OPTIMIZING THE THERMAL STABILITY OF INFLUENZA VACCINE FOR MICRONEEDLE DELIVERY

A Thesis
Presented to
The Academic Faculty

by

Miraj Desai

In Partial Fulfillment
of the Requirements for the Degree
Bachelors of Science with Research Option in the
School of Chemical and Biomolecular Engineering

Georgia Institute of Technology
April 2016

OPTIMIZING THE THERMAL STABILITY OF INFLUENZA VACCINE FOR MICRONEEDLE DELIVERY

Approved by:

Dr. Mark R. Prausnitz, Advisor
School of Chemical and Biomolecular Engineering
Georgia Institute of Technology

Dr. Andreas S. Bommarius
School of Chemical and Biomolecular Engineering
Georgia Institute of Technology

Dr. Clifford L. Henderson
School of Chemical and Biomolecular Engineering
Georgia Institute of Technology

Date Approved: 04/26/16

ACKNOWLEDGEMENTS

I would like to thank my parents for their support and guidance in the writing of this thesis, especially for the scientific expertise provided by my father and the moral support from my mother.

I would also like to thank Novartis for generously providing monovalent influenza vaccine stock. This work was supported in part by the National Institutes of Health (NIH Quantum Grant #EB012495). The work was carried out in the Center for Drug Design, Development and Delivery, and the Institute for Bioengineering and Bioscience at the Georgia Institute of Technology. Matthew Mistilis, Andreas Bommarius, and Mark Prausnitz are inventors of patent(s) that have been or may be licensed to companies developing microneedle-based products, and Mark Prausnitz is a paid advisor to companies developing microneedle-based products and is a founder/shareholder of companies developing microneedle-based products. This potential conflict of interest has been disclosed and is overseen by Georgia Tech and Emory University.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

CDC	Centers for Disease Control and Prevention
WHO	World Health Organization
IV	Intravenous
ELISA	Enzyme-Linked Immunosorbent Assay
DLS	Dynamic Light Scattering
CD	Circular Dichroism
HA	Hemagglutinin
PBS	Phosphate Buffered Saline
PDMS	Polydimethylsiloxane
PBST	Phosphate Buffered Saline with Tween-20

SUMMARY

The purpose of this study was to create a thermally stable formulation of influenza vaccine that can be delivered intradermally using a microneedle patch. By altering drying conditions, storage conditions, and formulation components, vaccine activity can be preserved at room temperature for several months in a dried state. Delivery via a soluble, biodegradable polymer microneedle patch is the method of choice in this study because it allows for self-administration of the drug, creates no sharps waste, is pain free, has high bioavailability, and shows potential for removing influenza vaccine from cold chain dependency. By optimizing the combination of stabilizing techniques previously studied, preliminary results have shown that excipient solutions made up of sucrose, trehalose, and arginine, to name a few, in an ammonium acetate buffer are able to preserve close to 100% of vaccine activity for at least six months at room temperature when patches are loaded with a full human dose. These results show much promise for the eventual removal of many vaccine and drug formulations from cold chain dependency.

CHAPTER 1

INTRODUCTION

According to the Centers for Disease Control and Prevention (CDC), influenza is a contagious respiratory illness that is spread by a viral vector; however, the CDC also states that it is an easily preventable illness if proper immunization is conducted prior to being infected [1]. A study conducted by the World Health Organization (WHO) found that only 42% of Americans are vaccinated against influenza yearly, and as a result, influenza causes close to 4000 (1.2 in every 100,000) American deaths annually. The death toll from influenza worldwide is much higher with a range from 250,000 to 500,000 deaths per year [2]. Getting a simple flu shot significantly decreases the chance of contracting the virus and eliminates the chance of death with reasonable statistical significance.

The most commonly used and traditional method for influenza vaccination today is via a syringe fitted with a hypodermic needle. The vaccine used in this method of delivery is prepared by reconstituting a lyophilized powder/crystalline form of the vaccine. Lyophilization is the process of freeze-drying vaccine in order to make the vaccine more thermo-stable and increase its shelf life; however, there are several downsides to lyophilization. The major issue is that the powders made by the process must still be stored at low temperatures to keep the proteins from denaturing, and thee powders are sensitive to changes in humidity. Lyophilized vaccines can also denature during the freeze-drying process due to the rapid phase transition to a crystalline form [3].

Aside from lyophilization, very little research has been done to find alternative methods for delivery of influenza vaccine. At the moment, the major alternative is vaccination by nasal mist with parenteral delivery used in extreme cases. Delivery via nasal mist is minimally invasive, has a high bioavailability, and is painless and easy to

administer, but also has several issues. To name a few limitations, pregnant women, patients with lung diseases and asthma, and the elderly may be adversely affected by intranasal vaccination [4]. On the other hand, parenteral delivery, which involves intravenous (IV) delivery, is able to deliver flu vaccine with the best rate of delivery and with instant entry into the bloodstream. The major downside to this method is that the patient must be hooked up to an IV and may require local anesthesia in certain cases [5]. This method of delivery also bypasses the many antigen presenting cells found in the outer tissue layers leading to a decreased immune response.

A much better alternative to these methods is to use a soluble, drug loaded, microneedle patch. Microneedle patches consist of a small array of soluble needles that range anywhere from 50 – 750 microns in length. The stratum corneum is the outer layer of skin consisting of dead cells, and drug delivery is impossible if the drug cannot make it past this layer. Since the stratum corneum is about 10 – 15 microns in thickness, most microneedle patches are easily able to penetrate past this layer of skin. The added benefit of this method is that needle length can be targeted to be just enough to reach past the stratum corneum, but not any further. This keeps the needles from reaching any nerves in the deeper layers (~1 mm is where pain can be felt) of the skin making this method of drug delivery completely painless [6]. Also, the microneedles are made of a sugar/polymer blend, which dissolves when contacted with skin. The drug is incorporated into the microneedles and releases when the needles dissolve into the skin's interstitial fluid. Because the needles are soluble, are so much smaller than syringes, and contain no sharps, the amount of waste from delivering drugs through soluble microneedle patches is significantly reduced. The lack of pain and the ability to self-administer the patch without professional medical assistance also motivates more people to get vaccinated. A study found that 7 – 8% of people choose not to vaccinate themselves due to needle phobia [7]. Another study found through a survey that offering microneedle patches as an alternative to vaccination by syringe increased the number of people who planned on getting their flu

shot from 44% to 65% and that 64% of people planning on getting vaccinated would have preferred to self administer their vaccination as opposed to going to a healthcare center [8].

