fluorescent microscope and the skin section was flash frozen and processed for histological examination. The 10 μm cryosections were analyzed using a fluorescent microscope to visualize microneedle based protein delivery. This can be seen in Figure 4.4C in Chapter 4 of this thesis.

3.1.5 Mechanical and physical properties of polymer microneedles

3.1.5.1 Analysis of the mechanical strength (fracture force) of polymer microneedles

The mechanical strength of polymer microneedles was tested using a fracture force test. Previous work had been done to characterize the insertion force required for proper microneedle insertion [40, 53]. In order for insertion to take place, the fracture force of the microneedle needs to be greater than the insertion force. In addition, the influence of the presence of methacrylic acid (MAA) on mechanical strength of the copolymer PVP-MAA was investigated.

In this experiment, the failure force under axial load was measured using a displacement force test station (Model 921A, Tricor Systems, Elgin, IL) following established protocols [40, 53]. Polymer microneedles were created using the following molar monomeric ratios (VP/MAA): 100/0, 99/1, 90/10, 75/25, 50/50, 25/75. The polymer microneedles were taped to the flat bottom of a metal applicator, which has a cylindrical post that fits securely in the force displacement machine. Force versus displacement curves were generated by pressing the array of microneedles (20-25 needles per array) against a hard metal surface at a rate of 0.5 mm/sec. The average microneedle fracture force was calculated by dividing the maximum fracture force by the number of needles. A plot of the fracture force versus monomeric content can be seen in Figure 4.3B in Chapter 4 of this thesis.

3.1.5.2 Qualitative analysis of the dissolution kinetics of polymer microneedles

The dissolution kinetics of PVP and PVP-MAA microneedles was measured in vitro in pig skin and in vivo in mice. Polymers of the following different content of PVP/MAA: 100/0, 99/1, 90/10, 75/25, 50/50, were used. The needles were created using

the previously described fabrication process, and attached to a metal applicator. The needles were applied to the skin for 15 seconds. Pressure was removed and the microneedles were left in the skin for a set period of time, ranging from 30 seconds to 5 hours. The microneedles were then inspected to determine the amount of the polymer microneedle that was dissolved in the skin. This was approximated by a calculation of the volume of the microneedle array that was left after insertion.

3.1.5.3 Determination of the molecular weight of the PVP polymer microneedles

The molecular weight of the polyvinylpyrrolidone polymers was determined by aqueous size exclusion chromatography (ASEC) at 30°C. First, PVP microneedles were created using the process described previously. Then, the microneedles were dissolved in a 0.05 M Na_2SO_4 buffer, which was the mobile phase of the column. The sample concentration was 3 mg/mL PVP in the buffer solution. The ASEC system was comprised of a Shimadzu LC-20AD pump, a Shimadzu RID-10A RI detector, a Shimadzu SPD-20A UV detector, a Shimadzu CTO-20A column oven, and Viscotek TSK Viscogel PWXL Guard, G3000, G4000 and G6000 columns mounted in series. The mobile phase consisted of the Na_2SO_4 buffer and the flow rate was maintained at 0.5 mL/min. Poly(ethylene glycol) narrow standards (Sigma-Aldrich, St Louis, MO) were used to calibrate the ASEC by universal calibration method. The weight average molecular weight (M_W), number average molecular weight (M_N) and polydispersity index (PDI) were determined based on the PEG control standards.

3.1.6 Analysis of retention of enzymatic activity after encapsulation in polymer microneedles

3.1.6.1 Protein encapsulation within polymer microneedles:

Prior to determining the enzymatic activity of β-galactosidase within the polymer microneedles, it was important to determine the amount of the protein in the microneedles. Due to the small amount of volume and protein used in the polymer microneedles, it was difficult to accurately weigh the mass of protein using a balance prior to suspending it in the liquid monomer solution. One method of determining the amount of protein was to measure the absorbance of a protein solution at 280 nm. The relationship of absorbance ($A_{280\ nm}$) to protein concentration is linear[131]. In this experiment, a standard curve was made using bovine serum albumin as the reference protein. Approximately 200 μg of β-galactosidase (Grade VIII, 600-1200 units/mg, Sigma-Aldrich, St Louis, MO) was added to 300 μL of vinyl pyrrolidone monomer. The solution was vacuum pulled into the microneedle mold and the backing was replaced with vinyl pyrrolidone monomer without enzyme. The microneedles were polymerized and then dissolved in 3 mL deionized water. The absorbance of this sample was tested at 280 nm. Unfortunately, it was not possible to determine the protein concentration using this assay because the polyvinylpyrrolidone polymer provided too large of a background signal due to amine groups in the chemical backbone of the polymer.

Another assay to determine the amount of protein was the fluorescamine assay. In this experiment, the non-fluorescent molecule fluorescamine binds to primary amines of proteins to form fluorescent compounds which can be measured [132]. However, as with the absorbance test, the polyvinylpyrrolidone polymer provides too large of a background and makes this result difficult to analyze.

All further determination of the amount of protein was done in one of two ways. First, if the protein was in solid form, a large amount, greater than 1 milligram, was weighed out using a sensitive balance (Mettler Toledo XP26 microbalance, Columbus, OH), that can accurately weigh to 0.1 mg. After forming polymer microneedles, the unused protein was stored in the liquid monomer solution for future use. For drugs that are found in liquid solutions and need to be lyophilized, the amount of protein was determined by first measuring the protein concentration in solution via standard methods, including the Bicinchoninic Acid (BCA) assay and the Bio-Rad protein assay, which is based on the Lowry protein assay[133]. These solutions had PBS or deionized water as solvents, which do not interfere with the more common protein determination methods. Then, the solution, of known concentration and volume, was lyophilized to remove all of the solvent, and the mass of the resultant powder could easily be calculated. This powder was then suspended in the liquid monomer solution and the dose of protein in the polymer microneedles was determined. This process was used for all of the influenza work involving PVP microneedles in this project.

3.1.6.2 Enzymatic activity of β-Galactosidase after encapsulation and release from PVP microneedles

The activity of β-galactosidase was measured after encapsulation within polymer microneedles to determine if the fabrication and dissolution processes damage the enzyme. A "dose" of 1.0 mg/100 μL of β-galactosidase was suspended in a liquid monomer solution of vinyl pyrrolidone. 100 μL was added to a microneedle mold, a vacuum was applied and the system was placed under a UV light where polymerization took place. The resultant PVP polymer microneedles were then dissolved in cold (4^{o} C)

PBS, and the activity of the released enzyme was measured, using the manufacturer's protocol [134]. This involves the enzymatic reaction of β-galactosidase with the substrate ONP β-D-galactopyranoside (Sigma-Aldrich, St Louis, MO), which produces nitrophenol, a yellow product whose absorbance can be measured at 410 nm. Positive controls were tested, containing β-galactosidase in cold PBS, and β-galactosidase in PBS containing previously dissolved, placebo PVP microneedles.

3.1.6.3 Enzymatic activity of β-Galactosidase in pig skin in vitro after encapsulation and delivery via polymer microneedles

Delivery of enzymatically active β-galactosidase was also studied in pig skin *in vitro*. In this case, 50 μg of β-galactosidase was encapsulated in an array of polymer PVP microneedles. Theses needles were inserted into pig skin and then removed after 18 h. The length of time was much longer than previous work, due to the particle nature of the β-galactosidase in the needles. While the β-galactosidase particles were delivered to the skin quickly, previous studies showed that the diffusion of the particles in the skin took a longer amount of time. This was allowed for a better visualization of the delivery profile. Next, the skin was flash frozen and 10 μm sections were taken using the microcryostat. The sections were visualized under a microscope to find the holes from microneedle insertion. Next, the selected sections were stained with X-Gal by the manufacturer's protocol. X-Gal binds to active β-galactosidase and produces a blue product that can be visualized using a stereomicroscope. The sections with microneedle insertion sites were fixed in cold formalin for 10 minutes at 4 ^{o}C washed 3 times with PBS for five minutes apiece and then incubated in an X-gal working solution overnight at 37 ^{o}C. The X-gal The X-gal working solution was made by diluting the X-gal stock solution in a 1:40 ratio

in warmed X-gal dilution buffer (37 ^{o}C). The X-gal stock solution was made by dissolving 20 mg of X-Gal (Sigma-Aldrich, St Louis, MO) in 0.5 mL of DMF (N, N Dimethylformamide, MP Biomedicals, Solon, OH**)**. The X-gal dilution buffer included 5mM crystalline potassium ferricyanide (Fisher Scientific, Pittsburgh, PA), 5 mM potassium ferricyanide trihydrate (Fisher Scientific, Pittsburgh, PA) and 2 mM magnesium chloride (Sigma-Aldrich, St Louis, MO) in PBS. The next day, the sections were rinsed twice in PBS for 5 minutes and then counterstained with nuclear fast red solution for 5 minutes. The nuclear fast red solution includes 0.5 g nuclear fast red (Sigma-Aldrich, St Louis, MO) and 25 g Al_2SO_4 (Sigma-Aldrich, St Louis, MO) in 500 mL of deionized water. Finally, the slides were rinsed in DI water and mounted directly with aqueous mounting medium. The sections were visualized using a stereomicroscope to determine if the delivered β-galactosidase was active and bound to X-gal, with was shown by a blue product. Successful delivery of active β-galactosidase via PVP microneedles can be seen in Figure 4.4D in Chapter 4 of this thesis.

3.1.7 Analysis of in vivo polymer microneedle delivery

3.1.7.1 Polymer microneedle delivery to hairless rats in vivo

PVP polymer microneedles were inserted into hairless rats in vivo to test the insertion capabilities. The microneedles were created, as described above, by polymerizing a liquid monomer solution of vinyl pyrrolidone. Sulforhodamine was encapsulated within the polymer microneedles to allow for an easier evaluation of the insertion. The hairless rat was purchased from Charles River and all work was done in accordance with the Georgia Tech Institutional Animal Care and Use Committee (IACUC). The rats were anesthetized in an isofluorane chamber and the anesthesia was

maintained using an isofluorane mask. The animal was placed on its belly and all insertions were performed on the back. The insertions were done using differing conditions. The length of time of application of the microneedles varied from 5, 15, and 60 seconds. The length of time the microneedles were inserted into the skin varied from 30 seconds to 15 minutes. In addition, various insertion techniques were used. This included mounting the microneedle array on multiple applicators to increase the force applied for insertion or using the index finger for insertion. Also, the skin of the rat was handled in different ways, including pinching the skin, spreading the skin on the ground or applying to the back without any handling.

After each insertion was finished, the microneedle array was removed and the insertion site was inspected and marked to allow for histology later. Also, the polymer microneedles are set aside for inspection under a microscope to determine if the needles failed (bent) or inserted and dissolved in the skin. After all of the insertions were finished, the skin of the rat that includes the insertion sites was removed and inspected under a fluorescent microscope. The rats were then euthanized under the IACUC protocols via a CO_2 chamber. Next, the removed skin was separated into smaller pieces for each insertion site and each piece was flash frozen and processed for histology, as described previously. The sample was cut into 10 μm sections, which are inspected using a fluorescent microscope to visualize the microneedle insertions and delivery of the fluorescent compound sulforhodamine.

3.1.7.2 Polymer microneedle delivery to mice in vivo

PVP polymer microneedles were inserted into mice in vivo to test the insertion capabilities. Polymer microneedles were created out of polyvinylpyrrolidone and

sulforhodamine was preferentially encapsulated within the needles to easily visualize the delivery in the skin. The mice were purchased from Charles River (Female, Balb/c) and all work was done in accordance with the Georgia Tech Institute for Animal Care and Use Committee (IACUC). First, the mice are anesthetized similar to the process used for the hairless rats described previously. Alternatively, the mice can be placed under anesthesia using an intramuscular injection of ketamine (10 mg/kg) and xylazine (1 mg/kg). This allowed for a deeper anesthesia and easier access of the mice for microneedle insertion, where the mice are not constricted by the anesthesia mask.

Prior to microneedle insertion, the hair of the mouse was removed to allow for better access to the skin of the mouse. This involved the application of a small amount of hair remover cream (Nair brand), 1 in^2, to the back of the mouse. This cream was rubbed into the mouse skin thoroughly and left for 1 minute. Then, a cotton swab was used to remove the cream and the hair. The skin was washed thoroughly with a damp towel to remove all excess cream, which can cause skin irritation if left on the skin for too long. The microneedles were inserted into the mouse using different application times, left in the skin for different insertion times and inserted using different applicators. All of these variations are the same as the hairless rat insertions detailed previously. In addition, for the insertions that lasted more than one minute in the skin, double sided tape was applied around the microneedle array. This tape held the microneedles onto the surface of the mouse skin after application without the need for constant force for the full insertion time. After each microneedle array was removed, the skin was marked and the microneedles are set aside for later inspection under the microscope. After all of the insertions are complete, the skin surrounding the insertions sites was removed. This skin

was viewed under the fluorescent microscope to analyze the microneedle delivery and then flash frozen and processed for histological inspection. After the skin samples are cut using the microcryostat, the sections are observed using a fluorescent microscope to determine the microneedle insertions and delivery profile.

3.1.8 Quantitative analysis of polymer microneedle delivery

3.1.8.1 Quantitative analysis of polymer microneedle delivery of sulforhodamine

To determine the delivery efficiency of the polymer microneedles, sulforhodamine was encapsulated within the microneedles and the percent delivery was measured. The basis for this experiment was the measurement of the emission spectrum of sulforhodamine, which was linearly proportional to sulforhodamine concentration over a range of 0.001 μg/mL to 1 μg/mL. In this experiment, sulforhodamine was dissolved in vinyl pyrrolidone monomer and added to the microneedle mold. It was important to have no sulforhodamine in the base of the microneedles because it would not be delivered to the skin and alter the delivery results. The liquid monomer with sulforhodamine was carefully pipetted off the surface of the mold and the mold was observed under a fluorescent microscope to verify that little to no sulforhodamine was found in the base. Next, a vinyl pyrrolidone monomer solution without sulforhodamine was added to the mold to provide the base for the polymer microneedles and the system was placed under the UV lamp where polymerization takes place.

The basis for the delivery efficiency is as follows. First, control pre-insertion polymer microneedle arrays (n=8) with sulforhodamine in the needles were placed in containers of 3 mL of deionized water, where they dissolved. The average value of this signal will stand for the total amount of sulforhodamine in a microneedle array. Next,

more control polymer microneedles (n=6) with sulforhodamine in the needles will have the microneedles removed and only the base remains. The remaining array bases were also dissolved in separate 3 mL of deionized water each. They act as a background if any sulforhodamine was in the base of the array. Finally, the other microneedle arrays with sulforhodamine (n=6, per condition) were inserted into the skin and left for a set amount of time. After each array was removed, it was visually inspected and then placed in 3 mL of deionized water to determine the amount of sulforhodamine left in the microneedle array. Next, the insertion site was tape stripped twice to remove all sulforhodamine that was left on the surface of the skin and was not delivered properly. The tape was applied to the skin with force and pulled off. These pieces of tape were also washed with 3 mL deionized water to determine the sulforhodamine amount left on the skin surface. The amount of sulforhodamine in each solution was measured by spectrofluorometry. 2 mL of each solution was added to a cuvette and the emission scan was read from 580 nm – 620 nm after an excitation at 550 nm. The area under the curve was determined and the concentration was calculated via a standard curve. The mass of sulforhodamine in each solution was found by multiplying the concentration by 3 mL. Finally, the delivery efficiency of the microneedles was determined by the following equation

$$\textit{Mass delivered} = \textit{Average(Preinsertion controls)} - \textit{mass on tape} - \textit{mass left in needle array}$$

3.1.8.2 Quantitative analysis of microneedle delivery to pig skin in vitro

The delivery efficiency of polymer microneedles was tested *in vitro* using pig skin. Frozen pig skin was thawed to room temperature, shaved to remove all hair and trimmed to remove excess fat from beneath. Polymer microneedles with sulforhodamine

encapsulated within the microneedles only were fabricated as described previously. The microneedles were inserted into the pig skin using different insertion times, ranging from 1 minute to 15 minutes. Also, the microneedles were applied using different applicators, which allow for different insertion forces. After each microneedle insertion, the array was observed under the microscope to see if the needles failed to insert (bent) or inserted and how much of the microneedle dissolved in the skin. The insertion site was observed for microneedle delivery using a fluorescent microscope and then tape stripped to remove the sulforhodamine left on the skin surface. The tape and postinsertion microneedles were placed in containers of 3 mL of deionized water. The amount of sulforhodamine delivered was determined as described in the previous section.

3.1.8.3 Quantitative analysis of microneedle delivery to mice in vivo

The delivery efficiency of polymer microneedles was tested on mice in vivo. The mice were anesthetized using an isofluorane chamber, and their hair on their back was removed via the Nair cream, as described previously. The mice were then given anesthesia by intramuscular injection of ketamine and xylazine, to allow for easier handling of the mice during the microneedle insertions.

Polymer microneedles were made with sulforhodamine in the needles. The microneedles were applied to the skin for 15 seconds for each insertion and left on the skin for times ranging from 5 to 20 minutes.

After the microneedle insertions, the delivery efficiency was evaluated as described previously. This includes observation of the microneedles and skin post insertion and the quantitative analysis of the percentage of delivery by

spectrofluorometry. Figure 7.3 in Chapter 7 of this thesis shows the delivery efficiency of the PVP microneedles to a mouse in vivo.

3.2 DELIVERY OF THE INFLUENZA VACCINE VIA COATED METAL MICRONEEDLES

3.2.1 Characterization of inactivated influenza virus

3.2.1.1 Determination of the protein concentration of inactivated virus solution

Prior to coating onto metal microneedles, the properties of the inactivated virus solution must be characterized, including the protein concentration and the antigenicity of the virus in solution. In most cases the protein concentration of the virus solution after inactivation is around 1 -3 mg/mL. The proper assay to determine the exact concentration would be a Bio-Rad protein assay which can accurately determine protein concentrations between 0.200 mg/mL to 1.6 mg/mL[133]. In this assay, 5 μL of the unknown solution was added in triplicate to a 96 well plate. In addition, a 2:1 and 4:1 dilution of the unknown solution was also added in triplicate. Next, 5 μL of the bovine serum albumin (BSA) standard was added in triplicate for the standard curve. The following standard concentrations are used, 0.2 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1.0 mg/mL and 1.5 mg/mL. Next, 25 μL of working reagent A in the Bio-Rad protein assay kit was added to each well. Then, 200 μL of working reagent B was added to each well and each solution was well mixed. The plate was left at room temperature for 15 minutes. When the incubation was complete, the standard solutions should have varying shades of blue. The absorbance of each solution was read at 750 nm using a microplate reader (Gemini SPECTRAMAX, Sunnyvale, CA). Using the standard curve, the protein concentration of the inactivated virus solution can be determined.

If the protein concentration was lower than 0.2 mg/mL, the bicinchoninic protein Assay (BCA, Thermo Scientific, Rockford, IL) was used [131]. This assay can measure protein concentrations between 0.5 μg/mL and 20 μg/mL. In this, assay 150 μL of each standard or unknown sample was added to a microplate well. Then 150 μL of the working reagent solution was added to each well and the plate was mixed thoroughly on a plate shaker for 30 seconds. The working reagent solution was made by 25 parts of micro BCA reagent MA, 24 parts of reagent MB and 1 part of reagent MC. The plate was covered and incubated for 2 hours at 37 ^{o}C. After incubation, the plate was cooled to room temperature and the absorbance was measured at 562 nm on a plate reader. The protein concentration can be found from the linear standard curve.

3.2.1.2 Analysis of the antigenicity of the inactivated influenza virus.

There are two methods for testing the activity or antigenicity of the inactivated influenza virus solution. An Enzyme-Linked ImmunoSorbent Assay (ELISA) analyzes the activity of the virus solution and the ability of the surface proteins to bind to specific antibodies. The Hemagglutination assay (HA) analyzes the antigenicity of the inactivated virus solution. This assay measures the ability of the surface hemaglutinin protein to agglutinate or bind to red blood cells.

3.2.1.2.1 ELISA to test the antigenicity of the inactivated influenza virus

The ELISA used in this study was qualitative and only allowed for the comparison of the activity of the virus solution with controls. In this study, 100 μL of 10 μg/mL of inactivated virus solution in sodium bicarbonate coating buffer was added to a well on a 96 well NUNC plate. The plate was left overnight at 4 ^{o}C. Next, the plate was

thoroughly washed 3 times with PBS + 0.05% Tween (PBS-T). Then, each well was coated with 200 μL blocking buffer, PBS-T with 3% Bovine Serum Albumin (BSA). The plate was left at 37 ^{o}C for one hour and then washed again 3 times with PBS-T. 100 μL of the primary antibody specific to the virus strain was then added to the wells in 1:100 or 1:1000 dilutions in PBS-T and the plate was incubated at 37 ^{o}C for 1.5 – 2 hours. After another washing 3 times with PBS-T, 100 μL of the secondary antibody (IgG-HRP, Horseradish Peroxidase-Conjugated Goat Anti-Mouse IgG, 1:1000, Southern Biotech, Birmingham, AL) was added to the plate and incubated for 1 – 1.5 hours at 37 ^{o}C. While the plate was incubating, the substrate solution was made of o-Phenylenediamine dihydrochloride (1 tablet of OPD + 12 mL citric buffer + 12 μL of 30% H_2O_2. This solution reacts with peroxidase on the 2nd antibody to form a yellow orange product. After the plate was washed 3 times again with PBS-T, 100 μL of the substrate solution was added to each well. The solutions are allowed to develop for one minute, and then 50 μL of 1 M H_3PO_4 was added to each well to stop the reaction. The absorbance was read at 450 nm using an absorbance plate reader (BIORAD Model 680 microplate reader, Hercules, CA). The absorbance of the processed virus solution was compared to the unprocessed solution to compare the activity.

3.2.1.2.2 HA assay to test the antigenicity of the inactivated influenza virus

The hemagglutination property of the virus solution was also tested. In this experiment, 25 μL of the virus stock solution was added to well 1 with 75 μL of PBS to make a 4:1 dilution. Serial dilutions (2:1) in PBS are made from wells 2 – 12 with a final volume of 50 μL in each well. Next, 50 μL of 0.5% chicken red blood cells (RBC, Lampire Biologicals, Pipersville, PA) was added to each well, and the solutions are

mixed thoroughly. The plate was left for one hour at room temperature. In addition, a positive control was done of 50 μL of PBS with 50 μL of 0.5% RBCs. When the virus was in too low of a concentration or activity, it cannot agglutinate or bind to the red blood cells. Instead, the red blood cells bind to each other and a red button was observed. When the virus was in higher concentration, it bound the red blood cells and prevented them from binding to each other. This was observed by a pink sheet. Once the positive control of PBS and 0.5% RBCs formed a red button, the assay was over. The HA value was determined by reading from left to right where the pink sheet becomes a solid red button. This value was the HA/50 μL. The activity of the virus in HAU/μg was determined by dividing the HA value by the protein concentration.

3.2.2 Influenza delivery via coated metal microneedles: in vitro delivery and virus processing

3.2.2.1 Fabrication of metal microneedles for influenza delivery

Rows of five in plane metal microneedles were fabricated by a process previously published[2, 135]. Briefly, the microneedle geometry was designed using a computer drafting software (Autocad), with rows of five microneedles of 750 μm height and 200 μm width. These rows of microneedles were cut from stainless steel metal sheets (75 μm thick, Trinity Brand Industries, SS 304, McMaster-Carr, Atlanta GA, USA) using an infrared laser (Resonetics Maestro, Nashua, NH, USA), which was operated at 1000 Hz, 20 J/cm^2 energy density and 40% attenuation of laser energy. A cutting speed of 2 mm/s and air purge at a constant pressure of 140 kPa was used and three passes were required to completely cut through the stainless steel sheet

Next the microneedle arrays were manually cleaned with detergent (Contrex, Decon Labs, PA, USA) to de-grease the surface and remove deposits left during laser cutting. The needles are then washed thoroughly with deionized water. To further sharpen and clean the microneedles and remove the burrs on the edges, microneedles were electropolished in a solution containing glycerin, ortho-phosphoric acid (85%) and deionized water in a ratio of 6:3:1 by volume (Fisher Scientific, Fair Lawn, NJ, USA) The electropolishing was performed in an electropolisher (model no. E399-100, ESMA, IL, USA) using a voltage of 6 volts for 15 minutes The microneedles are then cleaned by dipping three times in deionized water and 25% nitric acid (Fisher Scientific) for 30 s each. This was followed by another washing step in hot running water and a final wash in running deionized water. The electropolishing process reduces the thickness of the microneedles to 50 μm. Microneedles were dried using compressed nitrogen before storing in airtight containers until later use.

