ABSTRACT

Title of Document:	AN INVESTIGATION OF THE STABILITY AND DIFFUSIVITY OF FLEXIBLE LIPID VESICLES FOR TRANSDERMAL INSULIN DELIVERY
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Flexible lipid vesicles have the potential of complementing or even replacing traditional needle injection methods for insulin delivery. Vesicles are made flexible by the incorporation of a chemical surfactant which may also hinder their stability. We studied the changes in the size and apparent flexibility of vesicles with varying surfactant concentrations over time and the effects these changes have on vesicle diffusion. We found that increased surfactant concentrations lead to greater size fluctuations. In addition, we witnessed a significant decrease in the flexibility of vesicles over six weeks, while the diffusivity of surfactant infused liposomes increased over a single week. Our data suggests that while surfactants are necessary in vesicles for transdermal drug delivery, their long-term stability is uncertain. Using our diffusion data, we developed a model to estimate the insulin delivering capacity of a hypothetical insulin patch which has the potential to stabilize vesicles for extended periods of time.

AN INVESTIGATION OF THE STABILITY AND DIFFUSIVITY OF FLEXIBLE LIPID
VESICLES FOR TRANSDERMAL INSULIN DELIVERY

By

Team No More Needles

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1. Introduction

1.1 What is Diabetes, and Why is it Important?

According to the American Diabetes Association (ADA), diabetes is a disease in which the body does not produce or properly utilize insulin ("All About Diabetes", 2009). Insulin is a hormone necessary for cells to intake glucose, which in turn provides energy for proper cellular function. An untreated diabetic can suffer from conditions ranging from heart disease, stroke, high blood pressure, renal failure, nervous system disease, as well as a gamut of other complications. Synthetic insulin, amongst other treatments, can be used to help diabetics control their blood glucose homeostasis.

Diabetes is the sixth leading cause of death in the United States and affects an estimated 20.8 million people (7% of the population), with an additional 40.1 million Americans who have been identified as at-risk for developing the disease. In the year 2000, approximately 170 million people worldwide were affected with diabetes, and this number is expected to double by the year 2030 (Hadjiyanni & Drucker, 2007). In 2003, IMS Health reported that insulin sales alone reached $2.2 billion dollars, with the total diabetes therapy product sales reaching $7.8 billion. However, this is a small amount compared to the $132 billion (2002 statistic) in medical expenditures for treating diabetes-related conditions and ailments reported by the ADA. Such a large gap in spending for medications versus treatment of diabetes-related complications demonstrates the potential for enhanced diabetic therapy.

Both the widespread impact of diabetes and the complexity of care for diabetics warrant greater attention paid to the development of more effective diabetes treatments and the improvement of the quality of life for diabetics.

1.2 Why Was Team No More Needles Formed?

Team No More Needles was conceived under the blessings of the Gemstone Undergraduate Research Program at the University of Maryland, College Park. Consisting of cross-disciplinary scholars with interests in the synthesis of research, technology, and ethics, the Gemstone Honors program aligned a group of students united by the goal of removing needle usage from diabetes care. Team No More Needles has spent the last four years tackling the varied facets of this challenge, exploring angles from dense technical perspectives to the many slants of business opportunities.

While the expansive pharmaceutical sector has generally been successful in developing various new drugs and delivery devices, it was surprising that no clear alternative existed on the market for diabetics who require daily doses of insulin. After careful debate, the team decided that liposomes, microscopic molecules capable of encapsulating drugs, were the most appropriate candidates for research as a transdermal insulin delivery method.

As laboratory research progressed, the team decided to pursue the business potential of an insulin delivery device by designing a transdermal insulin patch that used liposomes as the delivery medium. As a result, the team wrote a business plan to advertise both the societal advantages and potential business opportunities of bringing an insulin patch to market (See Appendix A).   In the spring of 2008, Team No More Needles entered three business plan competitions with this potential product, under the name SymViva Technologies. They placed third in the Greater Baltimore Technological Council's Mosh Pit Business Plan Competition, second in the undergraduate category for the University of Maryland 50k Business Plan Competition, and were semi-finalists in the Mid-Atlantic Business Plan Competition, earning a total of $4,200 for their start-up business.

1.3 The Problem with Needles

Although needles have served as the traditional method of delivering insulin, this technique continues to present several problems, mainly due to its invasive nature. Needle-based delivery is not only tedious and painful, but also a potential biohazard. Patients who reuse needles and syringes risk the transmission of diseases as well as an increased chance of infection. Despite recommendations from manufacturers which state that needles should be discarded after a single use, some patients find it more convenient and cost-effective to reuse needles (Insulin Administration, 2000).

Frequent use of hypodermic needles can also cause physical deformations. Fat hypertrophy, or the formation of fatty growths at injection sites, is an unsightly problem. It is sometimes accompanied by fat atrophy or indentations on the skin caused by loss of fat under the skin's surface (BD, 2008). Also, scarring can occur if is the same injection site is used too often (BD, 2008).

Needle injections can also cause psychological discomfort for their users, which include anxiety and phobias associated with needles. The fear of needles can inhibit patients from following a strict regimen of self-injections and blood glucose tests (Mollema et al., 2001). In fact, multiple studies have correlated poor glycemic control in type 1 diabetes patients with high levels of anxiety and phobic symptoms (Berlin et al, 1997, Zambanini et al., 1999). Patients with needle-phobia experience a lowered quality of life due to these conditions (Mollema et al., 2001).

1.4 Why is More Research Needed?

While current alternatives on the market address certain issues associated with needle delivery, there has been no breakthrough technology that brings a greater degree of normalcy to

diabetic life. Currently, diabetics may choose between modified needles and insulin pumps. For a short period of time, Exubera, a system of inhalable insulin delivery, was also available, but has since been removed from the market due to lack of acceptance by doctors and patients.

Modified needle-delivery techniques consist mostly of insulin pens and automatic injectors, which do not circumvent the invasive nature of traditional needle injections. In the case of automatic injectors, the needle is typically surgically implanted and hidden from view (FDA, 2008). This is favorable for patients who are most uncomfortable with penetrating the skin on a regular basis (BD, 2008). However, automatic injectors still present many of the physical and psychological disadvantages associated with traditional needle syringes.

The most popular alternative on the market is the insulin pump. This small device is carried outside the body and is connected to subcutaneous tissue via a narrow flexible catheter (FDA). The pump allows for close control of insulin levels without multiple injections. It is also programmable to administer insulin when needed and delivers insulin with greater precision than traditional needle injections (ADA, 2008). One major disadvantage of the system is that the device must be on body at all times. Insulin pumps are relatively bulky to wear and the implanted catheter has in some cases been reported to become dislodged from a patient's body. This poses obvious dangers as users should not go without insulin for extended periods of time (ADA, 2008). Insulin pumps can be a hindrance to exercise, athletic activity, and even sleeping. They are also expensive, with the cost of a pump at around four to five thousand dollars (ADA, 2008).

In 2006 Pfizer introduced inhaled insulin and promised that their product, Exubera, would revolutionize insulin delivery. However, after approximately one year on the market, Exubera was withdrawn from the market with a 2.8 billion dollar loss by Pfizer (Weintrab, 2007). Use of the device had been associated with decreased lung function (D'Arrigo, 2007), and

the device itself was described as "cumbersome" by diabetics who used it. Moreover, a treatment regimen based on Exubera was estimated to cost 30% more than a regimen based on injections (Weintrab, 2007). With the decline of Exubera, no true alternative to needles exists on the market.

While this niche has yet to be filled, there are several promising candidate methods that are vying for the spot. Primary among these is that of lipid vesicles, known as liposomes. These vesicles are essentially bubbles of fats that have the capacity to encapsulate drugs and proteins such as insulin for transdermal delivery – an idea first proposed in the early 1980's (Mezei & Gulasekharam, 1980). While the skin serves as a potent boundary between the external environment and the interior of the body, vesicles have been found to have the capacity to pass through the stratum corneum (the outermost layer and main barrier of the skin) and ultimately reach capillaries below (Bouwstra & Honeywell-Nguyen, 2002). However, these results are not yet conclusive as many researchers have recorded contrasting results. As such, liposomes treatments are still largely in the experimental phase and the ultimate question as to whether or not liposomes are effective at transdermal drug delivery has yet to be answered.

1.5 What Does Team No More Needles Hope to Accomplish?

The primary objective of this project is to assess the viability of liposomes as a potential transdermal insulin delivery system for Type I Diabetics. We define 'viability' as stability of size, flexibility, and diffusivity of the liposomes over time, since changes in any of these three factors would dramatically alter the effectiveness of the liposome in delivering insulin. Another aspect in our definition of "viability" is testing of determined parameters in mathematical models. In order for the drugs to be viable, they must be able to withstand the manufacturing process, shipping, and have a reasonably long shelf life.

Our research questions are the following:

1. How do the size, stability, and flexibility of liposomes of different surfactant concentrations change over time?

2. How does surfactant concentration affect diffusivity of liposomes through a skin-like barrier?

3. How do rates of diffusivity of liposomes with different surfactant concentrations change over time?

4. Upon fitting the collected data to a mathematical model, what is the estimated extent of insulin delivery through the skin?

From preliminary data collected in the lab, a secondary objective emerged to produce a model liposome patch design, and evaluate the potential business opportunity for marketing such a design.

1.6 Outline of Study

A formulation for the vesicles was achieved through the mentorship of Dr. Oluwatosin Ogunsola. With the additional guidance of Dr. Srinivasa Raghavan and Dr. Nam Sun Wang, multiple liposome samples were generated and analyzed according to our objectives. Stability over time was measured using a dynamic light scattering (DLS) laser, and elasticity was measured using an extruder. The data collected was used to determine the ideal ratio of surfactant to add to the liposome in order to achieve superior stability and elasticity.

To achieve our second research question, agar gel samples were prepared and liposomes loaded with DII fluorescent dye were applied. The penetration of the liposomes through this membrane was measured using an optical microscope.

1.7 General Study Hypotheses

1. Increasing surfactant concentration of the liposome will result in more size fluctuations over time.

2. The flexibility of the liposome will decrease over time, regardless of surfactant concentration.

3. The diffusivity of liposomes through a skin-like barrier will decrease over time.

4. Combining these three hypotheses, we ultimately predict that the viability of liposomes for insulin delivery will decrease over time.

2. Literature Review

2.1 Anatomy of the Skin

Human skin is composed of three major layers, the cellular epidermis, the dermis, and the subcutaneous (Figure 1). The outer most layer of the cellular epidermis is the stratum corneum. It is composed of 15-20 layers of corneocytes, keratin-filled dead cells, which are surrounded by crystalline lipid lamellae. (Bouwstra and Honeywell-Nguyen, 2002 & El Maghraby, 2008). These lipid lamellae of the stratum corneum are composed of lipids different from those of the plasma membrane of living cells. The main lipids found in the stratum corneum are ceramids, cholesterol, and free fatty acids. The chain lengths of these lipids are much longer than the chains of the plasma membrane's phospholipids (Bouwstra and Honeywell-Nguyen, 2002). The organization of the stratum corneum is often described as a wall with the corneocytes as bricks embedded in a lipid mortar. These structures are 10-15 um thick when dry and up to 40 um thick when hydrated (El Maghraby, 2008).

Figure 1: Layers of the Skin
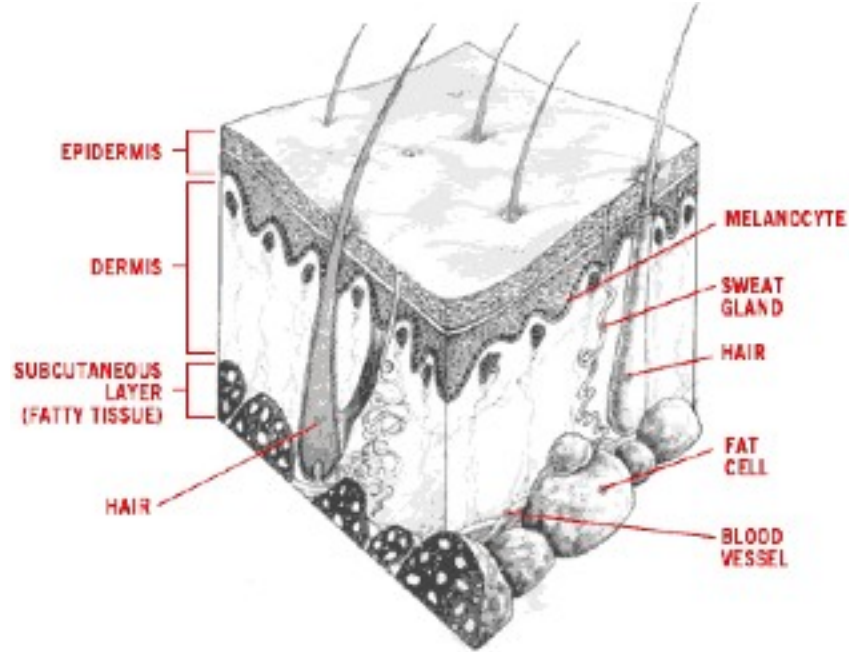
The 3 major layers of the skin. Taken from Cancer Council Victoria. Common Skin Cancers. Brochure. Melbourne: 2007.

The next layer, the dermis, is three to five nm thick and is composed of collagen and elastin proteins. Blood and lymphatic vessels, nerve endings, hair follicles, and sweat glands are all found in the dermis. The hair follicles and sweat glands are embedded in the dermis, but they extend through the cellular epidermis all the way to the skin surface (El Maghraby, 2008). Since blood vessels do not reach the cellular epidermis, nutrients and waste products must diffuse across the dermal-epidermal layer to reach the cellular epidermis (El Maghraby, 2008).

To penetrate the skin, there are two main routes that molecules take: the trans-appendageal pathway and the transepidermal (or transdermal) pathway. The trans-appendageal route is penetration through the sweat glands and the sebaceous glands associated with hair follicles. The transdermal route entails penetration of a substance through intact stratum corneum, which includes either maneuvering around the keratinocytes and diffusing through the lipid domains (transcellular pathway), or penetrating the keratinocytes and then diffusing across

the lipid domains (intercellular domain) (Figure 2) (El Maghraby, 2008). In both pathways, the

stratum corneum is considered the rate-limiting barrier as its intercellular lipid domains present a

major barrier to any molecule trying to penetrate the skin.

Figure 2: Transdermal Route

Intercellular Lipids
Keratinocytes

Intracellular Pathway   **Trans-cellular Pathway**
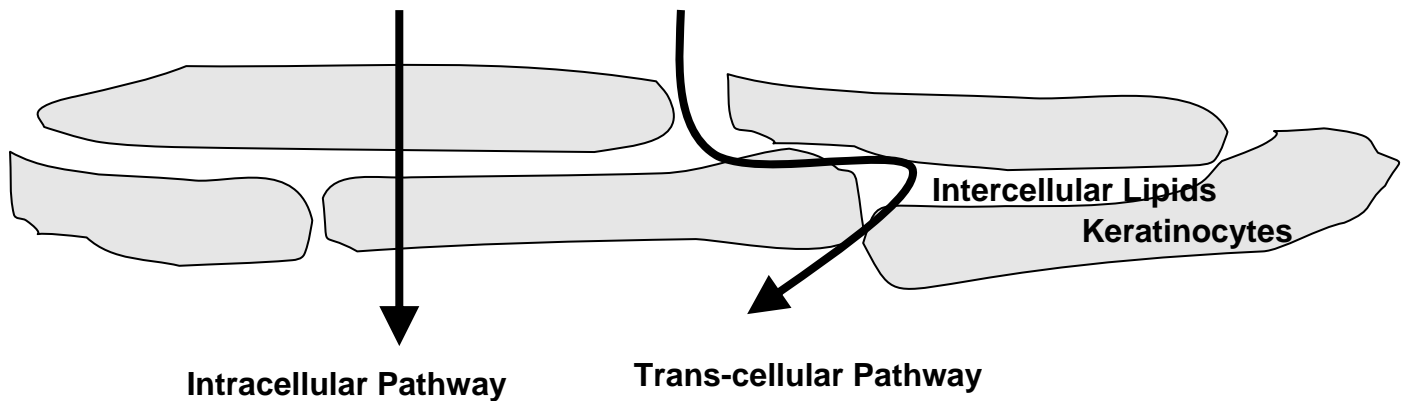
Figure 2: Bricks and mortar arrangement of the keratinocytes and intercellular lipid domains. The two sub-pathways of the transepidermal routes can be seen, with either penetration of the keratinocytes in the intracellular pathway or diffusion through the lipid domains while maneuvering around the keratinocytes in the transcellular pathway (adapted from El Maghraby et al., 2008).