The one downside to using microneedles as a medium of drug delivery is that they can only hold a relatively small volume of drug, and if left at room temperature for more than a few minutes, the drug solution will rapidly evaporate. This leads to the aggregation of proteins in the vaccine and the resulting conformational change often deactivates or damages the proteins to the point where they will no longer be able to elicit an immune response. However, there are ways to stabilize the vaccine. Adding excipients to vaccine formulations, then drying the vaccine under vacuum or at room temperature (which has to be done quickly to prevent denaturation from heat) leads to negligible loss in activity when reconstituted. Use of buffer salts, different microneedle materials, varied storage temperatures, and several other variables have a wide range of effects on vaccine activity while drying, but there are certain combinations of these variables where almost no loss of activity occurs. Some excipient stabilizers have even been able to stabilize dried vaccines at room temperature for at least one year. This means that the vaccine has the potential to be made completely independent of cold chain transport and storage resulting in energy savings and increased thermal stability [9].

This study will seek to determine the optimal excipient stabilizers among other variables that allow dried influenza vaccine to remain thermally stable and undamaged after long-term storage at room temperature. Activity will be tested using enzyme-linked immunosorbent assays (ELISAs). If time permits, ELISA data will be supplemented by dynamic light scattering (DLS) analysis and circular dichroism (CD) to monitor conformational changes. This study will also attempt to conduct an in vivo model in mice to ensure that immune response in mice that receive vaccine via microneedle patches is not statistically different from those that receive vaccine from a syringe. Long-term goals

and implications of this research are to promote and encourage vaccination, pain mitigation, energy savings, and waste reduction.

CHAPTER 2

LITERATURE REVIEW

The Influenza Virus

Influenza is a seasonal, highly contagious respiratory disease spread that spreads as a virus. The main symptoms consist of headaches, high fevers, coughing, and body aches to name a few, with death occurring in extreme cases. Death from influenza ranks number eight on the Centers for Disease Control and Prevention's list of most common causes of death in the U.S. The virus kills close to 4000 Americans and 250,000 to 500,000 people around the world each year. Globally, anywhere from 3 – 5 million people contract the virus and experience serious illness for some period of time each year. Children have a much higher risk of contracting the virus with the attack rate being 20-30%, yet only 46.9% of American children aged 6 months to 17 years were vaccinated in the last year. Adults aged 18-49 have a lower chance of being infected by the virus with a chance of 5-10%, yet only 29.6% of American adults are vaccinated against influenza annually [2 10 10b]. The easiest way to reduce the risk of death from influenza is through immunization two to three weeks prior to "flu season". A vaccine effectiveness study conducted by the CDC shows that getting immunized reduces the risk of contracting the virus by 70% on average [11]. Standard flu vaccines are either trivalent or quadrivalent, meaning they immunize against three or four strains of influenza respectively and are either live attenuated (a deactivated, live virus is introduced to promote immunity), or contain pieces of destroyed influenza virus. These strains consist of type A and type B strains. The different strains consist of different combinations of the two active molecules found on the surface of the influenza virus, hemagglutinin and neuraminidase. Hemagglutinin allows the virus to bind and enter cells and neuraminidase facilitates exit and spread of the virus to other cells. Vaccination helps promote the

creation of antibodies in the blood that are able to recognize and eliminate any virus particles with hemagglutinin and neuraminidase on their surface [12].

Influenza Vaccine

Influenza vaccination is currently the only method for significantly reducing the chance of contracting the influenza virus; however, the use of vaccine has limitations and care must be taken to ensure the sterility and potency of the vaccine. Since the vaccine is protein based, it is not very thermo-stable, i.e. it is extremely sensitive to changes in temperature. One study found that unaltered influenza vaccine loses almost all of its activity after storage at 55°C for three hours. Storage at room temperature increases the amount of time it takes the proteins to denature, but in the end, the protein is still unusable. The study was able to increase the thermo-stability period by using stabilizers in the formulation of the vaccine, but even the most promising of the formulations was only able to maintain 50% of its activity after three hours of storage at 55°C. Thermal stability in influenza vaccine is usually measured by assaying for hemagglutinin (HA) concentration after storage at various conditions [13]. Due to the relative lack of thermal stability of influenza vaccine, commercially used influenza vaccine is dependent on the cold chain. The CDC recommends that vaccine exposed to temperatures below 2°C or above 8°C for longer than a few minutes should not be administered because freezing and exposure to high temperatures result in denaturation of the proteins in the vaccine [14].

Syringe Delivery and Lyophilization

In today's world, hundreds, if not thousands, of drugs, vaccines, individual molecules, and various other chemical based compounds have been formulated for delivery to the human body, yet only a handful of delivery techniques have been researched and developed to facilitate their journey into the body. The vast majority of delivery techniques can be categorized into two major groups: invasive and minimally

invasive. For influenza vaccine, this narrows down to vaccination by hypodermic syringe and needle, intranasal vaccination, parenteral delivery, and transdermal delivery. The most common method of delivery by far is via hypodermic needle and syringe. This method of delivery relies on using influenza vaccine stored in vials at low temperatures to keep the vaccine from denaturing. Currently, very few methods exist to stabilize the vaccine at room temperature or higher. The most common method is to lyophilize the vaccine after adding excipients and stabilizers. One study tested the effects of freeze-drying influenza vaccine on vaccine stability after reconstitution and storage at various temperatures and found that thermal stability is greatly increased when the vaccine is stored as a powder in a sugar glass matrix. Drying of the vaccine reduces the mobility of the vaccine making it difficult for the molecules to rearrange and the use of a sugar glass matrix keeps the vaccine particles from aggregating; however, the study found that the shear stress introduced to the vaccine during freeze-drying resulted in some loss of activity and aggregation. The cause of this decrease in activity was most likely due to the loss of water, which is necessary to stabilize proteins when in the solid phase, during the drying process. The study also tested several other drying methods such as vacuum drying, desiccation, and supercritical drying, but many of these methods were unable to stabilize the vaccine any better than lyophilization. Also, the study found that most lyophilized vaccine powders were rendered completely impotent after storage at 45°C for four weeks [15]. Aside from the dependence of the influenza vaccine on the cold chain and lyophilization, delivery via hypodermic needle and syringe is also an invasive and painful method of drug delivery which results in about 5% of all patient non-compliance for vaccination [16]. There is also biohazardous sharps waste that must be properly disposed of post injection.

