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Increasing Vaccine Accessibility through Cost Alternative Manufacturing and Elimination of the Cold Chain

Jorgen Madsen
Utah State University

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Increasing Vaccine Accessibility through Cost Alternative Manufacturing and Elimination of the Cold Chain

By

Jorgen Madsen

Thesis submitted in partial fulfillment

of the requirements for the degree

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Approved:

_____	_____
Thesis/Project Advisor	Departmental Honors Advisor
Dr. Bart Tarbet	Dr. Kim Sullivan
_____	_____
Committee Member	Committee Member
Dr. Brett Adams	Dr. Dennis Welker

_____

Director of Honors Program
Dr. Kristine Miller

Utah State University
Logan, Utah
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Abstract:

Millions of people die each year from infectious diseases. This is partly due to the difficulty of transporting temperature dependent vaccines through what is called the cold chain in developing countries. I hypothesize that we can increase vaccine accessibility by finding cost effective alternatives to vaccine production and by eliminating the cold chain through vaccine stabilizers. The gold standard in purification of influenza virus is by means of ultracentrifugation. Although effective, this process is very expensive and thus impractical for developing countries. I hypothesize that column chromatography can be a cost efficient alternative that is as effective as ultracentrifugation. The purification ability of column chromatography was tested by comparing two different chromatography resins. The Capto Q resin separates proteins on the basis of protein charge. The Capto 700 resin separates proteins on the basis of both size and charge. It was found that while protein separation occurred, more research will be required to assure full viral protein purification.

It is hypothesized that vaccine stabilizers can be used to eliminate the cold chain. The effect of the gelatin on inactivated influenza virus vaccines was evaluated using hemagglutinin (HA) assays and neuraminidase (NA) assays. The assays evaluate viral protein activity in samples exposed to elevated temperatures for set periods of time (1 min, 5 min, 10 min, 20 min, 40 min, 60 min, 120 min, 240 min, 300 min, and 360 min). Elevated temperatures (45, 52, and 60 degrees Celsius) facilitated an accelerated stability test, which simulates extended effects of time on vaccines. In addition to testing different temperatures, the following concentrations of collagen were used as percent by total volume: 0.3%, 1%, 3%, and 10%. These were tested for optimizing the stabilization

of the vaccine. The results of the neuraminidase assay show that 3% collagen significantly increased

the stability of the vaccine by approximately 10,000 fold.

Acknowledgments

I would like to thank Dr. Bart Tarbet at Utah State University for his help and support in this project. Dr. Tarbet has given countless hours to train, discuss and teach me throughout the last year and a half. He was patient with me even during my mistakes. I have learned more from Dr. Tarbet than any other class I have taken on campus.

I would like to thank Brett Hurst for his patience and willingness to teach me the procedures and assays used in this experiment. I would also like to thank him for all of the help he has given me on checking my dilutions and math.

Finally I would like to thank Jason Fairbourn for his assistance in performing all of the assays, as well as brainstorming alternatives whenever our results did not work.

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Figures and Tables

Introduction

1.1 Background

It is estimated that 17 million people die each year from infectious diseases (Chen *et al.,* 2011). This accounts for more than half of the deaths found in the poorest of developing countries (Yager *et al.,* 2006). While our medical technology is advancing, pandemics such as HIV and Ebola are still rampaging through the developing world killing millions of people each year. Many infectious agents such as anthrax and small pox pose threats that could be used as weapons in bioterrorism. While antibiotics are used as a means of rescue, vaccines have been considered one of the greatest medical advancements in preventing such diseases (Kumru *et al.*, 2014). While the need for vaccines is crucial, many do not have access to them.

The lack of vaccine accessibility is partially the result of 1) the cost of manufacturing, and 2) the insufficiency of the transportation cold chain. I hypothesize that vaccine accessibility could be increased by addressing either of these two problems. By finding cost alternatives to manufacturing, such as column chromatography, developing nations could find ways to manufacture vaccines in their own country. If vaccines are manufactured on site, one could eliminate the costs of transportation. In addressing the insufficiency of the cold chain, I hypothesize that gelatin could be used as a vaccine stabilizer thus eliminating the need for a cold chain.

1.2 Costs of Manufacturing

A vital step in vaccine production is the purification of the virus in the vaccine. Normally this purification is done through ultracentrifugation. While effective, ultracentrifugation is extremely expensive making it impractical for developing countries. Column Chromatography is a purification alternative that is cost efficient. It is hypothesized that column chromatography may be as effective as ultracentrifugation and could be utilized in developing countries for vaccine production.

We used the influenza A/NWS/33 (H1N1) virus to test the purification ability of column chromatography. Madin Darby Canine Kidney (MDCK) cells were infected with virus, the virus was then inactivated by means of Binary Ethyleneimine (BEI). To compare the purification ability of column chromatography two different chromatography resins were used: Capto Q and Capto 700.

Capto Q is a resin that separates proteins on the basis of charge. This is done by anion exchange. The Capto Q resin uses a quaternary Amine group that has a positive charge. This allows proteins with a negative charge to bind to the column allowing them to be separated from the rest of the solution flowing through the column.
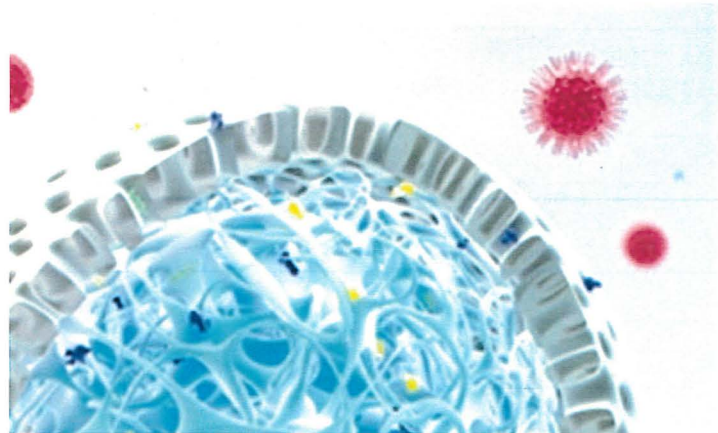
Figure 1: Artistic portrayal of the Capto 700 resin. Picture displays the ligand-activated core, as well as the outer membrane containing microscopic pores. Small proteins (in purple and yellow) are able to bind to the core while contaminants (shown in red) cannot penetrate the outer shell

http://wolfson.huji.ac.il/purification/PDF/HCIC/GE_CaptoCore700 DataFile.pdf

The second resin used was Capto 700 (Figure 1). This resin separates by size as well as charge. It has the ability to separate both size and charge because the resin is composed of beads that have a ligand-activated core and an outer layer that is composed of little microscopic pores. The outer shell prevents large molecules from entering and binding to the hydrophobic and positively charged ligands. Because both resins separate on the basis of charge, elution buffers with a gradient of increasing salt concentrations were used for elution of bound proteins.

1.3 Insufficiency of the Transportation Cold Chain

While vaccines are effective in preventing the spread of fatal diseases, they are rendered useless if exposed to conditions outside of the optimal range. The World Health Organization (WHO, 2006) has said that the only environmental factor to affect characteristics of all vaccines is temperature. This is why vaccine transportation relies so heavily on the cold chain.

Zhang *et al.* (2012) defines the cold chain as the distribution network that strives to keeps vaccines within the optimal temperature range during transport, storage, and handling. This not only means keeping the vaccine from getting too warm, but also from freezing. Vaccines contain molecules called adjuvants that increase the potency of the vaccine. Adjuvants such as aluminum, however, are inactivated if frozen (Kumru *et al.*, 2014). Accidental exposure of vaccines to freezing temperatures during the length of the cold chain occurs in over 75% of all shipments (Kumru *et al.*, 2014).

The process of the cold chain can often be lengthy. Most vaccine cold chains start with the manufacturer in a developed country. From there, the vaccine is typically flown to a central store. This central store will house the vaccine for a time, after which it sends the vaccine to regional stores, then to district stores, and finally to health clinics. Transportation between locations can be via plane, car, or even foot. It is estimated that the total process can take anywhere from 12-18 months (Cheyne, 1989). During this time the vaccines are housed in refrigerators, cold boxes, or simply put on ice.

As manufacturers often send vaccines to remote areas of the world, the vaccines are exposed to harsh climates and many unforeseeable challenges. Hurricanes, wars, or natural disasters can delay shipments for extended periods of time. Power outages or lack of fuel often render electric cooling units useless (Arya, 2000). Many times vaccines are lost in international flights, or central stores do not have room for them due to poor stock control (Cheyne, 1989). It is foreseen that global climate change will only add to the difficulties in maintaining the stability of vaccines (Arya, 2000). The logistics of the cold chain are often impractical in low resource settings, and the costs often become a major burden to developing countries (Wolfson *et al.*, 2007).

The burden of the cold chain on vaccine programs accounts for $200-$300 million dollars each year (Das, 2004). This alone can contribute to 80% of the vaccine program's cost in developing countries (Chen *et al.*, 2011). The money spent on simply transporting these vaccine could vaccinate an additional 10 million children (Das, 2004). Countries impacted the most by these costs are areas with harsher climates. Areas using kerosene powered coolers, can go through half a liter of kerosene a day just to maintain temperatures (Cheyne, 1989).

Exposure to increased temperatures causes denaturation of viral proteins. Conformational changes of membrane proteins can cause the vaccine to lose its potency decreasing its ability to elicit an immune response. These conformational changes can also cause the viral particles to aggregate, inactivating the vaccine (Zhang *et al.*, 2012). The decrease in vaccine potency requires an increase in dosages which in turn adds to the cost of vaccines for developing countries (Chen *et al.*, 2011). Zhang *et al.* (2012) estimates that failure of the cold chain results in the loss of almost half of all global vaccines.

1.4 Use of Collagen as a Vaccine Stabilizer

Having seen the insufficiencies of the cold chain, I have hypothesized that collagen could be used as a vaccine stabilizer thus eliminating the need for a cold chain. Collagen is a fibrous structure that is made up of three parallel polypeptide strands (Shoulders *et al.*, 2009). Collagen is one of the most abundant structural proteins, and makes up one third of all total protein. It is found in tendons, ligaments, vessels, as well as the extracellular matrix.