2.1 Diabetes

2.1.1 Pathology of the Disease

Diabetes is a disease characterized by abnormal glucose metabolism resulting from

defects in either insulin secretion or insulin action (Nathan, 1993). There are two classifications

of diabetes: type I and type II. Type II diabetes is non-insulin dependent diabetes and is the more

common form of the two, comprising 80-95% of all diabetes etiologies (Voltarelli, 2007). Type

II diabetes is caused by a resistance to insulin or an inability to produce sufficient amounts of

insulin. (Zimmet, 2001). In most cases, type II diabetes is treated with medication and exercise,

but up to one third of patients may ultimately require synthetic insulin supplements (Campbell &

Reece 268-269, Gerich, 2002).

Type I diabetes, also known as insulin-dependent diabetes, is a cell-mediated autoimmune disease that results in the destruction of insulin-producing pancreatic β cells (Silvera et al., 2006). The dwindling supply of insulin leaves the body unable to maintain glucose homeostasis, and the resulting hyperglycemia (high levels of glucose in the blood) can lead to progressive dehydration, reduction of blood, shock, and ultimately, coma. Severe and common complications of hyperglycemia include: retinopathy and the associated potential loss of vision, nephropathy and associated renal failure, and peripheral or autonomic neuropathy along with associated gastrointestinal, genitourinary, and cardiovascular symptoms (Report of the Expert Committee, 2002). In Western countries, type I diabetes is the leading cause of limb amputations and blindness (Hadjiyanni & Drucker, 2007).

In the early stages of type I diabetes, the patient's own T-lymphocytes infiltrate the β cells, and as the disease progresses the β cells begin to disappear (Babaya, 2005). Type I diabetes patients show a clear reduction in the total mass of these pancreatic cells compared with non-affected individuals (Davidson, 2000). The rate of β cell destruction varies among individuals, with rapid destruction common among infants and children, and slow destruction common among adults (Zimmet, 1994). Eventually, however, all patients suffering from type I diabetes must receive exogenous forms of insulin in order to survive (Zimmet, 2001). Studies have estimated that by the time type I diabetes is diagnosed only 10-20% of the patient's β cells are still functioning (Knip et al., 2006).

There is a genetic predisposition to type I diabetes (Bonen, 2002). Individuals with a first-degree relative suffering from type I diabetes are 15 times more likely to develop the disease than individuals whose immediate families are unaffected (Hakonarson et al., 2007). Eighteen human gene sequences have been associated with genetic susceptibility to type I

diabetes, but only three, HLA (human leukocyte antigen), VNTR (variable nucleotide tandem repeat), and LYP (lymphocyte tyrosine phosphatase) have been conclusively linked to type I diabetes predisposition (Babaya, 2005). Concannon et al. analyzed data from 1,435 families and found evidence for type I diabetes linkage to 10 other chromosome regions, suggesting that there are more genes linked to genetic predisposition for type I diabetes. Despite the understanding that type I diabetes clusters in families, its mode of inheritance remains unknown (Concannon, 2005).

In addition to genetics, specific environmental factors have also been associated with development of type I diabetes. According to a study conducted by the Diabetes Epidemiology Research International Group, there are significant differences in the incidence of type I diabetes among populations in different geographical areas. The group found that among patients 15 years of age, the average incidence of type I diabetes in Hokkaido, Japan was 1.7 per 100,000 people, while in Finland the incidence was 29.5 per 100,000 people (1998). Additionally, studies conducted on the incidence of type I diabetes in monozygotic twins revealed that only 13 - 33% are pair-wise concordant for type I diabetes (Kaprio, 1992). These results indicate the presence of differential exposure to environmental factors, as well as changes in lifestyle and environment, rather than post-conceptional genetic discordance. Moreover, approximately one-fifth of Caucasians carry the HLA diabetes susceptibility gene and only 5% of them develop overt type I diabetes (Knip et al., 2006). Knip et al. suggest that genetic susceptibility, a trigger, and exposure to an exogenous antigen are all required for progression to type I diabetes to occur; if any of these three factors are missing than the risk of developing type I diabetes is minimal. Potential exogenous environmental factors contributing to the development of type I diabetes include cow's milk, cereals, and viral infections. Cow's milk and cereals have been suggested to

trigger the production of insulin auto-antibodies, and the timing of their introduction to infants seems to be of importance (Knip et al., 2006; Virtanen, 2000). Viruses have also been implicated in the triggering of type I diabetes, and it has been hypothesized that they can act as self-antigens to accelerate the autoimmune process through molecular mimicry (Knip et al., 2006).

2.1.2 Insulin Treatment

2.1.2.1 Insulin

Insulin is a peptide hormone that is produced in the pancreas and facilitates the uptake of glucose by liver, muscle, and fat cells. The β-cells of the pancreas secrete insulin directly into the hepatic portal blood in response to hyperglycemia and sympathetic and parasympathetic stimulation. In healthy individuals the levels of insulin vary dramatically throughout the day, decreasing during times of exercise or fasting, and increasing during mealtimes (LeRoith, 2004).

Insulin can be isolated from pork pancreases or synthesized chemically through recombinant DNA technology so that it is identical to human insulin. Additionally, rapid-, intermediate-, and long-acting insulin analogs have been created by the modification of one or two amino acids of human insulin (Chen et al., 2003).

2.1.2.2 Insulin Delivery

Subcutaneous injection serves as the primary mode of insulin delivery (Owens et al., 2003). Insulin is injected into the subcutaneous tissue of the upper arm, thigh, buttocks, or abdomen, where it forms an insulin depot from which it can be absorbed into the blood (Chen et al., 2003; Insulin Administration, 2000). To be able to reach the blood stream, insulin molecules must dissociate from their natural hexameric form into monomers or dimers so that it is small enough to diffuse through the capillary wall. Once in blood circulation, insulin binds to insulin

receptors on target cells and performs its biological activity (Chen et al., 2003). However, the dissociation step results in a lag phase between the subcutaneous injection and the time of insulin action. Consequently, at meal times the absorption of insulin is too slow to mimic the body's rapid release of insulin (Brange, 1988). Through a single amino-acid substitution in human insulin, Brange et al. created an insulin analog that remains monomeric and is absorbed 2 to 3 times faster than unmodified insulin while retaining its biological activity (1988).

The modification of human insulin to produce insulin analogs, as well as the synthesis of human insulin through recombinant DNA technology, has allowed for the development of rapid-, intermediate-, and long-acting insulin (Insulin Administration, 2000). Rapid-acting insulin ("bolus" insulin) includes insulin lispro and insulin aspart, both of which differ from human insulin by one or two amino acids in positions 28 and 29. These modifications make it less likely that the insulin will self-aggregate into hexamers (Chen et al., 2003). Insulin lispro and insulin aspart have onset times of 5-15 minutes and can therefore be administered at mealtime, increasing the quality of life for patients with type I diabetes (Gerich, 2002). Studies by Home et al. have demonstrated that the absorption of insulin aspart is twice as fast as the absorption of human insulin and after injections of insulin aspart, plasma glucose levels fell twice as rapidly as they did after human insulin injections (1999). Studies on insulin lispro have found similar results, with one study finding that within 100 minutes of subcutaneous injection 90% of insulin lispro was absorbed compared to 180 minutes for unmodified human insulin (Gerich, 2002).

While it is important to control the surge in blood glucose immediately following a meal, type I diabetes patients must also be concerned with maintaining normal glucose levels throughout the entire day. Since rapid-acting insulin is only effective for up to five hours, two daily injections of intermediate-acting insulin (regular insulin) are also typically administered

(DeWitt & Hirsch, 2003). Intermediate-acting insulin has an onset time of four to ten hours after injection and is effective for 10 -18 hours (DeWitt & Hirsch, 2003). Intermediate-acting insulin refers to neutral protamine Hagedorn (NPH) or Lente insulin. NPH is regular insulin with the addition of protamine and lente insulin is regular insulin that has been bound to zinc. These additions make the insulin absorbed more slowly and thus are used as twice-daily basal insulin injections. Long-acting insulin, called ultralente insulin, is absorbed very slowly due to its zinc crystalline form. It has an onset time of 6-10 hours and is effective for 18-24 hours (DeWitt and Hirsch, 2003). The different forms of insulin can be administered separately, but administration of a mixture of the short and intermediate or rapid and long-acting forms can result in more normal blood glucose levels in some patients (Insulin Administration, 2000). Premixed insulin preparations composed of proportions of NPH and regular insulin or NPH and insulin lispro are commonly administered as they can produce a more normal blood glucose concentration over long periods of time (Insulin Administration, 2000).

2.2 Problems with Needles

In patients with type I diabetes, subcutaneous administration involves an average of four daily injections with syringes of varying lengths, in addition to regular self-testing of their blood glucose levels (Mollema et al., 2001). Since adherence to insulin self-injections and self-testing are essential for glycemic control, poor patient compliance is a serious impediment to the effectiveness of insulin therapies. Blood-injection-injury phobias and anxiety can keep patients from administering their own injections, thereby increasing the likelihood of unwanted health complications (Mollema et al., 2001). Berlin et al. found that type I diabetes patients with poor glycemic control also exhibited high levels of anxiety and phobic symptoms (1997). These findings were corroborated by a study conducted by Zambanini et al., who found that a

significant portion of type I diabetes subjects displayed high levels of anxiety in association with their insulin injections (1999). Mollema et al. attempted to estimate the magnitude of these blood-injection-injury phobias in patients receiving insulin treatments, and found that out of a research population of 1275 patients, 0.2-1.3% showed fears of self-injecting, and 0.6-0.8% showed fears of self-testing (2001). These results indicate that while phobias and anxieties do not prevail among type I diabetes patients, a significant number of patients may only receive decent glycemic control at the expense of their quality of life (Mollema et al., 2001).

Another problem associated with injected insulin is variation in the actual insulin absorption. Numerous studies have documented differing rates of absorption among patients with type I diabetes. One study conducted by Binder demonstrated that radioactively labeled insulin showed absorption rates varying from 19-104% within different patients (1969). Lauritzen et al. also demonstrated that the absorption rate among different patients varied by 50% (1982). Variations in absorption have also been shown to occur within an individual. According to the Health Technology Assessment, absorption rates in a single patient can vary by about 25% (Colquitt et al., 2004). A study by Heinemann corroborated this data and found that after subcutaneous insulin injection, the action of regular insulin within one individual varies by 15 to 25%, and the action of long-acting insulin varies by 25-35% (Heinemann, 2002). Lauritzen et al. also found that absorption within an individual varies by 25% (1982).

Further complicating the issue, Heinemann points out that variations in insulin absorption are likely greater than the reported 15-35%, as variations in patients leading normal daily lives are likely to be greater than variations in patients living under controlled experimental conditions (Heinemann, 2002).   Many factors, such as the site and depth of the injection, the amount of fat at the injection site, the patient's exercise habits, the patient's temperature, and the blood flow to

the skin, influence the absorption of insulin and could contribute to the aforementioned variations (Chen et al., 2003). This is a serious problem with subcutaneous injections, as the variations can lead to dosing problems; too little insulin can lead to hyperglycemia while too much can result in hypoglycemia. Intra-individual variation in insulin absorption may be responsible for the daily variation in blood glucose levels and is a serious impediment to the control of diabetes (Lauritzen et al., 1982).

In addition to the risks associated with phobia, poor patient compliance, and variations in absorption rates, the reuse of needles or syringes among patients introduces concerns of sterility and raises the risk of infection. While manufacturers recommend that the disposable needles and syringes be used only once, some patients claim that they find it practical to reuse needles (Insulin Administration, 2000).

2.3 Alternatives to Subcutaneous Injection

The aforementioned problems with needles have led researchers to investigate a variety of alternative mechanisms that could replace subcutaneous injections. While some of the following methods have reached the market and others have not, none of them have been widely accepted as a suitable substitute for needle-based methods.

2.3.1 Insulin Pump

One treatment method that has been presented as a replacement to daily insulin injections is that of continuous subcutaneous insulin infusion, commonly known as the insulin pump. The principle of the technology is simple; a small pump, weighing around 400 grams and containing a cartridge of insulin, is connected to a catheter that is inserted into the tissue (Lenhard & Reeves, 2001). This technique has been shown to reduce dangerous variations in glucose levels

throughout the day, decreasing the likelihood of the unexpected health complications (Biscoff et al., 1994). Also, the pump has the capability to deliver varying amounts of insulin throughout the day, allowing patients more freedom in their activities such as "skipping or delaying meals, sleeping late on weekends, or engaging in vigorous exercise" (Lenhard & Reeves, 2001).

Unfortunately, there are also several drawbacks to insulin pump therapy. Primary among them is the high risk of infection that exists at the point where the catheter enters the body; values as high as 11.3 events per 100 years of patient follow-up have been reported by the Diabetes Control and Complications Trial Research Group (1995). There is also the possibility that the catheter or tubing can become occluded or that the pump can exhaust its insulin supply. Since the pump relies solely on short-acting insulin, any break in delivery can result in rapid increases of blood glucose levels (Castillo et al., 1996). Finally, and most significantly for our team, the costs associated with an insulin pump are much higher than other methods for glucose control. The startup costs are about $5,000 with an additional annual cost of $1,500 (Lenhard & Reeves, 2001). This is prohibitive for many patients and does not meet the team goal for an alternative that can be widely accessible.

2.3.2 Jet Injection

Another method that has received considerable attention from researchers is that of jet injection. The jet injection technology was aimed at relieving patients' reluctance to accept multiple painful injections of insulin and their difficulty in synchronizing carbohydrate and insulin absorption (Alberti et al, 1980). These devices use compressed springs or gasses to shoot liquids containing the molecules of choice at high velocity into the skin, and have been shown to penetrate the stratum corneum (Stachowiak et al., in press). While physiologically significant amounts of insulin have been delivered to rats (Arora, et al., 2007), the benefits of the treatment

do not currently outweigh the costs. Bruising and pain have been reported by humans who have undergone jet injection therapies due to the deep penetration of the jets (Mitragotri, 2006), and delivery of insulin is not improved in comparison with subcutaneous injection (Anderson et al., 1980). While microjet injectors aim to minimize patient discomfort, they have yet to be standardized and the majority of current research utilizes custom-made technology. There are currently a number of jet injectors available commercially, but a quantitative understanding of the energetics of jet injection is still lacking (Mitragotri et al, 2004).

2.3.3 Inhalable Insulin

The idea of administering insulin in an inhalable form, similar to that found in inhalers used by asthmatics, blossomed in 2006 when Pfizer presented Exubera® to the market. Exubera was designed to deliver short-acting insulin to accommodate increases in glucose levels following meals. Like an asthma inhaler, Exubera required the patient to pump the handle, aerosolizing the insulin, and to then take a normal breath from the inhaler (D'Arrigo, 2007). While some initial reports from patients trying inhalable insulin products were positive (West, 2001) and others were negative (Weintrab, 2007), Pfizer decided to market their product. After approximately one year, Exubera was withdrawn from the market for a 2.8 billion dollar loss by Pfizer (Weintrab, 2007). Use of the device had been associated with decreased lung function (D'Arrigo, 2007), and the device itself was described as "cumbersome" by diabetics who used it. As a final blow, a treatment regimen based on Exubera was estimated to cost 30% more than a regimen based on injections (Weintrab, 2007).

Currently, there are no other forms of inhalable insulin on the market. In 1998, Eli Lilly and Co. announced that it was developing an insulin product based on the asthma inhaler, Albuterol Spiros. Eli Lilly stated the pulmonary delivery system would deliver a powder form of

insulin through the inhaler (Smith, 1998). However, in 2008, Eli Lilly announced that the trials of its insulin product were going to be cancelled due to high costs of production (Dagher, 2008).

2.3.4 Oral Insulin

Researchers have also investigated the possibility of administering insulin in a pill form similar to a vitamin supplement. The benefits of this approach are easy to see, as the patients' discomfort would be much less than it would be with needles, and pills are an easy and safe treatment that could travel with a diabetic. For these reasons, scientists have suggested that orally administered insulin would be advantageous to traditional injection methods (Ghilzai, 2003). Barclay found that "oral insulin was safe, well tolerated, more effective than placebo, and as effective as subcutaneous regular insulin at controlling postprandial glycemia" (2003). Additional support for these results has been presented by Xie et al. (2008), who found that blood glucose levels could be drastically reduced in diabetic mice when they were fed genetically altered rice seeds. The rice plants were mutated so that their seeds contained high levels of human insulin-like growth factor 1, a necessary precursor for insulin release in the body which could in turn reduce blood sugar levels.

Unfortunately, there are several challenges associated with delivering insulin through the gastrointestinal tract. Native proteolytic enzymes do not discriminate between insulin and other ingested proteins when degrading the contents of the tract, and permeation through the tract itself can be insufficient for insulin delivery (Hamman et al., 2005). Existing research has addressed a number of methods to circumvent these pitfalls such as absorption enhancers to increase the amount of insulin that passes through the intestinal epithelium, enzyme inhibitors to prevent insulin degradation, and targeted delivery systems that attempt to localize insulin to the areas of the intestinal tract where diffusion is easiest (Khafagy et al., 2007). Despite these efforts,

however, oral insulin has not yet reached its potential as a viable alternative to needle-based administration of insulin.