Common Alternatives to Syringe Delivery

Aside from delivery via hypodermic needle and syringe, the other two common methods of delivering influenza vaccine are via nasal mist and parenteral delivery. Intranasal delivery of influenza vaccine is a relatively new method of being immunized against influenza and offers several benefits, but also comes with a host of issues. First and foremost, intranasal drug delivery offers a much higher bioavailability than traditional delivery methods when delivering large molecular weight proteins and peptides. This is especially useful when delivering a drug like influenza vaccine because the vaccine proteins are live attenuated and mimic the uptake of pathogens through the nasal passage as it would occur naturally [17]. This method of drug delivery is also painless and is minimally invasive which could result in the decrease of patient non-compliance. While intranasal delivery of influenza vaccine flaunts high bioavailability and the absence of pain, it is not a viable option for everyone in the general population. The CDC states that people with respiratory issues, pregnant women, immune-compromised individuals (vaccine is live attenuated), the elderly, caretakers of immune-compromised individuals, and individuals that are allergic to eggs are not suitable for intranasal vaccination [4]. Intranasal vaccines are formulated in a similar fashion to vaccine used in bolus injection, and the FDA states that intranasal flu vaccine must be stored at similar conditions to that of regular liquid influenza vaccine. This indicates that the thermal stability of intranasal influenza vaccine is not any better than that of standard influenza vaccine and will require cold chain storage and transport. Also, the FDA regulation for storage of FluMist (one of the more common brands of intranasal influenza vaccine) is that the maximum shelf life is 18 weeks when stored between 2 and 8°C [18]. The other common (less common than intranasal and syringe) method for influenza vaccine delivery is parenteral delivery. Parenteral delivery boasts an almost 100% bioavailability and extremely fast delivery speeds since the drug is being inserted directly into the bloodstream. It is easy to control dosages and concentrations when delivering via

the parenteral route, but that is essentially the only useful attribute. One study found that while parenteral influenza vaccination significantly increased immune response and antibody levels in the blood and upper respiratory tract, the immune response was significantly weaker very shortly after vaccination, with antibody counts back to pre-vaccination levels after about two to three weeks [5]. Parenteral delivery is also an invasive, needle-based method of immunization that requires the patient to be hooked up to an IV bag. This method is not only uncomfortable, but is also inconvenient and expensive. This method of drug delivery most likely increases patient non-compliance even more than needle phobia. Since the vaccine used in parenteral delivery is practically the same vaccine used in bolus injection, it is similarly constrained to the cold chain and lacks significant thermal stability.

Transdermal Delivery

A new and novel approach for the delivery of influenza vaccine is transdermal drug delivery. Transdermal drug delivery focuses on delivering drug through the skin as the name suggests. There are currently no marketed transdermal influenza vaccinations available, but many have shown promise in laboratory and clinical trials. The one major barrier to transdermal drug delivery is the fact that only relatively small and hydrophobic molecules are easily able to pass through the outmost layer of the skin; however, transdermal drug delivery methods have found ways to bypass this outer layer of skin and access the underlying aqueous interstitial fluid. Also, because of the large number of antigen presenting cells, including Langerhans and dendritic cells present in the skin, extremely small doses of vaccine are able to achieve relatively high levels of bioavailability. The challenge in drug delivery is being able to get the drug past the stratum corneum, as mentioned earlier, which is a layer of dead skin cells just below the surface of the skin at the top of the epidermis. Once a drug makes it past the stratum corneum, it can be picked up by antigen presenting cells and invoke a cell mediated

immune response. Various methods of transdermal delivery have been studied with the focus on temporarily disrupting the structure of the stratum corneum in order to allow drugs to pass through undisturbed. The major contenders are iontophoresis, ultrasound, electroporation, thermal ablation, and microneedle injection. Iontophoresis focuses on using an electrical driving force to "push" molecules through the stratum corneum. This method has limitations in that it can only achieve high bioavailability for very small molecular weight drugs. Ultrasound uses high frequency oscillating pressure waves to disrupt the lipid structure of the stratum corneum to create a gap for molecules to pass through, but if not properly controlled, can damage lower level tissues in the skin. Electroporation uses high voltage pulses to increase cell membrane permeability allowing drugs to pass through the stratum corneum in a cell mediated transportation method, but engineering of devices to perform this task with proper accuracy is extremely difficult. Thermal ablation involves heating a localized area of skin to an extremely high temperature within the span of a few milliseconds. The high temperature and short duration minimize main and result in small micro craters that allow for enhanced drug delivery past the stratum corneum [19]. The aforementioned methods are all novel approaches to transdermal drug delivery, but all have major limitations that make it very difficult to deliver a drug like influenza vaccine. A much more effective method of transdermal drug delivery with less potential for injury is to deliver via a microneedle patch. This study will aim to optimize the long-term thermal stability of influenza vaccine through transdermal delivery using a soluble microneedle patch.

Microneedles

Microneedles injections are made up of an array of micron-sized needles that are able to painlessly pierce the outermost layers of the skin and deliver drug past the stratum corneum via several different methods. The major classes of microneedles include solid, coated, dissolving, and hollow. Solid microneedles create "holes" in the skin that allow

for increased delivery upon removal of the needles and secondary application of drug formulations. Coated microneedles consist of drug attached to the outside of the needles that detach once the needles enter the skin. Dissolving microneedles are fabricated with the drug as a part of the needle itself that involves the entire needle dissolving once it comes into contact with the skin. Finally, hollow microneedles consist of a backing filled with drug that releases through the needles leaving the needles intact upon removal. Microneedles are typically made of stainless steel or some form of polymer gel. Each type of needle has pros and cons associated with it, but since this study will focus on soluble microneedles, they will be discussed in additional detail.

Soluble Microneedles

Soluble microneedles are fabricated with water soluble and biodegradable polymers that hold the drug within the needles. Once the needle is contacted with the skin, the needles begin to dissolve and release the drug. Since the needles are made long enough to reach past the stratum corneum, bioavailability is high and most of the drug is able to make it past the layer. The needles are also made short enough so that they do not interact with pain receptors, which are usually found ~ 1 mm into the skin (well past the length of typical microneedles). The advantage of using this method of drug delivery is that it is painless and leaves no sharps waste behind. It is also a much easier method of drug delivery, potentially allowing for patients to self-administer the vaccine without relying on assistance from healthcare professionals [20]. This could lead to decreases in patient non-compliance in the future. One study found that offering a self-administrable patch for influenza vaccination increased participants' intent to vaccinate from 44% to 65% [8].

The issue with delivery via soluble microneedles is that the volume of drug is so small that it is prone to evaporating very quickly. Once the water has left the surface, the influenza vaccine particles begin to aggregate and render the vaccine useless. This means

that the thermal stability of unaltered soluble microneedle patches is very low; however, this drawback can be remedied. One study dried samples of influenza vaccine under various conditions and with different combinations of stabilizers and found that it was possible to store dried vaccine at room temperature for up to 26 weeks without reducing the concentration of HA significantly. The main method for drying used in the study was a lyophilization based freeze-drying operation after the vaccine formulation was mixed with various stabilizers. The study found that drying resulted in changes to the secondary structure of the virus subunits and that drying with stabilizers helped to reduce the conformational change and aggregation introduced by drying. This was verified by using a circular dichroism analysis to measure changes in secondary protein structure. Mice given the dried/reconstituted samples showed no significant difference in immune response from the control group. One of the major findings of the study was that upon freezing of the vaccine, the pH of the vaccine began to change and thus induced a conformational change [21]. Another study was able to implement the aforementioned stabilizer based drying method with microneedle patches. This study was able to dry vaccine with various stabilizer and buffer combinations in microneedle patches, store them at temperatures up to 40°C for six months without any significant loss of HA activity. The mechanisms for the drying are not fully understood yet, but it was found that the excipients introduced while drying were able to create a matrix to replace the water that had been evaporated away and that this was able to keep the vaccine subunits from aggregating excessively and denaturing. The major benefit of using this method to increase thermal stability is that the dried vaccine does not need to be reconstituted before use because it is designed for use in soluble microneedles.