The polypeptide strands of collagen typically consist of three repeating sequences with glycine being every third amino acid. The first two amino acids can vary, but the sequence typically goes as follows: Xaa Yaa Gly (Shoulders *et al.*, 2009). Because of the abundance of glycine (Figure 2) each strand, known as the alpha chain, is tightly wrapped
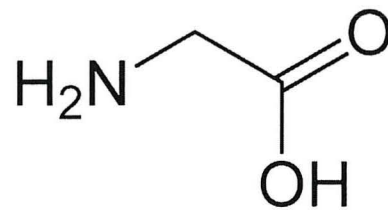
Figure 2: Structure of the amino acid Glycine

Photo taken from:
http://www.mpbio.com/product.php?pid=04808822&country=223

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Triple helix

Alpha chain

Amino acid sequence   – Gly – X – Y – Gly – X – Y – Gly – X – Y –

1.4 nm
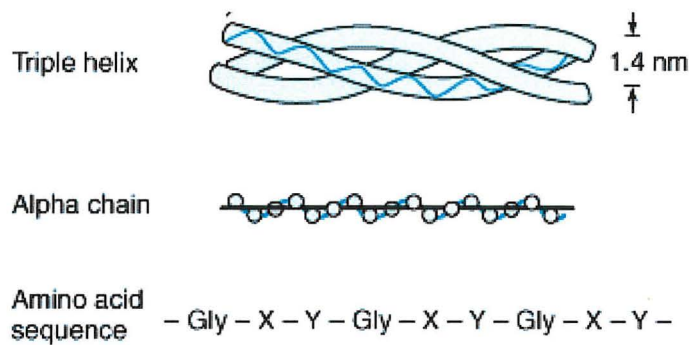
Figure 3: Structure of collagen fiber starting with the amino acid sequence, alpha chain, and finally the right handed triple helix. The triple helix is held together by hydrogen bonding between the Glycine and the X amino acid.

Photo taken from: http://www.namrata.co/category/chemistry-of-proteins/specialized-protein-collagen/

around itself. Three of these alpha chains form a helix (Figure 3) that is held together by hydrogen bonding between the alpha carbon of Glycine and the oxygen of the carbonyl group on the X amino acid of the repeating sequence. This causes a collagen strand to form a right handed triple helix composed of three left handed helical alpha strands, with two hydrogen bonds within each amino acid triplet (Shoulders *et al*., 2009; Ottani *et al*., 2001).

In our experiment, collagen was used in the form of gelatin. Gelatin is denatured collagen that was isolated from porcine skin. As Gelatin contains collagen strands of varying lengths going in all directions, this causes a meshwork with micro pores hence becoming a gel (Olsen *et al*., 2003).

1.5 Structure of Influenza

Influenza A/California/07/2009 (H1N1) virus and Influenza A/NWS/33 (H1N1) virus were both used in our study. Influenza is an enveloped virus, meaning it is surrounded by a cell membrane. This cell membrane makes influenza easy to kill, as one can disrupt the membrane with ethanol or simple sanitizers. While this is convenient for sanitizing, it presents a difficulty in stabilizing an influenza vaccine, as it is so easily disrupted. Inside the cell membrane are 8

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single stranded negative sense RNA segments that encode 11 different proteins (Kumru *et al.*, 2014). The virus is spherical in shape, and is typically 80-120 nm in diameter.

There are three different types of influenza viruses: A, B, and C. Influenza A and B are the types that are most associated with the annual or "seasonal" flu. These are further broken down into subcategories depending on the glycoproteins that are found on the outer cell membrane. Two of these membrane proteins were used in our assays to determine the vaccine potency. The two membrane proteins are: hemagglutinin, and neuraminidase.

Hemagglutinin (HA) and neuraminidase (NA) are the structural proteins that often elicit an immune response in our bodies. These proteins are often variable with 18 discovered HA subtypes and 9 NA subtypes. Influenza utilizes mutational drift to constantly change these membrane proteins, making it necessary for us to get flu shots annually. A dramatic change in the

Figure 4: Structure of Influenza H1N1. Virus is enveloped with 8 single stranded RNA molecules. Membrane proteins shown in blue are neuraminidase, and proteins shown in red are hemagglutinin.

Photo taken from:
http://micro.magnet.fsu.edu/cells/viruses/influenzavirus.html

hemagglutinin antigen is less frequent; however, changes in this protein are often responsible for larger pandemics (Kumru *et al.*, 2014).
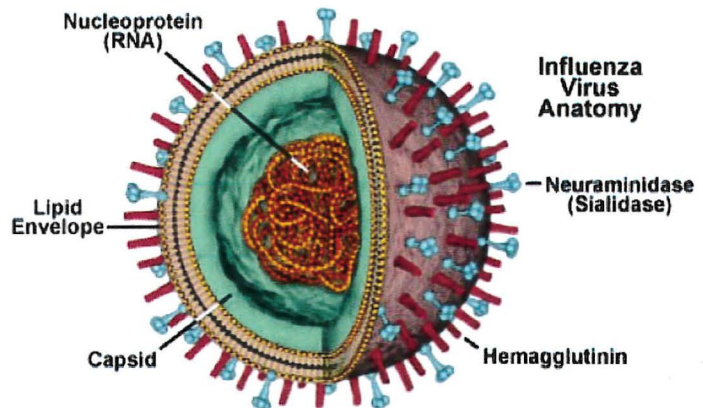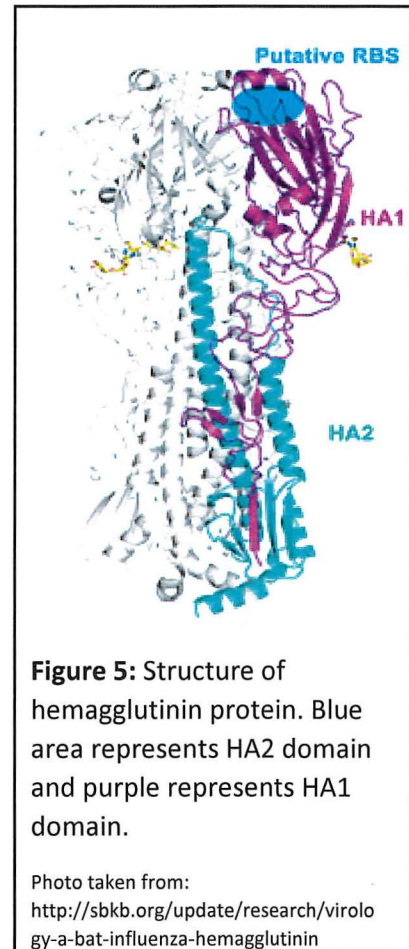
We classify the flu by using H_xN_x to specify which subtypes are found on the membrane. Example Influenza A H1N1 contains HA subtype 1 and NA subtype 1. Vaccines use inactivated

viruses with these membrane proteins intact in order to elicit an immune response (Pushko *et al.*, 2005).

The membrane protein HA is a trimer composed of two different domains: HA1 and HA2 (Figure 5). HA has a head, which binds to sialic acid found on receptors of the cells within the body. These cells then engulf the virus via endocytosis. Once inside the cell as an endosome, the virus utilizes a channel that lowers the pH of the endosome. The lowering of the pH causes a conformational change to occur in HA. After the conformational change, hemagglutinin unfolds on itself and exposes a part of the protein that bursts the endosome. This releases the viral RNA into the cell (Skehel *et al.*, 2000). HA also binds to red blood cells causing them to agglutinate. This allows us to quantify the amount of HA in a sample, by simply mixing them with red blood cells.

Figure 5: Structure of hemagglutinin protein. Blue area represents HA2 domain and purple represents HA1 domain.

Photo taken from: http://sbkb.org/update/research/virology-a-bat-influenza-hemagglutinin

Once the virus has replicated and undergone self-replication, the virus leaves the cell via exocytosis. Upon leaving the cell, however, the HA again binds to the host cell membrane and cannot leave. The enzyme neuraminidase (NA) frees the virus by cleaving the sialic acid on the cell membrane. This frees the virus allowing it to find another cell to infect. NA inhibitors, such as Oseltamivir (also known as Tamiflu), prevent the cleavage of sialic acid. While this does not stop the virus from initially infecting the cell, it prevents the virus from spreading to other cells.

1.6 Accelerated Stability Test

As real time tests can take up to two years, we employed an accelerated stability test for our experiment. Accelerated stability tests assess the rate of change of vaccine potency over time by exposing the vaccine to increased temperatures that are above the normal storage conditions. While accelerated stability tests cannot be used to estimate real time, they provide important information regarding the stability and vaccine potency (WHO 2006).

Methods and Materials

2.1 Virus

Influenza A/NWS/33 (H1N1) virus was used for our column chromatography tests. Influenza A/California/07/2009 (H1N1) was the virus used for the accelerated time tests. Both viruses were provided by the Centers for Disease Control and Prevention in Atlanta, Georgia. The viruses were grown in MDCK cells after being infected in roller bottles. Infection was done by diluting the virus 1:1,100 in an infection media. Our flu infection medium consisted of: MEM (HyClone CAT# SH30024.02), 1 ml of Gentamycin (Sigma CAT# G1397), 10 U/ml of Trypsin (Sigma CAT# T6567), and 1 µg/ml EDTA (Sigma CAT# E9884).

2.2 Virus Inactivation

The virus was inactivated by Binary Ethylenimine (BEI). This was done by mixing 2-Bromoethylamine hydrobromide (BEA) (Sigma) with 0.2 M of NaOH in sterile water and incubated for approximately one hour at 37°C +/- 2° C. This results in a binary ethylenimine

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(BEI) product. The BEI was then added to the virus at a ratio of one part of inactivating agent to nine parts of virus suspension. The final concentration of BEI was 10.0 mM. The virus-BEI mixture was then slowly stirred at 24°C +/- 1°C for 12 hours. We removed 20 ml samples every 30 minutes for the first 7 hours, and then hourly until 12 hours. The BEI-Virus solution was neutralized by adding 1.0 M sodium thiosulfate, and the pH was brought to 7.2 by adding 1.0 M HCl. The inactivated virus was then filtered through a Pellicon XL Cassette (Biomax 100KDa Cat# PXB100V50) and put into 1 ml aliquots and stored at -80°C.