2.3.5 Microneedles

Another transdermal alternative that has received attention in recent years is microfabricated needles, or more conventionally, microneedles. Microneedles used in transdermal drug delivery are typically presented in arrays of five to fifty needles whose lengths usually range between 500 and 1,000 micrometers (one half to one millimeter) (Gill et al., 2008). They can be either hollow, allowing substances to be pumped through them, or solid, with the substance to be transferred coating the needles' surfaces (Prausnitz & Langer, 2008). Drugs, proteins, and DNA particles have all been tested with relative success using these methods (Gill & Prausnitz, 2007). In fact, several studies have indicated that microneedles can be used to facilitate the delivery of physiologically significant amounts of insulin. Among these are McAllister et al. (2003) and Martanto et al. (2004), both of whom recorded decreases in rat blood glucose levels following microneedle treatment.

While microneedles show promise as a potential means of transdermal drug delivery, several drawbacks still exist with the technology. Primary among these is that fact that the protection provided by the stratum corneum is compromised, allowing undesired agents to enter the body after the target drug has been delivered (Cevc 2003). In addition, it is also possible for microneedle tips to break off from the patch and become lodged in the skin, again compromising the integrity of the skin barrier (McAllister et al., 2003). In addition to these negatives, we found that the fabrication of microneedles required materials not readily available to our team.

2.3.6 Transdermal Patches

There are currently numerous drug-delivery patches available on the market today. In 2003, it was reported that patch sales totaled $1.2 billion. Current transdermal delivery systems are designed to provide continuous controlled release of medication through intact skin, therefore providing a constant level of a drug in the blood (Egan, 2003).

2.3.6.1 Birth Control Patch

The birth control patch, Ortho Evra, is for the prevention of pregnancy in women who want to use a transdermal patch as contraception. Developed by Ortho-McNeil-Janssen Pharmaceuticals, Ortho Evra was approved by the Food and Drug Administration in 2002. Ortho Evra's website states that the patch is worn on the body and works by delivering a continuous level of hormones (progestin and estrogen) into the bloodstream through the skin. Progestin and estrogen prevent ovulation by preventing the ovary form releasing an egg to be fertilized ("Ortho Evra, the Patch"). These hormones also thicken the cervical mucus, making it more difficult for sperm to enter the uterus. In addition, the endometrium, the mucus membrane that lines the uterus, is affected and reduces the chance of implantation ("Ortho Evra, the Patch").

A new patch is placed onto the body once a week for three weeks in a row each month. The site advises that the patch is placed on one of four places: buttocks, upper torso, abdomen, upper outer arm. The patch contains three layers: the first layer is an outer protective layer of polyester, the middle layer is a medicated adhesive and the third layer is a clear polyester release liner, which is removed just before application ("Transdermal Contraception," 2005).

The birth control patch has been relatively successful. In fact, women reported using the patch correctly more often than they use oral contraception correctly. In a comparative study,

women used the patch correctly in 88% of their cycles compared with 78% of cycles among oral contraception users ("Transdermal Contraception," 2005).

However, there has also been some controversy surrounding the patch. The site mentions that a person using Ortho Evra is exposed to about 60% more estrogen than a person using the typical birth control pill, which increases the risk of side effects. And in January 2008, the FDA approved an updated additional warning label to the Ortho Evra patch, to clearly state that women using the patch may have a higher risk of developing venous thrombo-embolism (blood clots in the legs and/or the lungs) because of their receiving a higher dose of estrogen (Kuehn, 2008). This is a result of study conducted by the Boston Collaborative Drug Surveillance Program (Kuehn, 2008).

2.3.6.2 Nicotine Patch

Nicotine patches deliver nicotine patches at a relatively steady rate and are applied once daily (Nicotine Delivery Systems). The nicotine patch is designed to satisfy smokers' nicotine cravings. Each 2 inch adhesive patch delivers a controlled release of nicotine for 24 hours. It is used as a temporary aid to help one quit smoking by reducing nicotine withdrawal symptoms.

The Nicoderm website states that the patch uses a patented technology called SmartControl™ on the dermal side of the patch. SmartControl™ ensures that all of the nicotine will not be delivered at once or run out too soon.

Experiments have been performed investigating the success rate of using the nicotine patch in order to quit smoking. One study, done in 2008, examined whether adherence with daily nicotine patch wear was associated with improved rates of smoking abstinence. The double-blind study had randomized subjects receive either an active nicotine patch or a placebo under simulated over-the-counter conditions. It was concluded that under the experiment conditions,

adherence to daily nicotine patch wear within the first three weeks of treatment was associated with an improved likelihood of achieving smoking abstinence at six weeks.

2.3.6.3 Nitroglycerin Patch

Transdermal nitroglycerin is indicated for the prevention of angina pectoris due to coronary artery disease. The pharmacological action of nitroglycerin is relaxation of vascular smooth muscle and consequent dilatation of peripheral arteries and veins. Dilatation of the veins promotes pooling of blood and decreases the return to the heart, which reduces left ventricular end-diastolic pressure and pulmonary capillary wedge pressure. In terms of transdermal nitroglycerin, the MINITRAN™ Transdermal Delivery System is currently available today. It is designed to provide continuous controlled release of nitroglycerin through intact skin. The rate of release of nitroglycerin is linearly dependent upon the area of the applied area (Daily Med).

There have been recent studies which show that the transdermal administration of nitroglycerin could be used as treatment for pregnant patients with preeclampsia. Transdermal nitroglycerin patches deliver nitric oxide, which is a potent vasodilator and an inhibitor of platelet aggregation. This allows more blood to flow through the blood vessels easier. In pregnancy, nitric oxide is synthesized in several uteroplacental tissues and in endothelial cells of umbilical vessels. A deficiency in nitric oxide is a factor in patients with preeclampsia. In a study performed on pregnant patients with preeclampsia who had increased uteroplacental impedance, the delivery of nitroglycerin through transdermal technologies resulted in the decrease of uterine artery impedance. (Halmesmaki et al, 1998).

2.3.7 Technologies in Progress

Currently, there are two main non-invasive insulin delivery technologies undergoing phase 2 clinical trials. Both the insulin gel and ultrasonic insulin patch have the potential to capture the market niche our team hopes to dominate with our technology. Therefore, all of our team's future works must take care to create a technology that distinguishes itself from these competitors. In Australia, Phosphagenics Limited has patented a gel delivery system for insulin, and Pennsylvania-based Encapsulation Systems, Inc has developed an ultrasonic patch to replace the catheter in an insulin pump.

Phosphagenics Limited initiated phase 1B clinical trials for their transdermal insulin product, and has since reported positive results. The trials utilize Phosphagenics' patented TPM-02 delivery system. The system has been adapted for use with both short and long term insulin and presents both types of the drug in gel form. Phase 1A trials in 2006 showed that the insulin gel was able to effectively deliver insulin through the dermal tissue and into the bloodstream, resulting in significantly lowered blood glucose levels in human test subjects. The 1B trial provided supporting data necessary for a new drug application from the U.S. Food and Drug Administration (PR Newswire, 2007). In 2008, the company initiated phase 2 clinical trials in type I diabetic patients (Phosphagenics, 2008).

Phosphagenics' proprietary TPM delivery system is a patented transdermal carrier technology that works with the skin's natural transport mechanisms. It facilitates the transport of variously sized molecules through the tissue without compromising the integrity of the dermis as a barrier to harmful environmental elements. The TPM-01 system delivers small molecules such as morphine and testosterone, while the TPM-02 system delivers both small and large molecules, such as insulin and proteins (Phosphagenics, 2007).

Encapsulation Systems, Inc has also put a breakthrough technology for insulin delivery into the development pipeline -- an ultrasonic patch the size of a matchbook for use with an insulin pump. The technology was originally developed by a team of engineers at Penn State, which ascertained that the sonic patch could safely deliver insulin to rats (DeNoon, 2002). Encapsulation Systems, Inc has taken the technology a step further and developed the U-Strip (TM) Insulin Patch. The patch uses an alternating sonic transmission to dilate the pores of the skin, allowing large molecules such as insulin molecules to be effectively deposited within the dermis, allowing for direct bloodstream absorption. The patch consists of three major components: a patch designed to contain the target drug, the programmable ultrasonic transmitter, fitted above the patch, and a rechargeable battery system. The U-Strip (TM) Insulin Patch can be used for both basal and bolus doses of insulin. However, this technology is currently in phase 2 clinical trials and has not yet been approved by the FDA (Marketwire, 2007).

2.4 Liposomes

2.4.1 Define Vesicle

At the simplest level, a vesicle is nothing more than a bubble formed by a special type of molecules called phospholipids. The basic structure of a phospholipid can be seen in Figure 3-A. The top, circular portions (called heads) of these molecules contain electric charges which interact strongly with each other and with water. The presence of charges in the molecules makes them polar, meaning they are naturally attracted to one another and tend to group together.

Figure 3: The formation of a vesicle from phospholipid to belayed to sphere
(adopted from Wang, Inex Pharmaceuticals Corporation).

A.   B.   C.
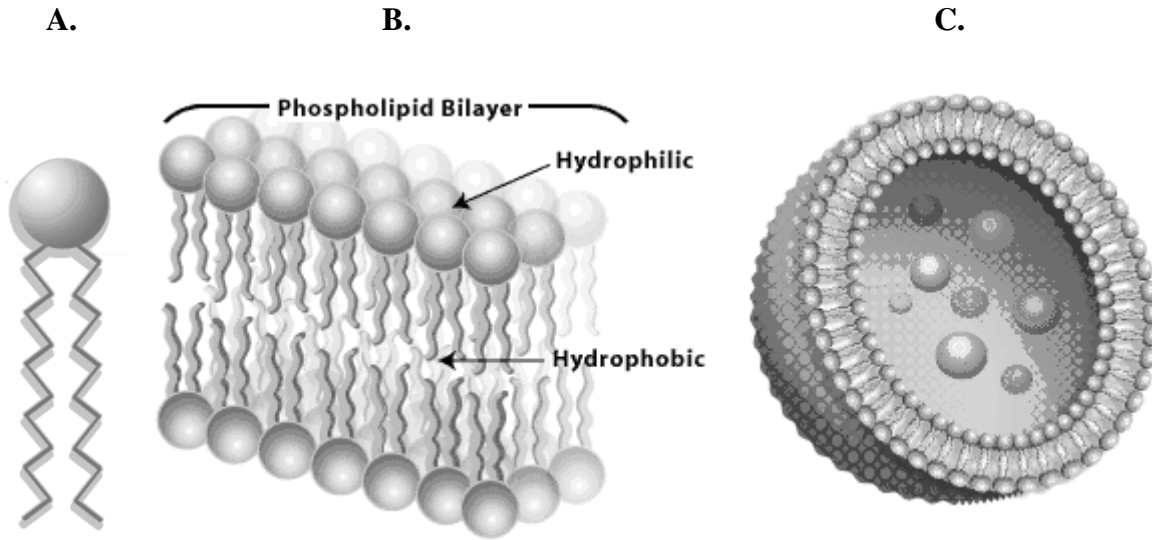
The lower portions (tails) of the phospholipid are formed primarily from electrically neutral elements which react with each other more than they do with water. These are described as nonpolar because they have a neutral charge so they are less likely to interact with charged molecules (as in the interaction between a magnet and a non-charged piece of metal such as a quarter). Because it is energetically favorable for nonpolar groups to associate with each other in a polar solution, phospholipids spontaneously arrange themselves so that their tails are aligned next to one another.

When both the head and tail ends of the phospholipids align with other polar and nonpolar ends, respectively, a sheet such as the one in Figure 3-B is formed. The symmetric arrangement of two layers (known as a belayed) is a further result of the interactions between heads and tails. The sandwich is formed because each portion of the molecule is surrounded by an environment where the tails are exposed only to tails, while the heads are exposed only to other heads and to the surrounding polar solution.

The final, bubble-like structure of the vesicle is a result of the work of these same forces. By closing the membrane into a sphere, any edges where the nonpolar tails would be exposed to the solution are eliminated (Figure 3-C). As with the previous steps, this arrangement occurs spontaneously in solution because of the inherent electrochemical properties of a phospholipid.

2.4.2 Define Liposome

A liposome is simply a lipid vesicle that encapsulates an aqueous solution (Williams, 2003). While they are the most common component of biological membranes, phospholipids are not necessarily the only types of molecules that can participate in vesicle formation. Other molecules, such as cholesterol, have similar polar and nonpolar components that allow them to participate in vesicle formation (Vemuri & Rhodes, 1995).

2.4.3 Define Surfactant

When liposomes are formed in the laboratory setting, the experimenter can choose what components are to be included in the membrane. By incorporating different types of molecules in addition to phospholipids, scientists can drastically alter the properties of the vesicles that are formed. Additional agents can be incorporated in order to add charge to a membrane, to adjust its flexibility or stability, or to make it respond to specific environmental stimuli.

One class of molecules that are commonly added to liposomes are known as "surface active reagents," or "surfactants" for short. Inclusion of surfactants in a liposomal membrane disrupts the native structure of the phospholipids, thus increasing the flexibility of the membrane (hence, these liposomes are called 'elastic'). To picture this, imagine that all of the phospholipid heads are cubes. Alone, they fit rather tightly together, side by side. When cylindrical surfactants are introduced to the membrane, the cubes will still roughly maintain their relationships;

however, there will be areas where a cube is bordered by a cylinder rather than by another cube. The resulting disorder is similar to that induced by surfactants in a phospholipid membrane.

A large number of surfactants have been investigated by scientists. Cevc et al. (1998) describe how the combination of multiple elements with different characteristics allows vesicles to adapt to stresses (such as squeezing through the tight pores in the skin). This "self-optimization" can occur because it is energetically favorable for all of the least stable molecules to associate at the stress point on the liposomal membrane. It has been argued that the localized increase in concentration of unstable molecules allows the vesicle to deform and enter the pore far enough to be drawn completely through (Cevc 2003).

Unfortunately, this increase in flexibility comes at a price. As the flexibility of a liposome increases, its ability to effectively hold its contents decreases. This results in a lower encapsulation efficiency (how much drug is actually held in the liposomes when they are created). Furthermore, the extreme deformations that occur while a liposome passes through a small pore result in some loss of carrying ability (Cevc et al., 2002).

An additional use of surfactants is to enhance the carrying abilities of liposomes for certain molecules. Three surfactants, sodium cholate, sodium deoxycholate, and Tween 80 were studied by Lee et al. (2005) in regards to their efficiency at encapsulating DNA (which carries a negative charge). Since Tween 80 is a neutral molecule, it was unable to complex with the DNA and was the worst performer of the three tested. However, this specificity can also work to the advantage of the experimenter.

Hayashi et al. (2003) attempted to use liposomes to deliver phosphatidic acid, a small fatty acid, to Chinese hamster ovary cells. If present in solution on its own, phosphatidic acid reacts with free calcium to form an insoluble complex, rendering it useless. By including the

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neutral Tween 80, the authors successfully prevented this undesirable reaction from occurring. In a similar experiment, Sudimack et al. (2002) used Tween 80 to stabilize their liposomes so that they would not be adversely affected by the proteins found in blood plasma/serum.

2.4.4 Conflicting Studies of Skin Penetration by Liposomes

One of the primary issues concerning the effectiveness of liposomes as a means of transdermal drug delivery is the lack of standardization that is employed throughout the field of research. A wide variety of surfactant combinations as well as the use of different model systems makes comparing different experiments difficult. Nonetheless, some consistent results have been obtained.

In their studies using both mice and humans as model systems, Cevc et al. (1998) observed similar trends in the effectiveness of liposomes at delivering insulin through the skin. This is significant because mice, unlike humans, are almost completely covered by hair follicles that could be serving as natural channels to allow liposomes to penetrate deep into the skin. The findings of Cevc and his colleagues indicate that the mouse can be used as an accurate model system despite this physiological disparity.

A study by Honeywell-Nguyen et al. in 2002 showed that rigid conventional (phospholipid only) liposomes did not penetrate the skin. In fact, intact vesicles were not even found at the skin's surface. This indicated that all of the vesicles either fused together or were ruptured by the forces acting at the skin's surface. While some level of fusion is expected, too much vesicular conglomeration will increase the size of the liposomes to the point that they will no longer be able to penetrate the skin. This result is corroborated by Zellmer et al. (1999), who found that conventional liposomes did not penetrate the stratum corneum. Furthermore, he found

that individual molecules could induce small barrier changes in the skin. However, these changes were deemed insignificant by the researchers.

This opinion is not universal, as some believe these barrier changes may actually have significant consequences. According to Bouwstra et al. (2002), proteins do not play a large role in deciding the organization of the stratum corneum. The authors found that model systems consisting solely of various fats (lipids) were remarkably accurate when compared with real stratum corneum. As such, anything that disturbs lipid organization can be considered significant in terms of skin permeability. In a study described previously, Honeywell-Nguyen et al. found that micelles (spheres similar to liposomes but with only one layer of phospholipids) disrupted the lipid portion of the stratum corneum. While the authors describe this in a positive light (micelles as "permeation enhancers"), the disruption of the stratum corneum could allow undesirable foreign molecules such as viruses or bacteria to enter the skin along with vesicles.