Once the microneedle is touched to the patient's skin, the drug will reconstitute into the skin itself and deliver [9]. The two above mentioned studies lay the foundation for non-invasive thermo-stable microneedle delivery of influenza vaccine and have shown that the cold chain is not necessary if the proper conditions are met while drying. The

implications of the various studies mentioned above are that it is extremely difficult to create a thermo-stable formulation of the influenza vaccine. The major contenders are lyophilization, which is the method of choice in the medical industry at the moment, and microneedle patches that deliver dried/reconstituted vaccine. Lyophilization does not increase thermal stability in a long-term sense and introduces shear-mediated stresses on the vaccine, but is a means to an end. On the other hand, microneedles are painless, leave no hazardous waste, can be self-administered, and can retain thermal stability for much longer periods of time at much worse conditions of storage. They also have higher bioavailability when properly delivered past the stratum corneum. This makes microneedle delivery of influenza vaccine the method of choice when it comes to preference and the future applications are endless once the cold chain dependency of the vaccine is removed.

CHAPTER 3

METHODS AND MATERIALS

Vaccines and Excipients

This study used both monovalent and trivalent vaccines. All monovalent vaccine used in this study was provided by Novartis Vaccines and Diagnostics (Cambridge, MA). Different strains of vaccine were used based on the type of storage condition and testing scenarios. For long-term storage, both monovalent and trivalent antigens from strains of [B/Brisbane/60/2008], [A/Brisbane/59/2007] (H1N1), and [A/Victoria/210/2009 (H3N2)] were used. For samples that were challenged with higher temperatures, [A/Brisbane/10/2010] (H1N1) antigens were used. All of the excipients employed were obtained from Sigma-Aldrich (St. Louis, MO). Vaccine/excipient methods were designed with Matthew J. Mistilis.

Formulations and Buffer Exchanges

All excipient based vaccine formulations were used at 10% w/v. Solution were prepared by dissolving the appropriate mass of excipient into a buffer solution. As mentioned earlier, certain samples required the use of different buffers for testing. Since stock vaccine was obtained in phosphate buffered saline (PBS), buffer exchanging was necessary. This was performed using Vivaspin 500, Vivaspin 20, or Vivaspin 2 centrifuge filters with molecular weight cutoffs of 100, 10, and 3 kDa, respectively (Sartorius AG, Göttingen, Germany) to concentrate the vaccine to 10x or greater concentration followed by reconstitution in the desired buffer (usually 150 mM ammonium acetate buffer). All vaccine formulations also contained 1% w/v carboxymethyl cellulose. Formulation methods were designed with Matthew J. Mistilis.

Using PDMS Chips for Drying Vaccine

In order to maintain a high throughput while testing samples, vaccine was dried on 6 mm diameter circular cutouts of polydimethylsiloxane (PDMS). PDMS was used in order to mimic the drying surface of a microneedle patch without having to make hundreds of patches. 2 μL droplets of vaccine were carefully pipetted onto the PDMS chips and weighed on a Sartorius SE2 Ultra Micro Balance (Sartorius AG, Göttingen, Germany) to account for pipetting inaccuracies. The drops were then allowed to dry and were stored at various conditions for stability testing in aluminum pouches (Oliver-Tolas Healthcare, Grand Rapids, MI) that were impulse heat-sealed (AIE-300, American International Electric, Industry, CA) and stored with desiccant. Once storage was complete, the chips were placed into plastic, 0.5, 2, or 5 mL tubes (depending on the volume of the solution) with a known volume of the same buffer that the vaccine dried in, allowing for the dried vaccine/excipient solution to reconstitute. A similar procedure was used for stainless steel microneedles, with the only difference being that stainless steel cutouts were used in place of the PDMS chips. PDMS chips methods were designed with Matthew J. Mistilis.

Microneedle Patches

This study did not test full stainless steel microneedle patches, but soluble microneedle patches were tested. The procedure for making the microneedle patches consisted of adding the vaccine and sealing the mold with a backing. Vaccine was placed onto a premade microneedle patch mold and was pulled into the holes under vacuum. Excess vaccine that did not make it into the holes was removed using a flat blade. Once the vaccine solution dried in the mold, a polymer backing was added to seal the holes. The backing was made of a solution of polyvinyl alcohol, sucrose, and water in a mass ratio of 8:6:15. The patch molds were then dried for two days with desiccant after which

they were demolded using scotch tape. After demolding, the patches were stored and reconstituted in the same manner as the chips as described above. Patch fabrication methods were designed with Matthew J. Mistilis.

ELISA

After reconstituting the chips/patches into buffer solutions, the solutions were assayed using a standard sandwich enzyme linked immunosorbent assay (ELISA) to test for active HA content. Polyclonal, strain-specific antibodies were received from the Center for Biologics Evaluation and Research of the Food and Drug Administration (Silver Spring, MD). Antibodies were conjugated to horseradish peroxidase with a Lightning Link conjugation kit (Innova Biosciences, Cambridge, UK). Unformulated vaccine stock was serially diluted in phosphate-buffered saline with 0.5% Tween-20 (PBST) and used as a reference standard curve. An Immulon 2HB 96-well microplate (Thermo Scientific, Waltham, MA) was used as the coated surface. The microplate was washed 3 times between each step with PBST with 3% w/v bovine serum albumin. The HRP substrate reaction involved SureBlue Reserve TMB solution (KPL, Gaithersburg, MD), which was stopped with TMB Stop Solution (KPL). The microplate absorbance at 620 nm was read using an iMark plate reader (BioRad, Hercules, CA). The standard curve was fit to a four-parameter function using the Microplate Manager 6 software (BioRad). ELISA methods were designed with Matthew J. Mistilis.

CHAPTER 4

RESULTS

The results from this study are all centered on vaccine activity remaining after changing some part of the formulation or storage of a formulation. The major parameters tested were buffer solutions, surfactants, excipient stabilizer solutions, storage temperature, and patch performance.