2.3 Column Chromatography

Inactivated influenza samples were passed through columns containing resins Capto Q and Capto 700, and then collected after buffer was added to the columns. The columns were initially washed with a phosphate buffer followed by 150 ml of the BEI inactivated virus. After the virus solution had been added to the columns they were washed again with the phosphate buffer. 100 ml increments of KCl containing phosphate buffers were added to each column. The columns were washed a third time and final eluates were collected.

Figure 6: Columns used for column chromatography. Columns contain Capto Q or Capto700 resin.

Photo taken in lab

2.4 Bradford Protein Assay

After inactivated influenza samples were taken from the column, a Bradford Protein Assay was used to evaluate total protein concentration. The inactivated influenza samples were compared to a standard containing Bovine Serum Albumin that ranged from 0 to 2000 µg of protein.
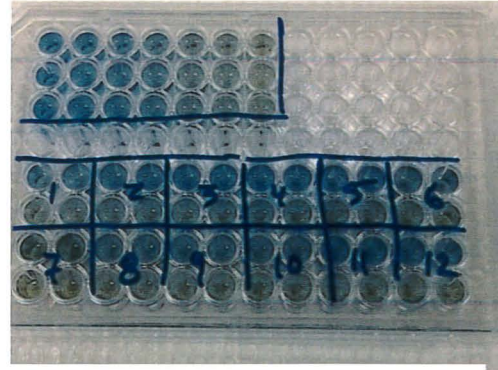
Figure 7: Plate used for a Bradford Protein Assay. Samples were placed on the bottom 4 rows. Bovine Serum Albumin control was placed in the top right corner.

Photo taken in lab

2.5 SDS Page

Once protein concentrations were determined, selected samples were passed through a 7.5% precast polyacrylamide gel. An electrical current of 200 V was applied for 10 minutes and then reduced to 150 V for 40 additional minutes. Total run time was 50 minutes.

2.6 Gelatin

Two different gelatins were used. The first was Gelatin from Porcine Skin (G9136-10 µg Type A lyophilized powder γ-irradiated BioXtra Sigma). The second gelatin used was Porcine Skin Gelatin (CAT# GELP12-N-10 Lot# SX0018 Alpha Diagnostic Intl. Inc).

2.7 Sample Preparation

Inactivated influenza samples were tested on four different variables: Viral protein concentration, temperature, time and gelatin concentration. Using an HA assay, the original viral protein concentration from our BEI inactivated stock was found to be 2.8 mg/ml. We used this stock to test protein concentrations at 1mg/ml and 0.3 mg/ml. Our gelatin concentrations

were tested as both percent by total protein concentration, as well as percent by total volume. Our gelatin percentages were 0.3%, 1%, 3%, and 10%. Temperature variables were 45°C, 52°C, and 60°C. Each sample was also made for the following time intervals: 1 min, 5 min, 10 min, 20 min, 40 min, 60 min, 120 min, 240 min, 300 min and 360 min. Inactivated influenza samples were prepared to a volume of 500 µl per sample. Two controls were utilized: the first was BEI-inactivated virus with no gelatin, and the other was BEI-inactivated virus with 3% of albumin by protein concentration/volume.

When testing the gelatin percent by total protein, we used the Type A lyophilized powder (γ-irradiated BioXtra) from Sigma. The gelatin stock was prepared at 1 mg/ml by mixing 10 mg of the lyophilized gelatin with 10 ml of phosphate buffered solution (PBS). Table 1 shows the volumes that were used to make the samples at a total viral protein concentration of 1.0 mg/ml. Table 2 shows the volumes that were used to make samples at a total viral protein concentration of 0.3 mg/ml.

It was found that the gelatin concentrations were not sufficiently high, so inactivated influenza samples with gelatin by percent of total volume were made. Porcine Skin Gelatin (CAT# GELP12-N-10 Lot# SX0018 Alpha Diagnostic Intl. Inc) was diluted to 200 mg/ml by mixing 1 gram of gelatin with 5 ml of PBS. As it has previously been demonstrated that optimal viral protein is at 0.3 mg/ml, samples of gelatin by percent of total volume were made with only 0.3 mg/ml of viral protein. Table 3 shows the volumes that were used to make these samples.

Table 1: Inactivated influenza sample volume preparation of 1.0 mg/ml viral protein concentration with gelatin by percent of total protein concentration

Gelatin by % of total protein concentration	Amount of BEI-Inactivated Virus added from stock (in µl)	Amount of PBS added (in µl)	Amount of Gelatin added from stock (in µl)
10%	167	193	140
3%	167	286	47
1%	167	317	16
0.3%	167	328	5
0%* Control	167	333	0
3% Albumin* Control	167	286	47** of Albumin

* Following samples contained NO gelatin but were used as controls

** 47 µl added were of Albumin NOT gelatin

Table 2: Inactivated influenza sample volume preparation of 0.3 mg/ml viral protein concentration with gelatin by percent of total protein concentration

Gelatin by % of total protein concentration	Amount of BEI-Inactivated Virus added from stock (in µl)	Amount of PBS added (in µl)	Amount of Gelatin added from stock (in µl)
10%	55	305	140
3%	55	398	47
1%	55	429	16
0.3%	55	440	5
0%* Control	55	445	0
3% Albumin* Control	55	398	47 of Albumin**

* Following samples contained NO gelatin but were used as controls

** 47 µl added were of Albumin NOT gelatin

Table 3: Inactivated influenza sample volume preparation of 0.3 mg/ml viral protein concentration with gelatin by percent of total volume

Gelatin by % of total volume	Amount of BEI-Inactivated Virus added from stock (in μl)	Amount of PBS added (in μl)	Amount of Gelatin added from 200 mg/ml stock (in μl)
10% (100 mg/ml)	55	195	250
3% (30 mg/ml)	55	362	83
1% (10 mg/ml)	55	420	25
0%* Control	55	455	0
3% Albumin* Control	55	362	83 of Albumin**

* Following samples contained NO gelatin but were used as controls
** 83 μl added were of Albumin NOT gelatin

2.8 Accelerated Stability Test

The accelerated stability test was performed by placing inactivated influenza samples in water baths and removing them after a set period of time. Water baths were set at 45°C, 52°C and 60°C. Samples were removed from the water baths at the following intervals: 1 min, 5 min, 10 min, 20 min, 40 min, 60 min, 120 min, 240 min, 300 min and 360 min. Once samples were collected, they were placed immediately in a -80°C freezer until further tested.

2.9 Hemagglutinin (HA) Assay

50 μl of PBS was added to each well of a round bottom 96-well micro-titer plate. In the first row 50 μl of the test sample was added, and serially diluted by transferring 50 μl from the

previous well to the successive well and so on. 50 μl of a

0.5% turkey red blood cell (RBC) suspension was added to

each well on the plate. Cell and virus controls were added

to the same plate. After viral sample addition, plates were

incubated at room temperature. Wells that contained viral

proteins, caused the red blood cells to form a lattice work

and stay in suspension. Wells without any viral proteins

formed "buttons" at the bottom of the well where the red

Figure 8: Microtiter plate on which hemagglutinin assay was performed. Wells with red dots show no viral proteins, while wells with no dots indicate hemagglutination has occurred.

Photo taken in lab

blood cells clumped due to gravity. Titers were recorded after 35 minutes. HA units were

expressed as the reciprocal of the maximum dilution of virus that resulted in complete
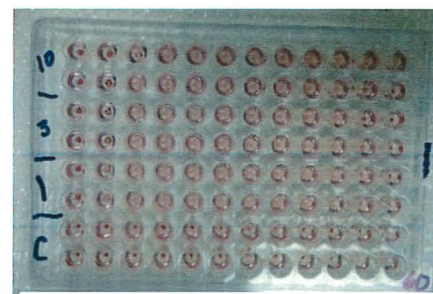
agglutination.

2.10 Neuraminidase (NA) Assay

Viral NA activity was assessed using a commercially available kit (NA-Star®Influenza

Neuraminidase Inhibitor Resistance Detection Kit, Applied Biosystems, Foster City, CA) in 96-

well solid white microplates. Inactivated influenza samples were serially diluted using a 1:2

dilution with the NA-Star Assay Buffer. NA-Star Substrate was then prepared by making a

1:1000 dilution in the NA-Star Assay Buffer. 10 μl of the diluted substrate were then added to

each of the wells. The plate was incubated at 37° C for 20 minutes. After incubation the NA

activity was evaluated using a Centro LB 960 luminometer (Berthold Technologies) for 0.5 sec

immediately after addition of NA-Star® accelerator solution. NA activity was determined by

plotting percent chemiluminescent counts versus $\log_{10}$ of the NA dilutions.

Results

3.1 Hemagglutinin (HA) Assay for Column Chromatography

Figure 9: Graph shows the HA value versus the Elution Buffer in Molarity. The blue line represents samples that were collected from the column containing the Capto Q resin on the 28th of September 2013. The orange line represents samples collected on the 2nd of November 2013.

Figure 9 shows HA concentrations from samples taken from the column containing the Capto Q resin. There was a peak of hemagglutinin in the 0.2M eluates collected on the 28 of September 2013.