3.2.2.2 Coating the influenza virus onto metal microneedles

In order to get a high dose of influenza virus on a row of metal microneedles, the protein concentration of the inactivated virus solution should be greater than 4 mg/mL. One way to accomplish this was to concentrate the virus solution using centrifugation through Vivaspin 30,000 Da molecular weight cut off membranes (Cole Palmer, Vernon Hills, IL). The virus molecular weight was much higher than 30,000 Da. Here, 1.5 mL of the virus solution was placed in the membrane tubes. The centrifuge was run at 7500 rpm for 10 minutes at 4 ^{o}C. Next, the bottom solution was removed and the tube was turned over and spun again at 2000 rpm for 5 minutes. This allowed for an easy retrieval of the concentrated virus solution.

The coating of the influenza virus solution to the metal microneedles was performed as described previously[2, 135]. The coating solution was composed of 1% (w/v) carboxymethylcellulose sodium salt (low viscosity, USP grade, Carbo-Mer, San Diego, CA, USA), 0.5% (w/v) Lutrol F-68 NF (BASF, Mt. Olive, NJ, USA) and a vaccine at 5 mg/ml. The coating-solution reservoir was slightly modified from the design described before[2]. Here, the reservoir consisted of two laminated parts: the 'bottom plate' and the 'cover plate', both of which were made of poly(methyl-methacrylate) (McMaster-Carr, Atlanta, GA). The bottom plate had a central feeding channel (1 mm deep×0.5 mm wide) machined into one of its faces. The cover plate had seven holes: five holes (400 μm diameter) drilled into it at the same interval as the microneedles in the in-plane row to be coated and two larger holes (1.5 mm diameter) at the end of feeding channel. These 'dip-holes' acted as individual dipping reservoirs to coat each of the microneedles in the row. The two plates (bottom and cover plates) were aligned and adhered to each other using solvent bonding with methylene chloride (Fisher Scientific, Pittsburgh, PA) as the solvent. Coating solution (40 μl) was filled into the channel using the standard 200 μl pipette.

The coating of the influenza virus onto the metal microneedles was performed manually while the dipping process was monitored by a video camera (DP71, Olympus America, Center Valley, PA) attached to a microscope (SZX16, Olympus America, Center Valley, PA). An X-micropositioner was used to control the length of the microneedle inserting into the coating solution reservoir. The diameter of the holes was made to be twice the width of the microneedles, to allow for slight misalignment issues.

3.2.2.3 Analysis of the antigenicity of the influenza virus after coating onto metal microneedles

Prior to immunization, it was important to determine the amount and quality of the influenza virus coated on the metal microneedles. Two rows of metal microneedles were placed in a flat bottom cuvette, and 200 μL of PBS was added and mixed vigorously. The protein concentration of this solution was determined by the BCA method described above. Also, the HA assay was used to determine the antigenicity of the coated virus. Figure 6.1 in Chapter 6 of this thesis shows the antigenicity of the influenza virus after coating and drying onto metal microneedles.

3.2.2.4 Delivery efficiency of coated metal microneedles

Finally, it was important to verify the delivery efficiency and kinetics of the coated metal microneedles. Here, vitamin B (Sigma-Aldrich, St Louis, MO) was coated onto the microneedles and the time required for complete delivery of the coating to skin was determined. First, the coated metal microneedles were inserted into pig skin in vitro and removed at specific time points. After removal, the needles were inspected using a bright field microscope to determine the amount of coating left on the microneedle. This experiment was also repeated in a mouse in vivo. Figures 6.2A and 6.2B in Chapter 6 of this thesis show the metal microneedles before and after insertion into pig skin.

3.2.3 Influenza delivery via coated metal microneedles: in vivo delivery and analysis

3.2.3.1 Coating of metal microneedles with inactivated influenza virus coating solution

The influenza virus was coated onto the metal microneedles in a process described above. After the coating process was complete, three rows of microneedles

were dissolved in 200 μL of deionized water to determine the protein concentration and HA value of the virus on the needles.

3.2.3.2 Delivery of influenza vaccine via metal microneedles to mice

The influenza vaccine was delivered to mice (female, 18g, 4 weeks, Balb/c,Charles River) by metal microneedles. The H3N2/A/Aichi strain of inactivated virus was used for these microneedle immunizations. The mice were anesthetized under the IACUC protocol by an intramuscular injection of ketamine (10 mg/kg) and xylazine (1 mg/kg). Next the hair covering the insertion site, approximately 1 in^2 on the back of the mouse, was removed by applying a Nair hair removal cream. This cream was applied deeply into the hair and left for 1 minute. The hair was removed by a damp paper cloth and the site was thoroughly cleaned to remove all of the excess cream. The mice were placed on a heat blanket after the washing in order to keep them warm. The rows of microneedles were inserted by hand one at a time and each left for 5 minutes in the skin. The number of rows of microneedles inserted into a mouse was dependent on the coating efficiency on the microneedles, to achieve the desired dose. After the microneedle insertions, the mice were transferred back into their cage and observed until they regain consciousness.

The following four groups were used in this study; microneedle immunization with 9.8 μg dose (4 MN rows with 1.95 μg on each), intramuscular immunization with 9.8 μg dose (100 μL), naïve group, and a separate naïve group where uncoated microneedles (3 MN rows) were inserted into their backs. Each experimental group had 12 mice total. Six of the mice of each group had one immunization on day zero and were challenged with a lethal dose of the virus 30 days after the immunization. The other six

mice had one immunization on day zero, and a boost immunization 28 days later. These mice were challenged 30 days following the boost immunization. The prime and boost doses were the same.

3.2.3.3 Measurement of the immune response of mice after vaccination of the influenza vaccine with coated metal microneedles

The immune response of the mice to the immunization of inactivated H3N2/A/Aichi influenza virus was measured by testing the IgG antibody levels and hemagglutination inhibition (HAI) of the mouse sera. The blood was collected 14 days after the prime and boost immunizations, when the sera IgG antibodies have reached a peak[136]. The mice were lightly anesthetized using an isofluorane chamber and approximately 200 μL of blood was taken from the orbital cavity. Next, the blood was spun in a centrifuge at 10,000 rpm for 15 minutes and the clear sera was removed and transferred into a separate eppendorf tube. This sera was stored at -20 ^{o}C until use for the HAI and IgG ELISA experiments.

3.2.3.3.1 Measurement of sera IgG antibodies of immunized mice via ELISA

The amount of sera IgG antibodies was measured via an ELISA. To begin, 100 μL of 5 μg/mL of purified H3N2/A/Aichi virus in Na_2CO_3 coating buffer was added to each well of a NUNC 96 well plate. A standard curve was made by adding 100 μL of 2 μg/mL of Ig(H+L) (Southern Biotech, Birmingham, AL) antibody solution to each well in the final row. The plate was left overnight to incubate at 4 ^{o}C.

The following day, the plate was washed three times with PBS-T. Next, 200 μL of blocking buffer (PBS-T + 3% BSA) was added to each well. The plate was incubated for 1 hour at 37 ^{o}C. After washing the plate again three times with PBS-T, the primary

antibodies from the sera was added to the plate. 100 μL of 1:100 dilutions of sera in PBS was added in duplicates. For the standard curve, 100 μL of 1000 ng/mL of the standard IgG antibody solution in PBS-T was added to the first well and serial dilutions (2:1) in PBS were made down to 1 ng/mL. The plate was incubated at 37 ^{o}C for 2 hours and then washed three times with PBS-T. Next, 100 μL of 1:1000 dilution of the secondary antibody IgG HRP in PBS was added to each well. The plate was incubated again for 1.5 hours at 37 ^{o}C. While this incubation was happening, the substrate solution was made by adding 1 tablet of o-Phenylenediamine dihydrochloride (OPD, Zymed Laboratories, Carlsbad, CA) to 12 mL citric buffer and. After the tablet completely dissolved, 12 μL of 30% H_2O_2 was added and the solution was kept out of the light. The plate was washed 3 times with PBS-T and 100 μL of enzyme solution was added to each well. The solutions were allowed to develop for approximately 30 seconds and 50 μL of H_3PO_4 was added to stop the reaction. The absorbance was read at 450 nm using the absorbance plate reader (BIORAD Model 680 microplate reader, Hercules, CA) and the concentration of the IgG antibodies in the sera was determined by the standard curve.

3.2.3.3.2 Measurement of the quality of antibodies of immunized mice via HAI assay

The quality of the antibodies in the sera was measured via the hemagglutination inhibition (HAI) experiment, which tests the ability of the antibodies to inhibit agglutination of red blood cells by a standard influenza virus solution. The first step of this experiment was to make the standard influenza solution that has an HA activity of 8 HA/50 μL. Also the sera must be treated prior to use in this experiment to remove the false positive signal of cryoglobulins which exists in the sera[137]. Here, 10 μL of sera was transferred to a new eppendorf tube and mixed with 30 μL of receptor destroying

enzyme (RDE). The samples are heated in a water bath at 37 ^{o}C 18 – 20 hours overnight. Next, the temperature was raised to 56 ^{o}C for one hour to deactivate the compliment. The samples are cooled to room temperature and 60 µL of deficient PBS was added. Next, 5 µL of packed chicken RBCs are added to the sample and set at 4 ^{o}C for one hour. The sample was then centrifuged at 15,000 rpm for 12 minutes to settle the RBCs and the supernatant was removed and transferred to a new tube. The sera was now in a 1:10 ratio and was stored at -20 ^{o}C until the HAI assay was performed.

For this experiment, 12.5 µL of the unknown sera was added to 37.5 µL of PBS to the first well, giving a 1:40 dilution. Serial 2:1 dilutions were made from the first well to the 6th well, making the 6th well 1:1280. Next, 25 µL of the standard H3N2/A/Aichi virus solution (8 HA/50 µL) was added to each well. The wells were well mixed and incubated at room temperature for 15 – 30 minutes. Then, 50 µL of 0.5% RBCs were added to each well and well mixed. The samples were incubated at room temperature for one hour, or until the positive control became a pellet. The positive control included 50 µL of PBS and 50 µL of 0.5% RBCs. The negative control contained 50 µL of the standard virus solution and 50 µL of 0.5% RBCs. This negative control should stay a pink sheet, due to the virus binding the red blood cells and preventing them from binding to each other. The positive control appeared as a red pellet, due to the binding of the red blood cells with one another. The value for the HAI was read from left to right when the sample with a red pellet became a pink sheet. A value of HAI of 1:40 or greater signifies seroprotection[138]. This was verified via a lethal challenge thirty days after the final immunization.

3.2.3.4 Measurement of the immunoprotection of mice to influenza vaccine via lethal challenge

The immunoprotection of the immunized mice was measured via weight loss in a lethal challenge using 5 LD_{50} of the live H3N2/A/Aichi virus. 20 μL of this virus solution was given intranasally to each of the immunized mice. The mice were then weighed (day = 0). Next, the mice were weighed each day until day 14, and then every few days until day 21. If the weight of a mouse dropped to below 25% of the original weight, the mouse was considered terminally ill and was euthanized using an isofluorane chamber. The mice that survived after 21 days were considered protected.

3.3 DELIVERY OF THE INFLUENZA VACCINE VIA DISSOLVING POLYMER MICRONEEDLES

3.3.1 Influenza delivery via dissolving polymer microneedles: in vitro processing

3.3.1.1 Preparation of the influenza virus solution for lyophilization

3.3.1.1.1 Concentrating process for the influenza virus solution

Prior to lyophilization, it was desired to have a high concentration of virus in solution to allow for a high encapsulation efficiency of lyophilized virus within the polymer microneedles. The main way of concentrating the virus solutions was by centrifugation with Vivapsin 30,000 Da MWCO membranes (Sartorius, Germany), as described above. In addition, if the inactivated virus was originally stored in PBS, this must be dialyzed out for deionized water due to previous work that has shown loss in activity of the influenza virus in PBS during lyophilization[139]. The virus was concentrated to approximately 2 mg/mL. After the virus was concentrated, the protein concentration and HA activity were determined.

3.3.1.1.2 Addition of sugars to act as cryoprotectants during lyophilization process

Previous work has shown that the lyophilization process, specifically freezing and drying stresses, can significantly damage viruses in solution [140, 141]. In order to retain activity of the virus, sugars, specifically trehalose (Sigma-Aldrich, St Louis, MO) and inulun (Sigma-Aldrich, St Louis, MO), were added to the virus solution in a 50:1 ratio to act as cryoprotectants [139, 142]. For the lyophilization processes detailed in this study, solutions of inactivated viruses with varying amounts of sugar were studied. This includes virus solutions with no trehalose, 5:1 trehalose by weight, 10:1 trehalose by weight, and 50:1 trehalose by weight. The virus solutions were placed in a 1.5 mL eppendorf tube and frozen quickly in dry ice at -80 ^{o}C for 15 minutes in preparation for lyophilization.

3.3.1.2 Lyophilization process for the inactivated influenza virus solution

The settings for the lyophilization of the inactivated influenza virus solutions were based on a published protocol [139]. To begin, the temperature of the lyophilizer (VirTis Wizard 2.0 freeze dryer, Gardiner, NY) was lowered prior to the addition of the frozen solution to prevent any thawing of the solution prior to lyophilization. The tops of the eppendorf tubes were removed and a plastic film was placed on top with small holes poked into the film. The samples were placed in the lyophilizer and the primary drying cycle was performed for 24 hours with a shelf temperature of -35 ^{o}C, a condenser temperature of -55 ^{o}C and a pressure of 160 mtorr. The secondary drying cycle followed for 24 hours at a shelf temperature of 20 ^{o}C, a condenser temperature of -55 ^{o}C and a

pressure of 50 mtorr. The samples were then removed, capped and stored in the refrigerator for later use.

3.3.1.3 Encapsulation of the lyophilized influenza virus in polymer microneedles

The lyophilized influenza virus was encapsulated into the polymer microneedles similar to the processes described previously for other molecules. To increase the efficiency of encapsulation of the virus within the microneedles, the amount of vinyl pyrrolidone monomer used to suspend the lyophilized powder was minimized. The monomer was added in drops of 20 μL until the lyophilized powder was suspended in the solution and does not settle in the bottom. The volume of monomer added was noted and was used to determine the amount of virus encapsulated within the microneedles. Typically, the approximate concentration of virus that was suspended in VP monomer was 4 μg/μL. The rest of the fabrication process of PVP polymer microneedles with encapsulated influenza virus follows the previously detailed protocol, including pipetting onto a microneedle mold, application of a vacuum, exchange of the base with an empty monomer solution and polymerization under the UV lamp. The microneedles were then removed and stored in a desiccator for later use.

3.3.1.4 Antigenicity and protein concentration of the inactivated virus was determined after lyophilization and encapsulation in PVP microneedles.

The lyophilized powder was dissolved in a set volume of deionized water and the HA and protein concentration were analyzed as described previously. The PVP microneedles with encapsulated influenza virus were dissolved in 2.0 mL of deionized water and the HA was determined. Unfortunately, the presence of the polymer PVP in solution introduced a large background signal for all of the protocols to determine protein

concentration. Thus, it was difficult to accurately determine an exact protein concentration for these solutions with PVP. As a result, the protein concentration was approximated based on the amount of protein in the lyophilized powder, the volume of monomer needed to suspend the powder, and the volume of deionized water required to dissolve the PVP microneedles. The mass of the lyophilized powder was determined by multiplying the protein concentration of the solution that was lyophilized by the volume of the solution. Next, the amount of virus in the microneedles was calculated by measuring the volume needed to suspend the virus. The concentration of lyophilized powder (μg) per μL of monomer solution was multiplied by a volume of 1.4 μL, which is the total volume of the microneedles, excluding the base. Finally, the protein concentration was found by dividing the mass of the protein found in the microneedles by the volume of water that was required to dissolve the microneedle array. In addition, an ELISA was performed, as previously detailed, to qualitatively test the virus after processing.

3.3.2 Influenza delivery via dissolving polymer microneedles: in vivo delivery and analysis

3.3.2.1 Measurement of the immunogenicity of the processed influenza vaccine via in vivo immunization

Before polymer microneedles were used in the immunization of the influenza vaccine, it was important to determine if the processing steps damage the immunogenicity of the virus. This included the lyophilization of the virus, suspension of the powder in liquid monomer and polymerization of the solution. This also involved the addition of the sugar, trehalose, to the virus solution prior to lyophilization, to act as a cryoprotectant[139].

In this work, the virus solutions, with or without trehalose, were frozen in dry ice and lyophilized using the conditions described above. The lyophilized solutions were done in duplicates: half of the solutions were resuspended in deionized water, and the other half were suspended in the vinyl pyrrolidone monomer, polymerized and the microneedles were dissolved in deionized water. The solutions were then injected into mice, and the immune response was tested using serum IgG antibodies, HAI and challenge studies. For each study, controls of the unprocessed inactivated virus solution and the unprocessed inactivated virus solution with dissolved, blank PVP microneedles were also tested.

The first in vivo test of the processed virus was a pilot experiment using the inactivated H1N1/A/PR8 virus. The following groups were used: lyophilized virus with no sugar, lyophilized virus with 5:1 trehalose by weight, lyophilized virus with 10:1 trehalose, lyophilized virus with 50:1 trehalose, and unprocessed virus with and without PVP, and naïve. In this study, mice (n=3) were injected intramuscularly with 20 µg of inactivated H1N1/A/PR8 virus in the solutions described above. The mice were bled fourteen days later and the serum IgG antibodies were measured.

The second pilot experiment involved testing the immunogenicity of the inactivated H3N2/A/Aichi virus after processing in polymer microneedles. The following groups were used: lyophilized no sugar, lyophilized with 5:1 trehalose, lyophilized with 10:1 trehalose, and unprocessed virus with and without blank PVP microneedles, and naive. As with the previous work, each lyophilized group was in duplicates: in half the lyophilized powder was resuspended in deionized water and in the other half, the lyophilized powder was suspended in vinyl pyrrolidone monomer,

polymerized and then the polymer microneedle system was dissolved in deionized water. In this study, mice (n=3) are injected intramuscularly with 10 μg of each of the above solutions. The mice were bled fourteen days after the immunization. The serum IgG antibodies and HAI titer were determined. Then, 28 days after the immunization, the mice were boosted with a second dose of 10 μg by intramuscular injection. Once again, the mice were bled fourteen days after this immunization and the serum IgG antibodies and HAI titer were determined. Finally, mice were challenged thirty days following the boost immunization with 20 μL intranasally of 5 LD_{50} of live H3N2/A/Aichi virus. The body weights were checked daily to test the protection against this lethal challenge.

The results of the above work showed that lyophilization, with or without trehalose, did not decrease the immunogenicity of the virus. However, there were some conflicting data with the PVP groups. Therefore, a larger study using a more antigenic strain of inactivated H3N2/A/Aichi virus was performed using the following 5 groups: lyophilized virus resuspended in deionized water, lyophilized virus processed through polymer microneedles and suspended in deionized water, unprocessed virus solution, unprocessed virus solution with blank PVP microneedles dissolved, and naïve. In this study, mice (n=10) were immunized with 10 μg of virus by intramuscular injection. All of the mice were bled on day 14 to test the serum IgG antibodies and HAI. Half of the mice (n=5) of each group were challenged with 10 LD_{50}, and their weights are measured to test for protection. The other half of the mice (n=5) were boosted with a 2^{nd} dose of 10 μg 28 days after the prime immunization. These mice were bled fourteen days later to test for serum IgG antibodies and HAI and challenged with 10 LD_{50} 28 days after the boost immunization.

3.3.2.2 Encapsulation of the influenza vaccine in polymer microneedles for in vivo delivery

H1N1/A/PR/8 virus was lyophilized and encapsulated in polymer microneedles for immunization to mice. The prior work on the effect of the polymer microneedle processing on the influenza virus in vivo involved the use of the inactivated H3N2/A/Aichi virus. However, there were issues concentrating and encapsulating enough inactivated H3N2/A/Aichi virus in the polymer microneedles to get a relevant dose. It required a much higher amount of monomer to encapsulate the H3N2/A/Aichi virus. This resulted in a much smaller amount of virus that could be encapsulated within the microneedles, approximately 0.5 μg. This was a much lower amount that had been accomplished using the H1N1/A/PR/8 virus. It was believed that the reason for this was the globular core structure of the H1N1/A/PR8 virus versus the filamentous structure of the H3N2/A/Aichi virus. It was discovered that the H1N1/A/PR/8 influenza virus was able to be concentrated and encapsulated in polymer microneedles in a high amount to reach a relevant dose, approximately 3 – 4 μg per microneedle array.

The antigenicity and protein concentration of the H1N1/A/PR/8 virus was tested prior to lyophilization. 500 μL of this inactivated virus was frozen in dry ice and lyophilized, using the same conditions described previously. This powder was covered and stored at 4 ^{o}C until encapsulation in polymer microneedles. Also, 25 μL of this virus was placed in a separate container and frozen and lyophilized to test the protein content and HA activity of the inactivated virus after the lyophilization process. This was done to verify that the lyophilization process did not damage the virus or result in a loss of virus.

The polymer microneedles fabricated for use in the immunization were made the same day as the immunization. Vinyl pyrrolidone monomer was added to the lyophilized

virus powder (1.1 mg) until it was completely suspended in solution and did not settle. The total volume used was 180 μL. Based on the volume of the pyramidal microneedles, this concentration of virus in the liquid monomer resulted in 6 μg of inactivated virus encapsulated in the polymer microneedles (pyramid, height = 600 μm, base width = 200 μm). This dose was based on the use of an 8x9 microneedle array with very sharp tips. The polymer microneedles with inactivated H3N2/A/X31 virus were created as described previously. After fabrication, the microneedles are inspected under a microscope and attached to a metal applicator for insertion. Also, double sided tape was applied to the base of the microneedles, surrounding the array. This held the array to the skin of the animal after application of the array. The microneedle arrays were stored in a desiccator prior to immunization.

3.3.2.3 Delivery of the influenza vaccine via dissolving polymer microneedles to mice

Mice were immunized with H1N1/A/PR/8 influenza virus delivered by PVP polymer microneedles. Three groups of mice (n=12) were used: polymer microneedles (6 μg), intramuscular immunization (100 μL, 6 μg), and naïve mice. All of the mice were also boosted 28 days after the first immunization. The mice that were immunized intramuscularly were injected with 50 μL of the H1N1/A/PR/8 virus in each of their hind legs. The microneedle group of mice was first anesthetized intramuscularly with xylazine and ketamine. After the mice were completely under anesthesia, a small amount of Nair hair removal cream was applied to their back, left for 1 minute and then thoroughly washed with warm water. The mice were dried completely and placed on a heating blanket for the immunization. For each mouse, one array of PVP polymer microneedles was inserted into their back in the hairless region. The microneedles were

applied to the skin for 15 seconds and left for 15 minutes, based on delivery kinetics shown previously. After the microneedles are removed, the mice were observed in their cage until they awake. Also, the insertion site for the microneedle delivery was examined daily for one week to observe any possible inflammatory response. There were no responses after any of the microneedle insertions.

3.3.2.4 Measurement of the immune response in mice after vaccination of the influenza vaccine via polymer microneedle delivery

The immune response of the mice to vaccination of inactivated H1N1/A/PR/8 virus by polymer microneedles was measured. Fourteen days after each immunization the mice were bled. Here, they were placed under light anesthesia using an isofluorane chamber and blood was taken from the orbital cavity. The blood was placed on ice, centrifuged and the clear sera was removed and transferred to a new eppendorf tube. The concentration of the serum IgG antibodies was measured via the ELISA process that was previously described. Also, the quality of the antibodies was measured via the hemagglutation inhibition (HAI) assay.

3.3.2.5 Measurement of the immunoprotection of mice to influenza virus via lethal challenge after polymer microneedle delivery

The immunoprotection of the mice immunized with inactivated H1N1/A/PR/8 or H1N1/A/PR/8 via polymer microneedles was tested by a lethal challenge. The mice were lightly anesthetized using isofluorane and challenged nasally with 20 μL of 5 LD_{50} of the live virus. The weights of the mice were measured daily for 14 days after challenge. If the weight of the mouse drops below 75% of the original weight, the mouse was considered terminally ill and was euthanized using isofluorane by IACUC standards. The

immunoprotection of the experimental groups of mice was determined based off of the survival rate and the average weight loss.

3.3.2.6 Measurement of the efficacy of lung clearance of the lethal influenza virus in mice after immunization via polymer microneedles

The ability of the immunized mice to effectively clear the live virus from their lungs was measured. These mice had been immunized with the inactivated H1N1/A/PR/8 via polymer microneedles or intramuscular injection. Here, the mice were lightly anesthetized using isofluorane and challenged nasally with 20 μL of 5 LD_{50} of the live H1N1/A/PR/8 virus. Four days after the challenge, the mice were sacrificed and their organs were collected for processing. This protocol is based off of previously reported analysis[127]. Lung homogenates were prepared to determine the viral titers (pfu per gram of tissue). For the plaque assay, serial dilutions of the lung supernatants were incubated with confluent MDCK cells and after treatment and three days of culture, the cells were fixed and the plaques were counted.