2.4.5 Evidence Supporting Skin Penetration by Liposomes

Opposing the studies denouncing skin penetration by liposomes is an abundance of evidence indicating that liposomes (particularly elastic liposomes) can serve as effective transdermal drug carriers. According to Cevc, et al. (1998), once the liposome reaches a certain distance within a pore, it is effectively sucked through by a transdermal osmotic force. To support this theory, the authors used Transfersomes®, a specific type of elastic liposome, to deliver insulin to mice. They found that within two to four hours of such attempts, the glucose levels of the mice decreased by 20-30%.

The authors were able to confirm that this decrease in blood sugar was a direct result of the transdermal insulin because the concentration of C-peptide, a protein produced in conjunction with natural insulin, did not increase as sugar levels increased (indicating an increase

in insulin). Further support for this claim comes from the *in vitro* portion of the same experiment. It was found that ninety percent of Transfersome-associated insulin remained after passing the liposomes through an artificial membrane barrier. As a result of their findings, the authors propose that their compound could be applied to forty square centimeters of skin (roughly the size of a palm) over the course of a day for treatment of Type I diabetes (Cevc 1998).

These findings are supported by Honeywell-Nguyen et al., who found that elastic vesicles can penetrate deep into human skin within one hour (2002). Freeze frame electron microscopy was used to visualize intact vesicles in channel-like regions that ran well below the surface of the skin. These channel-like regions were also present in control subjects, indicating that they are a natural phenomenon not created by the application of liposomes.

Further support for the beneficial contribution of liposomal flexibility to skin penetrance and ultimately drug delivery is presented by Kujik-Meuwissen et al. (1998). The authors found that fluorescently labeled vesicles in their liquid state (which are more flexible) penetrated deeper into rat skin than their gel state (more rigid) counterparts. This study also examined the penetrance of micelles into the skin and surprisingly found that they did not penetrate as well as the more flexible vesicles.

2.4.6 Liposome Stability and Penetrance

Various surfactant formulations and concentrations can alter the state of liposomes in solution. One of the primary ways in which these changes take place is the formation of micelles that can occur following the addition of surfactant to a liposome formulation. The results of a cryo-transmission electron microscopy study, which allows an extremely close view of a sample without disrupting its native structure, indicated that as the concentration of surfactant was

increased the resulting vesicles decreased in size, tended to open to solution more often, and collapsed more frequently into cylindrical and spherical micelles (Vinson et al. 1989).

Walter et al. (1991) provide further evidence of the transition that occurs between liposome and micelle when surfactants are present. They found that as the amount of surfactant was increased liposomes would be destabilized from hollow spheres into flat sheets, causing cylindrical micelles to break off from the edge of the sheets. This disintegration of the elastic liposomes results in smaller vesicles, which have been reported to have better penetrative properties than their larger counterparts. Verma et al. (2003) reported that liposomes with a radius of 120 nanometers were much more effective at traversing human abdominal skin than larger vesicles.

The fact that the sizes of the liposomes in a solution have a significant effect on penetration ability means that the size of a liposome over time is an important factor to consider in the study of liposome-based drug delivery methods. One group, led by Gritand (1992) used an excessively warm environment (60°C) to simulate the aging process, which results in partial hydrolysis of the lipids and ultimately membrane degeneration. They found that while the liposome size did not change appreciably over time, the stability (gauged by the capacity of the liposomes to keep a substance in solution outside of the membranes) decreased significantly after 15% of the membrane had been hydrolyzed.

3. Methods

3.1 Liposome Size and Stability Studies

3.1.1 General Methods

The objective of this experiment was to assess the stability and rigidity of liposomes over time. The first step in this process involved the construction of both rigid and flexible liposomes with different compositions of egg phosphatidylcholine (egg PC) and Tween 80 surfactant. We used a stainless steel extruder to filter the liposomes through polycarbonate membranes with defined pore sizes in order to produce a homogenous population of vesicles. Next we measured the mean radius of our liposome samples using dynamic light scattering. DLS relies on the scattering of light when a laser beam passes through particles in a colloidal suspension. Typically, such particles undergo random thermal motion, known as Brownian motion, which results in fluctuations of light intensity detected by the DLS machine. A computer program was used to calculate these size distributions based on the intensity fluctuations of the particles in a sample.

During the first trial, the temperature of the sample chamber within the DLS machine was not taken into consideration. This resulted in extreme fluctuations in size over the two-week trial. After trouble-shooting our experimental protocol, we concluded that temperature of the DLS needed to be strictly regulated and kept constant during size measurements to ensure uniform conditions throughout the experiment. Ultimately, we decided to make measurements at room temperature, or 25°C, under the same conditions which the liposomes were stored.

Both a two-week trial and a six week trial were conducted for the size measurements of the different molar ratios. The same revised protocol accounting for constant temperature was used for both trials.

3.1.2 Experiment Protocol

On the first day of liposome preparation, 7.4 grams of 20mg/ml egg phosphatidylcholine (egg PC) in chloroform solution was measured, placed into a large, glass vial, and dried with nitrogen at 6-7 psi until there was no longer any visible liquid. This process took approximately twenty minutes. The sample was then placed into the vacuum desiccator.

On the second day, the egg PC was removed from the vacuum desiccator. Tween 80 surfactant, and ten grams of de-ionized water were added to the glass vial, and the solution was stirred for thirty minutes. The solution was then frozen and thawed six consecutive times to ensure uniformity in the liposomes. Each time, the vial was dipped into liquid nitrogen for approximately 15 seconds, and then immediately thawed in a beaker of warm water. After each thaw, the warm water was replaced.

The sample was then forced through two 100 nm polycarbonate membranes in a stainless steel extruder ten to twelve times. After passing through these membranes, the sample was immediately transferred back into the vial, while simultaneously ensuring that no bubbles were introduced into the sample, as this could have affected the DLS measurements. Afterwards the vial was wiped free of smudges and placed into the DLS machine.

Once inside the sample chamber of the DLS machine, the temperature control was set to twenty-five degrees Celsius. The duration between size measurements was then recorded, as well as intensity, coherence, mean radius with standard deviations, and the polydispersity index. This was repeated three times and the values from these three measurements were averaged.

After collecting the size distribution measurements, the sample was separated into two 5 milliliters aliquots. The first aliquot was weighed and prepared for a flexibility test, while the second aliquot was stored for long-term size measurements. Aliquot one was then extruded through three 50 nm polycarbonate membranes, and the time taken for extrusion and mass of the extruded sample were recorded. The size distribution of this sample was then measured again. These values were used to calculate extrusion rate and flexibility index of our samples. The mean radius of liposomes in aliquot two was measured regularly for the duration of the experiment.

Upon completion of two weeks of measurements, the second aliquot was extruded through three 50 nm polycarbonate membranes. The duration of the extrusion, mean radius of vesicles before and after extrusion, as well as the weight of the sample before and after extrusion were recorded to calculate the flexibility index of the sample after the two-week time period.

The same experiment was conducted across a span of six weeks. Measurements were taken of the samples every other day over the course of the first two weeks, and then once a week after that. For the two-week trial we prepared vesicles with 0% surfactant, 25% surfactant, and 50% surfactant by molar ratio. In the six-week trial, we tested an additional liposome sample with 75% molar ratio surfactant.

The molar ratios used are summarized in the following table:

Table 1: Liposome Compositions

Liposome Compositions		
Molar Ratio of Tween 80 to Egg PC	**Tween 80 (grams)**	**Egg PC (grams)**
1 to 3	0.0570	7.40
1 to 1	0.1729	7.40
3 to 1	0.5188	7.40
1 to 0	0.1729	0.00

3.1.3 Data Analysis

For both the two and six week experiments, fluctuations in the mean radius of each sample were graphed with Microsoft Excel. The extrusion rate and flexibility of our various samples was also calculated with the following equation adapted from Hiruta et al. (2006):

$$\textbf{Elasticity} = \textbf{\textit{J}}_{\textbf{Flux}} \textbf{ x } (\textbf{\textit{r}}_{\textbf{v}}/\textbf{\textit{r}}_{\textbf{p}})^{2}$$

J_{Flux} represents the rate of penetration through a permeability barrier in units of grams per second. This value was calculated as the mass of sample per unit time that was extruded through 50 nm membranes.

r_v is the mean radius of the vesicles after extrusion. This value is equivalent to the mean radius of the sample measured by the DLS after the final extrusion.

r_p is the radius of the pores in the barrier, which in our case was 50 nm.

3.2 Gel Permeation Studies

3.2.1 Preparation of Slides

A custom slide setup was designed and constructed to allow for the preparation of gels directly on microscope slides. A microscope slide was cut ½ inch from the ends, generating two ½ inch pieces. These pieces served as spacers that were ultimately sandwiched and fixed with epoxy between two new slides. Finally, the gap at the bottom of the two slides was sealed with epoxy and the slides were allowed to dry overnight. This construction created an opening at the top of the slides 1 - 1.2 mm thick that could be filled with gel.

Agarose (Fisher Scientific Co.) was used to make gels that were 1.3% agarose by weight. A beaker was filled with about 50 ml of water and the water weight was multiplied by 0.013 to

obtain the mass of agarose that should be added to create a gel that was 1.3 wt% agarose. The

actual numbers used can be seen in Table 2.

Table 2: Actual values of gel compositions

Actual values of gel compositions		
Water (g)	**Agarose (g)**	**True Gel Percentage**
45.5960	0.5930	1.3006
55.2534	0.7189	1.3011
44.8858	0.5834	1.3000
33.9194	0.4429	1.3057
43.0450	0.5598	1.3005
49.2375	0.6405	1.3008
37.4190	0.4899	1.3092

Mean gel composition was 1.30 wt% with a standard deviation of 0.003.

The water and agarose were microwaved for 50 seconds and a micropipette was used to fill the

slide construction with the liquefied gel. A 12 mm spacer was then inserted into the middle of

the cooling liquid and the whole preparation was placed in 4°C for 4 minutes. The spacer was

then removed, leaving a well in solidified gel. The preparation was wrapped in parafilm and used

within 10 minutes in the permeation studies. An example of the slide construction can be seen in

Figure 4.

Figure 4: Example of slide construction

The red area in this image is the well after it has been filled with DII-Liposome solution. The lighter gray rectangle in the middle of the slide construction is the area of agarose gel.

These slide constructions were important because they allowed for the preparation of gels directly on microscope slides, which in turn allowed for convenient transfer of the gel and vesicles to the microscope for image capture.

3.2.2 Permeation Studies

In all permeations studies 70 µl of liposome-DII solution were added to the well in the gel. The slide preparation was then placed under the fluorescent light and images were captured at 30, 60, 90, 120, 180, 240, 300, 360, 420, 480, 540, and 600 seconds. All images were taken at room temperature.

3.2.3 Image Analysis

Images were analyzed with ImageJ, free imaging software provided by the National Institutes of Health. With the software's line drawing tool, a single line was drawn from the top of the image, corresponding to the top of the well on the microscope slide, to the bottom of the image. A sample image was taken 30 seconds after loading the fluorescent liposomes containing

50% surfactant onto the gel, and can be seen in Figure 5. The red arrow represents the direction of liposome migration.

Figure 5: Example of images visualized with Image J
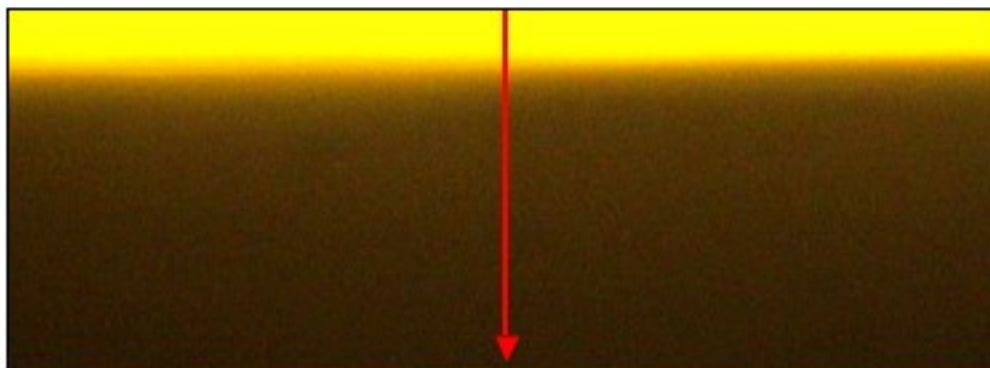
The program uses the red line to construct a profile plot of the fluorescence intensity (gray scale value) as a function of distance from the top of the image to the bottom of the image. A sample graph can be seen in Figure 6, with the red arrow representing the direction of liposome migration.

Figure 6: Example of profile plot of fluorescent intensity as a function of distance
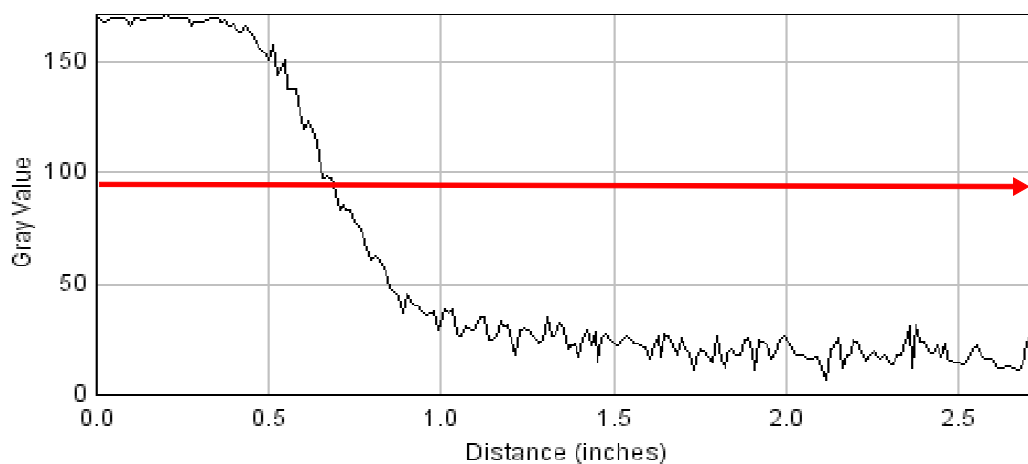
The x and y coordinates (position in inches and gray value, respectively) for the graph were extracted and pasted into a notepad file. These values were then used for extensive data analysis.

3.2.4 Determination of Diffusion Coefficients and Graphs of Diffusion Coefficients

Data obtained from the ImageJ photographs were fitted to the solution of Fick's Law of Diffusion (see below) with a least-squares regression done using the mathematical software package called MathCAD.

$$\frac{\partial \phi}{\partial t} = D \frac{\partial^2 \phi}{\partial x^2}$$

Fick's Law of Diffusion

$$n\left(x, t\right) = n(0)\operatorname{erfc}\left(\frac{x}{\sqrt{4Dt}}\right)$$

One Dimensional Solution to Fick's Law of Diffusion

A least squares regression fits a curve to a set of data by minimizing the square of the difference between the value of the dependent variable and the value given by the curve fit. Data was inputted from a text file, and through a series of matrix manipulations and minimizing functions, we determined the diffusion coefficient for each set of data points.

All the diffusion coefficients were compiled and plotted over time in Microsoft Excel. Using their regression package, we fitted our data to the power law and recorded the appropriate coefficients. We also plotted our diffusion coefficients using the Excel graph functions.

3.2.5 Modeling Methodology

Using the steady-state value of the equilibrium constant, the final diffusion coefficient value, we created an iterative model using Excel describing drug depletion from a patch reservoir and delivery through a skin barrier into the subcutaneous tissue. The model works by recalculating the amount of insulin in the patch reservoir for each time interval and applying

those amounts into determining the delivery rate for that interval. The delivery rate then affects the amount of insulin in the reservoir for the next time interval and etc.

The model starts with the diffusion coefficient, a patch volume and surface area, the barrier thickness and an initial concentration of insulin in the patch. The diffusion coefficient is taken from the plot of diffusion coefficients over time for 50% surfactant liposomes. After the initial drop, the diffusion coefficients eventually level off to a near-constant value and that value was taken to be the coefficient for our model. The patch volume and surface area are arbitrarily chosen values based on typical and reasonable dimensions for drug patches. The barrier thickness was chosen to reflect the more tortuous pathways of the skin compared to agarose gel. The initial concentration of insulin was calculated from the assumptions of 10% vesicle loading efficiency and of each loaded vesicle only having one repeating unit of the standard insulin crystal. Vesicle concentrations were calculated from initial lipid concentrations, our vesicle size study, and the work of C. Huang et al. on geometric packing of egg phosphatidylcholine vesicles (1978).