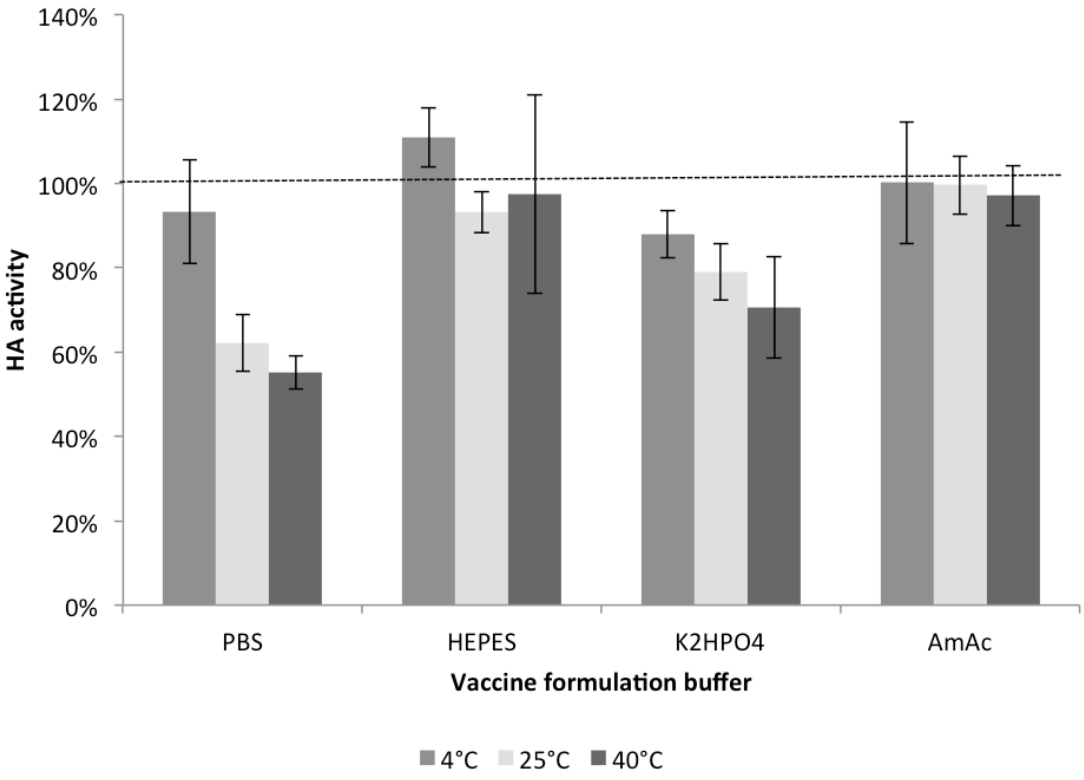

Figure 1. Activity of samples that were buffer exchanged and dried on PDMS chips at various temperatures. Some buffers were able to retain activity even at higher temperatures. Data represent averages of n = 6 replicates, with standard deviation bars shown. This data was obtained with Matthew J. Mistilis.

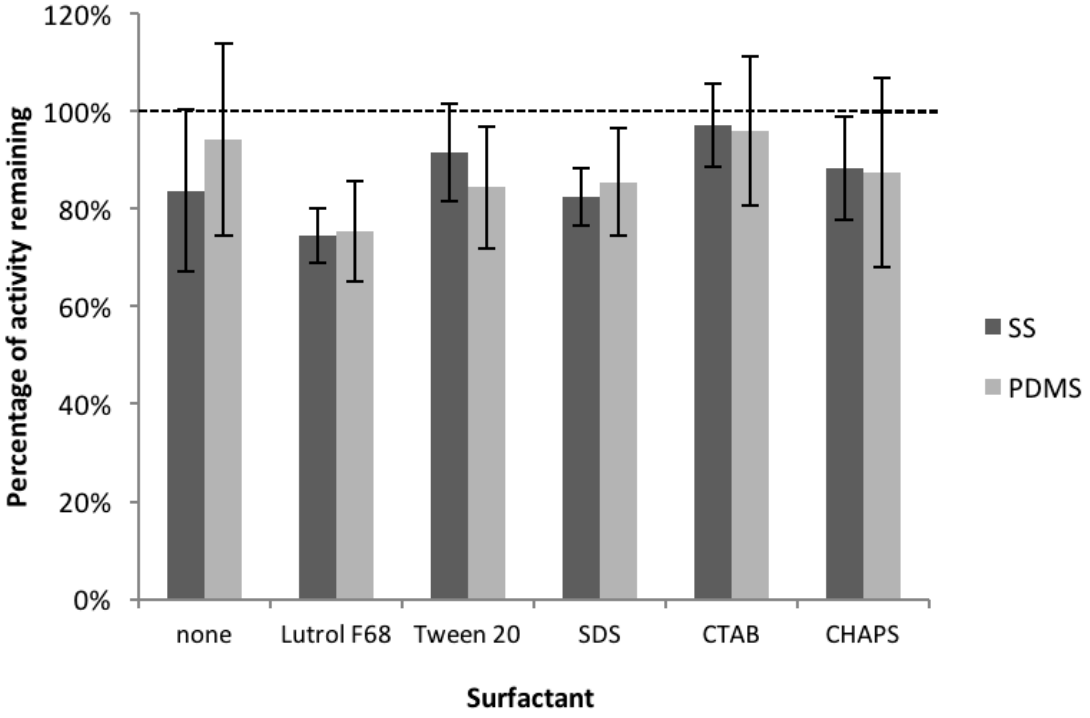

Figure 2. Effects of various surfactants on vaccine stability and comparison of PDMS as drying surface with a stainless steel surface. Surfactant was added to liquid vaccine, which was dried on chips for 20 minutes at room temperature. Most surfactants did not significantly affect activity retention in the short term. Data represent averages of n = 6 replicates, with standard deviation bars shown. This data was obtained with Matthew J. Mistilis.

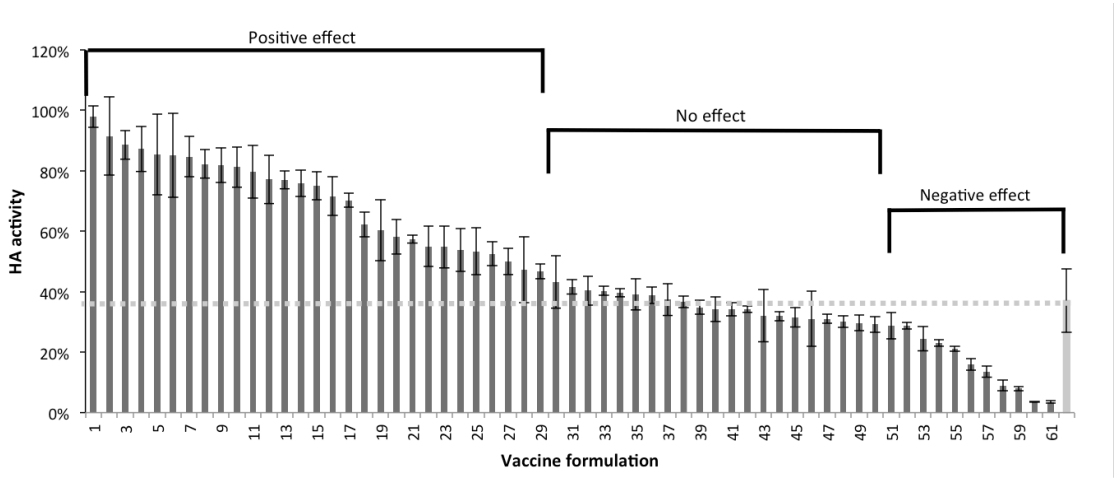

Figure 3. A screen of various stabilizers and their effects on vaccine activity after drying and storage at room temperature for 24 hours. All excipients were used at 15% w/v. *p-value $\leq$ 0.05 when compared to vaccine with no added stabilizer. The light gray bar on