CHAPTER 4

DEVELOPMENT OF A NEW FABRICATION PROCESS FOR POLYMER MICRONEEDLES FOR THE DELIVERY OF BIOPHARMACEUTICALS[1]

4.1 INTRODUCTION

Biomolecules, including proteins, peptides and vaccines, make up a large and potent part of all new drugs and hold great promise for the future of therapeutics [143, 144]. Although oral delivery of these biotherapeutics would be desirable, there is low bioavailability of biomolecules administered by this route due to enzymatic degradation and poor absorption in the GI tract, as well as first-pass metabolism of the liver [8]. As a result, most biotherapeutics are administered by hypodermic injection, which causes pain, can lead to infection, requires trained personnel and often needs frequent, repeated injections for the patient. Consequently, there exists the need for a minimally invasive, self-administered delivery system for biomolecules.

An attractive non-invasive option is the transdermal patch, which has become well-received for the delivery of nicotine, estrogens and other drugs [25]. However, delivery across intact skin permits transport only of small, lipophilic molecules and excludes transport of biotherapeutics, due to their large size.

This study presents a novel, hybrid delivery approach to achieve the delivery efficacy of injections and the safety and patient compliance of the patch. We designed

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[1]This work was published in Advanced Materials: Sullivan SP, Murthy N, Prausnitz MR. (2008) Minimally invasive protein delivery with rapidly dissolving microneedles, Advanced Materials 20:933-938

and synthesized rapidly dissolving polymer needles of micron dimensions for the painless, self-administered delivery of biomolecules. In this design, the drug is encapsulated within these polymer microneedles, and after insertion into the skin, the biocompatible polymer dissolves within minutes to release the encapsulated cargo and leave behind no biohazardous sharps or need for removal.

Previous work has shown that microscopically piercing the skin with micron-scale needles offers an effective and convenient alternative for the delivery of biomolecules, due to the efficient delivery [35, 36], lack of pain[10, 37], ease of use and the expected low cost of fabrication. Microneedles have been shown to deliver proteins, DNA and vaccines in vivo using devices small enough to be integrated into a low-profile, self-administered patch [46, 145, 146].

To date, most microneedles have been made of silicon or metal [43, 147] with little work involving polymers [53, 55, 148]. There are, however, safety concerns if microneedles made of these materials were to break off in the skin, or if they were accidentally or intentionally reused. In contrast, the use of biocompatible polymers could eliminate these concerns, because the needles completely and safely dissolve within the skin, and the needle free patch backing could be safely discarded, leaving no biohazardous sharps.

Achieving this goal presents significant material challenges. The ideal polymer material would be strong enough to penetrate the skin, dissolve rapidly once in the skin, and be safely excreted by the body. Also, the fabrication process for these microneedles should take place at ambient temperatures, without organic solvents, and avoid damaging fragile biomolecules during encapsulation. No current design allows for polymer

microneedles to be fabricated in this manner. Previous studies have relied on either high-temperature molding processes that risk damaging biomolecules [53, 148] or methods unsuitable for fabrication of micron-scale structures [55].

In this study, we have developed the first rapidly dissolving polymer microneedles. This advance required developing a new fabrication process to produce mechanically robust microneedles that encapsulate biomolecules under gentle processing conditions using methods suitable for inexpensive mass production. Here, we detail the new fabrication process, based on room temperature *in situ* polymerization, and study the mechanical, encapsulation, dissolution and delivery properties of the resulting polymer microneedles for the delivery of biomolecules to the skin.

4.2 RESULTS AND DISCUSSION

4.2.1 Development of a new fabrication process for polymer microneedles

To develop rapidly dissolving polymer microneedles, we first prepared master structures made of a polymeric photoresist epoxy (SU-8) by a photolithography method, from which we created reverse molds out of polydimethylsiloxane (PDMS). Each master structure was able to be copied into hundreds of molds, and each mold was able to be reused to produce at least a dozen microneedle arrays. PDMS was chosen as the mold material because it is flexible, lacks surface adhesion to the master structure and allows for the removal of the polymer microneedle array. These microneedle molds were then used to fabricate replicate microneedles by a new microfabrication process developed in this study, which involves the room-temperature photopolymerization of a liquid monomer within the microneedle mold (See Section 3.1 in Chapter 3 for more details). The gentle nature of this process allows for the encapsulation of biomolecules within the

microneedles, and its universality allows for the formation of a multitude of polymers and copolymers as the structural material of the needles. We believe that this is the first example of an *in situ* polymerization of microneedles, and represents a novel approach that could be broadly applied to *in situ* polymerization of other microstructures as well. Figure 4.1 below shows a diagram of the new fabrication process.

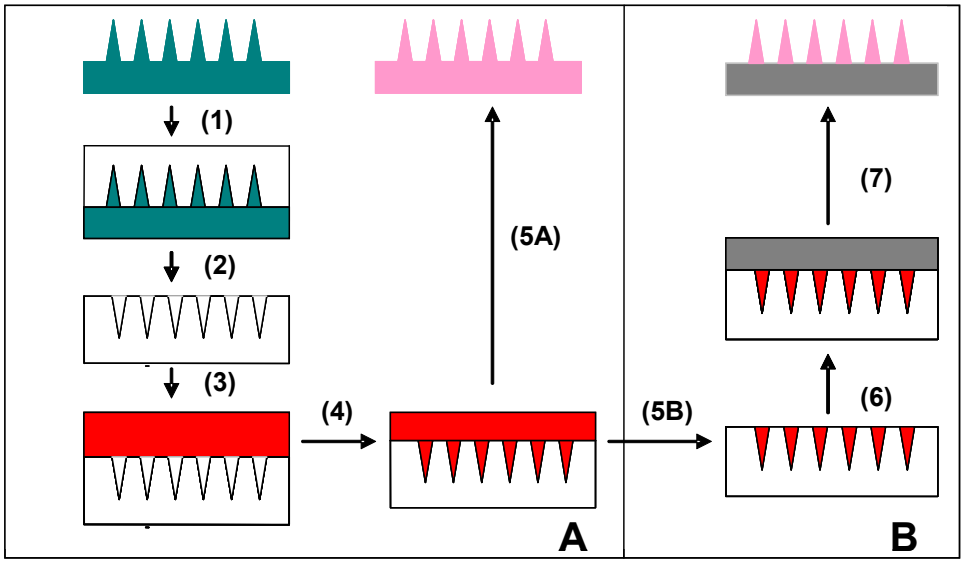

Figure 4.1: New *in situ* fabrication process for polymer microneedles: 4.1A (1) PDMS is poured onto microneedle master structure. (2) PDMS microneedle mold is cured and peeled off. (3) Liquid monomer and drug are pipetted onto the mold. (4) Vacuum is applied to pull the solution into the microneedle mold. (5A) System is placed under a UV lamp to polymerize microneedles, which are subsequently peeled out of the reusable mold. 4.1B (5B) Excess solution is removed from the surface. (6) A liquid monomer solution with no drug is applied to the surface. (7) System is placed under UV lamp to polymerize the microneedles, which are then peeled off

We chose to synthesize microneedles by polymerizing monomeric vinyl pyrrolidone using ultraviolet light. The resulting polyvinylpyrrolidone (PVP) microneedles are shown in Figures 4.2A and 4.2B. We used PVP as the structural material for microneedles for four reasons. First, the chemical backbone structure of the

vinyl pyrrolidone monomer contains a ring, which increases intramolecular rigidity and thereby provides mechanical strength to the polymer, which is important for microneedle insertion into skin. Second, PVP has high water solubility, which facilitates rapid dissolution once inserted into the skin. Third, PVP already has a long history of clinical use as a blood plasma expander [75, 149]. Finally, the vinyl pyrrolidone monomer is liquid at ambient conditions, which facilitates processing at mild temperatures without the need for an organic solvent to fill the microneedle mold.

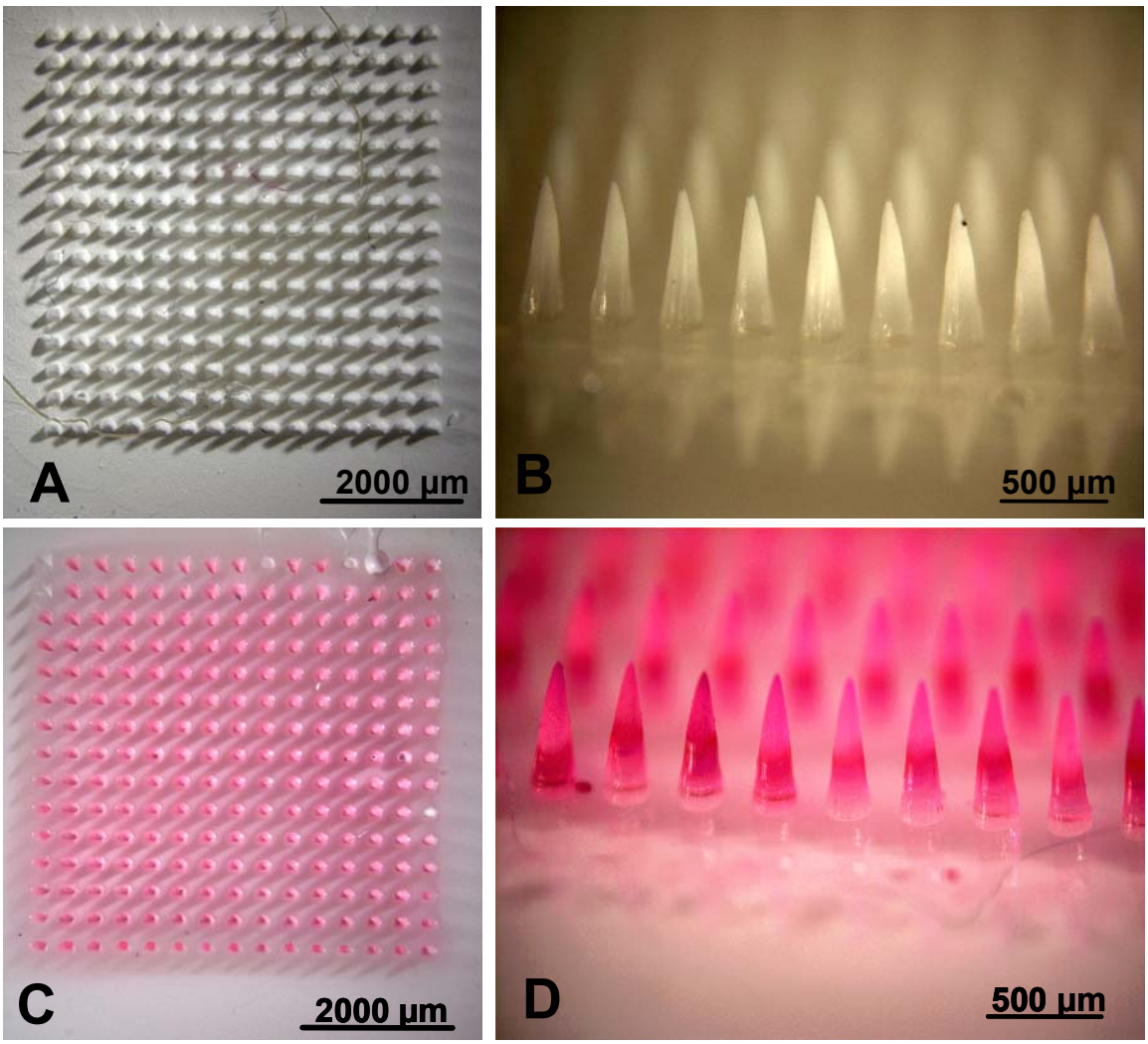

Figure 4.2: PVP polymer microneedles made by new *in situ* polymerization process 4.2A: Overhead view and 4.2B side view of pure PVP microneedles. 4.2C: Overhead view and 4.2D: side view of PVP polymer microneedles with sulforhodamine encapsulated within microneedles, but not in the base substrate. Each microneedle measures 750 µm in height, 100 µm in base radius and 5 µm in tip radius.

Using this approach, microneedles were produced to have a range of micron-scale feature sizes, depending on the mold geometry. For example, the conical microneedles shown in Figure 4.2 measure 750 μm in length, 100 μm in radius at the base and 5 μm in radius at the tip. These microneedles represent an excellent reproduction of the geometry of the master structure and the micromolds used to prepare them (data not shown). As discussed below, this *in situ* micromolding approach produced similarly faithful reproduction results when creating microneedles of pyramidal geometry, microneedles using a mixture of monomers to produce a copolymer structural material, and when encapsulating model drugs within the microneedles.

For the first generation of microneedles produced by this new fabrication process, both the microneedles and their base substrate are made of the same PVP polymer. Using this process to encapsulate a drug within the microneedles would result in the drug being distributed throughout the microneedles and the base. However, any drug encapsulated in the base would not be efficiently delivered into the skin because only the microneedles insert into the skin. Thus, an adaptation is required to encapsulate the drug exclusively within the microneedles. In this adaptation, after filling the mold with the monomer and drug mixture, all liquid on the base of the mold is carefully pipetted off, leaving liquid only in the cavities of the mold to form the microneedles. Then, a liquid monomer solution with no suspended drug is placed on the mold to form the base substrate and the setup is placed under ultraviolet light where photopolymerization takes place. This produces microneedles with drug exclusively encapsulated within the microneedles and not the base. Figures 4.2C and 4.2D show a representative PVP microneedle array with sulforhodamine encapsulated only within the microneedles, which have the same

sharpness as the PVP microneedles shown in Figures 4.2A and 4.2B. This adaptation is especially important when delivering expensive biomolecules and in scenarios where precise dosing is required.

4.2.2 Insertion capabilities and mechanical and dissolution analysis of PVP polymer microneedles

PVP microneedles are hypothesized to be sharp and strong enough to insert into the skin without breaking. We tested this hypothesis by inserting 100-microneedle arrays into porcine skin *in vitro* and then staining the skin after removing the microneedles to identify the sites of insertion. Figure 4.3A shows a representative image of the skin surface after microneedle insertion and staining. This image shows that all 100 microneedles inserted into the skin. Subsequent microscopic examination of the microneedles showed that the needles were not broken or deformed during the insertion process (data not shown.)

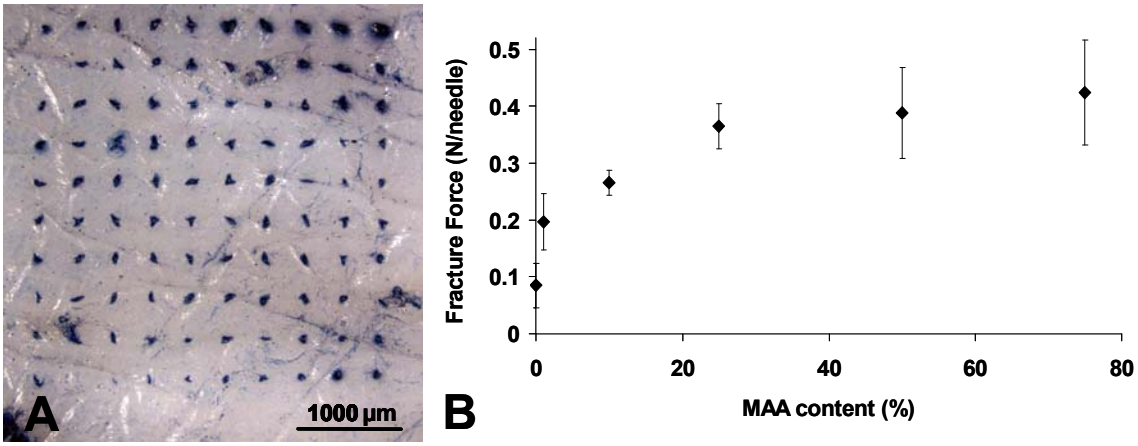

Figure 4.3: Insertion capabilities and mechanical properties of polymer microneedles. 4.3A: Evidence of insertion of PVP polymer microneedles into porcine cadaver skin via skin marking test. 4.3B: The mechanical strength (fracture force) of copolymer PVP-MAA microneedles increases with increasing methacrylic acid (MAA) content.

In addition, it is important to determine the microneedle dissolution kinetics in order to know the length of time the microneedles need to be left in skin prior to removal of the base. The dissolution kinetics of PVP microneedles were measured by inserting the needles into porcine skin *in vitro* and inspecting them after removal, which showed that the entire PVP microneedle array was dissolved in the skin within one minute (data not shown).

Although PVP microneedles are strong enough to insert into skin and then rapidly dissolve within the skin, it could be important to increase microneedle mechanical strength, prolong dissolution time, or otherwise tune microneedle properties for specific needs. To achieve this control over microneedle properties, we fabricated microneedles by copolymerizing two liquid monomers – vinyl pyrollidone (VP) and methacrylic acid (MAA) – to form poly(vinylpyrrollidone-co-methacrylic acid) (PVP-MAA). We chose MAA as the second monomer because it is nontoxic, is liquid in monomeric form, has been used in the past for drug delivery purposes and has a high mechanical strength due to the rigidity of its chemical backbone [79]. In addition, a copolymer of PVP-MAA could have additional mechanical strength from hydrogen bonding between the side chains of the VP and MAA monomeric units of the polymer [80].

As shown in Figure 4.3B, the mechanical strength (fracture force) of the copolymer PVP-MAA containing just 1% MAA is nearly double the mechanical strength of the homopolymer PVP and steadily increases as the methacrylic acid content is increased (ANOVA, $p<0.001$), such that PVP-MAA microneedles containing 75% MAA exhibit more than a four-fold increase in strength. Stronger polymer microneedles could

be advantageous for drug delivery to tougher tissue sites of the body where insertion is more difficult.

In addition, dissolution studies showed that the dissolution rate decreases with increasing MAA content, such that PVP-MAA microneedles containing 25% MAA dissolve after approximately 4 h within porcine skin *in vitro* (data not shown). Polymer microneedles with fast dissolution rates would be attractive for rapid delivery scenarios, such as vaccinations, where microneedles can be inserted, removed, and discarded without making the patient wait. Polymer microneedles with slower, controlled dissolution rates could be desirable for situations where controlled release of a drug over time is optimal. These slower dissolving microneedles could be designed in the future to quickly deposit within the skin by separating the base from the microneedles, which then dissolve slowly over time within the skin

Concerning safety, gel permeation chromatography analysis of PVP microneedle dissolution products determined that the average molecular mass of PVP is 8,970 Da with a polydispersity of 1.42. Given that PVP with molecular mass less than 20,000 Da has been shown to be safe for human use due to efficient clearance by the kidney [149], the low measured molecular mass suggests that PVP microneedle dissolution products can be safely excreted from the body.

As shown in Figure 4.3A, PVP microneedles are sharp and strong enough to insert into the skin. However, this assay does not determine the depth of insertion. Due to the elastic nature of the skin, even microneedles which are strong and sharp enough to insert will first deform the skin surface prior to insertion. Since delivery from these polymer microneedles requires needle dissolution within the skin to release the

encapsulated cargo, it is important to determine the depth of insertion. In addition, it could be beneficial to deliver drugs to specified depths within the skin, for example targeting dendritic Langerhans cells found in the epidermis for vaccination purposes [150]. To determine the depth of insertion, polymer microneedles were inserted into porcine cadaver skin *in vitro*, and histological sections were processed from the frozen samples. Figure 4.4A shows a cross section of skin after insertion of a PVP microneedle with encapsulated sulforhodamine. Figure 4.4B shows the same tissue sample after needle removal and H+E staining to visualize the layers of the skin and the hole left by microneedle insertion. The 750 μm-long microneedles inserted almost completely into the skin, which suggests that the entire drug encapsulated within would be efficiently delivered.

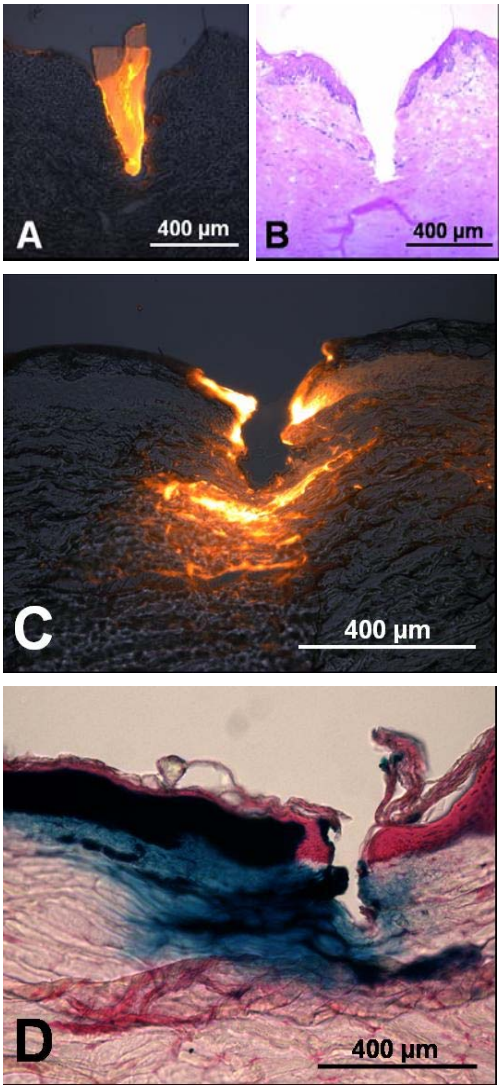

Figure 4.4: Microneedle insertion and protein delivery into skin. 4.4A: Fluorescence microscopy image of a PVP polymer microneedle with encapsulated sulforhodamine inserted into porcine skin. 4.4B: Brightfield microscopy image of the same skin section after microneedle removal showing the depth of microneedle insertion, stained with hemotoxilin and eosin. 4.4C: Fluorescence microscopy image showing delivery of fluorescently labeled bovine serum albumin by PVP polymer microneedles to porcine skin. 4.4D: Brightfield microscopy image of delivery of enzymatically active β-galactosidase via PVP polymer microneedles to porcine skin. The blue color represents the enzymatic conversion of X-gal by the delivered β-galactosidase

4.2.3 Delivery of active biomolecules via PVP polymer microneedles

The ultimate goal of this study was to produce polymer microneedles that can successfully encapsulate and deliver active biomolecules. To assess this objective, red-fluorescent bovine serum albumin was encapsulated within PVP polymer microneedles and delivered to porcine skin. Figure 4.4C shows a histological section prepared 15 min after microneedle insertion. The fluorescent protein has been delivered to both the dermis and epidermis and it has diffused a short distance away from the insertion site. This demonstrates the ability of the new polymer microneedles to deliver a biomolecule to the skin.

To assess if biomolecules can retain activity after encapsulation within polymer microneedles, we encapsulated another model protein, β-galactosidase, in PVP microneedles; dissolved them in PBS; and measured enzymatic activity of the resulting solution (further details can be found in Section 3.1.6 in Chapter 3 of this thesis). The normalized activity of β-galactosidase after encapsulation and release from polymer microneedles was 0.99±0.01, (n=5) which was statistically indistinguishable from (i) a solution of β-galactosidase in PBS (1.00±0.00) and (ii) a solution of β-galactosidase in PBS containing dissolved PVP from empty microneedles (0.99±0.01). This demonstrates that the *in situ* polymerization fabrication and microneedle dissolution processes are gentle enough to retain the activity of an encapsulated biomolecule. As further validation of this result, Figure 4.4D shows a histological section of porcine skin after delivery of β-galactosidase from PVP microneedles and exposure to X-gal. The enzymatic conversion of the X-gal substrate by β-galactosidase to its blue-colored product demonstrates that the β-galactosidase delivered in the skin is enzymatically active.

4.3 CONCLUSIONS

These findings suggest that rapidly dissolving polymer microneedles offer an exciting new drug delivery alternative to the hypodermic needle. They combine the painless, self-administrative abilities of the transdermal patch with the ability to deliver biotherapeutics, which is possible in current clinical practices only using hypodermic needles in most cases. The polymer microneedles created by the new *in situ* polymerization fabrication process developed in this study dissolve within the skin within a minute, thereby delivering the encapsulated cargo and leaving behind no biohazardous sharps associated with dirty needles.

The gentle nature of this new fabrication process allows for the encapsulation of fragile biomolecules and its universality allows for the use of many different copolymer systems, which could lead to the creation of other molded drug delivery devices. In addition, this process allows tuning of the mechanical strength and dissolution rate of the structural polymer material depending on the delivery site and the time course for the molecule to be delivered. These polymer microneedles were shown to successfully insert into the skin and deliver an encapsulated active protein. This new drug delivery platform shows future promise for the delivery of a range of biomolecules, including vaccines, proteins, peptides and nucleotides.

CHAPTER 5

IN VITRO AND IN VIVO ANALYSIS OF THE REFORMULATED INFLUENZA VACCINE FOR DELIVERY VIA DISSOLVING POLYMER MICRONEEDLES

5.1 INTRODUCTION

The influenza virus is responsible for tens of thousands of deaths each year in the United States and causes millions of illnesses worldwide[113]. While over 100 million individuals were vaccinated against the virus last year, there are some major issues with the vaccination process. It has low patient compliance, requiring a painful injection via hypodermic needle and can be time consuming due to the need for trained medical personnel for delivery[14, 15]. Also, there can be issues with accidental re-sticks of biohazardous sharps and the need for cold chain storage of the liquid solution of the vaccine[16, 151]. One exciting alternative delivery method is the use of microneedles for the influenza vaccine. Microneedles offer a patient compliant drug delivery alternative that is effective, relatively painless and could be self-administered in the future [1, 10].