Table 3: Parameters for Drug Delivery Patch Model

Model Parameters	
Initial Concentration (g/cm^3)	7.77E-04
Diffusion Coefficient (cm^2/min)	1.88E-04
Barrier Thickness (cm)	0.5
Patch Volume (cm^3)	1
Patch Contact Area (cm^2)	4
Patch Thickness (cm)	0.25

From the concentration, flux is calculated by multiplying the diffusion coefficient with the derivative of the concentration profile, which in this case is just the concentration divided by the thickness of the barrier. The mass of the drug remaining in the patch is also calculated from

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concentration by multiplying it with the volume of the patch. Flux is then multiplied by the area of the patch that is in contact with the skin, which gives the delivery rate. The new mass of drug in the patch for the next time iteration is then calculated by subtracting the delivery rate. From there, a new concentration can be calculated and then a new flux can be calculated, and etc. The results were plotted using Excel's graphing function.

3.2.6 Model Assumptions

1. The drug well in the patch is well mixed and uniform at within time intervals.

2. The steady-state between time intervals is reached in a short amount of time compared to the time interval

3. The drug, when it reaches the end of the barrier, is removed immediately by the blood stream and does not build up outside the barrier.

4. The model is based on data collected for agarose gel, which is the synthetic model for the skin. Agarose and skin are assumed to behave similarly for this model

5. The model also assumes that a patch of the given dimensions can be made

3.2.7 Implications of the Assumptions

Our team made simplifying assumptions in order to complete a ball-park assessment of the transdermal drug delivery technology using the vesicles we had chosen. If further study is warranted, a more detailed and refined solution can be performed at the cost of more time and money. More complex software and education would also be required.

Assumption 1: The theoretical patch is well mixed.

Assuming this allows for a simpler solution to Fick's law of diffusion. It also allows us to simply subtract the amount delivered from the drug well. Making this assumption will artificially increase the amount of drug delivered in our model compared to the amount that can be delivered in a real patch. In a real patch, the drug concentration within the drug well will also be at a gradient and will be less than a well-mixed (average) value. Having a lower drug concentration at the point of contact with the skin will decrease the rate of diffusion through the skin.

Assumption 2: A steady-state solution would be reached relatively quickly.

Assumption 2 is made so that we could use an iterative procedure to estimate the solution of a time-dependent transient problem. Since the concentration of the well is only changing by a very small fraction of the total concentration for each time interval, we assumed that a steady-state solution would be reached relatively quickly and that it would be an acceptable estimation to the more difficult transient solution. Our assumption would artificially decrease the amount of drug delivered compared to a real patch.

Assumption 3: Drugs delivered using the patch will immediately diffuse into the bloodstream.

Once the drug defeats the skin barrier, it is assumed that it is immediately carried away from the end of the barrier by blood vessels that circulate blood through the body. Any accumulation at the end of the barrier would decrease the amount of drug that is delivered over time. Our assumption is true for insulin that reaches blood vessels but may not be true for insulin accumulating in the fat before reaching the blood vessel. Overall, this assumption artificially increases the amount of drug delivered by our model.

Assumption 4: Agarose gel is a good model for the skin.

Since agarose and the skin have very different compositions and architectures we cannot truly judge the exact effects of using agarose as our skin model. We can safely assume that agarose will be easier to penetrate with vesicles than the skin. As such, using agarose gel as our skin model will artificially increase the amount of drug delivered by our model.

Assumption 5: The dimensions of the patch we used for our model are reasonable and feasible to produce.

4. Results

4.1 Liposome Size and Stability Studies

4.1.1 Vesicle Stability

We carried out two sets of experiments to test vesicle stability. In the first experiment, we measured the mean radius and scattering intensity of 0% surfactant liposomes, 25% surfactant liposomes and 50% surfactant liposomes over a two-week period. In each of these measurements, we averaged three mean radius values and three intensity values. We then plotted the average mean radius (*r*) and average scattering intensity (*I*) versus days elapsed after vesicle preparation. These graphs are presented below.

Figure 7: Changes in Mean Radius and Scattering Intensity over Two Weeks

Average Radius of Vesicles over Two Weeks
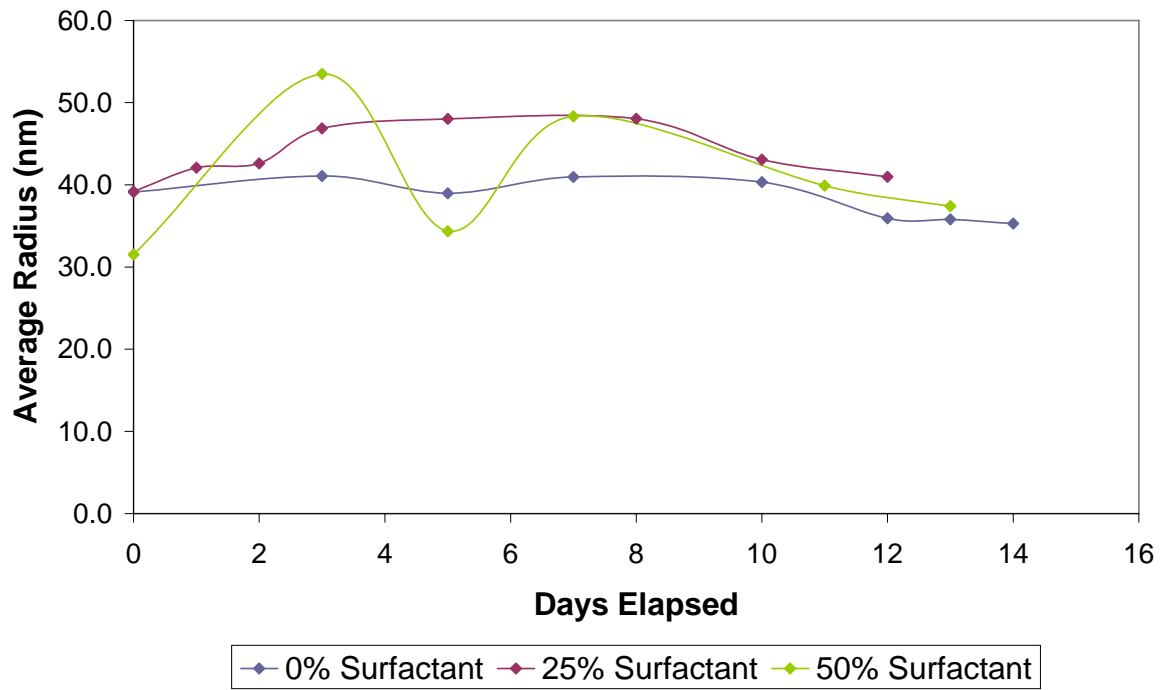
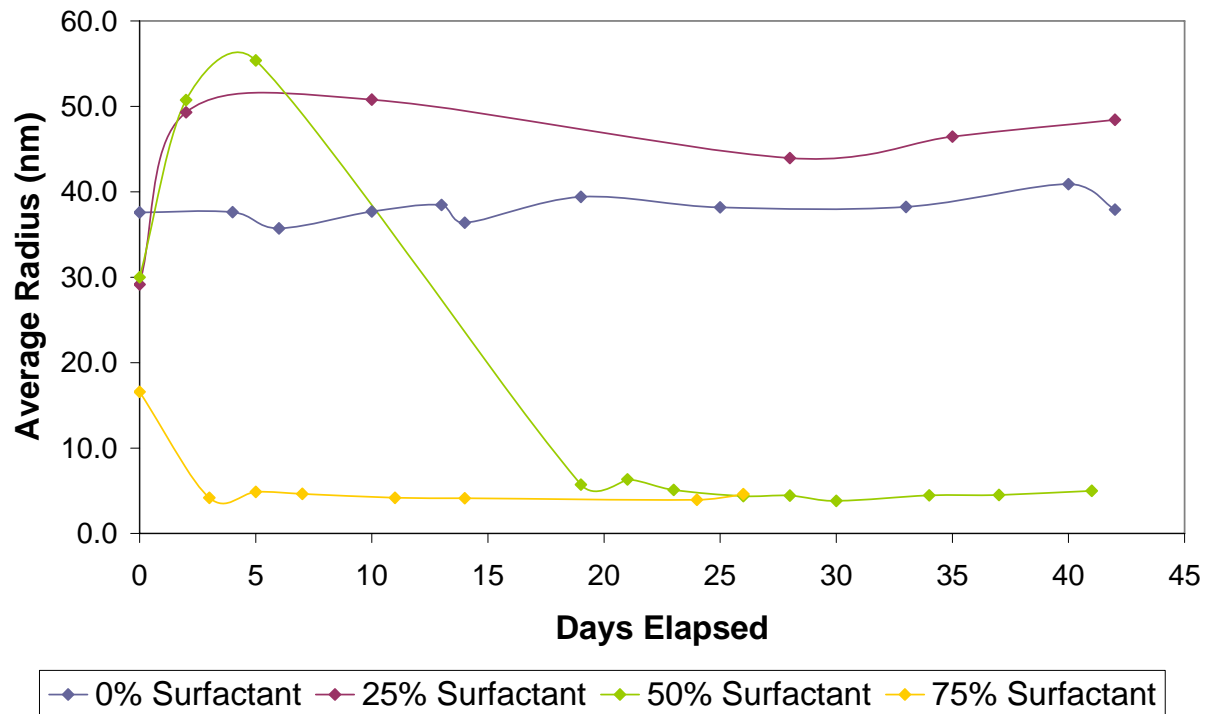# Changes in Scattering Intensity over Two Weeks
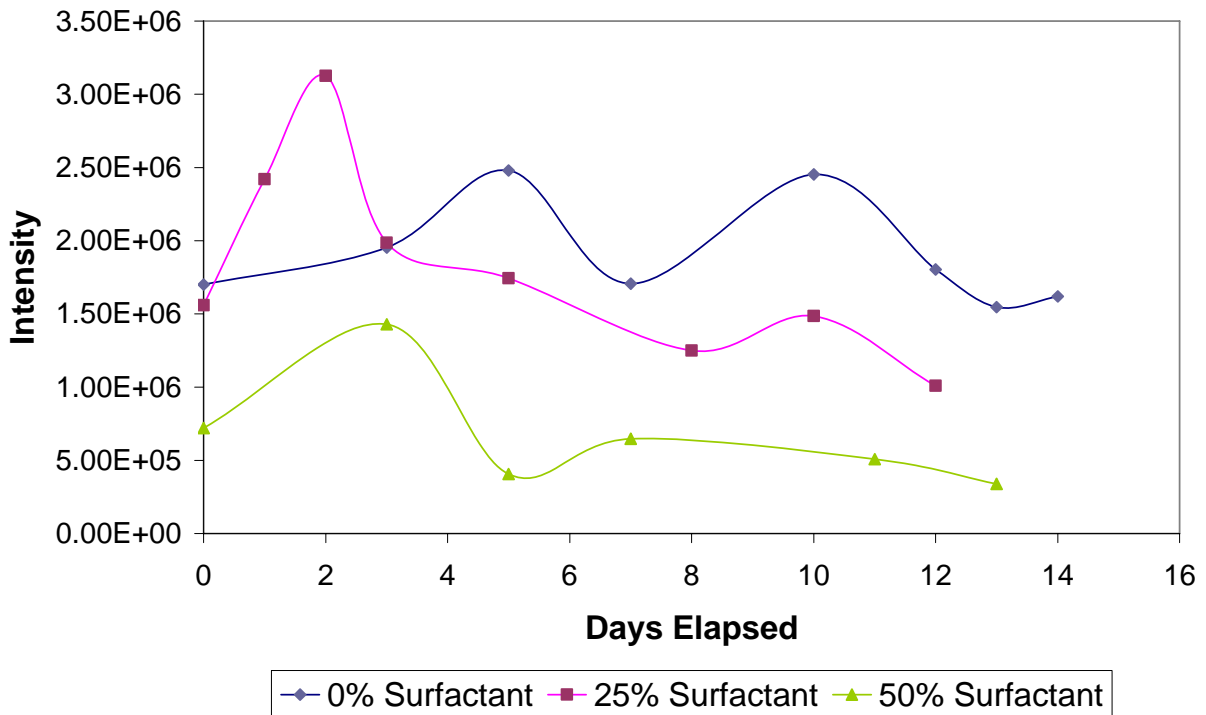
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The data for both average mean radius and average scattering intensity display a clear trend that higher surfactant concentration leads to greater fluctuations in size. The radius of the 0% surfactant liposomes started at an average of 39.3nm and only fluctuated between 41.1nm and 35.3nm, a range of just 5.8nm. Also, the radius of the 0% surfactant liposomes did not drop below 39nm until day 12 of the 14 day trial. The scattering intensity of the 0% surfactant liposomes started at $1.70*10^6$ and fluctuated between $1.55*10^6$ and $2.48*10^6$, a range of $9.3*10^7$. The radius of the 25% surfactant liposomes started at an average of 39.2nm and fluctuated between 39.2nm and 48.0nm, a range of 8.8nm. The scattering intensity of the 25% surfactant liposomes started at $1.56*10^6$ and fluctuated between $1.01*10^6$ and $3.13*10^6$, a range of $2.12*10^6$. The radius of the 50% surfactant liposomes started at an average of 31.5nm and fluctuated from 31.5nm to 53.5nm, a much larger range of 22.0nm. The scattering intensity of the 50% surfactant liposomes started at $7.2*10^7$ and fluctuated between $5.1*10^7$ and $1.43*10^6$, a range of $9.2*10^7$.

In order to achieve a better understanding of the trends beyond two weeks, we then conducted a six-week trial of the same experiment. In addition to the formulation in the previous trial, we added a 75% surfactant liposome sample to the six-week study. Again, we averaged three mean radius values and three intensity values for each measurement. The graphs of these data are presented below.

Figure 8: Change in Mean Radius and Scattering Intensity over Six Weeks

Average Radius of Vesicles over Six Weeks

Changes in Scattering Intensity over Six Weeks
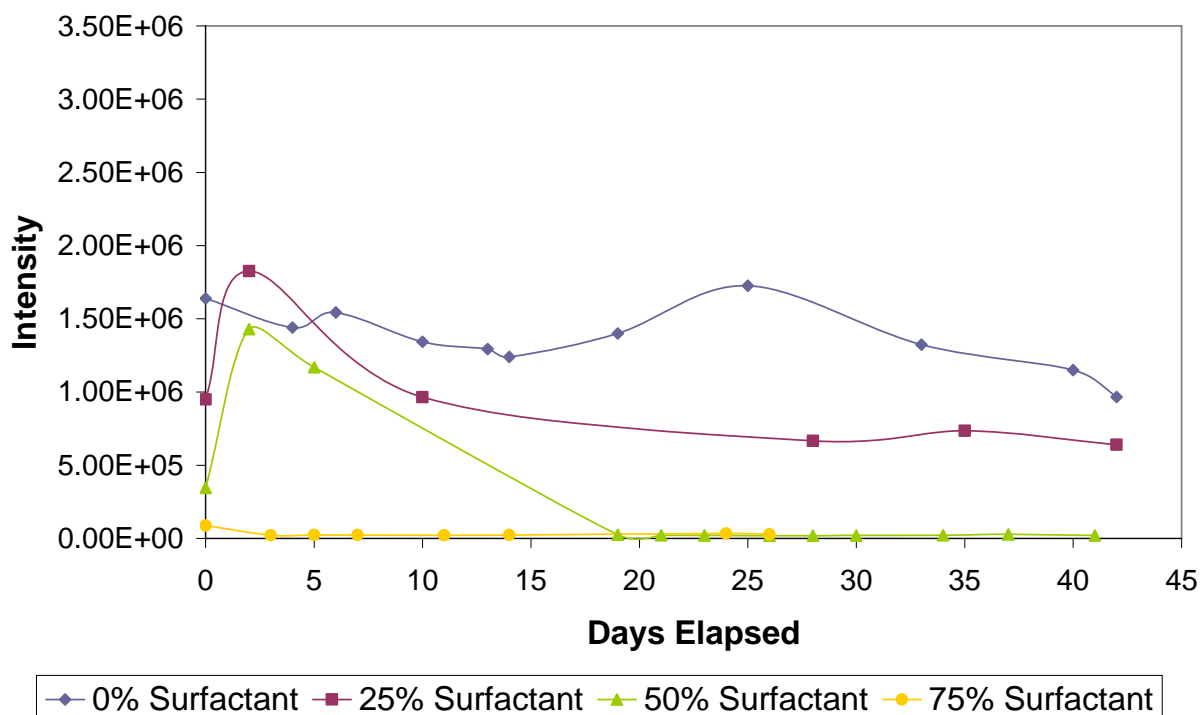
Once again, the data for both average mean radius and average scattering intensity display a clear trend that higher surfactant concentration leads to greater fluctuations in size. The radius of the 0% surfactant liposomes started at an average of 37.6nm and only fluctuated between 35.7nm and 40.9nm, a range of just 5.2nm over the six-week period. The scattering intensity of the 0% surfactant liposomes started at $1.64*10^6$ and fluctuated between $9.7*10^7$ and $1.73*10^6$, a range of $7.6*10^7$. The radius of the 25% surfactant liposomes started at an average of 29.2nm and fluctuated between 29.2nm and 50.8nm, a range of 21.6nm. However, there was a large increase in size from day-zero to day-two after which the 25% surfactant liposomes only fluctuated between 43.9nm and 50.8nm, a range of 6.9nm. The scattering intensity of the 25% surfactant liposomes started at $9.5*10^7$ and fluctuated between $6.4*10^7$ and $1.83*10^6$, a range of $1.19*10^6$. The radius of the 50% surfactant liposomes started at an average of 30.0nm and

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increased in five days to 55.4nm before dropping to 5.7 nm after 19 days and fluctuating between 3.8nm and 6.3nm for the remainder of the six-week period. The scattering intensity of the 50% surfactant liposomes started at $3.5*10^7$ and increased in two days to $1.43*10^6$ before dropping to $2.58*10^4$ after 19 days and fluctuating between $1.99*10^4$ and $2.8*10^4$ for the remainder of the trial. The radius of the 75% surfactant liposomes started at an average of 16.6 nm, dropped to 4.2nm after three days, and fluctuated between 3.9nm and 4.9nm for the remainder of the trial. The scattering intensity of the 75% surfactant liposomes started at just $8.88*10^4$ and fluctuated between $2.27*10^4$ and $3.43*10^4$ from the third day to the end of the trial.