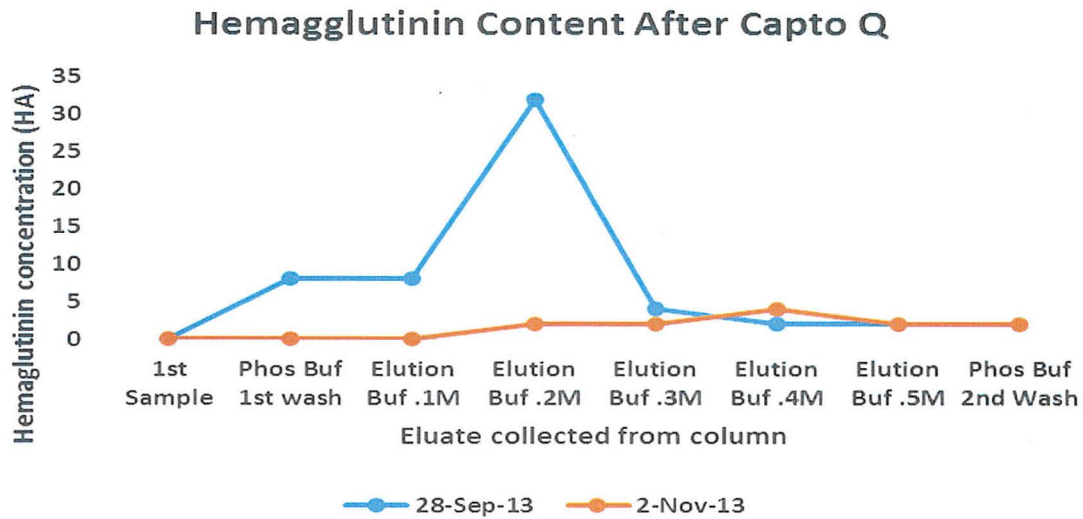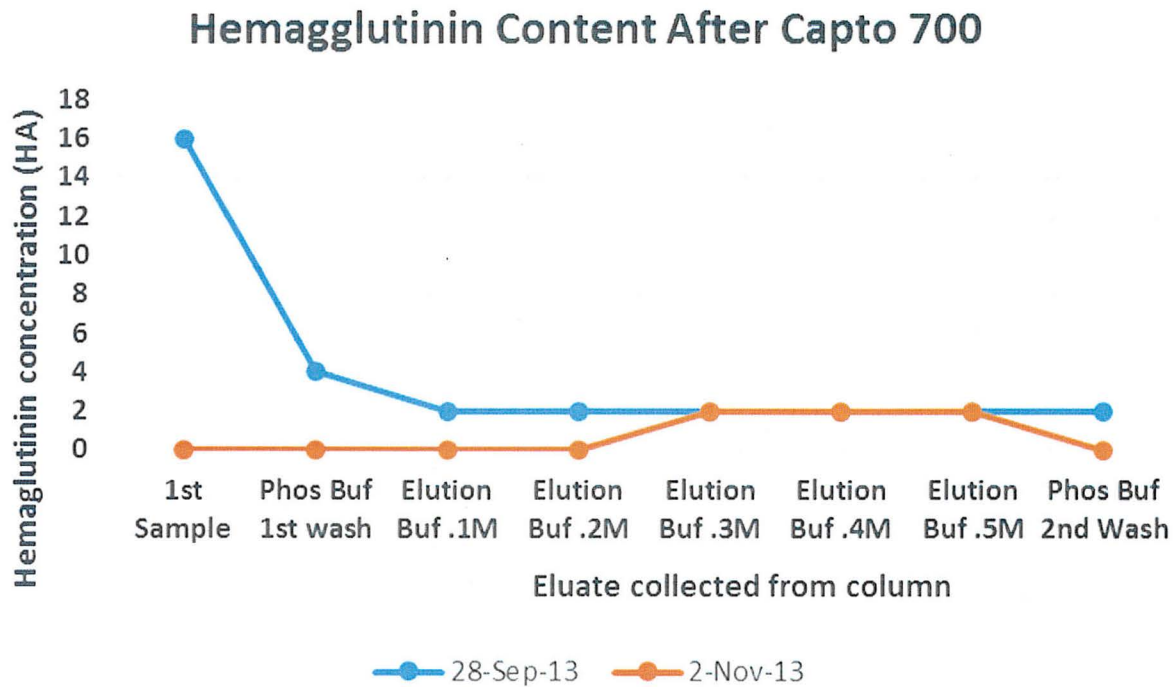# Hemagglutinin Content After Capto 700

Figure 10: Graph shows the HA value versus the Elution Buffer in Molarity. The blue line represents samples that were collected from the column containing the Capto 700 resin on the 28th of September 2013. The orange line represents samples collected on the 2nd of November 2013.

Figure 10 shows the eluates collected from the column containing the Capto 700 resin. The graph displays the concentration of the viral hemagglutinin protein. The data collected from the 28 of September 2013 suggests that the viral protein did not bind to the column, but rather flowed through the column.

3.1 Bradford Protein Assay for Column Chromatography

Figure 11 comes from the Bradford Protein Assay which shows the total protein
concentration present in the eluates collected. This assay showed a peak in protein
concentration. When comparing this graph with figure 9, one can see that the protein peak
corresponds to the hemagglutinin peak concentration.

Figure 11: Graph shows the total protein concentration from each of the eluates collected from the column. The blue line represents samples that were collected from the column containing the Capto Q resin on the 28th of September 2013. The orange line represents samples that were collected on the 2nd of November 2013.
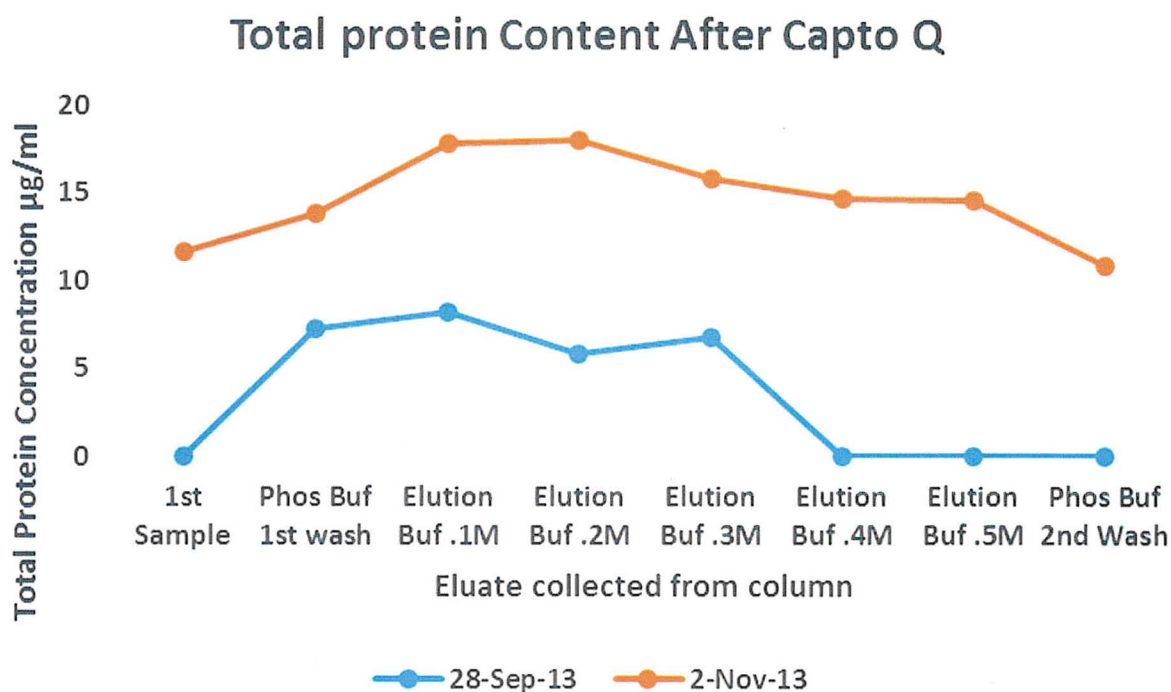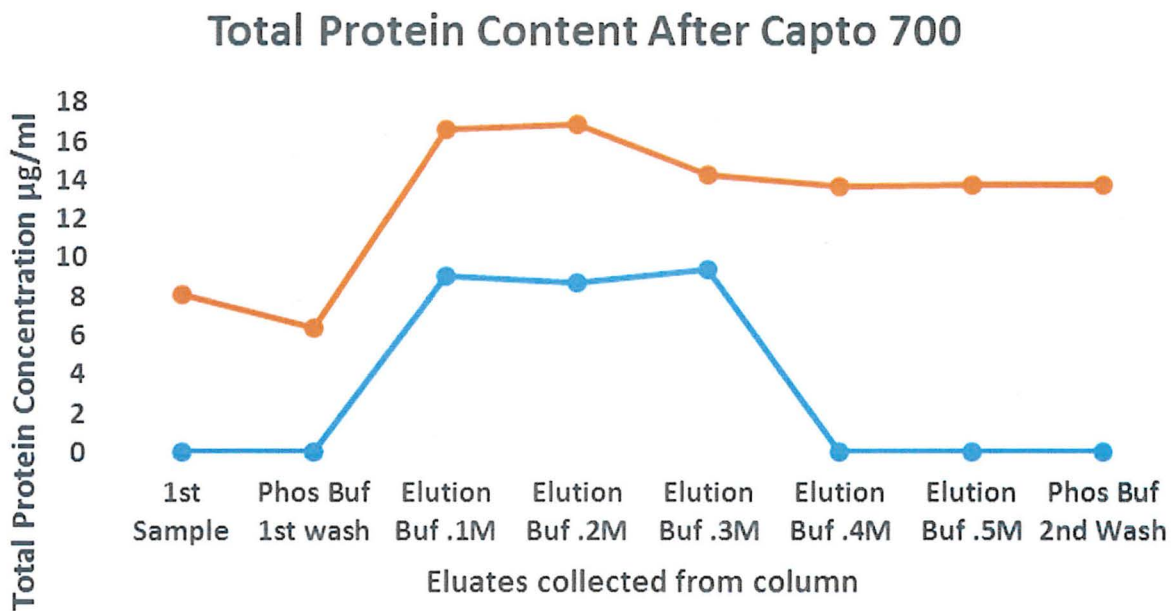
Total Protein Content After Capto 700

Figure 12: Graph shows the total protein concentration from each of the eluates collected from the column. The blue line represents samples that were collected from the column containing the Capto 700 resin on the 28th of September 2013. The orange line represents samples that were collected on the 2nd of November 2013.

Figure 12 shows the total protein concentration present in the eluates collected from the Capto 700 column. This graph indicates a similar peak of total protein concentration on both occasions in which the column was run. There does not seem to be a correlation between the total protein concentration and the hemagglutinin content (Figure 10) from the eluates collected from the Capto 700 resin.