One microneedle-based option involves rapidly dissolving polymer microneedles made of the biocompatible polymer, polyvinylpyrrolidone (PVP). The influenza vaccine would be encapsulated within the PVP microneedles and after insertion, the polymer needles dissolve in the skin, delivering the encapsulated vaccine and leaving behind no biohazardous sharps waste. Also, since the vaccine is in solid form in the microneedle, the system may bypass any need for the cold chain and could be stored at room temperature. However, there are formulation and processing steps that should be analyzed prior to the use of PVP polymer microneedles for the delivery of the influenza

vaccine. In this section, we will discuss the formulation and processing steps required for the encapsulation and delivery of the influenza vaccine in PVP microneedles. Also, the use of sugars will be analyzed for their importance in retaining the activity of the virus during the processing steps.

The new fabrication process for polymer microneedles involving photopolymerization of a liquid monomer solution within a microneedle mold was used in this study. Using this process, the drug needed to be in solid form prior to encapsulation within the microneedles. The form of the influenza vaccine that was used in this study was the inactivated form of the virus, which is typically stored in solution. Thus, the virus must first be processed into solid form, and the primary method used was lyophilization.

Lyophilization, or freeze drying, has become a widely used process in the biopharmaceutical industry[107]. The main use of this process is to increase the stability and long-term lifespan of biologically related molecules, especially proteins, by converting a liquid solution into a solid powder[94, 95]. For our application, the main use of lyophilization was to convert the liquid solution of inactivated virus into stable solid particles that retain full activity. After the virus solution is lyophilized, the solid particles were dissolved or suspended in the vinylpyrrolidone liquid monomer solution. This monomer-virus mixture was then used in the fabrication process detailed in Chapter 4; including UV initiated free-radical polymerization, to create PVP polymer microneedles with the influenza vaccine encapsulated within the microneedle tips. Figure 5.1 below shows a representative image of PVP microneedles with an

encapsulated molecule. Here, the pink dye represents the influenza vaccine, and it is only encapsulated in the microneedle tips and not the base of the microneedles.

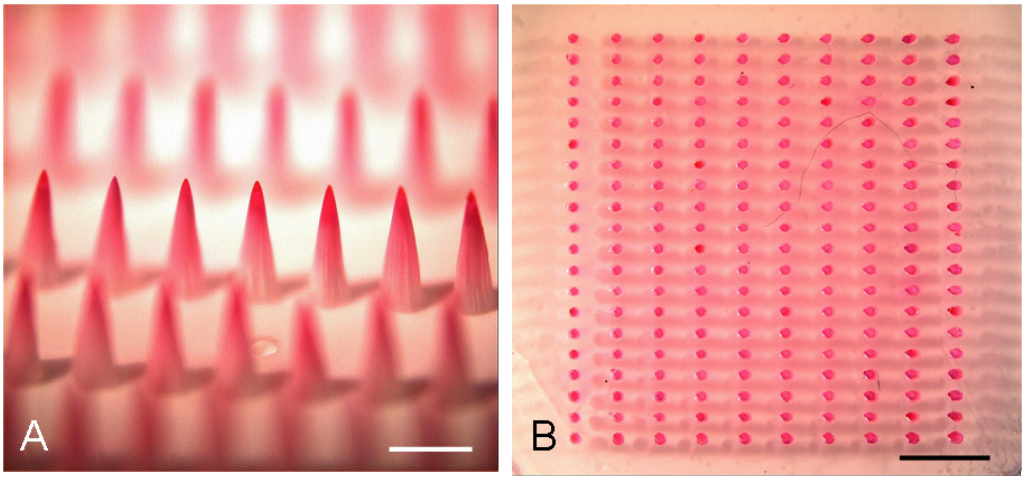

Figure 5.1: PVP Microneedles. Polyvinylpyrrolidone microneedles with model vaccine (sulforhodamine) encapsulated within tips of microneedles. 5.1A: Side view of microneedles, scale bar = 500 µm. 5.1B: Overview of array of microneedles, scale bar = 2 mm.

The main goal of this study was to determine if the inactivated influenza virus retains activity after lyophilization and encapsulation within the new PVP polymer microneedles. This was tested in vitro by qualitative ELISA and by the hemagglutination assay (HA) and in vivo by injecting solutions of the processed virus into mice, and measuring the antibody levels and survival of mice against a lethal challenge.

5.2 RESULTS

5.2.1 Reformulation of the influenza vaccine in polymer microneedles

Typically, the inactivated influenza virus is found in a liquid solution for delivery via the hypodermic needle. Due to the low stability of the virus in liquid solution[97, 99], it needs to be stored in the cold chain, which can be expensive and cumbersome in

transport and delivery of the vaccine[151]. Thus, alternate storage methods have been developed involving lyophilization to convert the liquid virus solution to a solid form[94, 95].

Previous work involving the lyophilization of the influenza virus included the use of sugars as stabilizers to help protect the virus during the freezing and drying steps of lyophilization. In this work, the influenza virus retained full activity during the lyophilization process when 50:1 of the sugar trehalose was added to the original solution[101]. Thus, in this study, the sugar trehalose was used as an excipient in varying amounts to test the effect of the excipient on retaining the activity of the influenza virus during lyophilization.

5.2.2 Antigenicity and immunogenicity of the H1N1 influenza virus after lyophilization and polymer microneedle processing

In the first pilot study, the antigenicity and immunogenicity of the inactivated H1N1/A/PR/8 influenza virus was measured after lyophilization and encapsulation within PVP polymer microneedles. The inactivated H1N1/A/PR/8 influenza virus was dialyzed against water prior to lyophilization. The virus was initially stored in PBS, but it has been shown that lyophilization of the influenza virus in a PBS solution resulted in a large decrease of activity due to pH effects [101]. The following groups were lyophilized: inactivated virus with no sugar, inactivated virus with 5:1 by mass trehalose, inactivated virus with 10:1 by mass trehalose, and inactivated virus with 50:1 by mass trehalose. Full details of the lyophilization process can be found in Section 3.3.1 in Chapter 3 of this thesis. After lyophilization, for each of these groups, half of the samples of lyophilized powder were reconstituted in water for determination of the

antigenicity. The other half of the samples was encapsulated within PVP polymer microneedles. Here, the virus with or without sugar, was suspended in liquid vinylpyrrolidone monomer, added to the microneedle mold and placed under UV lamp, where free radical polymerization occurred. The polymer microneedles with encapsulated influenza virus were then dissolved in water. In addition, the following controls were tested. Unprocessed influenza virus and unprocessed influenza virus with blank PVP polymer microneedles dissolved in solution. This latter control was used to determine if the presence of the polymer PVP in solution decreased the antigenicity of the virus alone.

The antigenicity of the inactivated H1N1 virus after processing was tested by measuring the binding of specific antibodies to the virus in an ELISA. Figure 5.2 below shows the relative antigenicity of the different virus solutions compared to the unprocessed control.

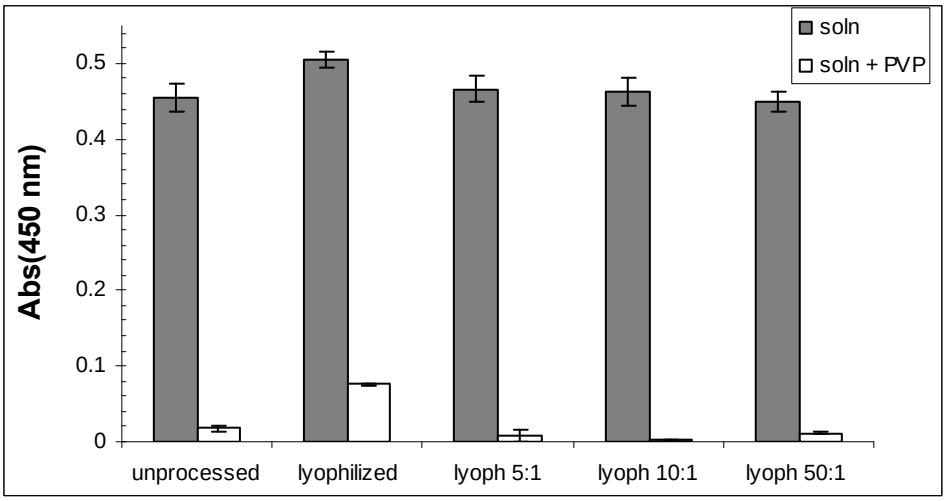

Figure 5.2: Virus Antigenicity after PVP polymer microneedle processing. Absorbance from ELISA read at 450 nm testing the binding of the processed virus to antibodies specific against the H1N1/A/PR/8 inactivated influenza virus. The effect of lyophilization and processing through PVP polymer microneedles on the virus is measured, as well as the inclusion of excipients. The ratios represent the amount of (sugar : virus) in the sample. Gray bars represent unprocessed virus solutions or virus that was lyophilized. White bars represent processed virus that was encapsulated in PVP polymer microneedles, or unprocessed virus with blank PVP polymer in solution. Virus concentration = 10 µg/mL, (n=3).

In general, the lyophilized samples retained full activity of the virus while the samples including the PVP polymer had a sharp decrease in activity. The presence of the sugar trehalose did not have any affect on the antigenicity of the virus (t test, $p>0.05$). Also, the unprocessed virus solution including PVP showed a large decrease in antigenicity (t test, $p<0.05$). It is possible that the presence of PVP in the solution provided a false negative in this assay due to improper coating of the plates with the virus solutions that included PVP. It was proposed to take this work in vivo to further analyze the effect of lyophilization and PVP polymer microneedle processing on the virus.

For this first in vivo pilot study, 20 µg doses of the H1N1 solutions detailed above were injected into mice (n=3). The mice were bled fourteen days later and the specific

IgG antibodies were determined. Figure 5.3 below shows the IgG titers after one injection.

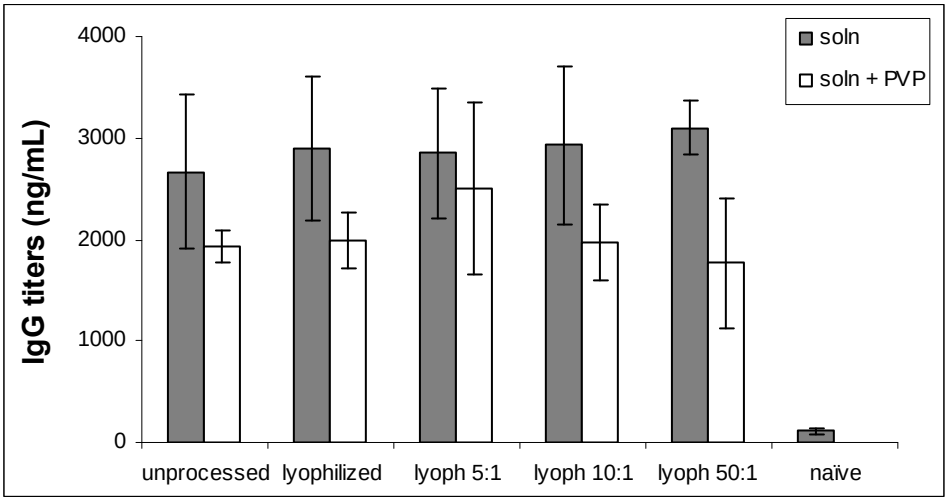

Figure 5.3: Measurement of the antibody immune response after one immunization with processed H1N1 influenza virus. Sera IgG antibodies. Mice (n=3) were immunized with 20 µg of the inactivated H1N1/A/PR/8 inactivated virus and bled 14 days after immunization. The gray bars represent the unprocessed virus or virus that was lyophilized and then dissolved in solution. The white bars represent virus that was lyophilized and encapsulated in PVP microneedles, or unprocessed virus with blank PVP polymer in solution

Overall, lyophilization had no influence on the immunogenicity of the virus, with each of the lyophilized samples producing similar IgG titers (t test, $p>0.05$). In addition, the inclusion of the excipient sugar did not have a noticeable influence on the immune response. On the other hand, the interaction of the polymer PVP in solution with the virus resulted in a slight decrease in the immune response compared to the lyophilized sample (t-test, $p<0.05$). This occurred in both the virus solutions that were processed through PVP polymer microneedles and the unprocessed virus that had blank PVP in solution. However, this immune response was still considerable and may be enough to instill protection against lethal challenge.

5.2.3 Antigenicity of the H3N2 influenza virus after lyophilization and polymer microneedle processing

The initial pilot study showed that the inactivated H1N1 influenza virus remained immunogenic after lyophilization and processing through PVP polymer microneedles. Since the current influenza vaccine contains both the H1N1 and H3N2 strains of the virus[124], it was important to confirm these studies with an H3N2 stain of the virus. Here, we tested the effect of lyophilization and encapsulation within polymer microneedles on the inactivated H3N2/A/Aichi virus. Once again, the effect of the stabilizer trehalose was also investigated. The following groups were lyophilized (n=6): inactivated virus with no sugar, inactivated virus 5:1 trehalose by mass and inactivated virus with 10:1 trehalose by mass. For each of these groups, half of the lyophilized samples (n=3) were encapsulated within PVP microneedles using the photopolymerization process. The microneedle arrays were then dissolved in water and tested for antigenicity and immunogenicity. The other half of the lyophilized samples (n=3) were also dissolved in water and tested. The positive control was the unprocessed virus and a solution of the unprocessed virus that included blank PVP polymer dissolved.

The antigenicity of the H3N2/A/Aichi virus was analyzed using the hemagglutination (HA) assay, which measures the ability of the virus to agglutinate red blood cells. The HA activity of the unprocessed virus was 12.8 HAU/µg. All of the lyophilized samples had identical activities, showing again that lyophilization does not decrease the antigenicity of the virus. The solutions that contained the PVP polymer all had decreased HA values, of 0.8 HAU/µg, including the unprocessed virus in solution with blank PVP microneedles. However, the presence of a large amount of PVP solution (1000**:**1 by weight, PVP**:**virus) may have produced a false negative for this assay similar

to the results shown in the ELISA assay used with the H1N1 virus. Thus, once again it will be necessary to evaluate the influence of the PVP polymer microneedle processing in vivo. In addition, since it was reconfirmed that the presence of sugars was not needed to protect the virus during lyophilization; the in vivo analysis did not include any of the groups with trehalose.

5.2.4 Immunogenicity of the H3N2 influenza virus after lyophilization and polymer microneedle processing

For the in vivo analysis of the immunogenicity of the virus after processing, a larger experiment was planned, including 10 mice per group. The mice were injected with 10 μg of virus and bled 14 days later to test the IgG antibody levels and the HAI inhibition antibodies. Thirty days after the immunization, half of the mice (n=5) were challenged with 10 LD_{50} of the live virus and the protection was measured by body weight. Figures 5.4, 5.5 and 5.6 below show the immune response after one immunization.

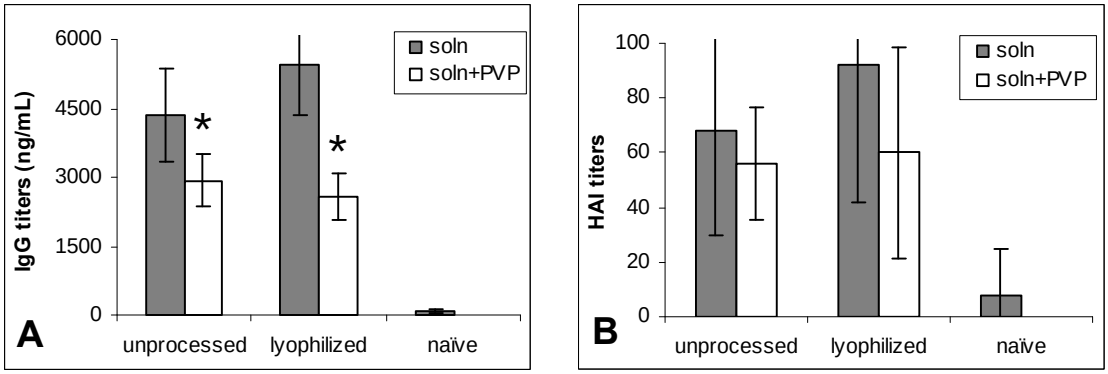

Figure 5.4: Measurement of the antibody immune response after one immunization with processed H3N2 influenza virus. 3A: Sera IgG antibodies. 3B: HAI titers. Mice (n=10) were immunized with 10 µg of the inactivated H2N2/A/Aichi influenza virus and bled 14 days after immunization. The gray bars represent the unprocessed virus solution or virus that was lyophilized and then dissolved in solution. The white bars represent virus that was lyophilized and encapsulated in PVP microneedles, or unprocessed virus with blank PVP polymer in solution. * indicates a statistically significant difference (t test, $p<0.05$) compared to the group without the PVP polymer.

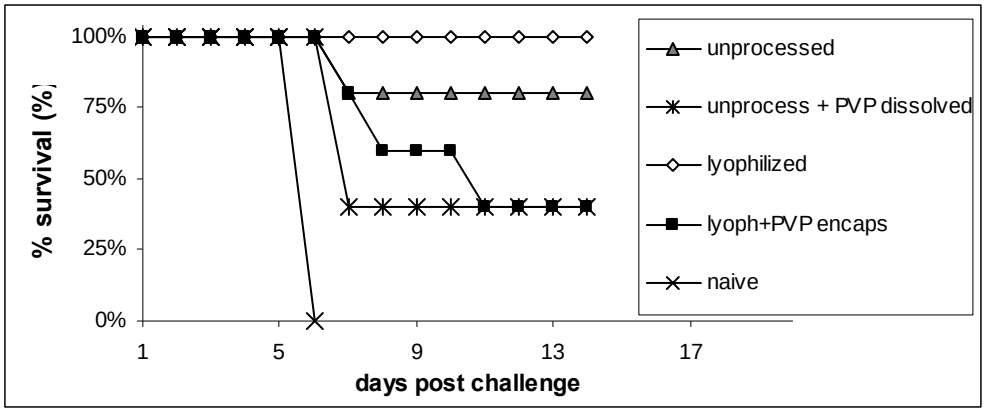

Figure 5.5: Protection against lethal challenge after one immunization: Survival analysis. Mice (n=5) were immunized with 10 µg inactivated H3N2/A/Aichi influenza virus and challenged with 10 LD_{50} of live H3N2 virus thirty days after immunization. Mice which lost more than 25% of their body weight were considered terminally ill and euthanized.

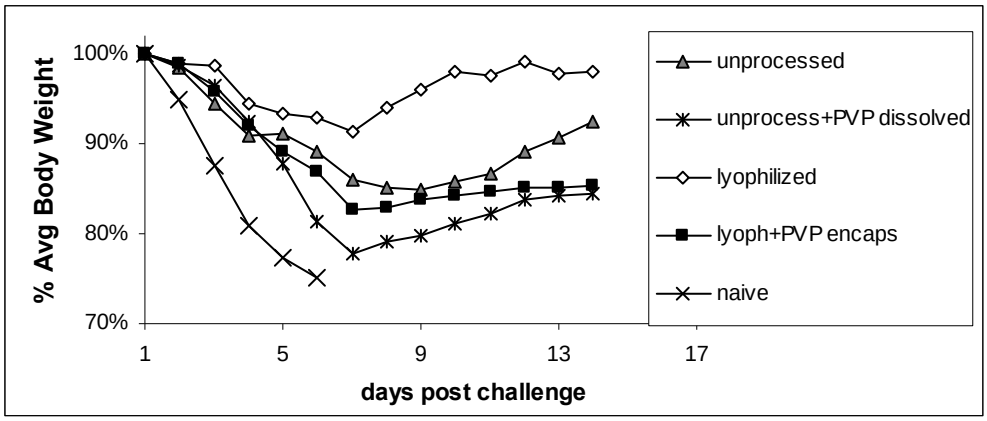

Figure 5.6: Protection against lethal challenge after one immunization: Average weight loss. Mice (n=5) were immunized with 10 µg inactivated H3N2/A/Aichi influenza virus and challenged with 10 LD_{50} of live H3N2 virus thirty days after immunization

After one immunization, it was shown that lyophilization had no effect on the immunogenicity of the virus, with the lyophilized group having slightly higher IgG and HAI titers than the mice immunized with the unprocessed virus (t test, $p>0.05$). The survival experiment further confirmed these results with all of the mice immunized with lyophilized virus surviving the challenge

While lyophilization had no negative effect on the immunogenicity of the virus, the presence of PVP in solution showed a decrease in the immune response. The group including the virus processed through PVP microneedles showed lower IgG titers than the positive control of the unprocessed virus (t test, $p<0.05$). Also, the unprocessed virus that included blank PVP polymer in solution had decreased IgG titers. However, the IgG titers for these two groups that had PVP in solution were statistically the same (t test, $p>0.05$). The HAI titers were inconclusive due to a false positive signal in the assay. Following the IgG titers, similar results were shown in the challenge experiment. The groups with PVP present, either in blank polymer in solution or encapsulated virus in the

polymer, had a lower survival weight, with only 2/5 mice living after a prime immunization and a maximum weight loss of around 20%. It appeared that the presence of PVP in solution had a deleterious effect on the immunogenicity of the virus. However, it is unclear whether the encapsulation process had a harmful effect on the virus or if the lower immune response was simply due to the presence of PVP polymer in solution with the virus.

Twenty eight days after the first immunization, the other half of the mice (n=5) were boosted with 10 μg of the different processed virus solutions. These mice were bled fourteen days after the immunization to measure the IgG antibodies and HAI inhibition antibodies. Twenty eight days after the boost, these mice were also challenged with 10 LD_{50} of the live virus and the protection was measured via body weight. Figures 5.7, 5.8, 5.9 below show the results after prime and boost immunizations.

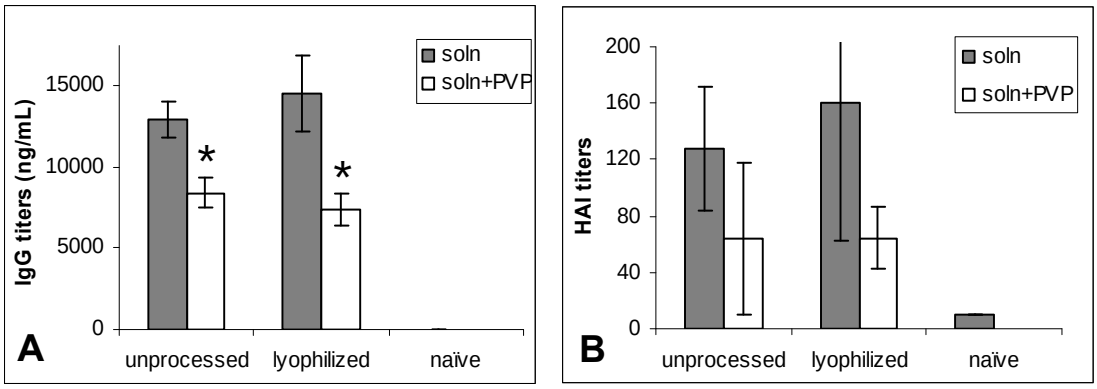

Figure 5.7: Measurement of the immune response after prime and boost immunizations with processed H3N2 influenza virus. 3A: Sera IgG antibodies. 3B: HAI titers. Mice (n=5) were prime and boost immunized with 10 μg of the inactivated H2N2/A/Aichi influenza virus and bled 14 days after immunization. The gray bars represent the unprocessed virus solution or virus that was lyophilized and then dissolved in solution. The white bars represent virus that was lyophilized and encapsulated in PVP microneedles, or unprocessed virus with blank PVP polymer in solution. * indicates a statistically significant difference (t test, $p<0.05$) compared to the group without the PVP polymer.

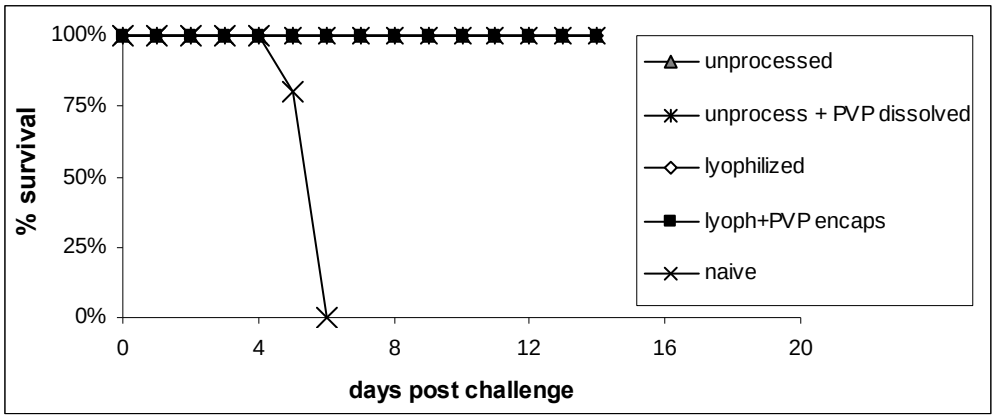

Figure 5.8: Protection against lethal challenge after prime and boost immunizations: Survival. Mice (n=5) were prime and boost immunized with 10 µg inactivated H3N2/A/Aichi influenza virus and challenged with 10 LD_{50} of live H3N2 virus thirty days after immunization. Mice which lost more than 25% of their body weight were considered terminally ill and euthanized, per the Emory IACUC.