4.1.2 Flexibility

The vesicles produced for the stability experiments were also tested for extrusion rate and flexibility both before and after a period of storage at room temperature. This period lasted 42, 41, and 26 days for the 25%, 50%, and 75% surfactant samples, respectively. The mass of each sample was taken before and after extrusion and the extrusion time was recorded. The extrusion rate was calculated simply as the mass after extrusion divided by the extrusion time. Flexibility index was then calculated for each sample from the extrusion rate and vesicle size measurements. The data and graphs of extrusion rate and flexibility for all of our liposome formulations are presented below.

Figure 9: Extrusion Rate and Flexibility

% Surfactant	Initial Extrusion Rate (g/sec)	Extrusion Rate Post-Storage (g/sec)	Initial Flexibility (g/sec)	Flexibility Post-Storage (g/sec)
0	n/a	n/a	n/a	n/a
25	0.04539	0.005816	0.08268	0.0146
50	0.07168	0.03702	0.09691	n/a
75	0.06369	0.06672	0.02653	n/a

Changes in Extrusion Rate after Storage
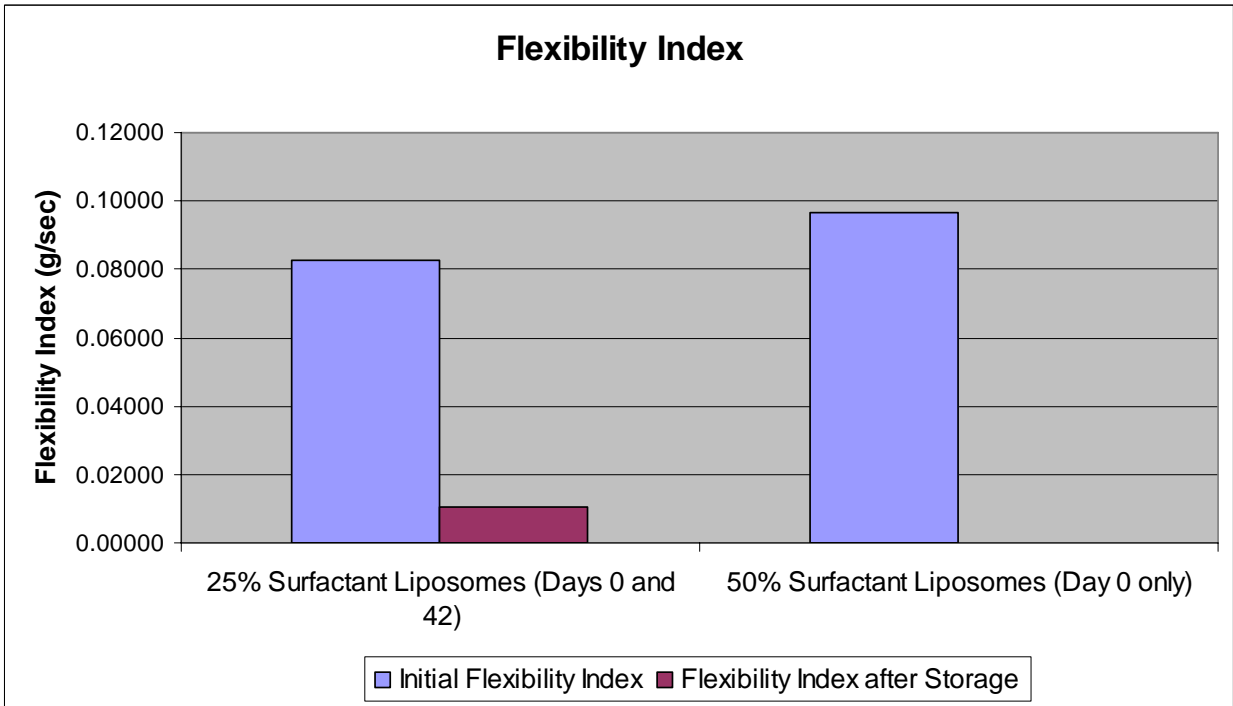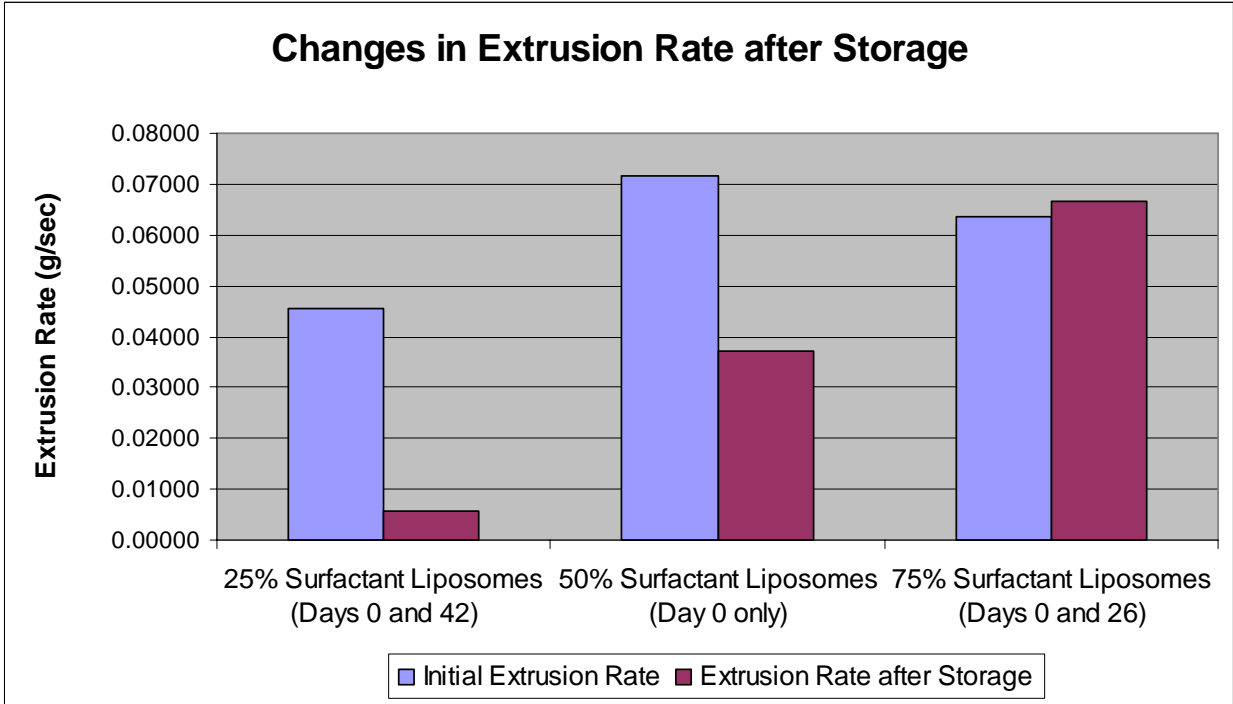
Flexibility Index

The 0% surfactant liposomes were not able to pass through the extruder, and therefore no extrusion rate or flexibility index was calculated for this sample. Our method of calculating

flexibility index assumes that the vesicles passing through the extruder are larger than pores through which they traverse. Thus the vesicles must deform in order to squeeze through the extrusion pores. The flexibility index then is the extent to which liposomes can undergo this deformation. We did not calculate the post-storage flexibilities of the 50% and 75% liposomes or the pre-extrusion flexibility of the 75% liposomes because the mean radius of these samples was smaller than the radius of our extrusion pores. Thus these vesicles would have passed through the extruder without any deformation, and consequently our flexibility indices for these samples would have been invalid.

Of the three samples, the 50% surfactant vesicles had the highest initial extrusion rate and flexibility at 0.07168 g/sec and 0.09691 g/sec respectively. After the storage period, the extrusion rates of the 25% and 50% samples dropped appreciably, with the larger decrease in the 25% sample. The extrusion rate of the 75% sample did not undergo any drastic increase or decrease. A complete set of pre and post storage flexibility values was obtained only for the 25% surfactant vesicles, for which flexibility decreased by a factor of 87% after 42 days.

4.2 Gel Permeation

We witnessed a clear diffusion of fluorescently tagged samples (0% surfactant liposomes, 50% surfactant liposomes, and 100% surfactant micelles) through agarose gel at 10x magnification. Sample images of this diffusion taken over the course of 600 seconds are shown below.

Figure 10: Sample Diffusion Images
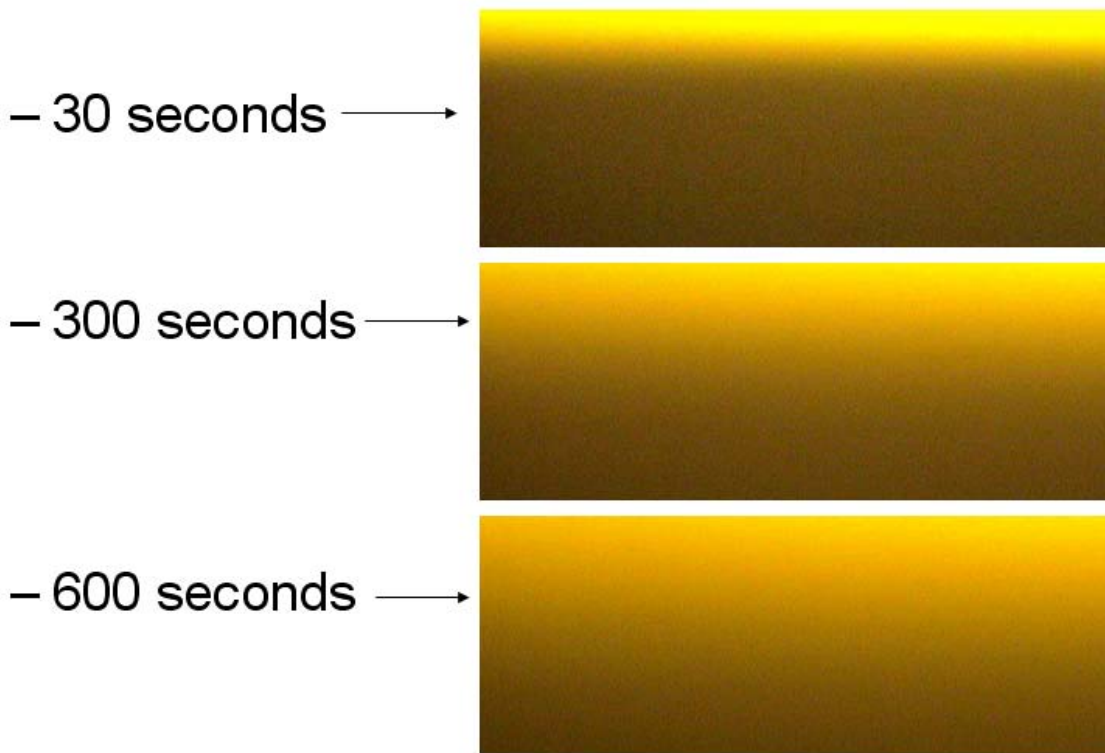
Captured images of diffusion of 100% surfactant micelles on day 0

The captured images were then quantitatively evaluated with ImageJ. We measured the intensity of fluorescence (represented by the "grayscale value" in our image analysis software) at various distances from the top of the image. Using these numbers, we were then able to calculate diffusivity values for each time point on each day that measurements were taken. In all samples we witnessed a constant decrease in diffusivity over the 600 second measurement period on all days. Shown below are the plots of diffusivity versus time for all three samples with data from every day of measurement.