3.3 SDS Page for Column Chromatography

Figure 13 is of an SDS gel that shows a single band of protein that was identified at approximately 60kDa. The fact that the band was identified after the 1st phosphate buffer wash
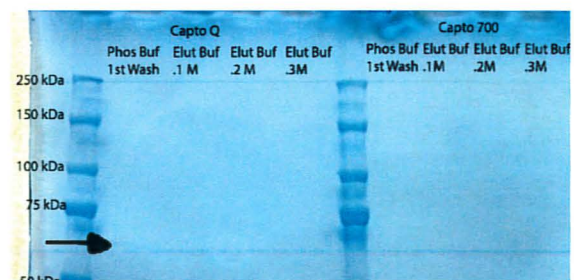

Figure 13: SDS Page of eluates collected from columns. A faint band can be seen coming from the 1st phosphate buffer wash at around 60 KDa.

Photo taken in lab

suggests that either the viral protein did not stick to the column, or that the protein visible on the gel was due to a contaminant (possibly bovine serum albumin.) As only one band of protein was visible, a more sensitive dye will be needed to detect lower concentrations of protein.

3.4 Hemagglutinin (HA) Assay for Accelerated Stability Test

Figures 14-16 show the HA results from the accelerated stability test with a viral protein concentration of 1.0 mg/ml. Figure 14 shows samples that were placed in a water bath at 60°C. The results show, that the temperature was too high, denaturing the viral proteins at all gelatin concentrations.
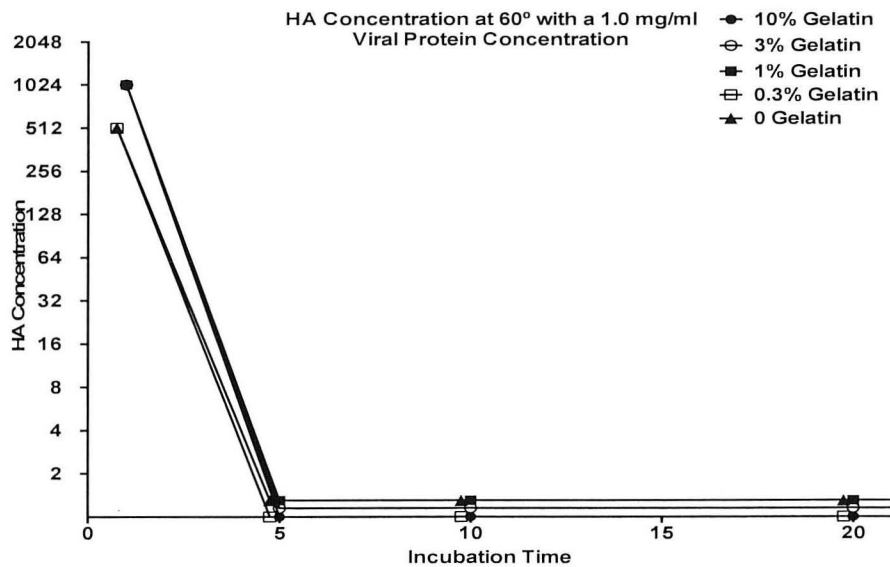

Figure 14: Graph shows the HA concentrations of samples taken from 60°C water bath at different intervals. As shown in the graph, all viral proteins were denatured after 10 minutes indicating that our water bath temperature was too high.

Figure 15 shows the HA concentrations of samples taken from a 45°C water bath at different time intervals. This shows that there no difference of HA was observed between the samples, suggesting that our water bath temperature was too low.
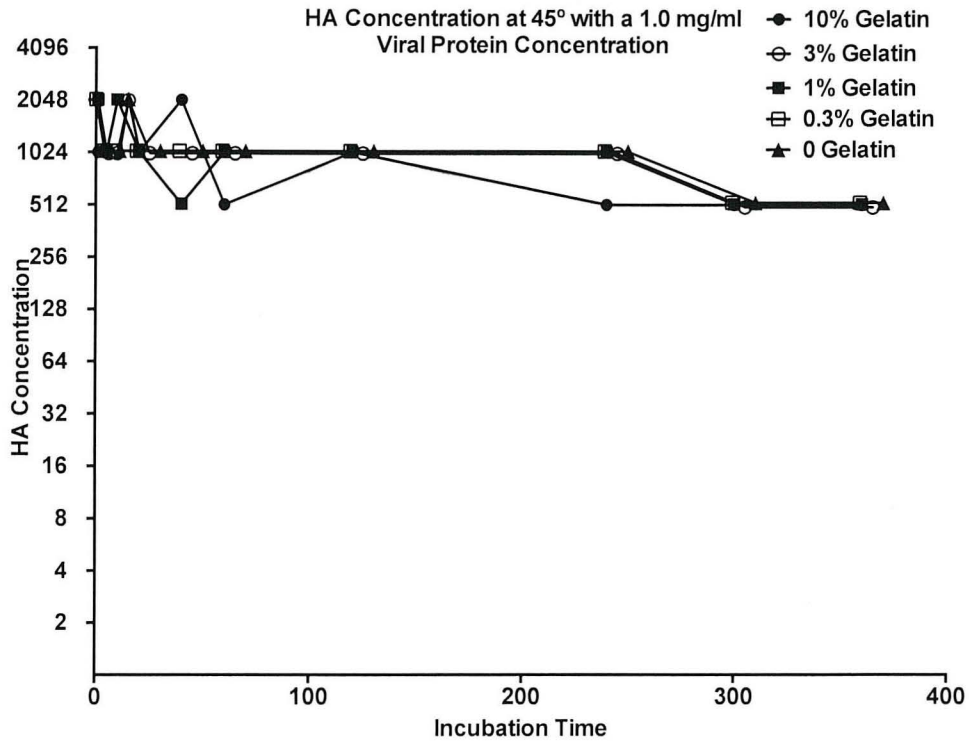

Figure 15: Graph shows the HA Concentrations of samples taken from 45°C water bath at different intervals.

Figure 16 shows the HA concentrations of samples taken from a 52°C water bath at different time intervals. The graph shows a drop in viral protein concentration after a set period
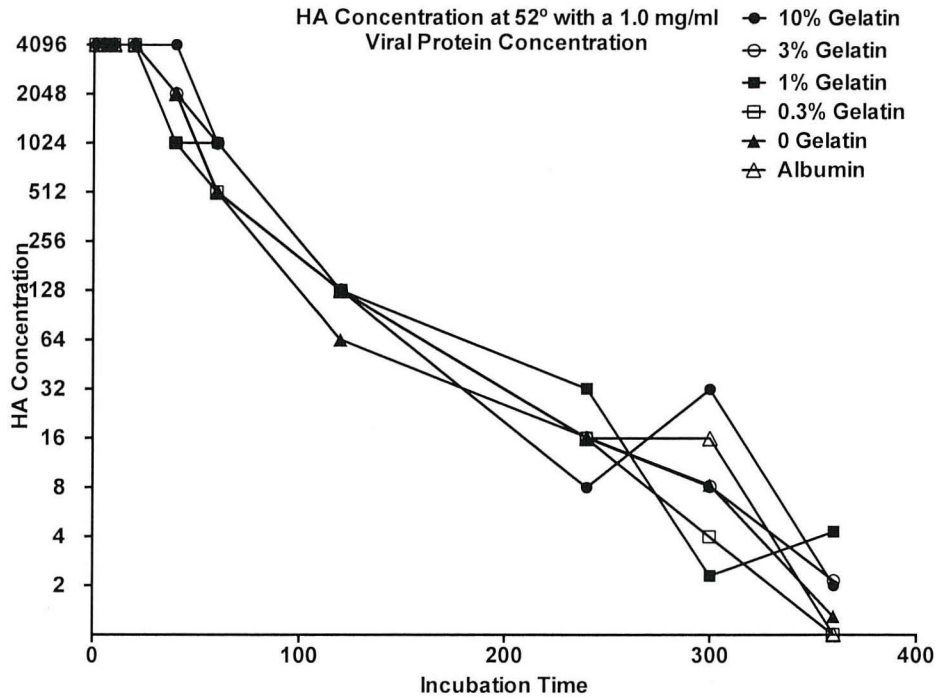
Figure 16: Graph shows the HA concentrations of samples taken from 52°C water bath at different intervals. There is a drop in hemagglutinin concentration around 60 minutes indicating 52°C as an optimal temperature for accelerated stability test.

of time, indicating that 52°C is optimal for our accelerated stability test. The graph also shows that no difference in stability was observed between the different gelatin concentrations.