the far right is unformulated vaccine. Data represent averages of n = 6 replicates, with standard deviation bars shown. This data was obtained with Matthew J. Mistilis.

Stabilizer	Activity remaining	Stabilizer	Activity remaining
maltodextrin 4	98%	sorbitol	76%
arginine	91%	arabitol	75%
trehalose	89%	fructose	72%
maltose	87%	cyclodextrin, γ	70%
histidine	85%	potassium gluconate	62%
calcium heptagluconate	85%	adonitol	60%
maltodextrin 13	85%	xylitol	58%
sucrose	82%	sodium thiosulfate	57%
glucose	82%	asparagine	55%
heparin	81%	cyclodextrin 2-hydroxypropyl-β	55%
raffinose	80%	TRIS	54%
myo-inositol	77%	sodium citrate	53%
lactose	77%	dulcitol	53%

Table 1. Activity values from Figure 3 (% of liquid vaccine activity remaining).

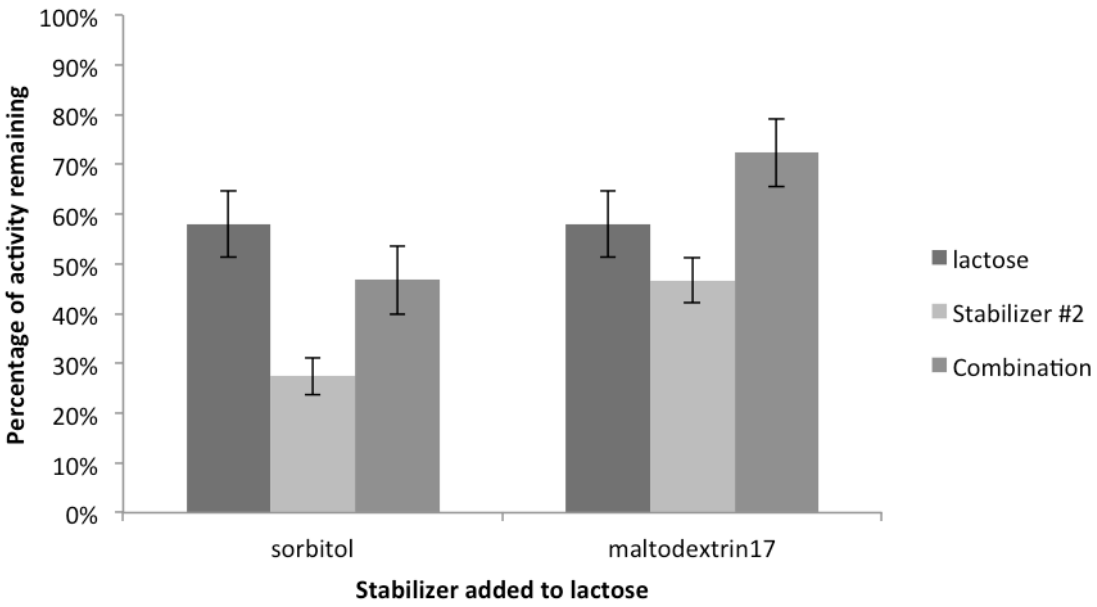

Figure 4. Effects of combining lactose with a second stabilizer, either sorbitol or maltodextrin on vaccine activity. Data represent averages of n = 6 replicates, with standard deviation bars shown. This data was obtained with Matthew J. Mistilis.

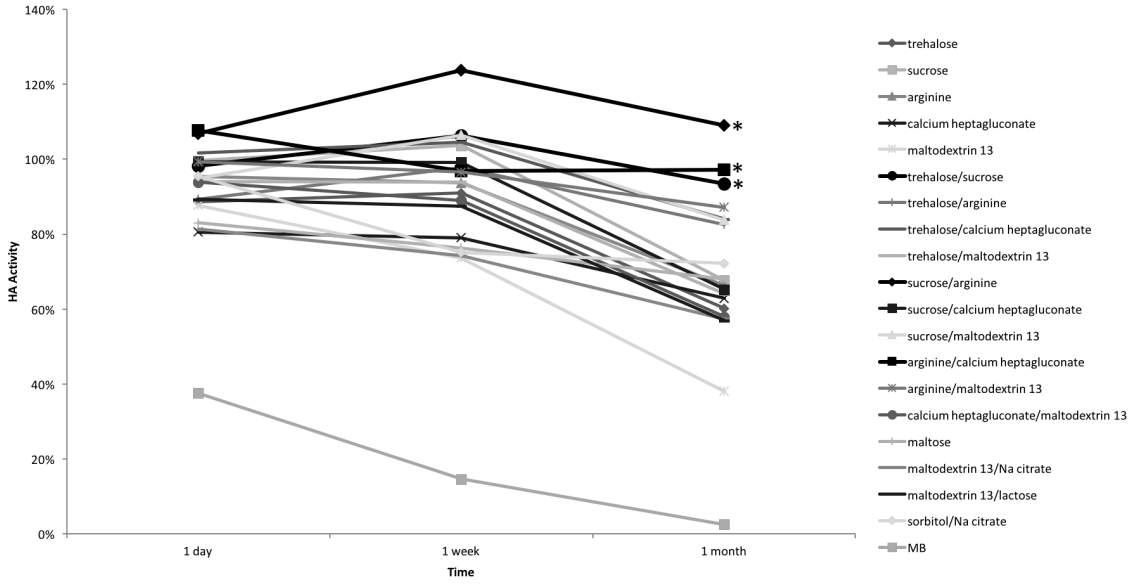

Figure 5. Effects of stabilizer combinations on vaccine activity as opposed to using only one excipient at a time. Certain stabilizer combinations were statistically stable on PDMS chips for up to 1 month at 40°C. Asterisks (*) represent formulations which showed no statistical difference among the time points tested (Student's t-test; $p \leq 0.05$). MB stands for monobulk vaccine, which is vaccine that was left without added stabilizer as a control. Data represent averages of n = 6 replicates, with standard deviation bars shown. This data was obtained with Matthew J. Mistilis.