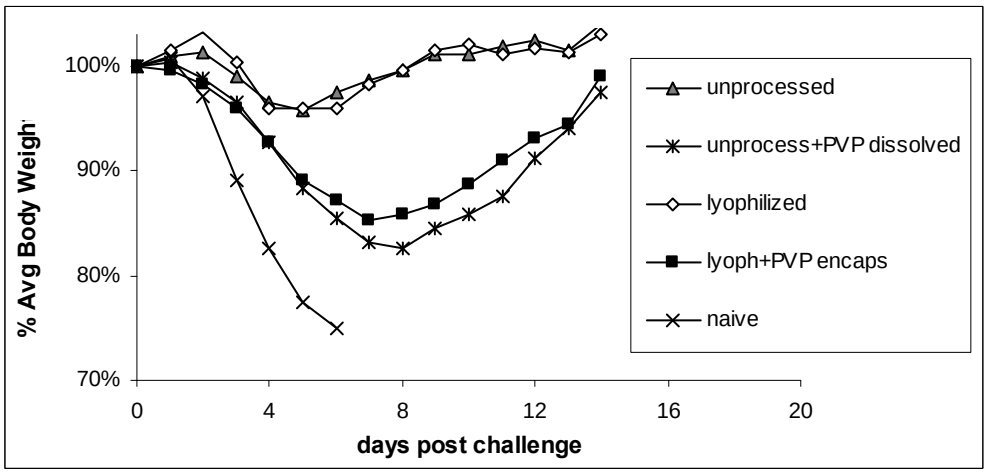

Figure 5.9: Protection against lethal challenge after prime and boost immunizations: Average weight loss. Mice (n=5) were prime and boost immunized with 10 µg inactivated H3N2/A/Aichi influenza virus and challenged with 10 LD_{50} of live H3N2 virus thirty days after immunization.

The IgG and HAI results after the boost immunization were similar qualitatively to the prime sera results. There was no decrease in the IgG or HAI levels for the

lyophilized group compared to the unprocessed virus (t test, $p>0.05$). Similarly, the groups with PVP in solution had decreased antibody levels from the positive controls (t test, $p<0.05$). This included both the unprocessed virus in solution with blank PVP polymer and the virus that was lyophilized and encapsulated in PVP polymer microneedles.

The level of immunoprotection was determined via a lethal challenge. Here, all of the mice in the groups for the lyophilized virus and the unprocessed virus survived and had very little loss of body weight (4% of original weight). The groups with PVP in solution had a greater loss of body weight (15%), but all of the mice in these groups also survived the challenge. All of the naïve mice died rapidly in six days. These results show that while there is a decrease in immune response with virus in solution with the PVP polymer, this virus is still immunogenic and can provide protection against the lethal challenge after boost.

5.3 DISCUSSION

Polymer microneedles offer an exciting delivery option for the influenza vaccine. In fact, they offer a number of advantages over the hypodermic needle. This includes minimal pain, self-administration, easy intradermal delivery, and no biohazardous sharps after delivery. Also, these microneedles could be administered in a mass immunization setting with more ease than the current needle injection due to easier disposal and the lack of need for medically trained personnel. However, there are a number of reformulation steps that were required for the influenza vaccine to be encapsulated and delivered via polymer microneedles. This included lyophilization of the liquid virus

solution into a solid powder and encapsulation within polymer microneedles, which involves free radical polymerization.

The goal of this study was to test the antigenicity in vitro and immunogenicity in vivo of the influenza virus after lyophilization and processing through polymer microneedles. This is an important analysis prior to the use of polymer microneedles for the delivery of the influenza virus in vivo. Also, the addition of sugars to the virus solution was tested to determine the optimal formulation for the virus solution for protection during the freeze drying process. Since the current influenza vaccine contains the H1N1 and H3N2 strains of the virus, both of these strains were tested in this study. The analysis into the processed influenza virus can be divided into three parts: in vitro assays to test the antigenicity of the virus, analysis of the sera after immunization to test the immunogenicity of the virus, and lethal challenge experiments to analyze the protection imparted by the delivery of virus to the mice.

The in vitro assays had similar results for both the H1N1/A/PR/8 and H3N2/A/Aichi viruses. The in vitro studies showed that lyophilization, with or without the addition of sugars, did not affect the antigenicity of the virus. However, it was difficult to determine the effect of the encapsulation process on the in vitro activity of the influenza virus since the PVP polymer conflicted with the assays used to measure the antigenicity.

Due to the possible complications with the in vitro assay for virus solutions that include PVP, the in vivo analysis was important to determine if lyophilization and encapsulation within PVP microneedles affects the activity of the influenza virus. For the H1N1 virus, the in vivo analysis showed that lyophilization did not decrease the antibody

response, with or without the presence of the trehalose. However, all of the groups that included PVP had decreased IgG antibody levels. This included the group with the unprocessed virus with blank PVP polymer in solution, and the group with the virus processed through PVP polymer microneedles. These groups had very similar IgG titers leading to the analysis that the presence of PVP in solution with the virus decreases the virus activity.

In vitro and in vivo analysis showed that the influenza virus retains full activity after lyophilization with or without the presence of excipients. Thus, it was decided that sugars would not used for any further experiment. The lack of sugar is quite important for the final delivery of the virus through PVP polymer microneedles. There is a limit to the amount of total mass that can be encapsulated in the microneedles. If sugars were required for lyophilization, especially in high amounts, than the encapsulation efficiency of the vaccine in the microneedles would be severely diminished. The lack of sugars in the lyophilized powder allows for a much higher amount of vaccine to be encapsulated within the microneedles, on the order of 3-10 μg per array, which has been shown to be enough to elicit a protective immune response in animals. By altering the design of the microneedle array, this dosage could be increased even further to approximately 30 – 50 μg/array.

A larger experimental plan was carried out using the H3N2/A/Aichi virus, including lethal challenge experiments to determine if the processed virus could impart immunoprotection. These results were quite similar to the results using the H1N1 virus. The in vivo experiments also showed that lyophilization had no impact on the immunogenicity of the influenza virus. After prime and boost immunizations, the sera

IgG and HAI titers were actually higher for the lyophilized group than the unprocessed solution. Once again, the presence of PVP decreased the immune response in regards to both the IgG and HAI titers. However, these titers were still quite high (IgG =5600 ng/mL and HAI = 64), especially compared to the naïve group (IgG = 72ng/mL, HAI = 10). The lethal challenge showed corresponding results. After prime and boost immunizations, all of the mice in the processed or unprocessed virus groups lived, with all of the naïve mice dying within seven days of the challenge. However, while the lyophilized and unprocessed groups after prime and boost lost little to no weight (4% of original body weight) the PVP processed groups lost more weight (14-18% of original body weight). This showed that while the virus was still immunogenic after the interaction with the PVP polymer, the response was not as strong as the lyophilized and unprocessed groups.

Overall, these results show that lyophilization does not decrease the antigenicity or immunogenicity of the H1N1/A/PR/8 or H3N2/A/Aichi viruses. The presence of the polymer PVP in the virus solution decreases the immunogenicity of the virus, but the virus still induced a strong immune response. This included the unprocessed virus in solution with blank PVP polymer, which did not include any lyophilization or encapsulation with PVP polymer microneedles. Prior research has shown that the polymer PVP in solution can degrade proteins, specifically by deamination. In fact, the rate of protein denaturation in a solution with PVP polymer can be up to 10,000 times faster than the denaturation in solid PVP form[152]. This could be the source of the decrease in immunogenicity. Typically, the PVP and virus interacted in solutions for a few days prior to immunization. There are two reasons why this should not affect PVP

microneedle delivery of the influenza virus. First, for microneedle-based delivery, the virus and polymer will not interact in solution for an extended period of time. During the encapsulation process, the virus will only interact with the PVP polymer in solid form and not in solution. Also, when the polymer microneedles dissolve in the skin and the virus is released, the polymer will diffuse away and have little interaction with the virus in the body. Second, the amount of PVP/virus ratio that will be in contact will be decreased from 1000:1 ratio in this experiment to a 100:1 ratio with microneedle delivery since there is no polymer backing to be dissolved as was used in this study. It is believed that these two factors should allow for a higher immune response when the virus is delivered via solid PVP polymer microneedles.

5.4 CONCLUSIONS

Polymer microneedle-based delivery of the influenza vaccine offers a number of advantages over the current hypodermic injection. This includes minimal pain, self-administration, lack of need for trained personnel, fast administration, and no biohazardous sharps waste. However, for the influenza vaccine to be delivered by this microneedle system, the virus must be reformulated, including lyophilization and encapsulation in the polymer microneedles. In vitro and in vivo analysis showed that lyophilization did not affect the antigenicity or immunogenicity of the H1N1/A/PR8 or H3N2/A/Aichi virus strains. Mice immunized with the lyophilized virus elicited high antibody titers and were completely protected against the lethal challenge. Mice immunized with the influenza virus in the presence of the PVP polymer in solution had a decreased immune response in comparison to mice immunized with the unprocessed virus. However, mice immunized with the virus processed through polymer

microneedles were fully protected against lethal challenge after a prime and boost immunization. In fact, it appeared that the decreased immune response was due to the deleterious interactions between the polymer PVP and the virus in solution and not the polymer microneedle encapsulation process. Looking forward, the next steps of this research should be use polymer microneedles to deliver the processed influenza vaccine and determine if this delivery method can induce a significant immune response, including full protection against a lethal challenge. These experiments are covered in Chapter 7 of this thesis.

CHAPTER 6

DELIVERY OF THE INFLUENZA VACCINE VIA COATED METAL MICRONEEDLES TO MICE TO INDUCE A PROTECTIVE RESPONSE AGAINST LETHAL CHALLENGE

6.1 INTRODUCTION

The influenza virus is responsible for tens of thousands of deaths each year in the United States and causes millions of illnesses worldwide[113]. While over 100 million individuals were vaccinated against the virus last year, there are some major issues with the vaccination process[115]. It has low patient compliance, requiring a painful injection via hypodermic needle and can be time consuming due to the need for trained medical personnel for delivery[14, 15]. Also, there can be issues with accidental re-sticks of biohazardous sharps and the need for cold chain storage of the liquid solution of the vaccine[16, 151]. One exciting alternative delivery method is the use of microneedles for the influenza vaccine. Microneedles offer a patient compliant drug delivery alternative for the delivery of the influenza vaccine that is effective, relatively painless and could be self-administered in the future [1, 10].

Coated metal microneedles offer a reliable and effective delivery method for the influenza vaccine. In this delivery scenario, the influenza vaccine is coated onto the metal microneedles, and after the needles are inserted into the skin, the vaccine coating dissolves off of the needle within minutes and is delivered to the skin. Coated metal microneedles have been used effectively clinically for the delivery of ovalbumin[48], as a model antigen, and desmopressin[49], as a peptide therapeutic. One additional benefit of

using coated metal microneedles is that the vaccine coated onto the needles is in solid form. This may allow for the storage of the microneedle system at room temperature, which is not possible with the current influenza vaccine solution that requires refrigeration[151].

The goal of this study was to evaluate the capability of coated metal microneedles to deliver the inactivated influenza virus in vivo and induce a protective immune response. First, the delivery efficiency of the metal microneedles was tested in porcine skin in vitro and in mice in vivo. Also, the antigenicity of the virus was tested in vitro after coating onto the microneedles to determine if there was any loss of activity during the coating and drying process. Finally, mice were immunized with the influenza vaccine via metal microneedles and the immune response was tested via sera antibodies and protection against lethal challenge.

6.2 RESULTS

6.2.1 Antigenicity of the H3N2 influenza virus after processing and coating onto metal microneedles

Metal microneedles offer a promising delivery alternative to the hypodermic needle for the delivery of the influenza vaccine. However, there are some reformulation steps that need to be evaluated prior to the use of these microneedles for influenza vaccine delivery. In this project, the inactivated H3N2/A/Aichi influenza virus was used for vaccination purposes. For this virus to be delivered via metal microneedles, it must first be coated onto metal microneedles. Previous work has been done to optimize the coating conditions for metal microneedle based delivery [2, 153]. The coating solution contains carboxymethylcellulose and Lutrol F-68 as a viscosity enhancer and surfactant,

respectively [153]. These are two critical properties needed for effective coating onto the microneedle. This coating method has been shown to be quite versatile, with the ability to coat small molecules, proteins, DNA, viruses and microparticles onto metal microneedles[2]. Prior to the use of metal microneedles for influenza delivery in vivo, it was important to test the activity of the virus after coating onto the microneedles

The hemagglutination assay (HA) was used to test the antigenicity of the inactivated influenza virus after coating onto metal microneedles. This assay is primarily testing the activity of the surface hemagglutinin protein (H) of the virus, which is vital for viral entry into cells. A proper antibody response against this protein is thought to provide protection against the virus.

For all microneedle influenza work in this section, rows of five metal microneedles, 750 μm in height, 200 μm in width and 75 μm in thickness were used. The microneedles were fabricated using an infrared laser as previously described[2]. Inactivated H3N2/Aichi influenza virus was added to the coating solution and dip coated onto the rows of metal microneedles (Further details on the fabrication and coating of the microneedles can be found in Section 3.2.2 in Chapter 3 of this thesis). The coated microneedles were allowed to dry for approximately one hour and then the virus coatings were dissolved off using DI water and the resulting solution was tested for HA activity. Also, the effect of the coating solution on the influenza virus was tested by measuring the HA activity of a solution of unprocessed virus in coating buffer. Figure 6.1 below shows the relative activities of the virus solutions (n=3).

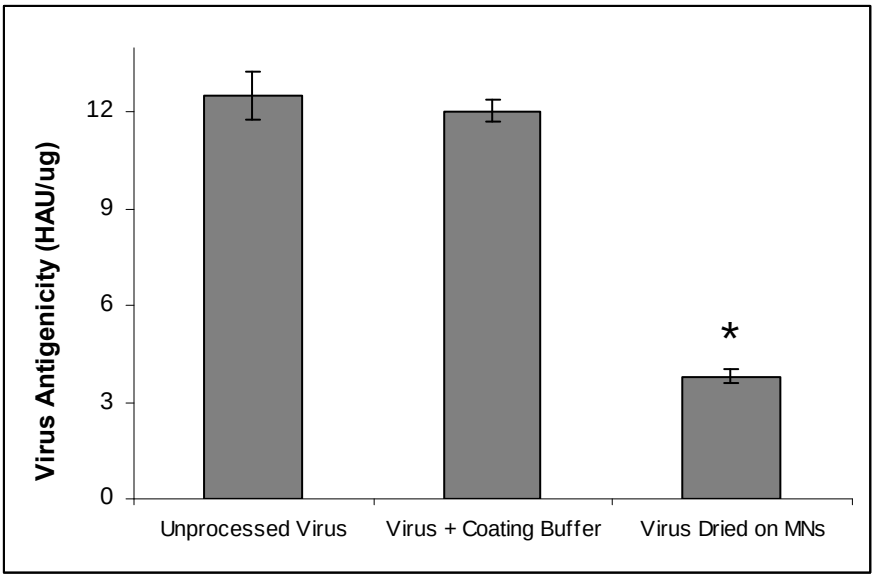

Figure 6.1: Virus Antigenicity (HAU/ug) after coating onto metal microneedles. (n=3 for each group.) Antigenicity is determined by hemagglutination (HA) assay normalized by protein concentration. * indicates a statistically significant difference (t test, $p<0.05$) compared to unprocessed virus.

As determined by the HA assay, the activity of the influenza virus decreased after coating and drying onto metal microneedles (t test, $p<0.05$). However, the virus that was placed in coating buffer, in a wet state, did not show a decrease in activity versus the unprocessed virus (t test, $p>0.05$). Since the virus coated onto the microneedles retained some activity (30%), it was decided to take this work in vivo to test the ability of the metal microneedles to deliver the influenza vaccine and induce a protective immune response.

6.2.2 In vitro and in vivo delivery efficiency of coated metal microneedles

Prior to the use of coated metal microneedles for the delivery of the influenza vaccine in vivo, it was important to evaluate the delivery capabilities of the metal microneedle rows. For this, two experiments were done. First, metal microneedles were

coated with vitamin B using the same coating solution and process as described previously. Vitamin B was used as the representative vaccine because it was easy to see visually, and thus easy to determine if the coating was delivered to the skin after insertion. Rows of metal microneedles were inserted into in vitro pig skin and left for one minute. Then, the needles were removed and inspected under a microscope. Figure 6.2 below shows the needles before and after insertion. After one minute, all of the coating was delivered to the skin and no coating was left on the row of microneedles, or the skin surface.

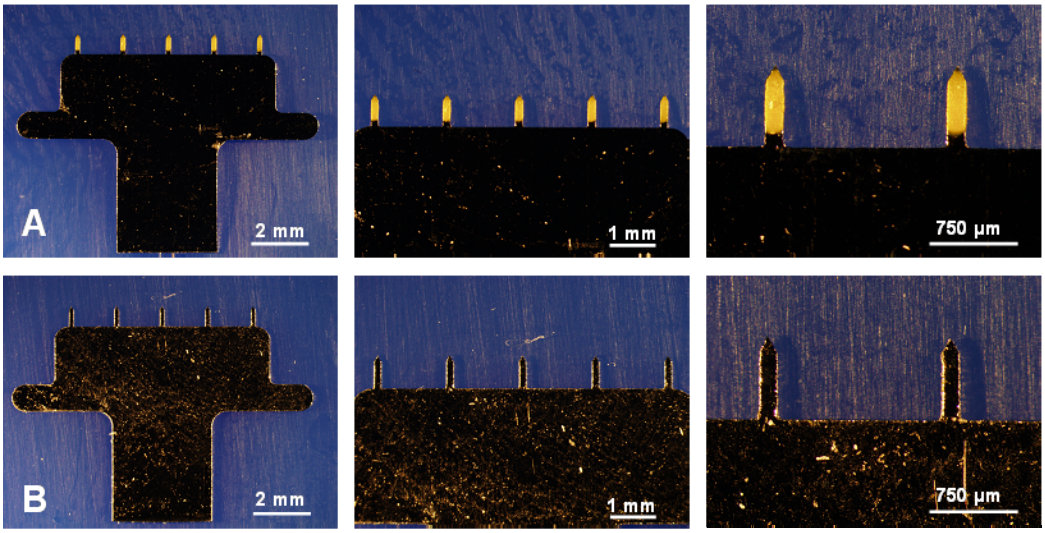

Figure 6.2: Metal Microneedle delivery in vitro: 2A: Row of metal microneedles coated with vitamin B preinsertion shown at different magnifications. 2B: Metal microneedles after insertion in pig skin for 1 minute. All microneedles are 750 µm in height, 200 µm in width and 75 µm in thickness.

The second delivery experiment involved evaluating the delivery efficiency of coated metal microneedles in vivo on a mouse. Mice are the most common animal used to evaluation the immune response for influenza delivery. In terms of delivery, mice offer challenges that do not exist in pig skin or larger animals, such as humans. This

includes preparation of the mouse skin and handling of the small animal during the insertion process. Thus it was important to evaluate the delivery efficiency of the metal microneedles on a mouse prior to the use of the influenza vaccine.

Prior to microneedle insertion, the hair of the mouse was removed using a topical cream (Nair). Next, rows of metal microneedles coated with sulforhodamine were inserted into mice. All of the coating was delivered after three to five minutes insertion into the mouse. Figure 6.3 below shows a mouse with metal microneedle arrays on its back. This shows the relative size of the mouse to the array. Also, Figure 6.3B shows the delivery site after insertion, showing pink dots where the insertion and delivery occurred. Overall, metal microneedles were easily inserted into the mouse and had efficient delivery. The next step was to coat the rows of microneedles with the inactivated H3N2 influenza virus and deliver it to mice to test the immune response.

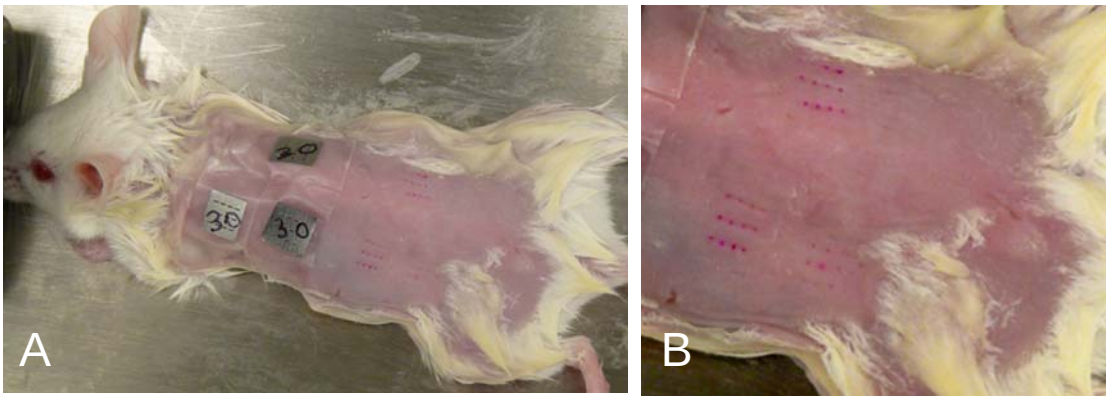

Figure 6.3: Mouse applied with arrays of metal microneedles: 6.3A: Full picture of mouse with multiple arrays of metal microneedles adhered to the back. 6.3B: Higher magnification picture of mouse skin after insertion and removal of coated metal microneedles. Pink dots signify delivery of sulforhodamine that was coated on metal microneedles. Photo courtesy of Vladimir Zarnitsyn.

6.2.3 Immune response after one immunization with the H3N2 influenza vaccine in mice via coated metal microneedles

For this study, inactivated H3N2/A/Aichi influenza virus was coated onto rows of metal microneedles. Mice (n=12) were immunized with 9.8 µg of the virus by microneedles or intramuscular injection. There were four experimental groups in this study: coated metal microneedles, intramuscular injection, naïve, and naïve-uncoated microneedles. The uncoated metal microneedles were used to test if the treatment of the mouse alone, including removal of hair and insertion of microneedles, would induce an immune response. After fourteen days, the mice were bled and the sera IgG antibodies and HAI antibody response were measured. Figure 6.4 below details the antibody immune response.

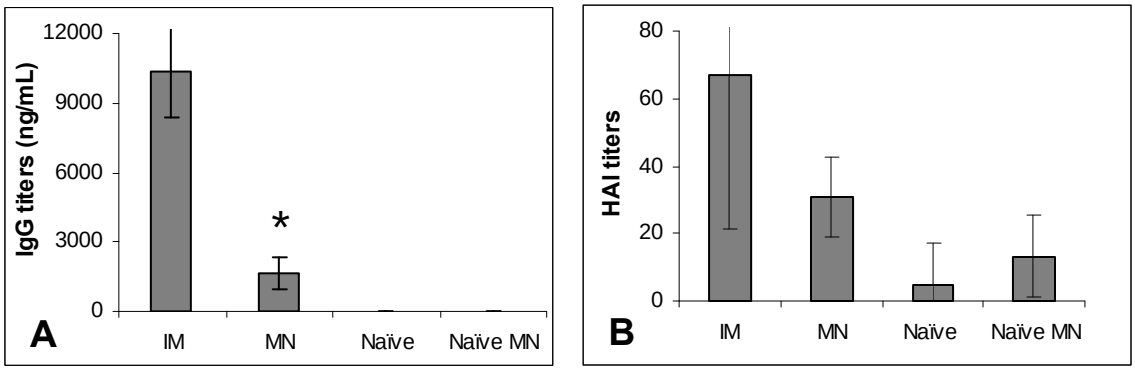

Figure 6.4: Measurement of the antibody immune response after one immunization with the H3N2 virus using coated metal microneedles: 6.4A: Sera IgG titers and 6.4B: HAI titers. Mice (n=12) were immunized with 9.8 µg of inactivated H3N2/Aichi influenza virus and bled 14 days later. The mice of the Naïve MN group were prepared the same way of the MN group, with removal of the hair. Uncoated MNs, with no vaccine, were inserted into these mice. * indicates a statistically significant difference (t test, $p < 0.05$) in comparison with intramuscular injection group.

Thirty days after immunization, half of the mice (n=6) from each group were challenged with a lethal dose of the live H3N2 virus (5 LD_{50}) to test the

immunoprotection after one immunization. After the challenge, the mice were weighed each day for fourteen days. If a mouse lost more than 25% of its body weight, it was considered terminally ill and euthanized as required by the IACUC protocol. Figure 6.5 below shows the average body weight loss and survival of the mice after this lethal challenge.