After looking at our results for the accelerated stability tests with a protein concentration of 1.0 mg/ml, we repeated our accelerated stability tests with a protein concentration of 0.3 mg/ml. Figure 17 shows the HA concentrations of those samples. While we saw some differences in our inactivated influenza samples, there was no evidence that gelatin extended the stability of the vaccine. The graph also show that there was not a drop in hemagglutinin in our control, suggesting that the temperature was too low to cause denaturation for the time intervals set.
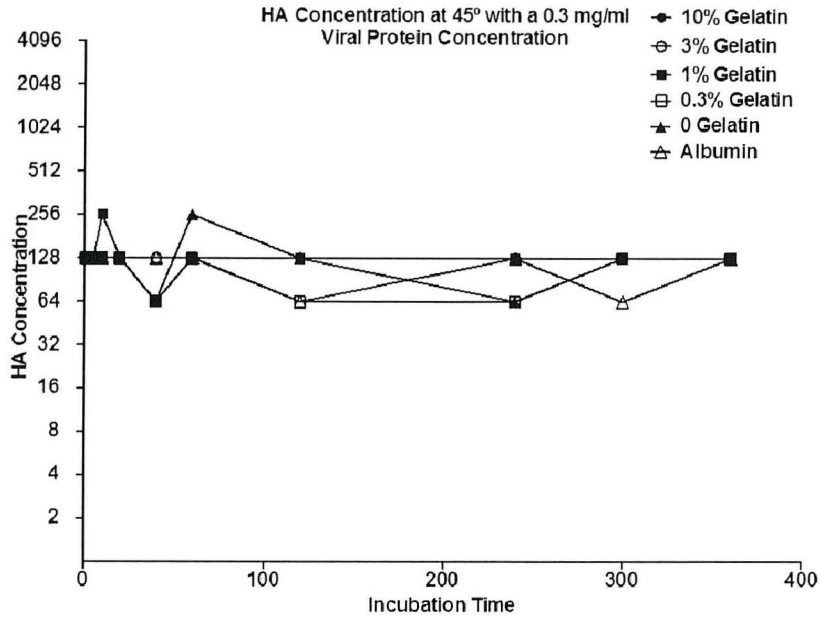
Figure 17: Graph shows the HA concentrations of 0.3 mg/ml viral protein samples taken from a 45°C water bath. While there is variance in the samples, there is not a difference between the values to suggest that any sample had an increase in stability.
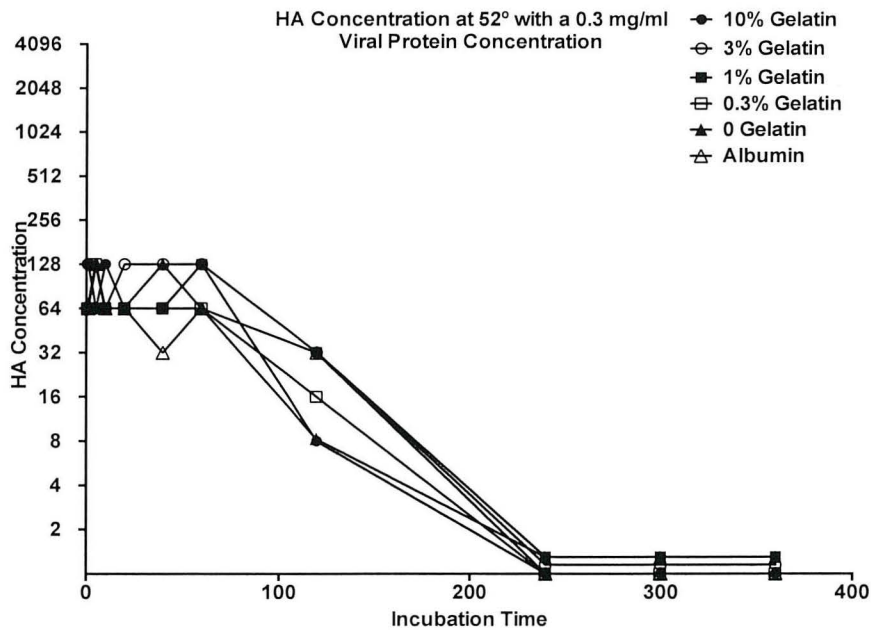

Figure 18: HA concentrations of 0.3mg/ml viral protein samples taken from a 52° C water bath. Despite variance between HA values in samples during the first 100 minutes, no difference in viral protein stability can be seen.

As we did not see a decrease in HA from the 45° C water bath, we tested the 0.3 mg/ml viral protein samples at 52 °C. Once again there was no significant difference between the inactivated influenza virus samples (Figure 18) to say that the gelatin had an effect on the stability of the viral proteins.

As no stabilizing effect could be seen from the gelatin, we hypothesized that the gelatin concentrations were too low. We increased our gelatin by using a percent by total volume. As we had found that 52°C was the optimal temperature for our tests, we performed an accelerated stability test with gelatin by percent of total volume at 52°C. As figure 19 shows there was yet again no difference between our samples and the control.
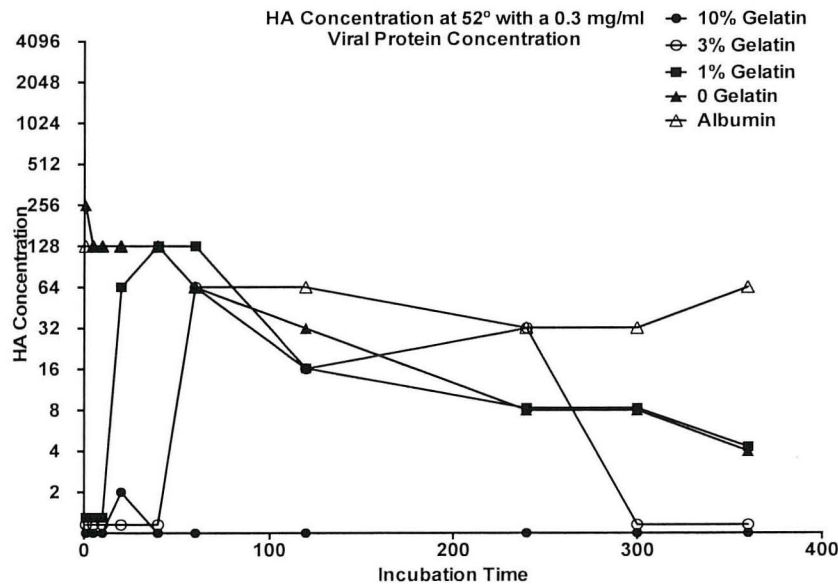

Figure 19: HA concentrations of samples containing a 0.3 mg/ml viral protein concentration. The gelatin samples are 10%, 3%, 1%, and 0% by total volume. Samples were taken from a 52° C water bath.

As no differences in vaccine stability could be seen between our samples and our control, we hypothesized that the gelatin might be interfering with the red blood cells used in our hemagglutination assay. To test this, we took samples of 0.3mg/ml viral protein that were not placed in water baths and performed an HA on them. As none of the inactivated influenza samples had been exposed to elevated temperatures, the estimated HA was around 258. The gelatin was measured as percent by total volume using 0.5%, 1%, 2%, 3%, and 4%. As figure 20 shows, our hypothesis was confirmed that the gelatin was interacting with the assay and interfering with our results.
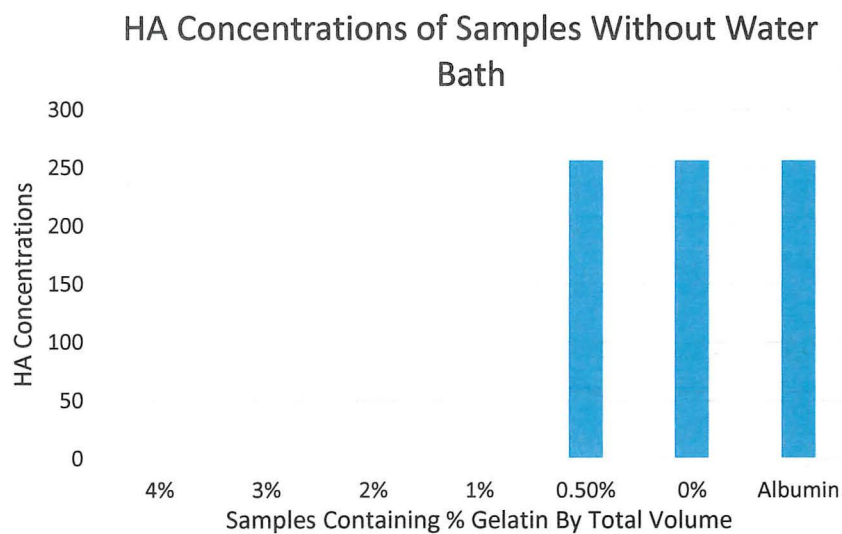
Figure 20: HA concentrations of samples containing a 0.3 mg/ml viral protein concentration. None of the samples were exposed to elevated temperatures, and were expected to have an HA value of 258. As the graph shows, the gelatin seems to affect the HA values of the assay.

3.5 Neuraminidase (NA) Assay for Accelerated Stability Test

As gelatin interfered with the results of our HA assay, a NA assay was performed on samples containing a 0.3 mg/ml viral protein concentration. Inactivated influenza samples contained 1%,

3%, and 10% gelatin by total volume and were placed in a water bath at 52°C. NA activity was determined by plotting percent chemiluminescent counts versus $\log_{10}$ of the NA dilutions. Graphs representing each time interval inactivated influenza vaccine was removed from the water bath. Figures 21-28 show the results of the neuraminidase activity.

Figures 21-28 show significant differences in chemiluminescence between gelatin samples and the control were seen within 10 minutes of exposure to elevated temperatures. The 3% gelatin sample has around a 10,000 fold increase in neuraminidase activity than the control after 240 minutes.

Figure 21: Neuraminidase activity of samples containing 0.3 mg/ml viral protein concentration after exposure to 52°C water bath for 1 minute. Graph shows chemiluminescence vs NA dilution