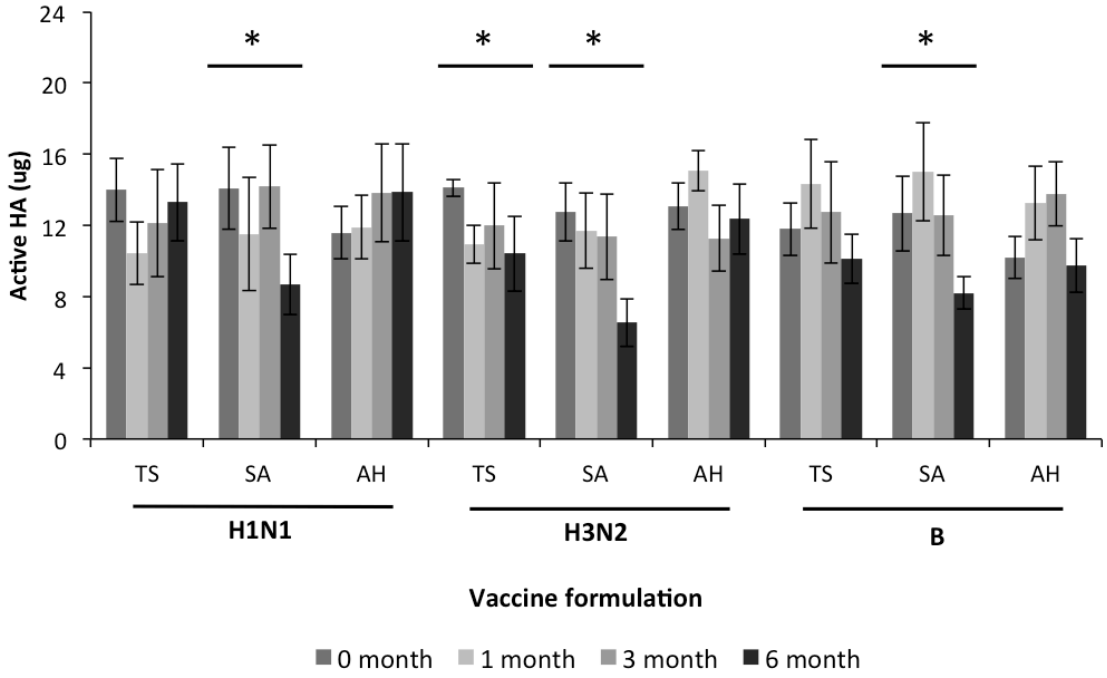

Figure 6. Vaccine activity after storage in microneedle patches at 25°C for up to 6 months. Patches were loaded with almost a full human dose of vaccine. (full human dose = 15 µg per strain) and three different strains of vaccine antigens were tested. The excipient combinations that were tested were the highest performers in Figure 3. Asterisks (*) represent formulations which showed statistically different activity between 0 month and 6 month time points (Student's t-test; $p \leq 0.05$). Data represent averages of n = 6 replicates, with standard deviation bars shown. This data was obtained with Matthew J. Mistilis.

CHAPTER 5

DISCUSSION

The results obtained from this study indicate that influenza vaccine can successfully be made thermally stable if formulated with an optimal combination of conditions in amounts as large as a full human dose. The data also closely matches the trends seen in stability testing during lyophilization.

Analysis of the effects of buffer solutions on activity revealed that ammonium acetate was the optimal buffer for activity retention, with vaccine activity unaffected even after storage at 40°C, as seen in Figure 1. This is why many of the formulations that were tested were buffer exchanged from their stock PBS to ammonium acetate. The mechanism for stabilization via buffer salts is still being researched, but the current hypothesis is that certain salts within the buffer are more effective than others in keeping protein molecules from aggregating with each other. As for surfactants, many were useful for the initial coating/wetting of metal microneedles, but based on the data presented in Figure 2, none of them had positive effects on activity, regardless of the drying surface. Since surfactants were not found to be helpful during drying, and there is evidence that including surfactant in microneedle formulations can lead to instability during long-term storage, they were not included in any of the other microneedle patch formulations. They were also not necessary for the proper function of soluble microneedles, which were the focus of this study.

A wide range of compounds were tested for possible stabilizing effects. After analyzing the excipient screen, it is clear that not all excipients had the same effect on

vaccine stability, but that there were several excipients able to maintain vaccine activity after drying. Of the stabilizers that were tested, there were several that improved stability (with respect to the vaccine without an added stabilizing excipient), but there were also a few that were detrimental to stability. Upon further testing, it was found that the activities in Figure 3 and Table 1 did not stay the same when samples were stored for longer periods of time. For this reason, combinations of stabilizers were tested alongside individual excipients. An example of one combination was when lactose was combined with two other stabilizers. Figure 4 shows that proper selection of the constituent stabilizers can actually yield a higher retention of activity than its constituent excipients (as well as a lower activity or an activity between the two constituents). Similar to the stabilizer screen in Figure 3, various combinations of the stabilizer combination were also tested, but this time at 40°C for up to one month of storage. The data for this is presented in Figure 5, and it is evident that certain combinations performed much better than others. Untreated vaccine was completely devoid of functional protein after one month of storage, but some of the combinations retained essentially 100% of their activity (no statistically significant difference). As controls, some of the high performing individual stabilizers were also tested, but were almost always outperformed by the combinations.

All of the previously mentioned data was obtained from dried vaccine on PDMS chips in an ammonium acetate buffer solution. As a further proof of concept, the vaccine was also incorporated into microneedle patches and assayed after production and storage. As seen in Figure 6, regardless of the strain of vaccine used, almost every excipient solution tested had no statistically significant difference between the vaccine at the day of drying and the vaccine six months later after storage at 25°C. Furthermore, the patches used a significantly larger volume of vaccine than the PDMS chips, and a full human

dose equivalent was loaded. This shows that the vaccine was effectively made thermally stable in the limits of a full human dose.

The implications of these results are that a proof of concept has been tested and proven for the stabilization of influenza vaccine for delivery via soluble microneedle patches. This technology holds the potential to reformat the administration of traditional vaccines and can also help remove many drug solutions from cold chain dependency. Hopefully, by making vaccination easier, more cost effective, and more accessible, this study's broader aim is to reduce the prevalence of sickness and deaths that are preventable by vaccination.