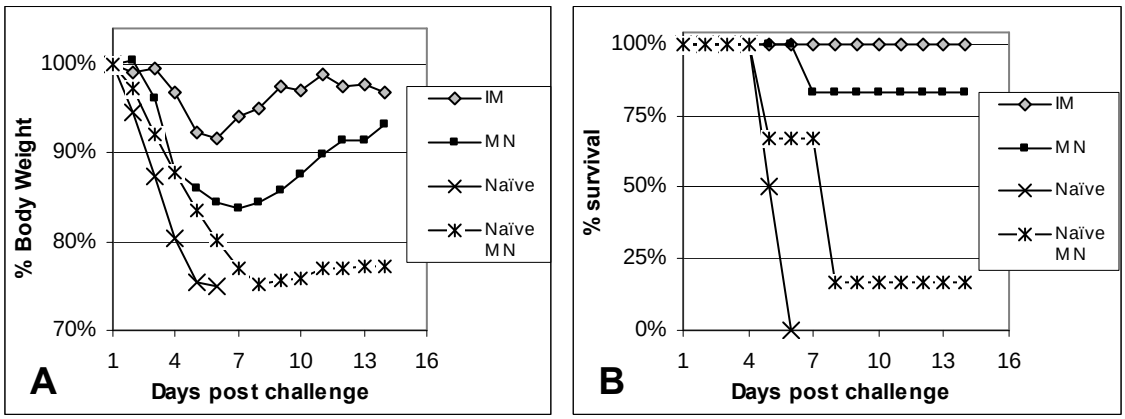

Figure 6.5: Protection against lethal challenge after one immunization of H3N2/Aichi influenza vaccine with coated metal microneedles. 6.5A: Average body weight after challenge. 6.5B: Survival data after challenge. Mice (n=6) were challenged with 5 LD50 of live H3N2/Aichi virus, 30 days after immunization. Mice which lost more than 25% of their body weight were considered terminally ill and euthanized, per the Emory IACUC

Overall, there are a few conclusions that can be made after one immunization. The mice immunized by coated microneedles had a lower immune response, in antibody levels and challenge, than the positive control (IM) group (t test, $p<0.05$). However, there still was a significant immune response in the microneedle group, with 5/6 mice surviving the lethal challenge, though with higher maximum weight loss (16%) than the IM group (8%). The mice that had blank microneedles inserted did have a slight immune response, with 1/6 mice surviving the lethal challenge, but had no significant IgG or HAI

antibody levels. It was determined that the remaining mice should be boosted to determine if prime and boost immunization by coated metal microneedles provided full protection against the lethal challenge.

6.2.4 Immune response after prime and boost immunizations with the H3N2 influenza vaccine in mice via coated metal microneedles

Twenty eight days after the first immunization, half of the mice (n=6) from each group were boosted with 9.8 µg of inactivated H3N2/A/Aichi virus. Fourteen days after this boost immunization, blood was collected and the sera IgG antibodies and HAI antibody response were measured. Figure 6.6 below shows the antibody response after prime and boost immunizations.

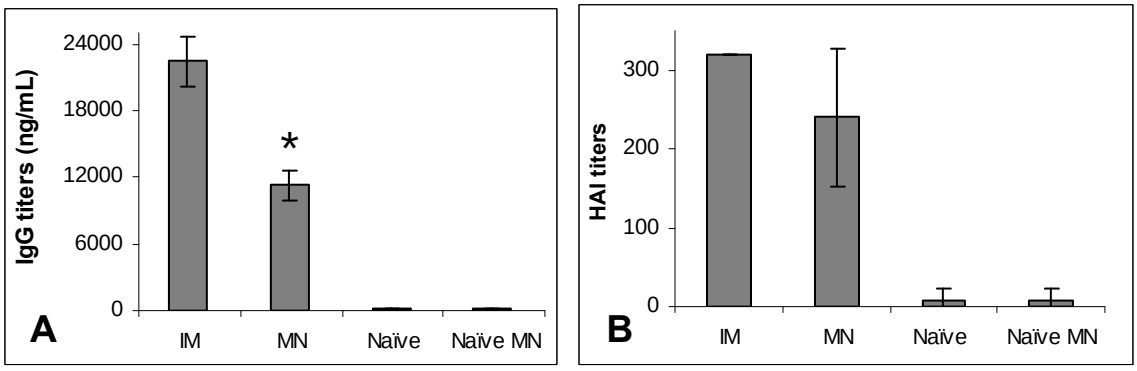

Figure 6.6: Measurement of the antibody immune response after one immunization with the H3N2 virus using coated metal microneedles: 6.4A: Sera IgG titers. 6.4B: HAI titers. Mice (n=6) were prime and boost immunized with 9.8 µg of inactivated H3N2/Aichi influenza virus and bled 14 days later. The mice of the Naïve MN group were prepared the same way of the MN group, with removal of the hair. Uncoated MNs, with no vaccine, were inserted into these mice. * indicates a statistically significant difference (t test, $p < 0.05$) in comparison with intramuscular injection group.

Thirty days after the boost immunization, these mice (n=6) were challenged with a lethal dose (5 LD_{50}) of the live H3N2/A/Aichi virus. As with the previous challenge, the mice were weighed each day for 14 days after the challenge. If a mouse lost more

than 25% of its body weight, it was euthanized. Figure 6.7 below shows the average weight loss and survival data for these groups.

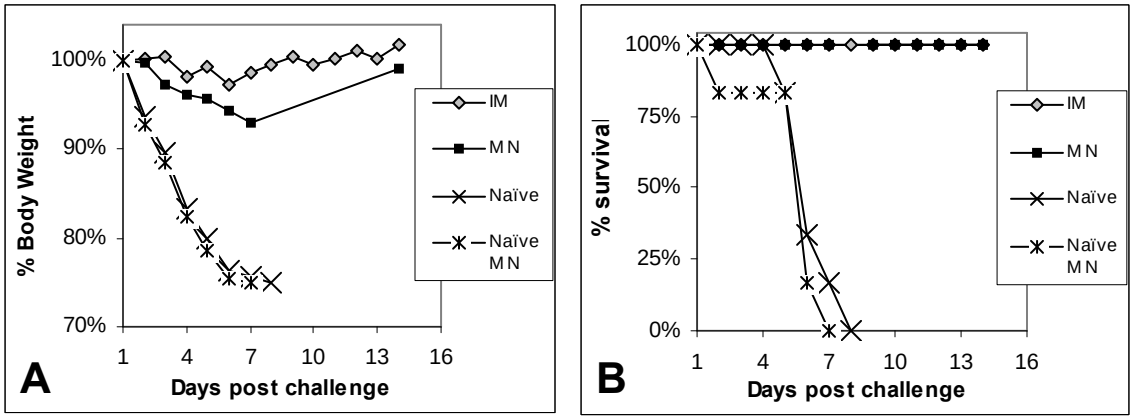

Figure 6.7: Protection against lethal challenge after prime and boost immunizations of H3N2/Aichi influenza vaccine with metal microneedles. 6.7A: Average body weight after challenge. 6.7B: Survival data after challenge. Mice (n=6) were challenged with 5 LD50 of live H3N2/Aichi virus 30 days after boost immunization. Mice which lost more than 25% of their body weight were considered terminally ill and euthanized, per the Emory IACUC.

After prime and boost immunizations, the immune response, as measured by HAI titers, for the coated metal microneedle group was statistically the same to as the positive control (IM) (t test, $p>0.05$). The IgG antibody levels for the IM group were much higher than the microneedle group, but this was most likely due to the levels that were induced after prime immunization (t test, $p<0.05$). Concerning the lethal challenge, all of the mice in the microneedle and IM groups lived, with the microneedle group only having a slightly higher weight loss (7%) compared to the IM (4%). These weight losses are quite low and represent an efficient clearance of the live viral challenge. The mice that were inserted with blank uncoated microneedles and the naïve mice had no significant immune response, with all of the mice in these groups dying within eight days. Overall, these

results show that coated metal microneedles can effectively deliver the inactivated influenza vaccine to mice and produce a protective immune response.

6.3 DISCUSSION

Metal microneedles offer a patient compliant delivery alternative to the hypodermic needle for the influenza vaccine. These microneedles allow for an efficient delivery for a number of molecules[2], are relatively painless[10, 37], require little waste disposal, offer an easy intradermal delivery and could be self-administered in the future. However, there are some processing and delivery steps that must be examined prior to the use of these microneedles for influenza vaccination purposes in vivo. This includes testing the antigenicity of the influenza vaccine after coating onto the microneedle and verifying the delivery efficiency of the microneedles in a mouse in vivo. The overall goal of this study was to determine if metal microneedles coated with the inactivated H3N2/A/Aichi influenza virus can deliver the vaccine effectively to mice in vivo, inducing a protective immune response. This analysis included testing sera antibodies after the vaccinations and protection against a lethal challenge. This analysis was conducted after one immunization as well as after a prime and boost immunization.

The virus that was coated and dried onto the metal microneedles showed a decrease in HA activity versus the positive control (30% retention). However, the virus that was in the coating solution, in the wet state, retained full activity. Therefore, the most likely cause of the decrease of activity was the drying process onto the microneedles and not any interactions between the coating buffer and the virus. Prior work has shown that the influenza vaccine can lose activity when it changes from the liquid to solid state

[101]. This loss of activity is thought to be due to the removal of water, which stabilize the surface proteins of the virus through hydrogen bonding. Since there was some retention of activity on the coated microneedle, it was determined to transition this work in vivo with the metal microneedles in mice and determine if this loss of antigenicity results in a lower immune response.

Prior to the use of metal microneedles for vaccination of influenza in mice in vivo, the delivery capabilities of the coated needles were tested. In pig skin in vitro, metal microneedles coated with Vitamin B successfully delivered all of the coating after one minute of insertion time. Since mice are the animal model used for influenza vaccination, it was important to verify delivery via metal microneedles. The coating was effectively delivered to the mouse after microneedle insertion for 5 minutes. This study determined the proper handling and delivery technique for successful delivery using coated metal microneedles. This included stretching of the mouse skin prior to insertion of the microneedles.

The ultimate goal of this study was to determine if coated metal microneedles could deliver the influenza vaccine and impart a protective immune response. This was tested after one immunization as well as a prime and boost immunization. After the first immunization, the antibody levels for the microneedle group were lower than the IM group. This included IgG antibodies and HAI antibody response. In terms of the challenge, 5/6 of the microneedle group survived the challenge versus 6/6 survival in the intramuscular group. Overall, after one immunization, microneedle based delivery of the influenza delivery imparted a significant immune response to the mice, but this protection was lower than the intramuscular group. This decrease in immune response was most

likely due to the loss of antigenicity during the drying step of the microneedle coating process.

After prime and boost immunizations, the immune response for the microneedle group was significantly higher than the prime immunization. The IgG antibody levels were a ten fold increase over the prime response, but still the level for the microneedle group was lower than the IM group (22400 ng/mL for IM vs 11200 ng/mL for MN) (t test, $p<0.05$). The HAI response for the MN group was statistically the same as the IM group (t test, $p>0.05$). Finally, all of the mice in the microneedle group survived the lethal challenge with little weight loss (7%). Overall, the immune response for the prime and boost immunization was significantly higher than after one immunization. This includes full protection against lethal challenge.

While microneedles offered full protection after prime and boost immunization, the immune response was still lower than the response for the intramuscular group. The most likely cause for this decrease was the loss of antigenicity of the virus during the coating and drying onto the metal microneedles. Thus, in order to increase the immune response via metal microneedle based delivery, the main challenge will be to retain full activity during the coating and drying process. There are a couple possible solutions to this problem. One would be to add a stabilizer to the coating solution to protect the virus during the drying process. One of the most common stabilizers is trehalose, which has been used during freeze drying to stabilize and retain activity of the influenza virus against freezing and drying stresses [101]. However, it will be important to minimize the amount of stabilizer required in the coating solution, since a large amount would decrease the mass (dose in μg) of the virus coated on the metal microneedles. The other

improvement would be to reduce the drying time of the coating on the microneedles. Previous work has shown that it is important to minimize the amount of time the virus is drying from the liquid and solid state. This could include the use of lyophilization during the drying process. As shown in Chapter 5 of this thesis, the inactivated influenza virus retained full activity after lyophilization. It is possible that using lyophilization directly after coating the virus onto metal microneedles may allow for the retention of the activity. As a result, a higher retention in virus antigenicity during the coating process could lead to a higher immune response after metal microneedle-based delivery.

6.4 CONCLUSIONS

Coated metal microneedles offer an exciting delivery option for the influenza vaccine, with many advantages over the current hypodermic needle based delivery. This includes a relatively painless injection, no need for trained medical personnel, a much smaller amount of biohazardous waste, possible storage at room temperature, and easier and faster administration in a mass vaccination scenario. In this study, coated metal microneedles were shown to be efficient in delivering the drug coating to in vitro pig skin within a minute and to mouse skin in vivo within five minutes. However, there were some processing steps that were required for coated metal microneedles to be used for influenza delivery. The antigenicity of the inactivated H3N2/Aichi influenza virus decreased 70% after coating and drying onto the metal microneedles. After one immunization, the metal microneedle-based immunization produced high antibody titer and had 5/6 mice survive a lethal challenge. However, this was lower than the immune response for the intramuscular injection, which had significantly higher antibody levels and full protection against lethal challenge. Most likely the lower immune response in

the microneedle group was due to the loss of activity during the coating and drying process. After prime and boost immunizations, metal microneedles coated with the H3N2 influenza vaccine produced a ten fold increase in antibody response compared to one immunization and provided full protection against a lethal challenge, similar to the results for the intramuscular injection. The next steps for this work should focus on protecting the influenza virus during the coating and drying process, possibly by adding sugar stabilizers to the coating solution or using lyophilization. A higher retention in activity of the influenza virus coated on the metal microneedles should lead to a greater immune response, including full protection against lethal challenge after a single immunization.

CHAPTER 7

DELIVERY OF INFLUENZA VACCINE VIA DISSOLVING POLYMER MICRONEEDLES TO MICE TO INDUCE A PROTECTIVE IMMUNE RESPONSE AGAINST LETHAL CHALLENGE

7.1 INTRODUCTION

The influenza virus is responsible for tens of thousands of deaths each year in the United States and causes millions of illnesses worldwide[113]. While an efficient vaccine exists for this virus, vaccination of all indicated individuals does not occur, and one area of improvement should be in the delivery vehicle. The hypodermic needle has low patient compliance, requires trained medical personnel, has limitations in a mass immunization effort and results in biohazardous sharps waste [14]. As a solution, dissolving polymer microneedles offer an effective delivery option that can meet all of the challenges mentioned above. In this study, we show that dissolving microneedles provide equivalent immune response to the intramuscular injection for the influenza vaccine, including full protection against lethal challenge after one immunization, while offering additional patient and logistical benefits. In addition, these microneedles offer a versatile delivery device that could be used in the future for the delivery of a variety of other biomolecules and vaccines. This includes mass immunization scenarios and the possibility of self-administration in the future.

7.2 RESULTS AND DISCUSSION

7.2.1 Use of dissolving polymer microneedles for the delivery of influenza vaccine

The seasonal influenza virus remains a serious problem each year, including limitations with the method of delivery, the hypodermic needle. An ideal delivery device for influenza vaccination would be patient compliant, allow for easy self-administration and mass immunization, possibly eliminate the need for cold-chain storage, and result in little to no biohazardous waste and sharps. One possible improvement to the current intramuscular injection is the transdermal delivery route, which is promising immunologically due to the vast number of dendritic cells that exist in the skin[154]. However, while intradermal injections are possible, they require highly trained personnel and can be unreliable due to the need for an insertion of a specific shallow depth. In order for alternative needle-free transdermal methods to work, the outer layer of the skin (stratum corneum) must be disrupted to allow for delivery of the large vaccine molecules. Some of the current methods for this delivery include thermal ablation[126], dermal abrasion[145] and epidermal powder immunization[155]. While these devices meet many of the criteria mentioned above and have been successful in vaccination scenarios, more work is required prior to the clinical application of these devices.

Microneedles offer another promising delivery option, which meets all of the delivery requirements mentioned previously. These microscopic needles have been shown to be relatively painless[10] and effective in vaccination scenarios[48, 49]. Specifically, dissolving polymer microneedles offer an even more exciting delivery option for the influenza vaccine, since the microneedles completely dissolve in the skin, resulting in no biohazardous sharps waste. Also, in this scenario, the vaccine is

encapsulated within the solid polymer microneedle, which may remove the need for cold chain storage. To date, polymer microneedle systems have been shown to effectively retain the activity of biomolecules after encapsulation and effectively deliver the cargo to in vitro skin [4, 156]. However, limited work has been done on the use of dissolving polymer microneedles for vaccination purposes in vivo. The goal of this study was to evaluate the use of dissolving polymer microneedles for the delivery of the influenza vaccine to mice in vivo.

There are specific design criteria that must be met for the use of dissolving polymer microneedles for the delivery of the influenza vaccine. First, the fabrication process for the polymer microneedles should be gentle so that it does not decrease the antigenicity of the influenza vaccine. Also, the polymer used for the microneedles must be biocompatible, encapsulate the proper dose of the influenza vaccine, be strong enough to insert into the skin and dissolve rapidly after insertion, to deliver the virus effectively to mice. Finally, the microneedles must induce a protective immune response against lethal challenge in the mice, hopefully equivalent or better than the response induced by intramuscular injection.

The fabrication process that was chosen for the microneedles involves room temperature photopolymerization of a liquid monomer solution within a microneedle mold to form the polymer microneedles. The specific steps of this process have been detailed previously and can be found in Chapter 4 of this thesis[4]. The encapsulation of the drug within the microneedles involves dissolving or suspending the solid drug within the liquid monomer solution, which is then vacuum pulled into the microneedle mold. The polymer polyvinylpyrrolidone (PVP) was chosen as the structural material for the

experiments in this study. This polymer can be created using the above fabrication process, is water soluble, has been used in the past for clinical applications[149], and has been shown to be strong enough for microneedle insertions in the skin[4]. Figure 7.1 below shows pictures of PVP polymer microneedles created via this fabrication process. The pink dye in the microneedle tips represents the model vaccine.

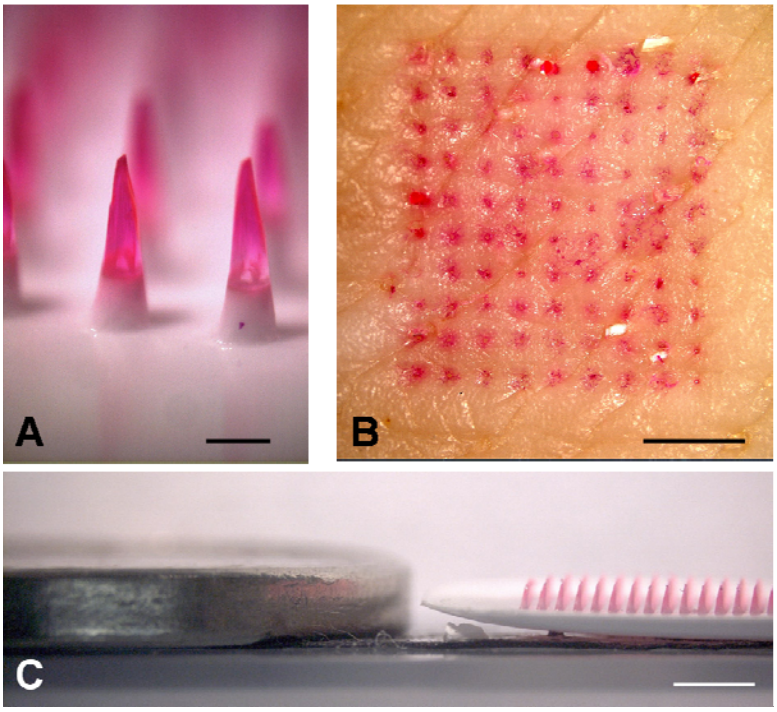

Figure 7.1: PVP polymer microneedles. 7.1A: **Side view of PVP polymer microneedles. Scale bar = 250 µm. 7.1B: Picture of skin, showing the delivery of sulforhodamine from the PVP microneedles. The red staining show sites of microneedle insertion and dissolution. Scale bar = 1 mm. 7.1C: Relative height of PVP polymer microneedles next to a nickel. Sulforhodamine is encapsulated within the tips of the microneedles. Scale bar = 2 mm.**

7.2.2 In vitro and in vivo delivery efficiency of dissolving PVP polymer microneedles

Prior to the use of PVP polymer microneedles for the delivery of the influenza vaccine, the dissolution kinetics of these microneedles were examined. When inserted

into in vitro pig skin, the microneedles completely dissolved within five minutes. Figure 7.2 A-C below shows the dissolution of the PVP microneedles over time in pig skin in vitro. This dissolution time was longer than the previous reported dissolution time for PVP conical microneedles of one minute. Here, pyramidal microneedles were used, which provide a more reliable insertion, but the insertion is more shallow. This can be seen in Figure 7.2C, 7.2D where the microneedle insertion holes were around 150 µm deep, which is only 25% of the length of the microneedles. It is believed that the shallow insertion results in a longer dissolution time, due to less interaction of the water soluble polymer microneedle with the aqueous skin environment.

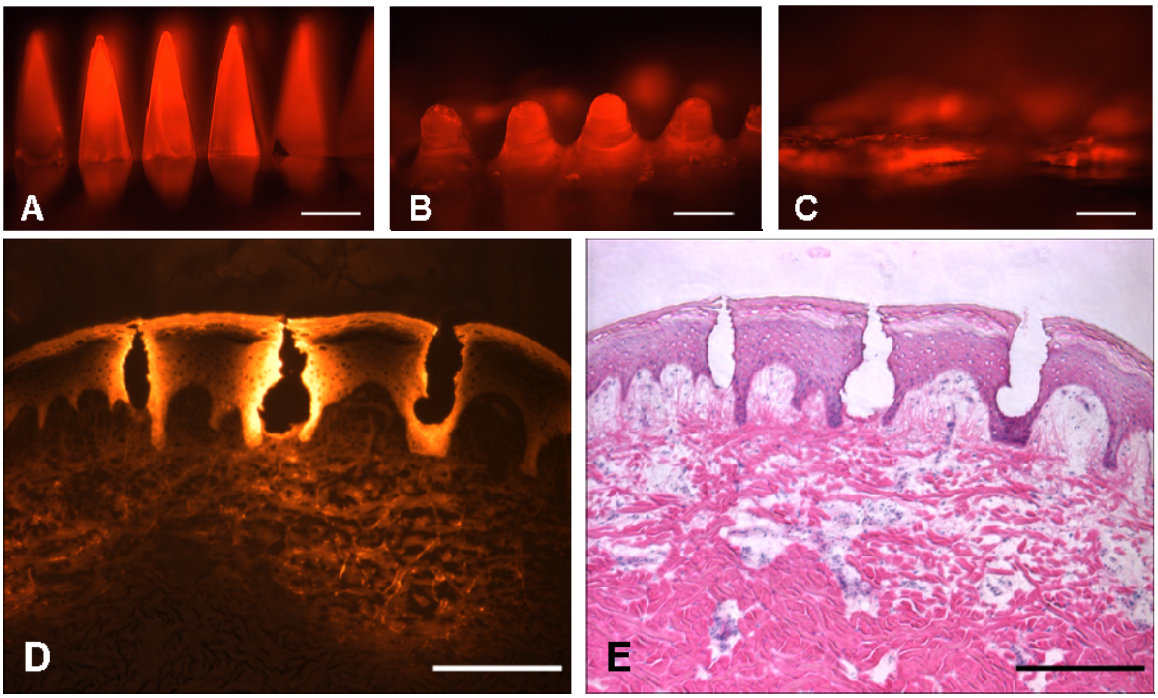

Figure 7.2: PVP polymer microneedle dissolution kinetics. 7.2A: Microneedle preinsertion. 7.2B: After one minute in skin. 7.2C: After 5 minutes in skin. Scale bars = 300 µm for microneedle images. 7.2D: Fluorescent microscopy of skin histology after insertion of pyramidal PVP microneedles, fluorescent image. 7.2E: Bright field microscopy of same image stained with H+E staining. Scale bars = 200 µm for skin histology images. All insertions performed in porcine skin in vitro.

The delivery kinetics were also tested in mice to determine the proper time the PVP polymer microneedles should be left in the skin to deliver the encapsulated cargo. Here, sulforhodamine was encapsulated within the PVP polymer microneedles and the needles were inserted into the mouse. The needles were removed at 5, 10 or 15 minutes, and the efficiency of delivery was determined. Figure 7.3 below shows a summary of the results. Here, the PVP microneedles effectively delivered the encapsulated cargo (>80%) in 15 minutes. This longer time for delivery versus pig skin was probably due to poor insertion and affixation of the microneedle array to the skin surface due to the small size and curvature of the mouse body. The pig skin insertion and delivery experiments offers a better representative of human skin, but the mouse delivery was also tested since that it was the animal model primarily used for influenza immunizations.