Figure 22: Neuraminidase activity of samples containing 0.3 mg/ml viral protein concentration after exposure to 52°C water bath for 5 minutes. Graph shows chemiluminescence vs NA dilution
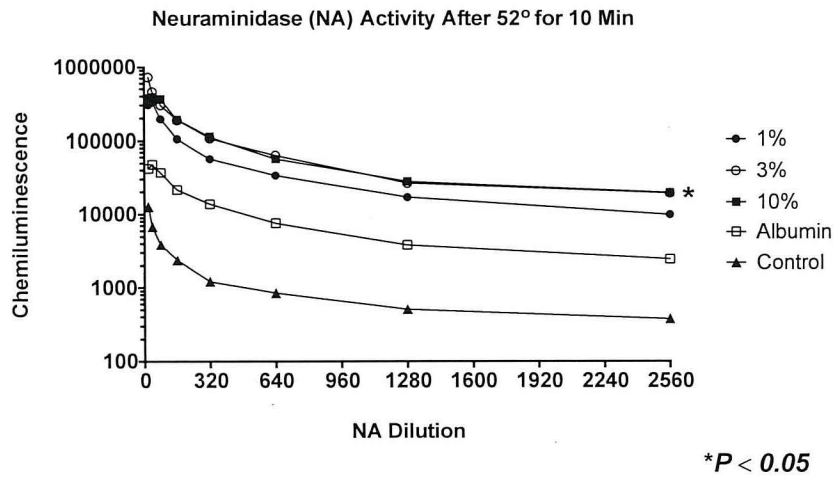
Figure 23: Neuraminidase activity of samples containing 0.3 mg/ml viral protein concentration after exposure to 52°C water bath for 10 minutes. Graph shows chemiluminescence vs NA dilution
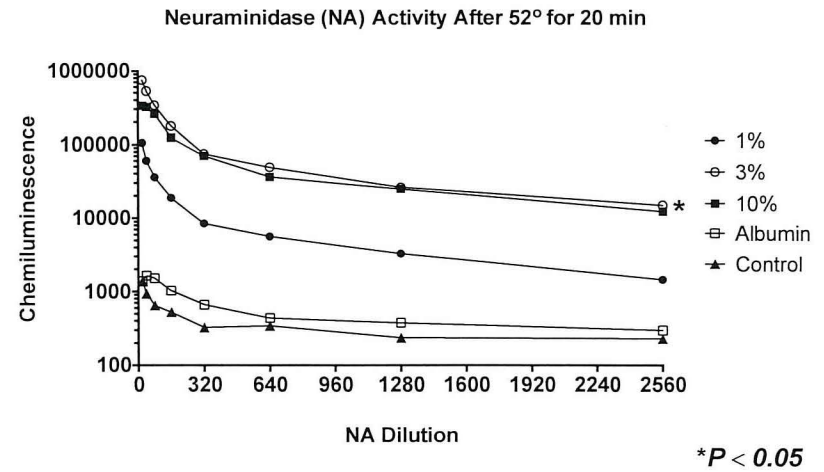
Figure 24: Neuraminidase activity of samples containing 0.3 mg/ml viral protein concentration after exposure to 52°C water bath for 20 minutes. Graph shows chemiluminescence vs NA dilution
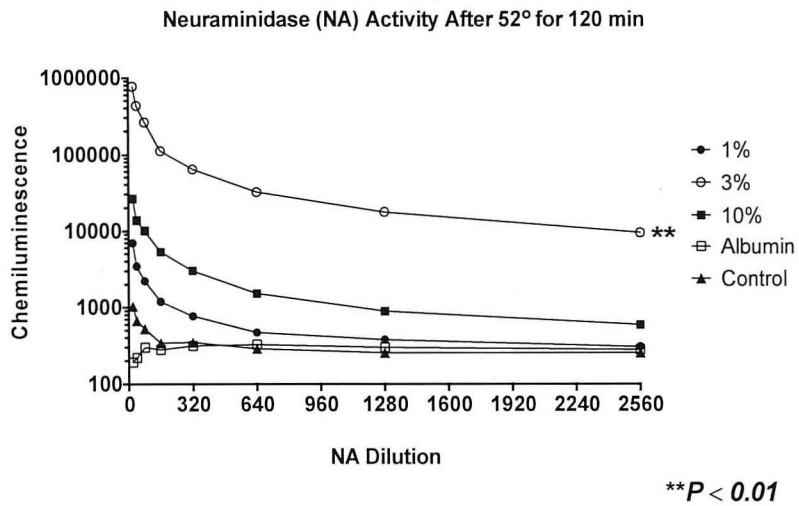
Figure 25: Neuraminidase activity of samples containing 0.3 mg/ml viral protein concentration after exposure to 52°C water bath for 40 minutes. Graph shows chemiluminescence vs NA dilution
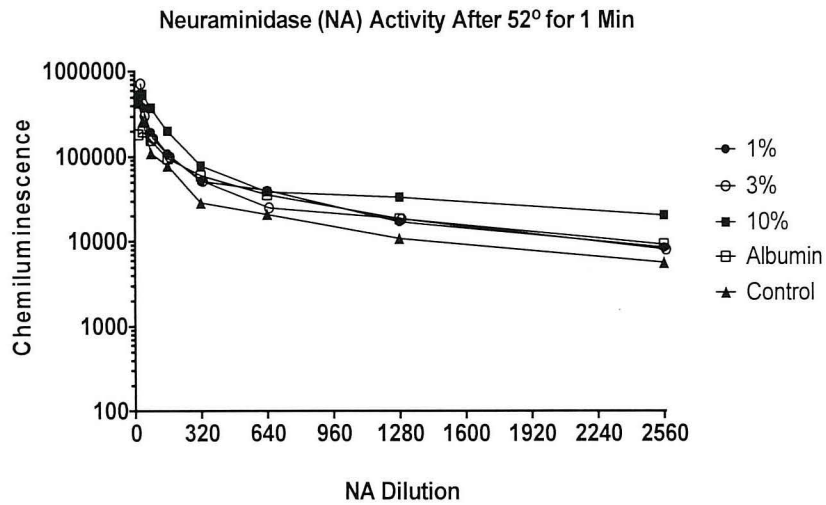
Figure 26: Neuraminidase activity of samples containing 0.3 mg/ml viral protein concentration after exposure to 52°C water bath for 60 minutes. Graph shows chemiluminescence vs NA dilution

Figure 27: Neuraminidase activity of samples containing 0.3 mg/ml viral protein concentration after exposure to 52°C water bath for 120 minutes. Graph shows chemiluminescence vs NA dilution
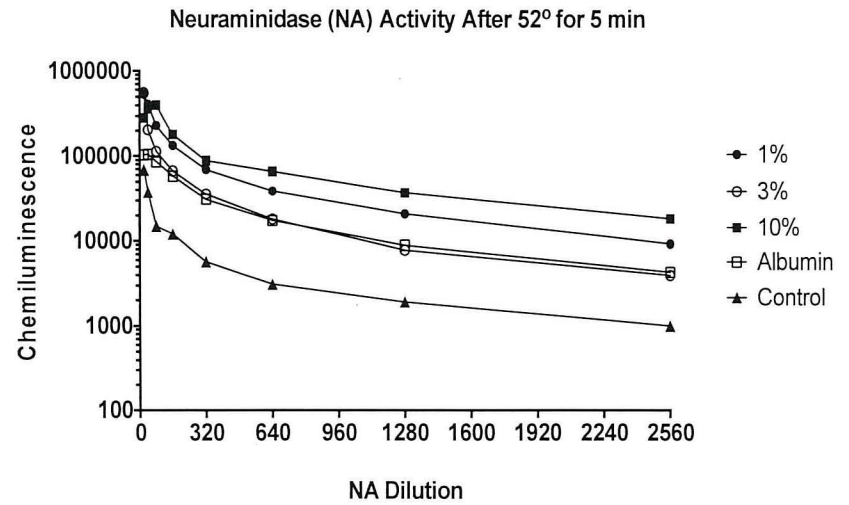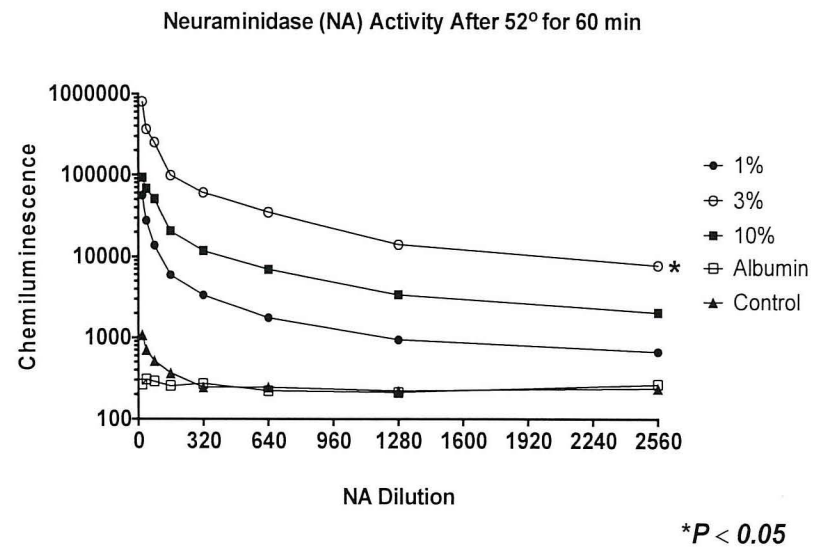

Figure 28: Neuraminidase activity of samples containing 0.3 mg/ml viral protein concentration after exposure to 52°C water bath for 240 minutes. Graph shows chemiluminescence vs NA dilution
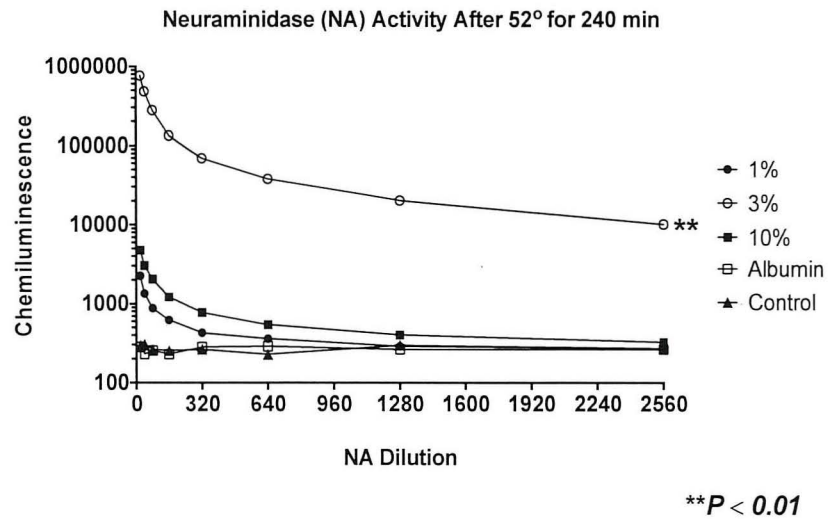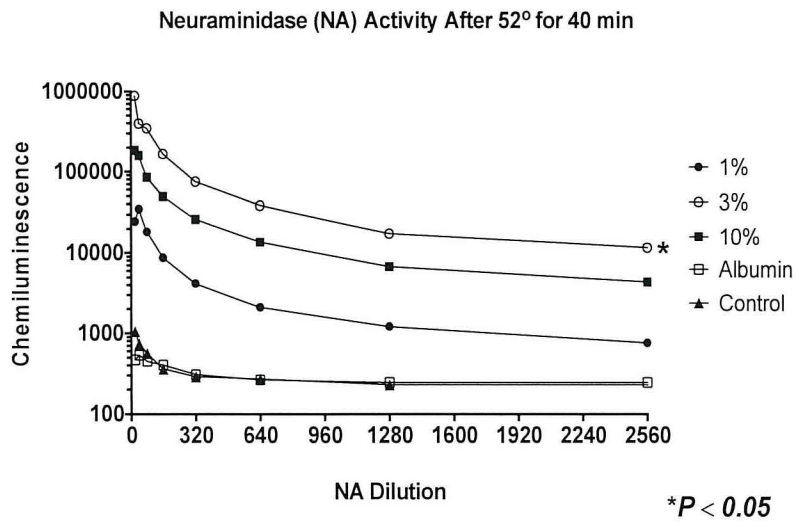
Conclusions

4.1 Column Chromatography

The results from our column chromatography study showed that a peak protein concentration appeared to correlate with peak HA content in both columns (Figures 11 and 12). This suggests that little protein purification has occurred. A band of 60 KDa protein was identified by the SDS-PAGE from the 1st phosphate buffer wash (figure 13). Because the HA assay identifies viral proteins in later eluates (Figure 9), the datum suggests that the band of protein is a contaminant and not the desired viral protein. It is hypothesized that the sensitivity of the Simply Blue dye used was too low. A more sensitive dye could be used, such as a silver stain, to identify any remaining proteins in the sample.