CHAPTER 6

CONCLUSIONS

Influenza is responsible for several thousands of deaths around the world each year, yet is also one of the most preventable pathogenic infections. By receiving an influenza vaccination once per year, chances of death can be reduced by up to 70%. Currently, the clinical methods for vaccination involve the use of a hypodermic needle and syringe or an intranasal mist, both of which are severely limited with regards to ease of administration and maintaining vaccine integrity. A novel alternative to these methods is the use of microneedle patches with excipient stabilization, which allow for enhanced vaccine thermal stabilization as well as painless administration, reduced biohazardous sharps waste, potential self-administration, and high immunogenicity.

The results of this study have shown that finding the optimal combination of vaccines, excipients, buffers, and surfactants can lead to a stabilized, microneedle incorporated delivery system. Using an ammonium acetate buffer with excipients solutions containing combinations of trehalose, arginine, sucrose, and heptagluconate has been shown to stabilize and retain the activity of a full human dose of influenza vaccine for over six months at room temperature. Combinations of stabilizers were found to stabilize vaccines in a much different manner than individual excipients, with the frontrunners being formulations that contained at least two excipients. This study is one of many first steps in development of this technology, and the hope is that it will serve as a fundamental proof of concept to promote further research in the area.

CHAPTER 7

FUTURE WORK

Plans for continuing research include using dynamic light scattering (DLS) and circular dichroism (CD) to monitor the mechanism of vaccine activity loss, as well as performing an animal study to verify *in vivo* immunogenicity. DLS is used to test for the size of particles in solution. Since influenza vaccine proteins tend to aggregate during drying, DLS can be used to better understand the mechanisms behind protein aggregation and denaturation. CD is useful for analyzing secondary protein structure. By following the changes in the arrangement of peptide chains in the HA protein, the mechanism of any protein conformational changes can be observed. Finally, an animal study utilizing mice is currently in progress. Mice are immunized either by standard vaccine via a syringe or by vaccine in a microneedle patch (some microneedle patches have been stored for various amounts of time). Antibody titers will be collected afterwards to compare levels of anti-influenza antibodies.

REFERENCES

[1] Srivastav, A.; Santibanez, T. A.; Kahn, K. E.; Zhai, Y.; Greby, S. M.; Bridges, C.; Lu, P.-J.; Williams, W. W.; Singleton, J. A.; Stinger, M. C.; Dever, J. A.; Lawrence, M. S. S.; Fahimi, M.; Davis, N., National Early Season Flu Vaccination Coverage, United States, November 2014. Prevention, C. f. D. C. a., Ed. 2014.

[2] WHO Influenza (Seasonal) Facts Sheet. http://www.who.int/mediacentre/factsheets/fs211/en/.

[3] Rexroad, J.; Wiethoff, C. M.; Jones, L. S.; Middaugh, C. R., Lyophilization and the Thermostability of Vaccines. *Cell Preservation Technology* **2002,** *1* (2).

[4] CDC, Vaccine Information Statements: Live Intranasal Flu VIS. **2014**.

[5] Brokstad, K. A.; Cox, R. J.; Olofsson, J.; Roland, J.; Haaheim, L. R., Parenteral Influenza Vaccination Induces a Rapid Systemic and Local Immune Response. *The Journal of Infectious Diseases* **1995,** *171* (1), 198-203.

[6] Henry, S.; McAllister, D. V.; Allen, M. G.; Prausnitz, M. R., Microfabricated Microneedles: A Novel Approach to Transdermal Drug Delivery. *Journal of Pharmaceutical Sciences* **1998,** *87* (8).

[7] Taddio, A.; Ipp, M.; Thivakaran, S.; Jamal, A.; Parikh, C.; Smart, S.; Sovran, J.; Stephens, D.; Katz, J., Survey of the prevalence of immunization non-compliance due to needle fears in children and adults. *Vaccine* **2012,** *30* (32), 4807-4812.

[8] Norman, J. J.; Arya, J. M.; McClain, M. A.; Frew, P. M.; Meltzer, M. I.; Prausnitz, M. R., Microneedle patches: Usability and acceptability for self-vaccination against influenza. *Vaccine* **2014,** *32* (16), 1856-1862.

[9] Mistilis, M. J.; Bommarius, A. S.; Prausnitz, M. R., Development of a Thermostable Microneedle Patch for Influenza Vaccination. *Journal of Pharmaceutical Sciences* **2015,** *104* (2), 740-749.

[10] (a) CDC Influenza. http://www.cdc.gov/nchs/fastats/flu.htm; (b) CDC Deaths and Mortality. http://www.cdc.gov/nchs/fastats/deaths.htm.

[11] CDC Vaccine Effectiveness - How well does the flu vaccine work? http://www.cdc.gov/flu/about/qa/vaccineeffect.htm.

[12] Laver, W. G.; Valentine, R. C., Morphology of the isolated hemagglutinin and neuraminidase subunits of influenza virus. *Virology* **1968,** *38* (1), 105-119.

[13] Bliu, A.; Lemieux, M.; Li, C.; Li, X.; Wang, J.; Farnsworth, A., Modifying the thermostability of inactivated influenza vaccines. *Vaccine* **2012,** *30* (37), 5506-5511.

[14] CDC Influenza A (H1N1) 2009 Monovalent Vaccine Storage, Preparation, Handling Q & A. http://www.cdc.gov/h1n1flu/vaccination/storage_handling_qa.htm.

[15] Amorij, J. P.; Huckriede, A.; Wilschut, J.; Frijlink, H. W.; Hinrichs, W. L. J., Development of Stable Influenza Vaccine Powder Formulations: Challenges and Possibilities. *Pharmaceutical Research* **2008,** *25* (6), 1256-1273.

[16] Harris, K. M.; Maurer, J.; Uscher-Pines, L.; Kellerman, A. L.; Lurie, N. Seasonal flu vaccination: why don't more Americans get it? http://www.rand.org.prx.library.gatech.edu/content/dam/rand/pubs/research_briefs/2011/RAND_RB9572.pdf.

[17] Illum, L., Nasal drug delivery—possibilities, problems and solutions. *Journal of Controlled Release* **2003,** *87* (1–3), 187-198.

[18] FDA; MedImmune FluMist Indications and Usage. http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm294307.pdf.

[19] Prausnitz, M. R.; Langer, R., Transdermal drug delivery. *Nature biotechnology* **2008,** *26* (11), 1261-1268.

[20] Kim, Y.-C.; Park, J.-H.; Prausnitz, M. R., Microneedles for drug and vaccine delivery. *Advanced Drug Delivery Reviews* **2012,** *64* (14), 1547-1568.

[21] Amorij, J. P.; Meulenaar, J.; Hinrichs, W. L. J.; Stegmann, T.; Huckriede, A.; Coenen, F.; Frijlink, H. W., Rational design of an influenza subunit vaccine powder with sugar glass technology: Preventing conformational changes of haemagglutinin during freezing and freeze-drying. *Vaccine* **2007,** *25* (35), 6447-6457.