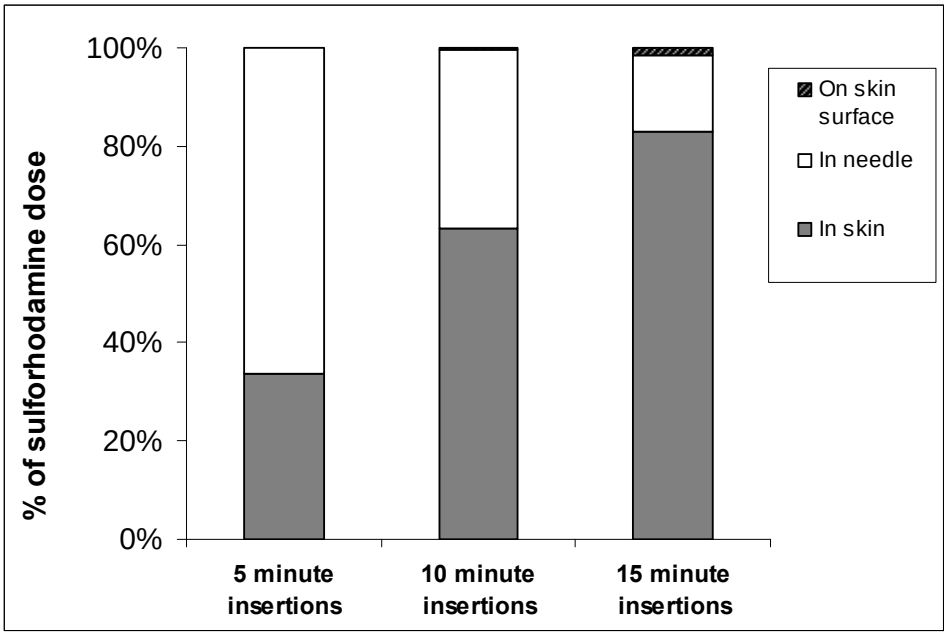

Figure 7.3: PVP polymer microneedle delivery efficiency to mice in vivo. Sulforhodamine was encapsulated within PVP microneedles and delivered to mice. The delivery efficiency was determined by measuring the amount of sulforhodamine left in the microneedle after insertion as well as on the skin surface of the mouse. The remaining sulforhodamine was considered to be delivered to the skin. n=5 for each time point. The delivery efficiency for the three time points were statistically different from one another (t test, $p<0.05$).

7.2.3 Immune response after one immunization with the H1N1 influenza vaccine in mice via dissolving polymer microneedles

For the influenza vaccine to be encapsulated and delivered via dissolving polymer microneedles, a number of reformulation and processing steps were required, including the lyophilization of the virus solution into solid particles and encapsulation within the PVP polymer microneedles. These processing steps were studied in depth in vivo and the results can be found in Chapter 5 of this thesis. In summary, lyophilization had no impact on the immunogenicity of the H1N1/A/PR/8 or H3N2/Aichi influenza virus while the encapsulation process within the polymer microneedles alone also did not affect the activity of the virus. These experiments resulted in an optimization of the reformulation process needed for the influenza vaccine to be delivered via polymer microneedles.

The inactivated H1N1/A/PR8 influenza virus was used for the immunizations in this study. Mice (n=12) were immunized with 6 μg of the influenza vaccine. Three groups were tested: intramuscular injection (100 μL, 60 μg/mL), PVP polymer microneedles (2 arrays, 3 μg/array) and a naïve group. The antibody levels were measured after fourteen and thirty days and are shown in Figure 7.4A and 7.4B below. The IgG and HAI antibody levels for the microneedle and IM groups were much higher than the naïve mice (t test, $p=5.6 \times 10^{-10}$) after one immunization and not statistically different from each other (t test, $p>0.05$).

Next, the immunoprotection of the mice was measured via survival against lethal challenge. Half of the mice (n=6) were exposed nasally to the live H1N1 virus (5 LD_{50}), and the weights of the mice were measured for fourteen days after the challenge. The average weights and survival data can be seen in Figure 7.4C and 7.4D below. The mice from both the IM and PVP microneedle groups all survived the challenge and had negligible weight loss (< 2%). This shows that the microneedles provided full protection against a lethal challenge with effective clearance of the virus after only one immunization.

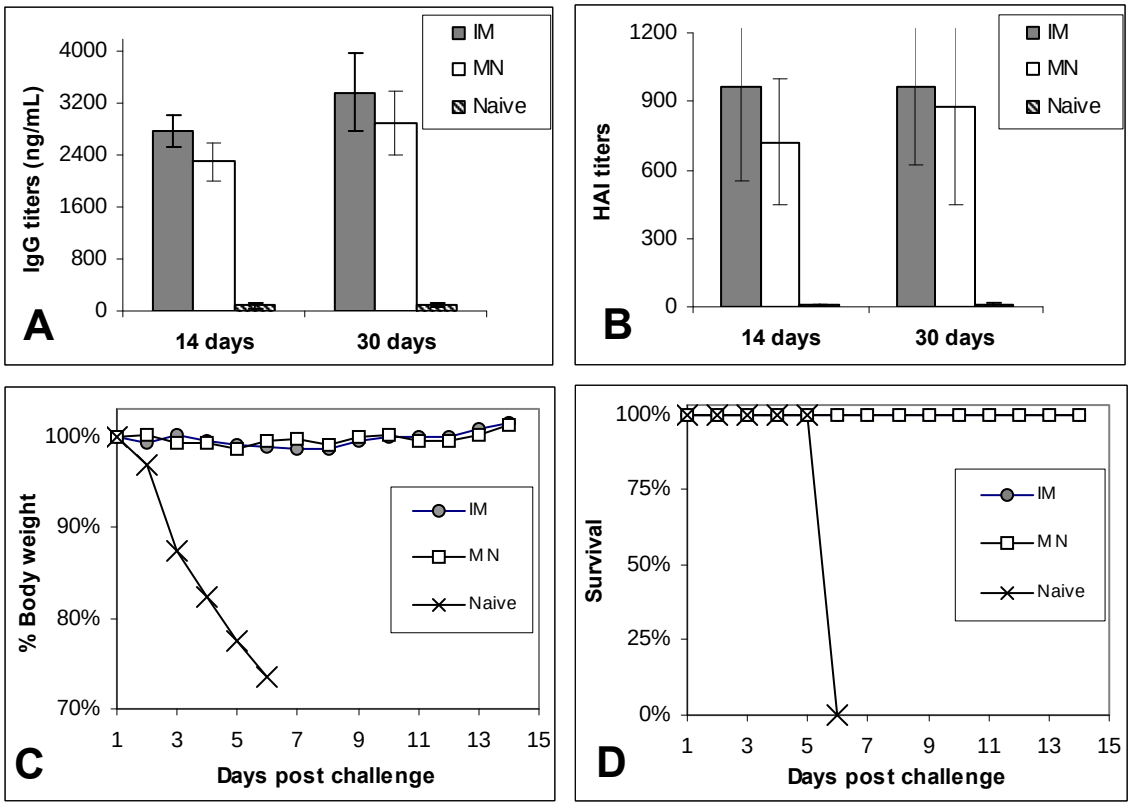

Figure 7.4: Measurement of the immune response after one immunization with H1N1 influenza virus with dissolving polymer microneedles. 7.4A: IgG antibody titers and 7.4B: HAI titers, 14 and 30 days after immunization. 7.4C: Average body weight after challenge. 7.4D: Survival after challenge. Mice (n=12) were immunized with 6 µg of inactivated H1N1/A/PR/8 influenza virus. Thirty days after the immunization, mice (n=6) were challenged with 5 LD_{50} of live H1N1 virus. Mice which lost more than 25% of their body weight were considered terminally ill and euthanized, per the Emory IACUC.

We then assessed the ability of immunized mice to clear the virus from their respiratory system upon infection. Humoral–systemic and mucosal- as well as cellular immune responses play important roles in limiting virus replication and spread in the lungs. The other half of the mice (n=6) from each immunized group were challenged with live H1N1 virus (5 LD_{50}) and four days later they were sacrificed and their lungs were collected and prepared in suspension. To measure virus clearance, we determined the number of plaque forming units that remained in the lungs after challenge (pfu/g lung tissue) (Figure 7.5). The mice immunized using PVP polymer microneedles had a much

higher clearance of the virus compared to the intramuscular injection, with a minimal amount of virus found in the lungs four days after challenge. In fact, the clearance of the virus in the mice immunized with dissolving microneedles was six orders of magnitude higher than clearance from the naïve mice and three orders of magnitude higher than the IM injection group(t test, $p<0.05$).

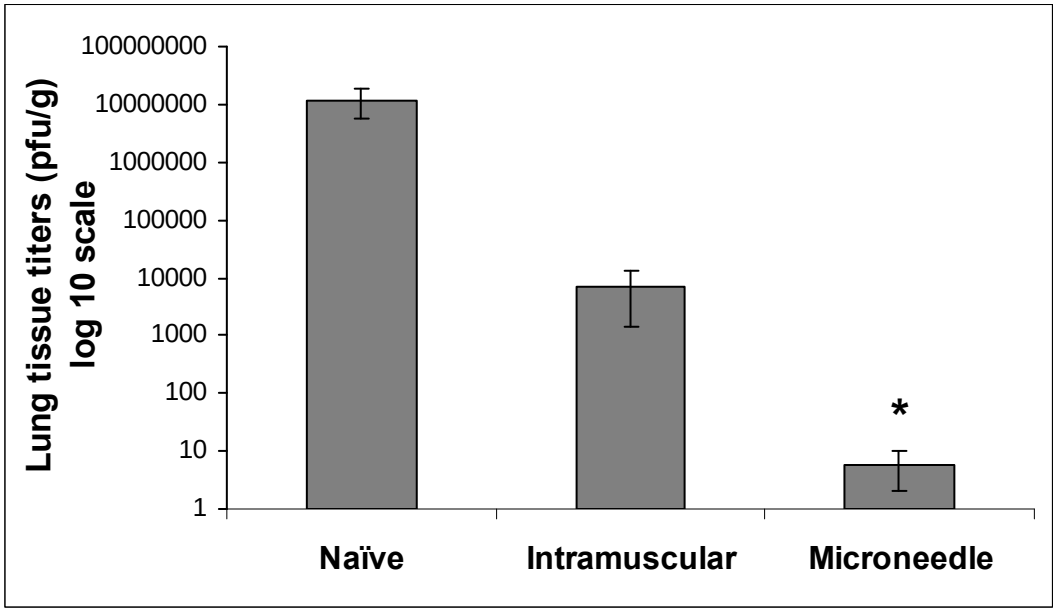

Figure 7.5: Clearance of virus from lungs of mice after lethal challenge. Mice (n=6) were challenged with 5 LD_{50} of the live H1N1 influenza virus 82 days after immunization and sacrificed four days after the challenge. The ability of animal to clear the lethal virus was determined by measuring the presence of the virus (pfu/g) in the lungs. A lower amount of the virus in the lungs signifies a better clearance and a stronger immune response. * indicates a statistically significant difference (t test, $p< 0.05$) in comparison with intramuscular injection group.

7.2.4 Immune response after prime and boost immunizations with the H3N2 influenza vaccine in mice via dissolving polymer microneedles

The polymer microneedle system was also evaluated in a separate immunization using an H3N2/X31 strain of the influenza virus. Once again, lyophilization had no impact on the antigenicity of the virus, with the same activity as the unprocessed virus

solution. Mice (n=6) were immunized twice, thirty days apart, with 6 μg of the X31 virus, via either intramuscular injection or PVP microneedles. The antibody response was measured fourteen days after each immunization. As shown in Figure 7.6 below, the immune response for the dissolving microneedles was statistically the same as the intramuscular injection for the IgG titers (t test, $p>0.05$) and for the HAI titers (t test, $p>0.05$). The HAI levels for both the MN and IM groups were significantly high (HAI>250) to predict full protection against lethal challenge. HAI values greater than 40 typically lead to full protection[138]. These results confirm that influenza delivery via dissolving polymer microneedles induces a significant immune response, quite similar to the intramuscular injection.

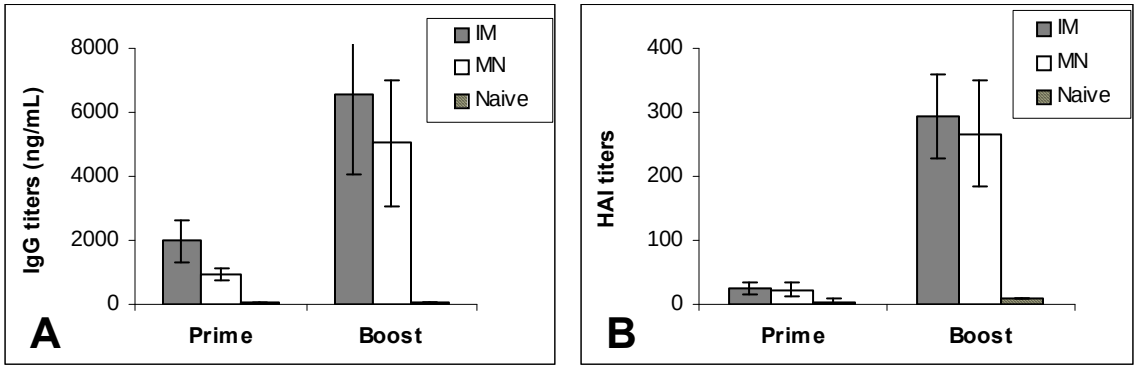

Figure 7.6: Measurement of the antibody immune response after prime and boost immunizations of H3N2/X31 influenza virus with dissolving polymer microneedles. 7.6A: IgG titers. 7.6B: HAI titers. Mice were immunized with 6 μg of H3N2/X31 virus and bled 14 days after each immunization.

7.3 CONCLUSIONS

The seasonal influenza virus remains a major medical problem in the United States with tens of thousands of deaths and hundreds of thousands of illnesses each year. One area of interest is to improve the delivery method of the influenza vaccination

process. Dissolving polymer microneedles offer an attractive option that is expected to be patient compliant, allow for self-administration without trained medical personnel, and result in no biohazardous sharp waste. Here, we have shown that dissolving microneedle-based immunization of the influenza vaccine to mice resulted in full protection against a lethal challenge after one immunization and produced antibody levels that were similar to the response for the intramuscular injection. In addition, the mice immunized with the polymer microneedles had a more efficient clearance of the virus from their lungs, in comparison to the control IM injection group. Subsequently, with no sharps left after immunization, the dissolving microneedle system would provide a unique solution to the problem of accidental or intentional reuse of hypodermic needles[16]. Also, this microneedle system could be self-administered in the future, possibly allowing for quicker and more efficient mass immunization efforts. Overall, the dissolving polymer microneedles discussed in this study offers an effectively delivery vehicle for the influenza vaccine while offering numerous logistical and patient advantages. This system could also be used for the delivery of a number of other biopharmaceuticals as a more desirable delivery method than the hypodermic needle.

CHAPTER 8

DISCUSSION

In this project, a new drug delivery system, based on dissolving polymer microneedle system, was developed. This delivery system is particularly exciting as an alternative delivery method to the hypodermic needle for the delivery of biopharmaceuticals. Here, the significance of this work will be discussed in comparison to other delivery methods for biopharmaceuticals as well as specifically against the other microneedle based delivery systems.

8.1 BIOPHARMACEUTICAL DELIVERY DEVICES

Biopharmaceuticals, including vaccines, DNA, peptides, and proteins, is one of the fastest growing sects of the overall pharmaceutical market. In fact, the global biopharmaceutical market exceeded 80 billion dollars in 2007 and is growing at an annual rate of 17%, a higher growth rate than the overall pharmaceutical industry[6]. However, there are limitations with the delivery of these molecules. Oral delivery is difficult due to poor absorption, enzymatic degradation and the first pass metabolism effect of the liver[8]. Therefore, the most commonly used method currently is the hypodermic needle, which is effective, but has issues with patient compliance, the need for medically trained personnel and the possibility of needle-stick injuries[14, 15]. Currently, there exists a large amount of work into new devices as alternatives to the hypodermic injection for the delivery of biopharmaceuticals. There are a number of factors that must be analyzed for a new delivery device, including microneedles. The optimal device should be equal to or better than the hypodermic needle and its

competitors in the following categories. These parameters are listed in order of importance.

8.1.1 Parameters for alternate delivery devices for biopharmaceuticals

- **Safety**: This is the most important parameter for any medical device. For the device to be used, it must pass FDA inspection, and thus be safe for use.

- **Effectiveness**: This is also a very important parameter for any medical device. The new device must deliver the biomolecule in full activity and induce an equal to or better biological response than an injection.

- **Low cost/Mass fabrication:** It is important that the new device can be mass fabricated at a relatively low cost. One hypodermic needle device costs approximately $0.10. While the new device may cost more than this, it should be similar on the order of tens of cents per delivery.

- **Patient Compliance**: For an alternative delivery device to be successful there must be a high uptake by patients. This includes relatively low pain and ease of use, possibly including an all in one device.

- **Biohazardous sharps waste**: The device should have limited biohazardous waste produced with no sharps. This is particularly important if the device will be self-administered at home, where the disposal of biohazardous waste can be difficult.

- **Versatility:** The device should be able to effectively deliver a wide variety of biomolecules, including vaccines/peptides/proteins/DNA.

- **For Vaccine Based Delivery:**
    - **Dose sparing**: The common intramuscular injection delivers the vaccine is a poorly immunogenic region. One possible advantage of a new delivery system would be to induce a greater immune response by delivering the vaccine to a highly immunogenic region, i.e. transdermal to the skin[11].

    - **Room temperature storage**: One requirement of vaccine delivery is the need to have refrigeration for the cold chain storage of the vaccine. It would be beneficial and cost effective if the new delivery device did not require refrigeration and could be kept at room or elevated temperatures.

Currently, there is no one delivery system on the market that is better than the hypodermic needle in all of the above parameters. However, a number of delivery

systems are currently in clinical and preclinical trials that would offer many advantages over injections. Specifically, there are a number of devices being developed for transdermal delivery. The dissolving microneedle system was analyzed in terms of these transdermal delivery systems. This includes systems for thermal ablation, dermal abrasion (sand paper based), ultrasound, chemical enhancers, and other microneedle-based delivery devices.

8.1.2 Safety

The dissolving microneedle system has been shown to be safe in limited animal experiments with no visible inflammation, and there should be no safety issues for the used in patients. However, since the polymer material is being deposited in the skin, it will be important to ensure that the polymer has no adverse interactions in humans and is completely cleared from the body. Polyvinylpyrrolidone (PVP) is biologically inert, has been used clinically in the past[72, 73, 78] and has been shown to be cleared efficiently from the body if the molecular weight is below 20,000 Da[71]. The molecular weight of the PVP polymer made in this study was well under this value. The other delivery devices have been used clinically and have been shown to be safe. The only limitation is chemical enhancers, which can cause some skin irritation. Overall, the dissolving microneedle delivery system should be safe, but further testing must be done to verify the safety prior to clinical use of this system.

8.1.3 Effectiveness:

The dissolving microneedle system has been shown to be as effective as the hypodermic injection for the delivery of the influenza vaccine in mice. This included

full protection against lethal challenge after one immunization. While these results are promising, preclinical experiments in larger animals and clinical trials are still required. The other transdermal delivery devices have already been shown to be effective in clinical trials for the delivery of a number of biomolecules. This includes the human growth hormone and insulin for thermal ablation[157], vaccine delivery for dermal abrasion[158], and lidocaine for ultrasound[159]. However, one disadvantage of these alternate delivery systems is in the speed and efficiency of delivery. These systems require diffusion of the biomolecule from a patch based reservoir into the disrupted skin, which can be slow, and result in low bioavailability. The dissolving microneedles insert into the skin, delivering the entire drug cargo in the skin faster (within minutes), and with a higher bioavailability (>80%).

8.1.4 Low cost/Mass fabrication:

Currently, the dissolving microneedle system is made on a singular basis. However, the fabrication process lends itself to mass fabrication. In fact, UV initiated curing of polymers is common in industry[160-162]. Concerning cost, it has been approximated that mass fabrication of microneedles could be on the order of tens of cents or less[5]. Also, the materials needed for creation of the dissolving microneedles are relatively inexpensive. Thus, it seems that the cost of fabrication may not be a limiting factor on the future use of the dissolving microneedle system. It is difficult to accurately determine what the cost of the other delivery systems is, but it could be assumed that the systems with mechanical equipment and power supplies (thermal ablation, ultrasound) would be more expensive than the microneedles and the simpler systems (dermal abrasion, chemical enhancers) may be less expensive.

8.1.5 Patient Compliance:

Low patient compliance is a major limitation of the hypodermic needle, and thus improving the compliance is one of the main focuses of the alternative delivery systems. The dissolving microneedle technology should be highly desirable with patients, with minimal pain or sensation and it can be self-administered with minimal force. The delivery mechanism can be explained as being as simple as the current transdermal patch technology, which is currently used by millions of patients in the United States. The minimally invasive nature of this product is one of the major advantages over the hypodermic injection.

The other transdermal delivery devices are also more patient compliant than the hypodermic needle. Each of them offers delivery with minimal or no pain. However, many of these devices require multiple components, which make them a little more difficult to use. Also, chemical enhancers and to a lesser extent dermal abrasion may lead to irritation on the skin surface, which may not be desirable for some patients. The dissolving microneedle delivery device should be more desirable than the hypodermic injection and appears to be equal to or better than the other transdermal delivery systems.

8.1.6 Biohazardous sharps waste:

This is another major limitation of the hypodermic needle; the production of biohazardous sharps waste with the risk of re-sticks of needles. This is another advantage of the dissolving microneedle system over the current needle based delivery. Since the microneedles completely dissolve within the skin, there is no biohazardous sharps waste produced. The other transdermal delivery devices do not involve needles and thus have no issues with biohazardous sharps waste.

8.1.7 Versatility:

One of the major benefits of the hypodermic needle is the ability to deliver the large number and variety of molecules effectively to the body. So far, the dissolving microneedle system has been able to successfully encapsulate and deliver a model protein (β-Galactosidase) in vitro and a virus (influenza vaccine) in vivo. However, since the fabrication process requires the drug molecule to be in solid form, this system is limited to biomolecules which can remain active after drying. Most likely, viruses and DNA should be able to be delivered by this method since they have been lyophilized successfully in the past. On the other hand, the delivery of fragile proteins will probably provide processing challenges. Testing and optimizing the versatility of the dissolving microneedle system is one of the key areas that needs to be analyzed in the future.

The other transdermal delivery devices require little to no reformulation of the biomolecules prior to delivery. This is an advantage over the dissolving microneedle system, since limited work will be needed to use these devices for the delivery of a new biomolecule. As for delivery, thermal ablation and dermal abrasion each remove some part of the barrier function of the skin and thus are able to deliver a large number of molecules. However, in terms of chemical enhancers and ultrasound, there is no mechanical disruption of the skin. Thus, it may be difficult to deliver larger molecules with these devices. In fact, since all of these devices rely on diffusion for delivery, there may be limitations in the speed and bioavailability of large macromolecules. While, delivery will occur, it may be slower and less efficient than dissolving microneedles.

8.1.8 Vaccine Based Delivery:

The dissolving microneedle system delivers the vaccine to the epidermis and dermis of the skin, which should be more immunogenic than the muscle, where the current standard injection occurs [12, 17]. Also, the vaccine is stored in solid form within the dissolving microneedle system. This may allow for room temperature storage, which is more convenient and less expensive than the current refrigeration for cold chain storage of vaccines. All of the transdermal delivery systems deliver the antigen to the skin, with the same benefits as the dissolving microneedle system. Also, the lack of cold chain storage is possible for the alternate delivery systems.

8.1.9 Overall analysis

The dissolving polymer microneedle system has many potential advantages over the hypodermic needle, specifically in terms of expected patient compliance, lack of biohazardous sharps disposal and possible removal of the cold chain storage for vaccines. The main next steps will be to test the versatility of this device for the delivery of a wide range of molecules. Also, the fabrication process should be optimized to minimize cost and allow for mass fabrication.

In terms of the alternative delivery systems, the dissolving microneedle system is similar in many of the categories listed above. However, in order for the microneedles to be widely adopted in the future, it should be highly desired by the patient and be shown to be able to deliver a wide range of molecules. The dissolving microneedles are very similar to the other systems in terms of benefits, so patient choice will be a major factor on the success of these devices. However, one concern for the dissolvable microneedle system is that it has only been shown to be successful in one animal experiment while the

other delivery systems have already been shown to be successful in some form of clinical trials. On the other hand, one advantage of the dissolving microneedle system is that the delivery is fast (within minutes) and efficient, with a high percentage of the encapsulated cargo delivered to the skin. The other delivery systems are dependent on diffusion of the molecule into the skin and can be slower and have a lower bioavailability. Finally, in terms of vaccination, it is possible that the transdermal delivery systems could be more effective than the intramuscular injection by delivering the vaccine to the skin. This needs to be further tested in the future, but could be highly beneficial and allow for a lower dose for vaccinations.