Figure 11: Diffusivity Coefficients for Various Liposomes

Diffusivity Coefficient of 0% Surfactant Liposomes

Diffusivity Coefficient for 50% Surfactant Liposomes

Diffusivity Coefficient for Surfactant Micelles
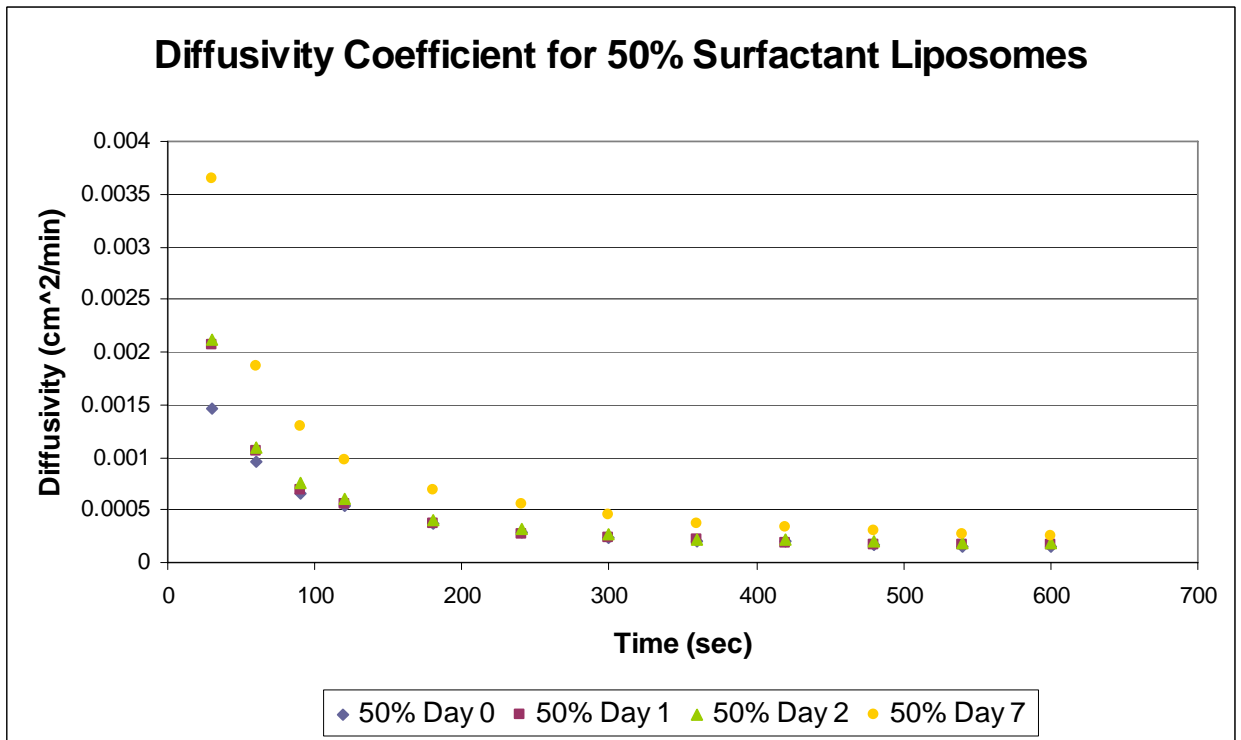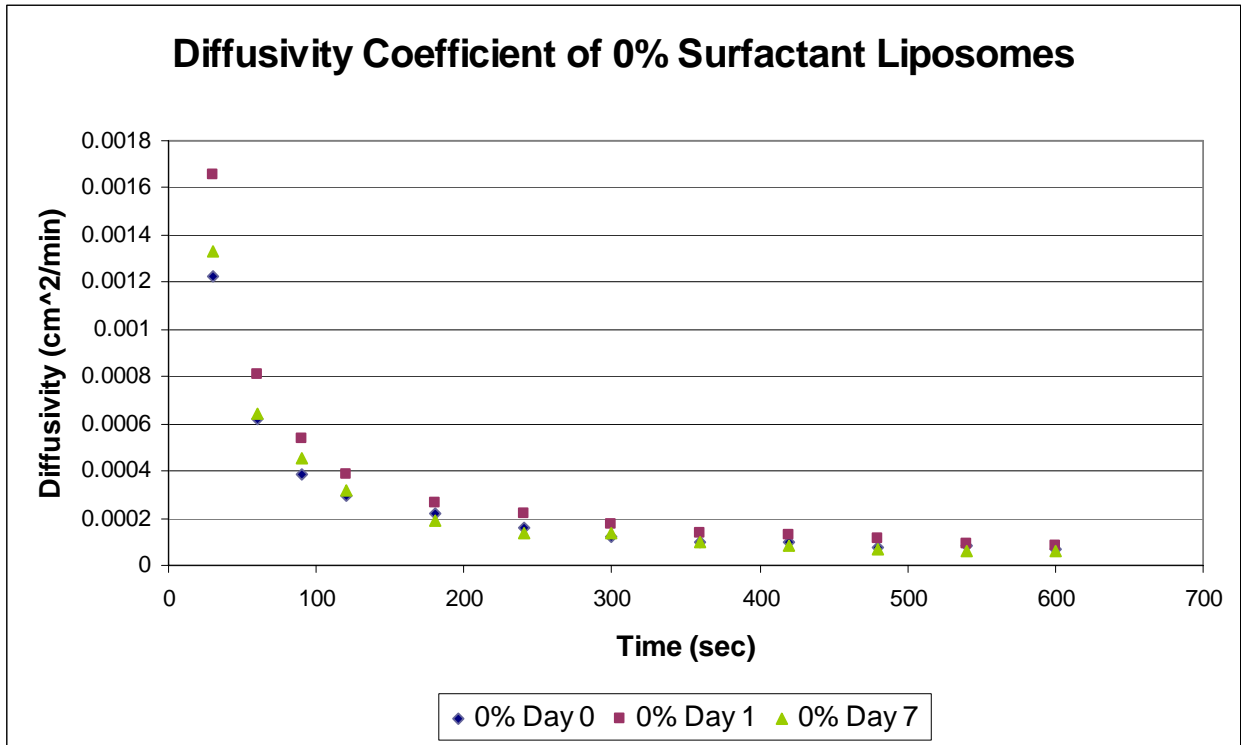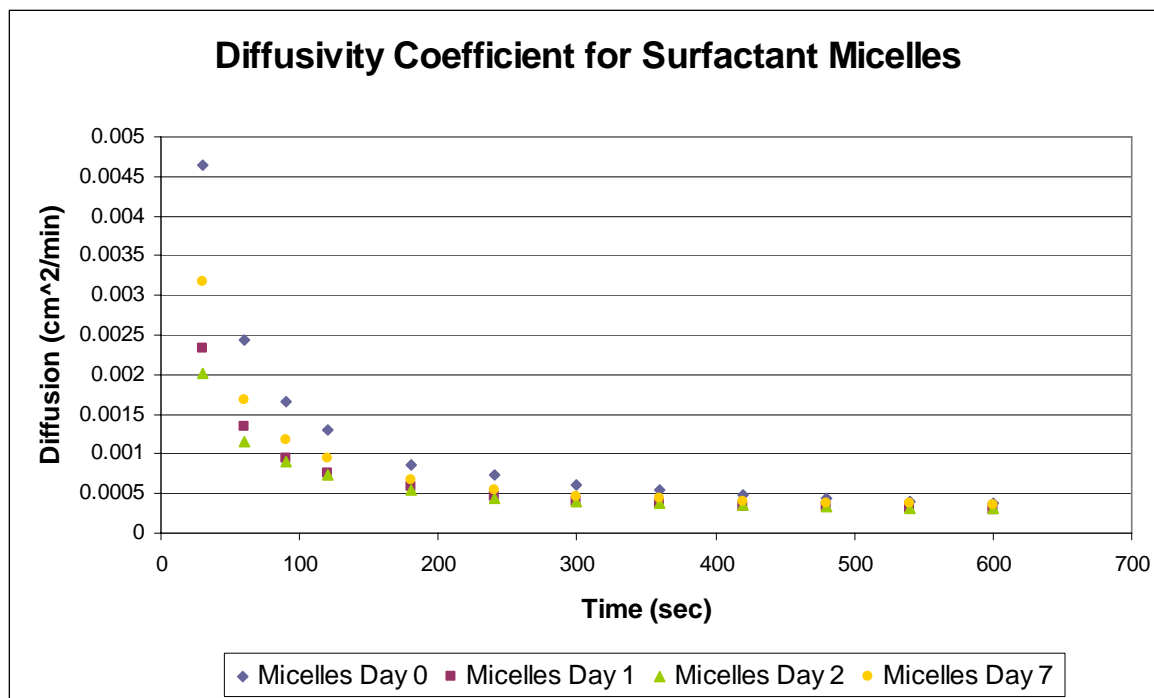
The 0% liposomes showed no clear trend in diffusivity over the one-week storage period. As can be seen above, diffusivity values increased between day 0 and day 1, but then decreased between day 1 and day 7 to a value close to the initial diffusivity. Similarly, we observed no unidirectional trend for the surfactant micelles. Diffusivity decreased drastically between day 0 and day 1, and showed a slight decrease between day 1 and day 2. However, by day 7 the diffusivity values were between those calculated for days 0 and 1. In contrast, the 50% surfactant sample showed a continuous increase in diffusivity between days 0 and 7.

Comparatively, the 0% surfactant sample consistently had the lowest diffusivity values for all days of measurement. Initially, diffusivity was higher in the 100% surfactant micelles than the 50% liposomes. Over the course of the storage period, however, the micelles decreased in diffusivity while the 50% liposomes increased in diffusivity. Consequently, the 50% surfactant sample and 100% surfactant micelles had similar diffusivities at day 7. These data are summarized in the following graphs of diffusivity versus time.

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Figure 12: Diffusivity Coefficient for Various Days Stored

Diffusiviity Coefficient of All Samples Day 0

Diffusiviity Coefficient of All Samples Day 1

Diffusivity Coefficient of All Samples Day 7
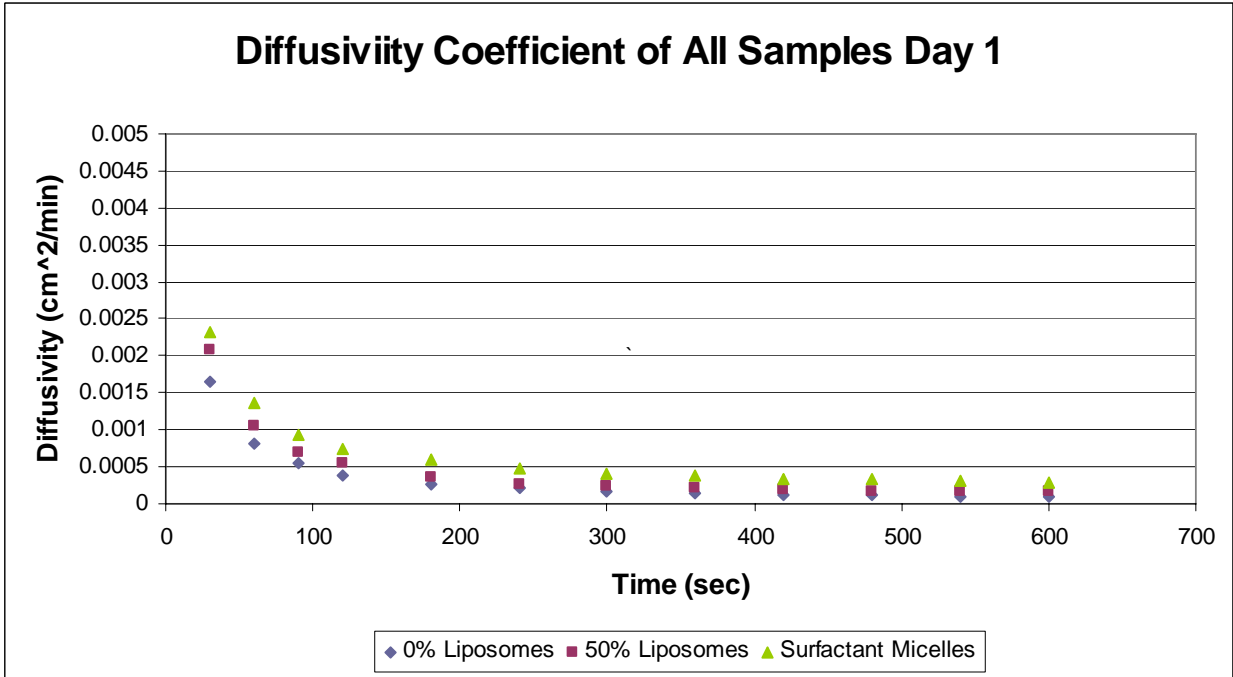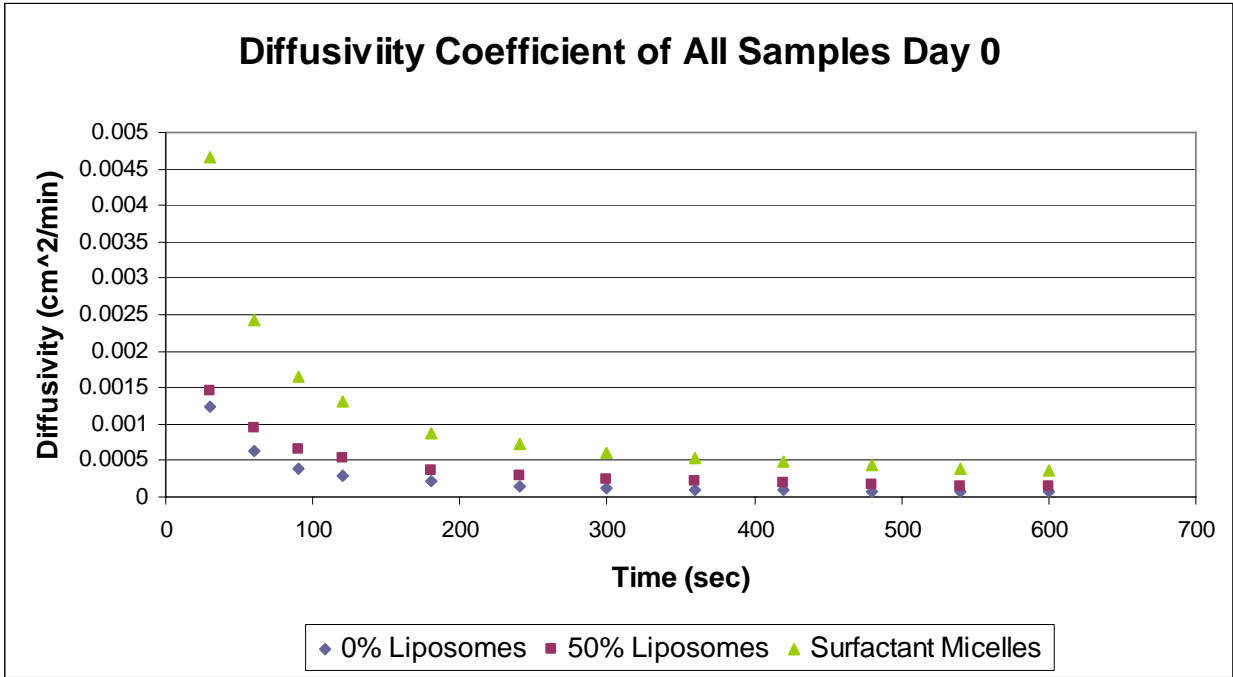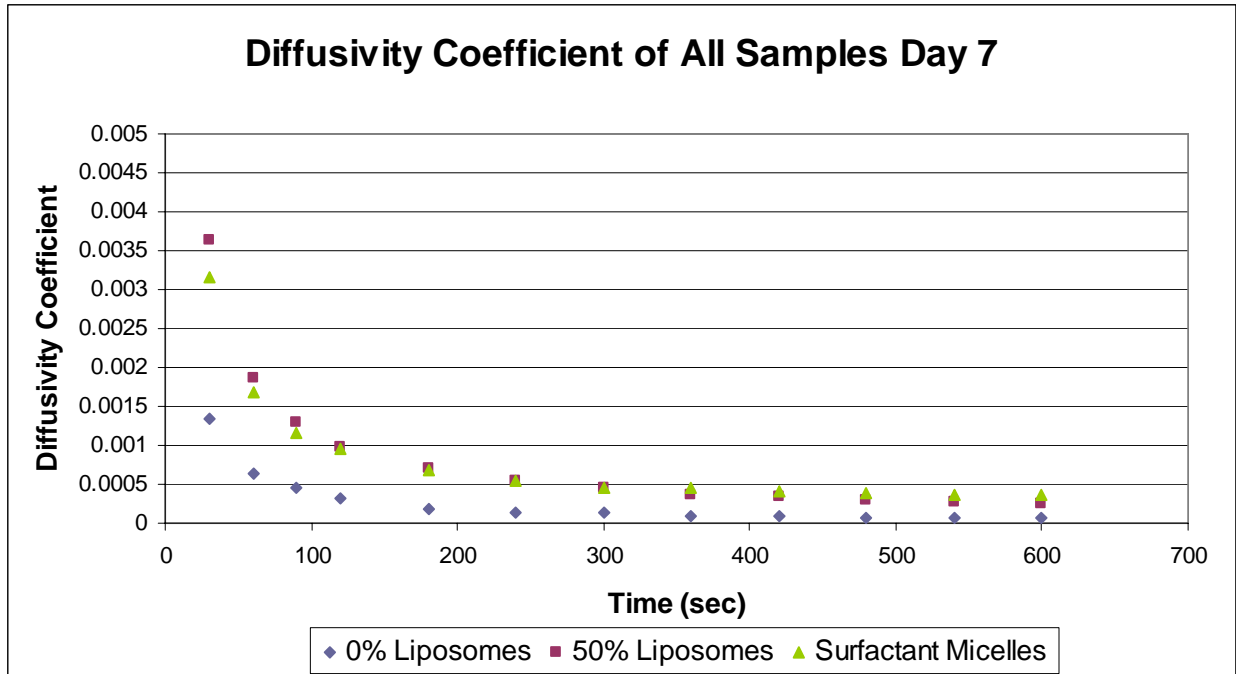
In all cases the diffusion coefficients eventually leveled off to a "final" value which we tabulated and performed some statistical calculations upon. As seen in the figures below, the different final diffusion coefficient values for each type of vesicle were distinct, with no overlap within one standard deviation of their averages.

Figure 13: Average Final Diffusion Coefficients and Standard Deviations

	Average	Standard Deviation
0%	1.775E-05	1.614E-05
50%	4.685E-05	5.431E-05
100%	8.317E-05	4.557E-05

Final Diffusion Coefficient Averages +/- 1 Std Dev
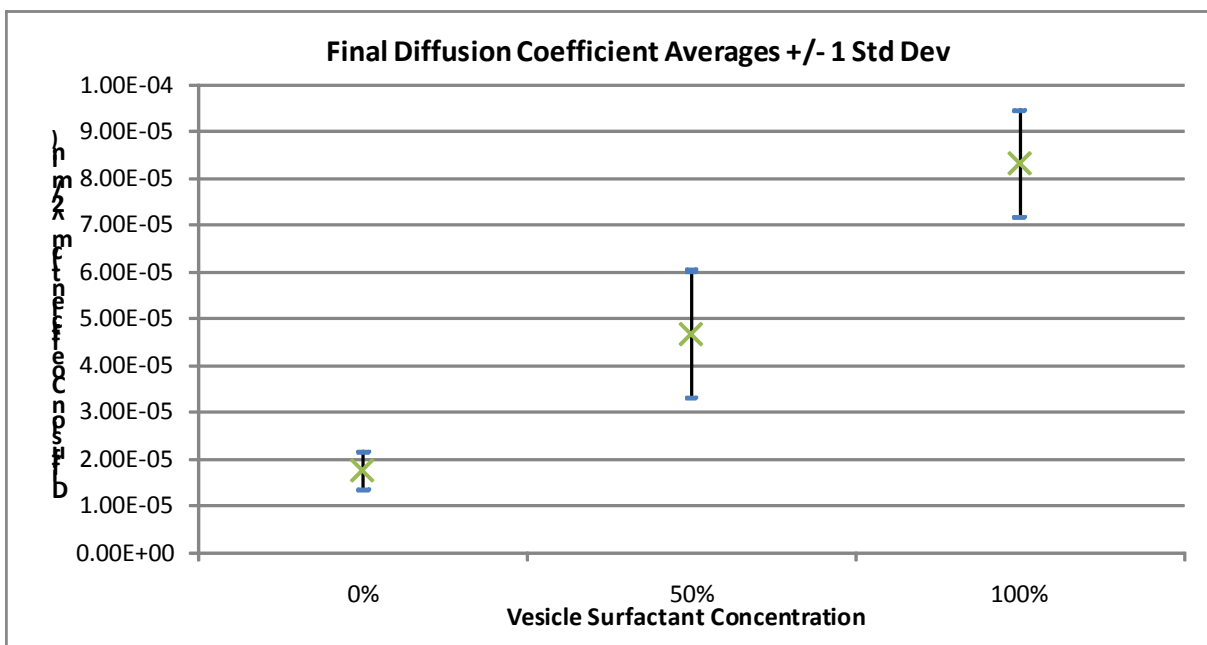
In addition, we also examined the correlation between the final diffusion coefficient and the number of days the vesicles were stored. Based on the data points we collected, we found a strong correlation between increasing diffusion coefficients with increased storage time for the 50% vesicles. There was a weaker negative correlation between storage time and days stored for 0% vesicles. The pure micelles had little correlation between days stored and the resulting diffusion coefficient. Correlations are contrained by our limited data size.