As we were not able to identify our protein of interest with the column chromatography, our results for the column chromatography still remains inconclusive. Such purification is theoretically possible, but more tests are required to achieve this.

4.2 Hemagglutinin (HA) Assay for Accelerated Stability Test

As shown in figures 14-20, there was no significant difference in the HA activity between the gelatin samples and the control. It was confirmed that gelatin was interfering with the HA assay by performing an HA on samples that had not been exposed to elevated temperatures (Figure 20). Since none of the sample had been exposed to elevated temperatures similar HA

values were expected. The results show that only the control, albumin, and 0.5% gelatin samples had recordable HA values.

Since the exact mechanism for the gelatin interference is unknown, I hypothesize that the gelatin could be causing some form of aggregation with the blood cells. It is known that collagen is a main stimulant for platelet aggregation. As the body is full of redundancies and multiple pathways, I hypothesize that the collagen could have caused agglutination in the erythrocytes that were used.

4.3 Neuraminidase (NA) Assay for Accelerated Stability Test

Our results from figures 21-28 show that viral protein stability was achieved by the use of gelatin. The 0.3% gelatin sample was shown to have approximately 10,000 fold increase in NA activity. Such increased activity suggests that gelatin could be used as an effective vaccine stabilizer.

Discussion

The cold chain has been the largest obstacle in distributing vaccines around the world. Because vaccines must be kept at low temperatures, many areas of the world struggle to keep the vaccine potency throughout the transportation process. If a vaccine stabilizer was used, vaccines could be distributed at room temperature without losing vaccine potency. Our results have shown that gelatin could be used as an effective vaccine stabilizer for the influenza H1N1 BEI inactivated virus.

The exact stabilizing mechanism of gelatin is still unknown. Based on the findings two hypothesis can be suggested. The first has to do with the structure of gelatin. As gelatin is simply denatured collagen, there is a meshwork of tiny pores that are formed from the different lengths of collagen strands. This could possibly form small nanoscale environments that are optimal for the viral particles. Such stabilization has been seen in the application of silk as a stabilizer (Zhang *et al.*, 2012).

Another hypothesis for gelatin's stabilizing ability, could be from binding of the gelatin to the virus. According to Liska *et al.* (2007) gelatin provides non covalent binding to virus particles that is non-specific. It is believed that this binding could enhance the stability of the viral particles. As we can only hypothesize the stabilizing mechanisms of collagen, more research is required.

While it has been shown that gelatin can increase the stability of a vaccine, it should be noted that one cannot inject a patient with the gelatin still inside. Many studies have shown that endogenous gelatin can cause serious allergic responses (Sakaguchi *et al.*, 2000). Because of this danger, we have collaborated with our partner Dr. Michael Seungju at the University of Utah. Dr. Seungju has developed a peptide that binds to gelatin. With this peptide, we could use gelatin as a stabilizer and then extract it from the vaccine after successful transportation. This could revolutionize the way vaccines are shipped around the world.

The cold chain is one of the most difficult problems we face in vaccine distribution today. 80% of funds for vaccine production is spent on the effective transportation of the vaccines. With the use of gelatin as a vaccine stabilizer, we can eliminate the cold chain

altogether. This could not only increase the accessibility of vaccines across the world, but has

the potential to save millions of lives each year.

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Jorgen Madsen

Honor's Thesis

4/29/15

Being a biology major, my classes have been very science oriented. There did not seem to be very much overlap in the humanities programs. My involvement with UNICEF, however, has made me very interested in areas such as global health. I found it difficult at times to combine my interests in UNICEF with my biology degree. It felt as if they were two separate aspects of my life that were completely polar from each other.

My capstone project was an opportunity to be able to bridge all of my interests during my college career into a single reflective project. Working at the Institute for Antiviral Research, I was introduced to Dr. Bart Tarbet. Dr. Tarbet has worked with vaccine manufacturing for a number of years, as well as BARDA. After talking with him, I discovered that we shared a common interest in global health. I found this as an opportunity to apply my interests from UNICEF with projects that I was already working on in the lab.

As I worked with Dr. Tarbet for over two years, I was able to learn a vast amount of assays and procedures. I started off basic, learning how to do something as simple as culturing cells and aseptic technique. Towards the end of my college career, I was able to do all of my own viral dilutions and sample preparations.

Of all of the lessons that I have learned from my project, the greatest is probably persistence. Many times our tests would fail and we would not get the data we wanted. I constantly had to go back and evaluate what I did, and find ways to improve. There were many 12 hour Saturdays spent in the lab,

only to find that our results did not work. This was frustrating at times, but it taught me that there is no such thing as a "failed" experiment. You take your results, and learn what you can do better.

There was a time during my undergraduate career, that I had to take a hiatus form my research in order to study for my MCAT. As I was applying for medical school, I was finding it difficult to spend the amount of time I needed to in the lab. This hiatus was strenuous on my project, and it is reflective on our column chromatography data. As the project required more time, we were not able to get the results we wanted. Once I finished my MCAT I had intended on returning to the column chromatography project but we were presented with a more promising alternative; vaccine stabilization.

While I found the vaccine stability project to be very exciting, it also came with many failed results. We had read a number of different journals that had said vaccine stability could be achieved through gelatin stabilization, yet we were not able to reproduce these results. I found myself constantly looking at our variables and seeing what we needed to change. This was anything from our temperature to our viral protein concentration.

My proudest moment, however, came from looking at another "failed" run and realizing that was not due to our error. After looking at the results closely, it became clear to me that the gelatin had to have been interfering with our assay. We made a hypothesis, and ran a tests to see if we were correct. One of the most gratifying feelings I have ever felt, was getting our results and seeing that it supported our hypothesis. While I have applied the scientific method many times in my college career, this was the first time that it was not laid out for us in a lab manual. I felt like I was a pioneer in a new field, or an explorer discovering something for the first time.

After taking our results, we were able to evaluate different membrane protein found on the virus. At this point, it was like something just clicked. Our results started coming back beautiful. I felt

giddy to finally, after two years, start getting results that were working. To anyone else, it might just look like lines on a graph, but to me it is a reflection of years of dedication and hard work.

My honor's capstone thesis has been one of the most rewarding achievements I have done in my entire undergraduate career. This project has pushed me farther than any other class I have taken. I have spent countless hours in a lab and reading journals, but it has been worth every second of it. Looking at the finished project, I am proud of how difficult it was.

If I could give advice to anyone starting a thesis, I would say this: Find something you are passionate about. The amount of time spent on this project was far more than a three credit class, but because I was invested in the results it never felt like a burden. By taking pride in your project, it will push you to do more than what is merely required of you. By thinking about your thesis early on in your undergraduate career, you can identify a project that will reflect not only what you did during your time at USU, but who you became as a person.

I am grateful to have had a mentor that was able to council me and help me to do more than I thought I ever could. Dr. Tarbet pushed me to strive for perfection. While at times I thought he was being nitpicky on little things, I have realized that he was helping me to be the best that I could be. I am also grateful for all of the faculty that have worked with me on my committee. I felt that the faculty on my project genuinely cared about me and my future plans. I do not think I would have gotten to know them at this level if it had not been for my project. This has project has truly been a capstone that served as an emotional finale for my time here at Utah State University. Thank you for the opportunity!

Autobiography

Jorgen graduated from Bear River High School in 2008. He initially started his undergraduate career at Southern Utah University majoring in theater. After serving an LDS mission in Antananarivo, Madagascar, Jorgen transferred to Utah State University and changed his major to Biology with a minor in Chemistry.

In 2012 Jorgen started the UNICEF club at Utah State University. The club focuses on educating students about global poverty, advocating for UNICEF, and raising funds to help children in need. In 2014 Jorgen was one of six students in the nation to serve as a national campus initiative leader for the US Fund for UNICEF. During that time, Jorgen oversaw 16 universities in the Midwest, and coordinated with the US Fund for UNICEF in New York City.

Jorgen has been actively involved on campus working as a teaching assistant for six semesters in Human Anatomy, Human Dissection, as well as Advanced Human Physiology. Jorgen has also served as a camp facilitator for the Hugh O' Brian Youth (HOBY) Leadership camps. From 2012-2014 Jorgen worked as a volunteer in the Logan regional emergency room.

From 2013-2015 Jorgen worked as the lead animal technician for the Institute for Antiviral Research at Utah State University. During that time he has assisted in a number of different in vivo studies, as well as in vitro. During his time with the Institute for Antiviral Research, Jorgen started working with Dr. Bart Tarbet with research projects that he felt related to his passion with UNICEF and global health. Jorgen has worked with Dr. Tarbet for over four months leading up to his thesis.

Jorgen has just recently been accepted to medical school at Penn State Milton Hershey Medical School, as well as the University of Utah. He plans on pursuing*- a medical career in Infectious Diseases with an emphasis in Pediatrics.