8.2 MICRONEEDLE-BASED DELVERY SYSTEMS

There are four main types of microneedle delivery systems. One is hollow microneedles, where the drug solution is flowed through the needle into the skin. Second, there are coated metal microneedles, where the drug is coated onto the microneedle, and after insertion the coating is dissolved off of the needle and into the skin. Third, there are metal microneedles which are applied to the skin and removed, creating microchannels, through which a drug solution could be delivered via a patch based system. Finally, there are solid dissolving polymer microneedles, where the drug is encapsulated within the polymer microneedle and after insertion into the skin, the polymer dissolves, delivering the drug. Each of these microneedle delivery systems should have a high patient compliance, with minimal pain or sensation and may offer a self-administered all in one device. Also, each of these systems has been shown to be effective for delivery in vivo. Solid and coated metal and hollow microneedles have been shown to be effective in animals and humans while the polymer microneedles have shown to work so far in

preclinical trials in animals. However, there are some differences between the microneedle systems, and specifically advantages and disadvantages of the dissolving polymer microneedle system.

The main advantage of the dissolving polymer microneedles is the lack of biohazardous sharps after delivery. This is especially important for the use of microneedles at home and in Third World situations where safe disposal of sharps may be a problem. After delivery, the hollow and metal microneedles remain intact and would require disposal of the sharps. While the possibility of re-sticks of microneedles is expected to be small compared to hypodermic needles, the disposal of the sharps must still be addressed.

The main limitation of the dissolving microneedle system is that reformulation of the drug is required to be encapsulated within the microneedles. While this may not be a problem after the reformulation process is optimized, it must be addressed prior to this system being used for a wider variety of biomolecules. The coated metal and hollow microneedle systems have been tested more in depth, with success in delivering a wide variety of molecules, including insulin, influenza vaccine, DNA, hepatitis B vaccine, and ovalbumin amongst others[45, 48, 51, 163]. In fact, in terms of delivery via hollow microneedles, there may be no reformulation required prior to delivery of a new molecule. As for metal microneedles, the coating process has been shown to be effective using a wide variety of biomolecules, including DNA, viruses and proteins [153].

Overall, the dissolving microneedle system offers an exciting delivery device that is expected to be more patient compliant and effective and especially promising for home use, with no biohazardous sharps disposal after delivery. However, further testing in the

reformulation process must be done for this system to be used for a wide variety of biomolecules.

CHAPTER 9

CONCLUSIONS

Biopharmaceuticals, including proteins, DNA, vaccines and other biologically related molecules, make up one of the fastest growing segments of the overall pharmaceutical market. However, there are issues with the main delivery method for these molecules, i.e. the hypodermic needle, such as low patient compliance, the need for medically trained personnel, disposal of sharps after injections, and limitations in a mass immunization scenario. There exists a need for an alternate delivery method for biopharmaceuticals that is patient compliant, possibly self-administered, has no biohazardous sharps after delivery, and would be easier to facilitate mass immunization efforts.

Dissolving polymer microneedles offer an exciting delivery option for biopharmaceuticals that is designed to meet all of the requirements listed above. While an effective polymer microneedle system has been developed previously, the fabrication processes for this system required elevated temperatures, which may lead to degradation of the encapsulated biomolecules. Also, no previous work involved a rapidly dissolving polymer system, instead focusing on slower releasing polymers like PLGA. The goal of this project was to develop a rapidly dissolvable polymer microneedle system that is capable of delivering an active biomolecule in vivo, producing an equivalent physiological response to the standard hypodermic injection. The influenza vaccine was chosen as the model biomolecule for this project.

9.1 DEVELOPMENT OF A NEW FABRICATION PROCESS FOR POLYMER MICRONEEDLES FOR THE DELIVERY OF BIOMOLECULES

The goal of this study was to develop a new fabrication process for polymer microneedles. This fabrication process must produce sharp microneedles and be gentle enough to allow for the retention of activity of active biomolecules during the encapsulation process. Also, the polymer chosen as the structural material for the microneedles must be strong enough to allow insertion in the skin, able to break down quickly in the skin, releasing the encapsulated cargo, and be biocompatible, allowing for human use in the future. The main findings from this research follow:

- UV initiated, room temperature free radical polymerization can be used to create polymer microneedles that have the identical geometry and tip sharpness as the original microneedle master structure.

- This fabrication process can produce polymer microneedles made of a variety of vinyl polymers and copolymers The choice of polymer can vary depending on the desired properties, including mechanical strength and rate of breakdown of the polymer, i.e. delivery in vivo

- The ideal material for polymer microneedles would be mechanically strong, dissolve quickly within the skin and have been used in the past clinically. Polyvinylpyrrolidone (PVP), the main structural material used in this project, met all of these requirements.

- The copolymer poly(vinylpyrrolidone-co methacrylic acid) PVP-MAA was also used as a structural material for polymer microneedles, primarily due to an increased mechanical strength from hydrogen bonding within this copolymer.

- Arrays of PVP polymer microneedles successfully inserted into in vitro porcine skin and delivered the encapsulated cargo within minutes.

- In terms of biocompatibility, PVP is biologically inert and can be cleared by the body if the molecular weight is below 20,000 Da. The molecular weight of the PVP polymer produced via the new microneedle fabrication process was less than 10,000 Da.

- The encapsulation process within PVP polymer microneedles resulted in no damage to a model enzyme, β-Galactosidase. In addition, β-Gal retained enzymatic activity after delivery to in vitro pig skin.

- In summary, the fabrication process developed in this study produced polymer microneedles that were sharp and strong enough to insert into pig skin in vitro and mouse skin in vivo and deliver the majority of the encapsulated cargo. Also, it was shown that proteins retained full activity after being encapsulated within polymer microneedles created using this new fabrication process.

9.2 IN VITRO AND IN VIVO ANALYSIS OF THE REFORMULATED INFLUENZA VACCINE FOR DELIVERY VIA DISSOLVING POLYMER MICRONEEDLES

The main goal of this study was to determine if the inactivated influenza virus retained activity after the processing steps required to be encapsulated and delivered via PVP polymer microneedles. These processing steps include lyophilization of the virus solution and encapsulation within PVP polymer microneedles. The analysis included in vitro testing of the antigenicity of the processed virus and in vivo testing in mice of the immunogenicity of the processed virus. Also, the effect of the stabilizer trehalose was tested to determine if it is required to retain the activity of the virus solution during lyophilization.

- Lyophilization of the inactivated H1N1 and H3N2 influenza viruses, with or without the excipient trehalose, resulted in no loss of antigenicity of the virus.
- The polymer PVP introduced an artifact in solution that prevented a meaningful analysis of the antigenicity of the influenza viruses.
- Lyophilization of the H1N1 and H3N2 influenza viruses resulted in no loss of immunogenicity as measured by IgG and HAI antibody titers.
- The presence of the polymer PVP in solution with the H1N1 and H3N2 influenza virus resulted in a lower immune response, as measured by IgG and HAI antibody titers. The encapsulation process of the virus within polymer MNs appeared to have no additional deleterious effect on the activity of the virus.
- Lyophilization of the H3N2 influenza virus results in no loss of protection against lethal challenge after one immunization (5/5 mice survive).

- The presence of the polymer PVP in solution with the H1N1 influenza virus resulted in a lesser protection against lethal challenge after one immunization (2/5 mice survive). However, this effect is less impactful after a boost immunization, which has full protection for the mice from lethal challenge (5/5 mice survive).

- Similar to prior results, the influenza virus that was encapsulated within PVP microneedles produced the identical immunoprotection against challenge as the virus in solution with the PVP polymer. Thus, it was determined that the encapsulation process alone did not have an effect on the immunogenicity of the influenza virus..

- In summary, lyophilization has no effect on the antigenicity or immunogenicity of the influenza virus. However, the presence of the polymer PVP in solution with the virus alone does result in a decrease in immunogenicity.

- In order to fully determine if the encapsulation process damaged the activity of the influenza virus, PVP microneedle-based delivery of this vaccine should be tested. This delivery scenario should not have any issues of the PVP polymer in solution with the virus for an extended period of time.

9.3 DELIVERY OF INFLUENZA VACCINE VIA COATED METAL MICRONEEDLES TO INDUCE A PROTECTIVE IMMUNE RESPONSE AGAINST LETHAL CHALLENGE

The main goal of this study was to evaluate the ability and efficiency of coated metal microneedles for the delivery of the inactivated influenza virus. This analysis included testing the delivery efficiency of the coated drug in vivo, the antigenicity of the virus after coating onto the metal microneedles and the immune response induced in mice after delivery via coated metal microneedles.

- The inactivated H3N2/Aichi influenza virus was used in this study.

- Metal microneedles were successful in delivering the entire coating of drug to in vitro pig skin within a minute. Full delivery in vivo in mice required three to five minutes of insertion.

- The act of drying of the inactivated H3N2 influenza virus onto metal microneedles resulted in 70% activity loss.

- Coated metal microneedle based immunization with the H3N2 influenza virus produced a lower immune response (IgG and HAI antibodies) than a control intramuscular injection.

- After one immunization, coated metal microneedles based delivery resulted in a lower protection against lethal challenge for the mice (5/6 survived) and a higher weight loss than the IM injection.

- After a prime and boost immunization, coated metal microneedle based delivery resulted in full protection against lethal challenge for the mice (6/6 survive) and similar weight loss as the IM injection, which was minimal.

- The insertion of blank metal microneedles with no virus coated produces no immune response or protection against lethal challenge (0/6 survive).

- In summary, metal microneedle based delivery of the H3N2 influenza virus to mice produced high antibody levels and full protection against lethal challenge after prime and boost immunizations. However, the immune response after one immunization was lower than the control IM injection.

- Most likely, the reduction in antigenicity of the influenza virus during drying onto the metal microneedles was responsible for the lower immune response. Future work should be conducted to minimize this loss of activity, which should lead to a higher immune response in vivo.

9.4 DELIVERY OF INFLUENZA VACCINE VIA DISSOLVING POLYMER MICRONEEDLES TO INDUCE A PROTECTIVE IMMUNE RESPONSE AGAINST LETHAL CHALLENGE

The main goal of this study was to evaluate the ability and efficiency of dissolving polymer microneedles for the delivery of the inactivated influenza virus. This analysis included testing the delivery efficiency of the dissolving microneedles in vivo, the antigenicity of the virus after lyophilization and microneedle processing, and the immune response induced in mice after delivery via dissolving polymer microneedles.

- Polyvinylpyrrolidone (PVP) was chosen as the structural material for the dissolving polymer microneedles used in this study. The immune response against the influenza vaccine via dissolving polymer microneedles was evaluated compared to the intramuscular injection using the H1N1 and H3N2 subtypes of the virus.

- The geometry of polymer microneedles was extremely important on the % of needles inserted and the depth of delivery, which affected the delivery rate of the polymer microneedles.

- Only ~50% of conical shaped PVP polymer microneedles inserted properly into porcine skin in vitro, due to a low failure force of this geometry. However, for the conical needles that did insert, the depth of insertion was ~ 90% of the length of the microneedle and allowed for rapid dissolution within the skin (1 minute).

- 100% of the pyramidal shaped PVP polymer microneedles inserted properly into porcine skin in vitro, probably due to a higher failure force of this geometry. However, these microneedles only insert ~25-50% of their length, resulting in a slower dissolution of the complete polymer microneedle and thus slower delivery (5 minutes). The insertion into mice in vivo was even shallower, resulting in a even longer delivery time (15 minutes).

- The lyophilization process had no effect on the antigenicity of the H1N1 or H3N2 subtypes of the inactivated influenza virus, as measured using the hemagglutination assay.

- Immunization of the H1N1 influenza virus with the dissolving PVP polymer microneedle system produced an equivalent humoral immune response (IgG/HAI) as the control intramuscular injection.

- Immunization of the H1N1 influenza virus with the dissolving PVP polymer microneedle system resulted in full protection against lethal challenge after one immunization with minimal weight loss. These results were also equivalent to the control intramuscular injection.

- Mice immunized with the H1N1 influenza virus via PVP polymer microneedles cleared the virus from their lungs more efficiently than the control intramuscular injection group.

- In summary, the dissolving microneedles based delivery of the influenza vaccine produced a protective immune response after one immunization that was equal to the intramuscular injection. In addition, this delivery method offers logistical, safety and patient compliance advantages over the intramuscular injection, including ease of administration, lack of pain, and no biohazardous sharps waste after delivery.

CHAPTER 10

FUTURE DIRECTIONS

The dissolving polymer microneedle system developed in this project, using the new fabrication process, was successful at delivering the influenza vaccine in vivo, inducing a protective and equal immune response compared to the intramuscular injection. However, more work needs to be done prior to this system being used clinically. The following are some of the areas of research that should be studied to optimize the polymer microneedle system for the delivery of biomolecules.

10.1 OPTIMIZATION OF THE POLYMER MICRONEEDLE FABRICATION PROCESS FOR USE WITH A WIDE VARIETY OF BIOMOLECULES

The current fabrication process necessitates the drug to be in solid form prior to the encapsulation process for the polymer microneedle array. Often this requires the lyophilization of the drug solution into a dry powder. So far, the inactivated influenza vaccine (H1N1 and H3N2 subtypes) can be lyophilized with no loss of activity. Also, the enzyme β-Galactosidase was encapsulated within PVP polymer microneedles and retained full activity. However, this may not be the case with other molecules. It will be important to determine if other biomolecules, proteins, peptides, DNA and other vaccines can be lyophilized and encapsulated within PVP polymer microneedles and retain full protection. Also, for those molecules that lose activity during lyophilization, it will be necessary to add cryoprotectants, like trehalose, which can protect the biomolecule during the freeze drying process. However, it will be important to minimize the amount of excipients added to the drug solution. Any excipient added will decrease the amount

of drug that can be encapsulated within the polymer microneedle system. Often, excipients are added in 100:1 ratio to the drug, which would severely limit the dose encapsulated and delivered using the polymer microneedle system.

In addition, it will be important to optimize the fabrication process to minimize the loss of drug during the encapsulation step. Currently, the drug of choice is suspended in liquid monomer and approximately 50-75 μL of solution is added to the microneedle mold. Only 2 μL enters the mold, and if solution on the surface is not fully recycled, significant wasting of the drug will occur. This loss of drug in the bulk solution was attempted to be minimized in this process, but further optimization is needed.

Also, it may be helpful to decrease the time of the polymerization step of the fabrication process. Currently, the microneedle system is left under UV curing for 30 minutes to one hour to complete the polymerization process for form the polymer microneedles. It would be beneficial to decrease this time to a few minutes if possible. This could be done by optimizing the conditions for the polymerization process, including but not exclusively the type and quantity of free radical initiator used, the purity of the monomer, and the distance between the UV lamp and microneedle system. This optimization may lead to lower mass fabrication costs for the dissolving microneedle system in the future.

10.2 OPTIMIZATION OF THE DESIGN AND GEOMETRY OF POLYMER MICRONEEDLES

In this project, two microneedle geometries were used, conical and pyramidal. There are advantages and disadvantages to each system. In summary, the conical system typically had a higher aspect ratio (height: base width) and was more prone to failure

before insertion than the pyramid microneedles. The pyramid microneedle system resulted into insertion of all microneedles in the array while the conical system often had insertion of only 50% of the microneedles. However, when insertion did occur, the conical microneedles inserted deep into the skin (80-90%). This is much higher than the pyramid microneedles, which had a shallow insertion into the skin (25 – 50%). This altered the length of time required for the different microneedle systems to dissolve and deliver the encapsulated cargo to the skin. The conical microneedles required less than one minute in pig skin, while the pyramid microneedles required 5 minutes of insertion to dissolve.

It would be optimal to combine the advantages of the two microneedle systems (pyramid/conical) and create a design that produced a stable structure with full insertion of the microneedle. Thus, it will be important to adjust the width, height and aspect ratio of the microneedles to determine the optimal design. In addition, a larger microneedle would allow for a higher dose of drug to be encapsulated and delivered. This may allow for a wider range of molecules to be delivered, which previously were limited due to dose constraints.

10.3 OPTIMIZATION OF THE DELIVERY EFFICIENCY OF THE POLYMER MICRONEEDLE SYSTEM

Currently, the dissolving microneedle system is applied to the skin with a small amount of force from a thumb. While the insertion technique can be taught easily to a new user, it should be automated in some way to allow for reproducible delivery with a small amount of error. This is especially important for self-administered use at home, where no trained personnel would be present. A possible solution would be an insertion

device. One example could be a spring loaded device, where the device is applied to the skin and a button is pushed, inserting the microneedles. Also, the insertion device should be reusable, simply applying a new microneedle patch to the end. The development of a new insertion device would be an important next step on the path of using the dissolvable microneedle system in a clinical setting on patients.

10.4 DEVELOPMENT OF ALTERNATE FABRICATION PROCESSES FOR POLYMER MICRONEEDLES BASED ON POLYMERIZATION TECHNIQUES

The new fabrication process, developed in this study, involved free radical photopolymerization of a liquid monomer solution within a microneedle mold to produce polymer microneedles. Since it uses a free radical mechanism, the polymers produced are constrained to vinyl polymers. It may be beneficial in the future to use a polymerization process to create microneedles made out of different polymers. The entire process would remain the same, including the use of a microneedle mold and liquid monomer, but the type of polymerization would change. This may involve addition or condensation polymerization. In addition, it is possible to develop alternate means of making polymer microneedles, outside of polymerization techniques. The main reason for a new fabrication process is to make a polymer microneedle system with specific desired properties. This may include faster or slower degradation in the skin, an adjuvant affect for vaccination purposes or other applications.

APPENDIX A:

BUSINESS PLAN FOR POLYMER MICRONEEDLES

During my graduate career, I had the opportunity to take part in the TI:GER (Technological Innovation: Generating Economic Results) program. The main goal of this program is to teach scientific, business and law students the tools required to take a product to market. Each team involved a PhD science/engineering student, an MBA student and two JD students. Their goal was to evaluate the potential of the PhD student's research technology for commercial use. The final step of this process involved the synthesis of a business plan, which was presented in multiple competitions nationwide. Below is the executive summary of the business plan for the polymer microneedle technology developed in this thesis.

A.1 EXECUTIVE SUMMARY

A.1.1 Business Description

PolyDerm Delivery Systems' PolyDerm Patch is an exciting new, painless drug delivery technology utilizing polymer microneedles. This technology consists of an array of microscopic needles large enough to deliver drugs effectively to the body, but small enough to avoid the nerves in the skin.

Currently, the world is struggling with limited quantities of vaccines and hypodermic needles and the inability to quickly and efficiently administer vaccinations in the case of a pandemic. The main advantages the PolyDerm Patch offers are:

- Reduction in the volume of vaccine needed to immunize, thus dramatically expanding coverage and reducing costs
- Decreased number of trained medical personnel for administration
- Elimination of medical waste and their associated risks
- Increased patient compliance due to ease of administration

Our mission is to provide an efficient, painless drug delivery system for large molecule drugs currently delivered by hypodermic injection. Our vision is to improve health worldwide while improving the environment by eliminating biomedical waste associated with dirty needles. To achieve these objectives, PolyDerm Delivery Systems (PDS) will partner with vaccine manufacturers and pharmaceutical companies to create custom drug delivery applications.

A.1.2 Development Status

Technology Accomplishment to Date:

- Prototype construction completed with FDA approved, non-toxic polymer
- Effective encapsulation and delivery of active protein to skin *in* vitro
- Successful animal trials showing effective delivery of influenza vaccine

Next Steps:

- Formulation of the final design including adhesive patch
- Passage through FDA clinical trials under Abbreviated New Drug Application with partner

A.1.3 Markets

PDS will focus on large molecule drugs that are currently delivered by the hypodermic needle. The flu vaccine market will be the initial focus with an eventual expansion into the broader biopharmaceutical drug delivery market.

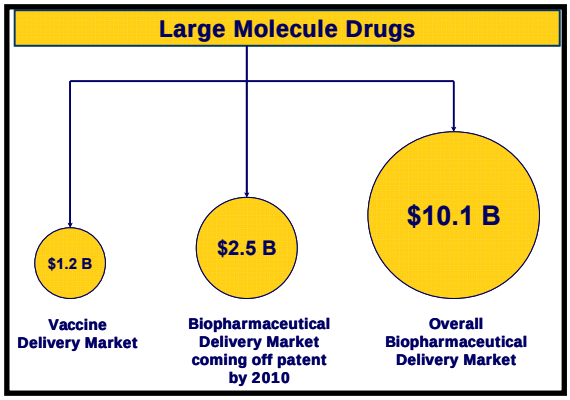

Figure A.1: Market for large molecule drugs

Microneedles offer an excellent technology for the vaccine market because they are painless, pre-packaged injections allowing for easier mass vaccination without extensive medical personnel. According to the New England Journal of Medicine, vaccine delivery to the skin will allow for five times the number of doses to be manufactured without increasing vaccine production.

A.1.4 Operations

PDS will operate as a drug delivery application development company creating individual applications and performing animal trials. After we have shown the effectiveness of polymer microneedle-based delivery to animals, clinical trials and production will be performed by our partner. PDS will assist in clinical trials and advise the partner how to

develop manufacturing. Because each application will require custom development and significant testing for approval, partnerships will be of utmost importance. Distribution will be the responsibility of the partner by utilizing their existing channels.

A.1.5 Risks

PDS must overcome two intellectual property risks. After comprehensive analysis and legal counsel, we anticipate receiving a strong patent for the polymer microneedles that are the basis of the PolyDerm Patch. Our unique technology encapsulates the drug in a polymer and effectively delivers the drug through the skin. An invention disclosure has been submitted to Georgia Tech; we expect that a patent application will be filed by June 2006. PDS will then secure a sublicense from Biovalve for their microneedle patent to avoid the risk of infringement lawsuits. Due to existing relationships between Biovalve and both Georgia Tech and one of our founders, PDS feels confident that a sublicensing agreement can be reached. Nelson Mullins is currently helping PDS develop a more comprehensive IP Strategy. Development, industry, and consumer risks are clearly displayed in the table above with corresponding mitigation strategies.

IP Risks	**Mitigation**
• Inability to secure Polymer Microneedle Patent and license • Inability to sublicense from Biovalve	• Invention disclosure submitted to Ga. Tech - File patent by June, 2006 • Leverage new patent application and existing relationships
Development Risks	**Mitigation**
• Inability to obtain FDA Approval through ANDA • Inability to scale manufacturing methods • Dosage limitations	• Partnerships for clinical trials • Simple manufacturing process • Less vaccine per dose • Biopharmaceuticals typically small dosages
Industry Risks	**Mitigation**
• Competition beats us to market • Competition has superior product	• Animal testing with flu vaccine delivery complete in Spring, 2007
Consumer Risks	**Mitigation**
• Lack of patient acceptance	• Enter the vaccine market first to limit risk to biopharmaceutical partners

Figure A.2: Risks associated with commercialization of polymer microneedle technology

A.1.6 Management

The PDS team currently consists of Steve Selfridge, James Stefanakos, and Sean Sullivan, all of whom attend the Georgia Institute of Technology. Steve and James are second year MBA candidates with experience in both consulting and chemical processes, and Sean is a PhD candidate in Biomedical Engineering and the inventor of the technology. The team brings excellent experience to the company as do the advisory board members which include experts in the fields of drug delivery and immunization. Recognizing the need for adding industry expertise to lead the company, the team will hire a seasoned CEO by the end of the first year and later bring in additional executives, scientists, and sales professionals.

A.1.7 Financials

The company projects profitability in the fiscal year beginning July 2009 and anticipates revenues in excess of $130 million by FY 2011 with a market penetration of 24% of the U.S. flu vaccine volume. We also expect to introduce the PolyDerm Patch to the biopharmaceutical market by FY 2011.

A.1.8 Exit Plan

The size of the identified markets and PDS's value proposition warrant an IPO if we grow rapidly and successfully enter the biopharmaceutical market. Slow growth or unanticipated capital needs would lead PDS to seek to be acquired by an existing drug delivery or biopharmaceutical company.

A.1.9 Offering

PDS is seeking seed level investment of $700,000 over the next year to solidify our IP position and find a vaccine partner. Subsequent investments estimated at $5.2 million will be required over the following two years to bring in a management team, assist our partner with FDA clinical trials, and enter the biopharmaceutical market. While the investment will take greater than three years to see any returns, we believe that the upside potential of this platform drug delivery technology makes the investment compelling.

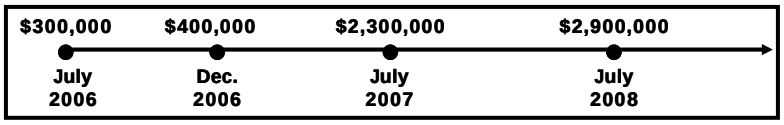

Figure A.3: Timeline for future investments

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