Figure 14: Final Diffusion Coefficients vs Days Stored

Days Stored	Final Diffusion Coefficient (cm^2/min) for Vesicle Surfactant Concentration		
	0%	**50%**	**100%**
0	6.963E-05	1.516E-04	3.710E-04
1	8.408E-05	1.625E-04	2.903E-04
2	n/a	1.774E-04	3.145E-04
7	5.933E-05	2.581E-04	3.548E-04
Correlation	-0.730	0.999	0.223

Final Diff. Coeff. Over Time Stored

Diffusion Constant Effected (m^2/h)

Days Stored

- 0% Surfactant Liposome
- 50% Surfactant Liposome
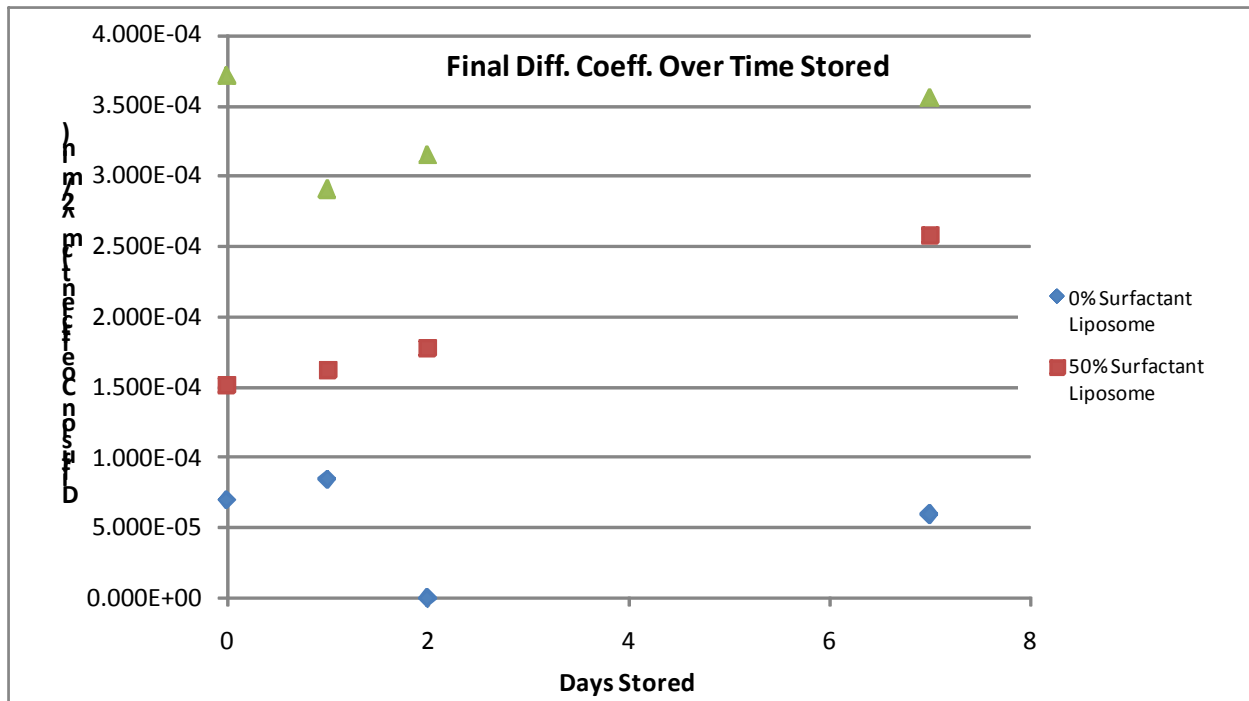
4.2.1 Insulin Patch Modeling

Using the parameters given in the methodology, a set of model delivery data was generated. As seen in the figures below, flux, delivery rate, and patch drug concentration decrease gradually over time as the amount of drug delivered reaches an asymptotic value. In general, increasing the surface area of contact of the patch and decreasing the barrier thickness tended to increase the curvature of the plots. Increasing the volume of the patch (while maintaining the same surface area) reduces the curvature of the graphs. Changing the initial concentration scales all of the plot values but does not change the shape of the graphs.

Figure 15: Patch Drug Delivery Model Results
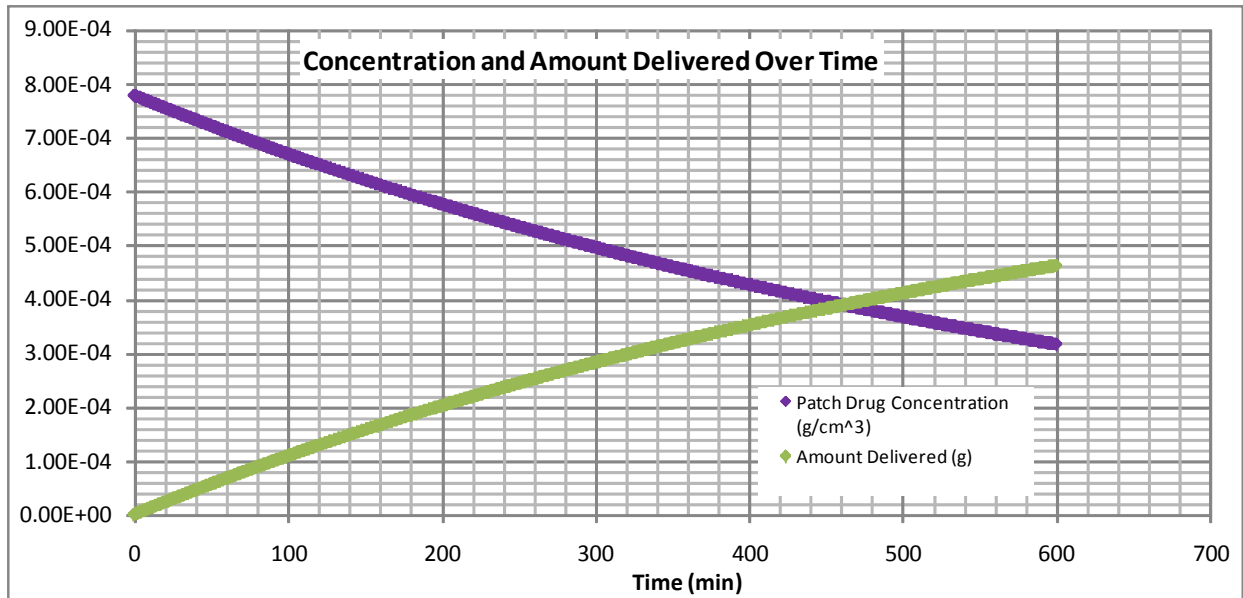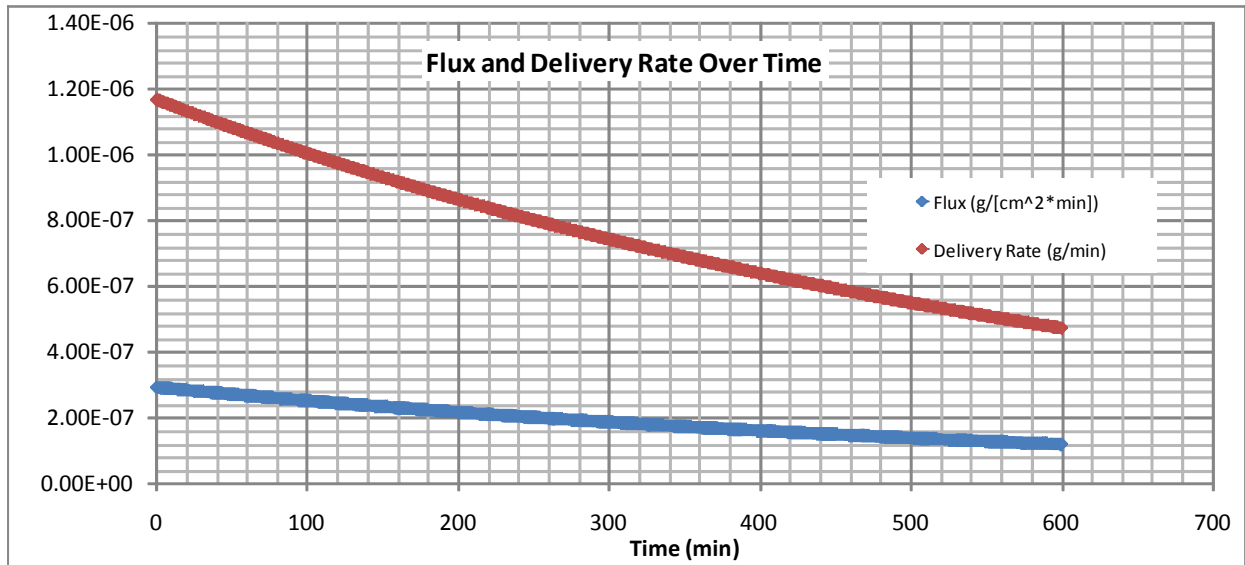
Delivery rates in gram units were converted to standard insulin units as defined by the

World Health Organization. According to model parameters, the patch can deliver in a relatively

linear fashion over ten hours, eventually delivering a total of around 10 units of insulin.

Figure 16: Hour-by-hour and Cumulative Delivery Data from Drug Model

Hour	Insulin Delivered (mg)	Insulin Delivered (IIU)	Cumulative Insulin Delivery (IIU)
1	0.068	1.496	1.496
2	0.061	1.344	2.840
3	0.056	1.228	4.067
4	0.051	1.122	5.190
5	0.047	1.025	6.215
6	0.043	0.937	7.152
7	0.039	0.856	8.009
8	0.036	0.783	8.791
9	0.033	0.715	9.507
10	0.030	0.654	10.160
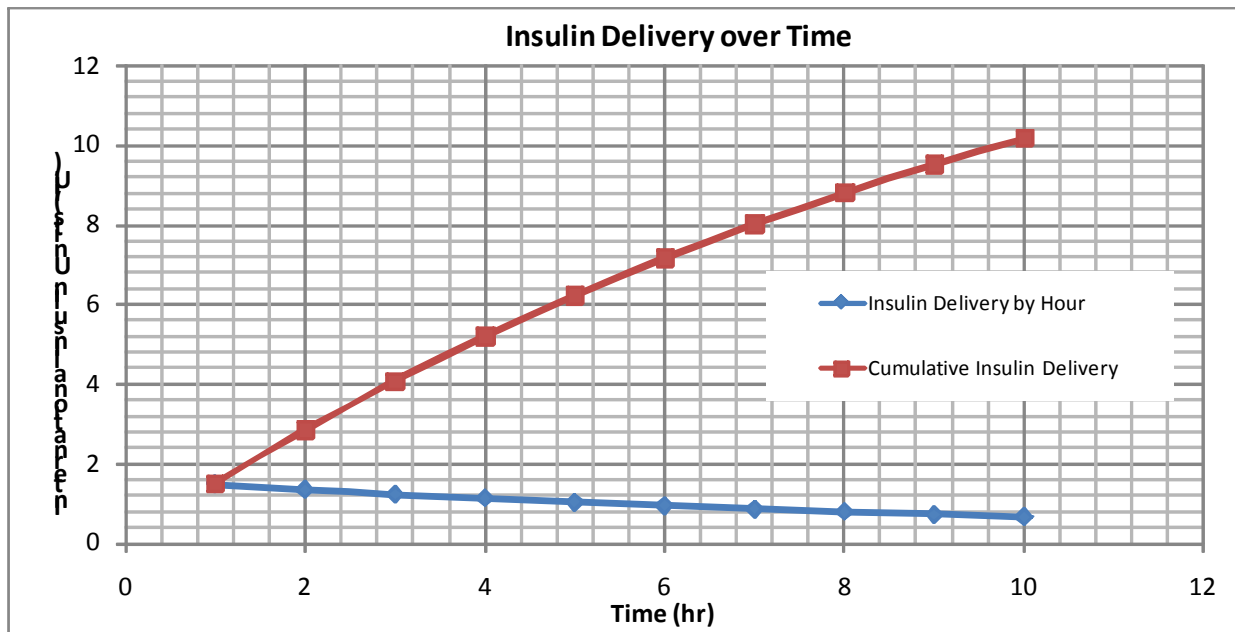
After testing our model with our initial configuration, we then tried more model parameters in order to change the delivery characteristics. The following three configurations involve either changing the surface area of contact or changing the volume of the drug reservoir.

Figure 17: Alternative 1- Increased surface area to 9 cm^2.

Model Parameters	
Initial Concentration (g/cm^3)	7.77E-04
Diffusion Coefficient (cm^2/min)	1.88E-04
Barrier Thickness (cm)	0.5
Patch Volume (cm^3)	1
Patch Contact Area (cm^2)	9
Patch Thickness (cm)	0.111111

Hour	Insulin Delivered (mg)	Insulin Delivered (IIU)	Cumulative Insulin Delivery (IIU)
1	0.145	3.186	3.186
2	0.116	2.554	5.740
3	0.095	2.085	7.824
4	0.077	1.702	9.527
5	0.063	1.390	10.916
6	0.052	1.135	12.051
7	0.042	0.926	12.977
8	0.034	0.756	13.733
9	0.028	0.617	14.350
10	0.023	0.504	14.854
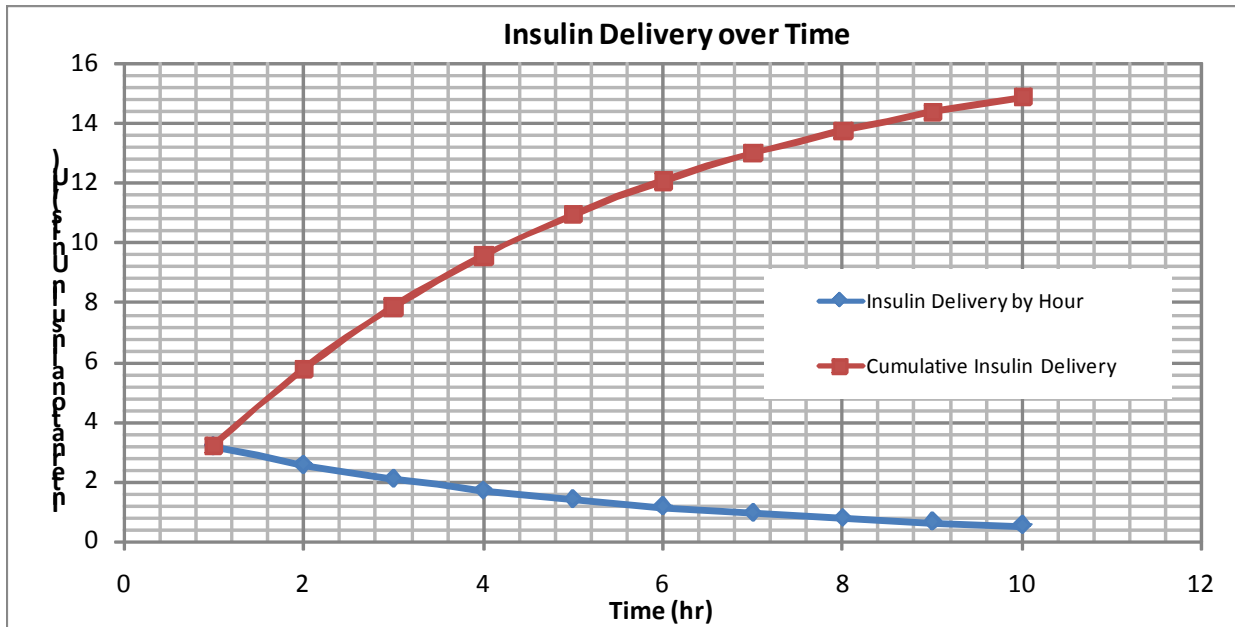
Figure 18: Alternative 2- Increased surface area to 12cm^2.

Model Parameters	
Initial Concentration (g/cm^3)	7.77E-04
Diffusion Coefficient (cm^2/min)	1.88E-04
Barrier Thickness (cm)	0.5
Patch Volume (cm^3)	1
Patch Contact Area (cm^2)	12
Patch Thickness (cm)	0.083333

Hour	Insulin Delivered (mg)	Insulin Delivered (IIU)	Cumulative Insulin Delivery (IIU)
1	0.187	4.112	4.112
2	0.140	3.078	7.190
3	0.107	2.348	9.539
4	0.081	1.792	11.330
5	0.062	1.367	12.697
6	0.047	1.043	13.740
7	0.036	0.796	14.536
8	0.028	0.607	15.143
9	0.021	0.463	15.606
10	0.016	0.353	15.959
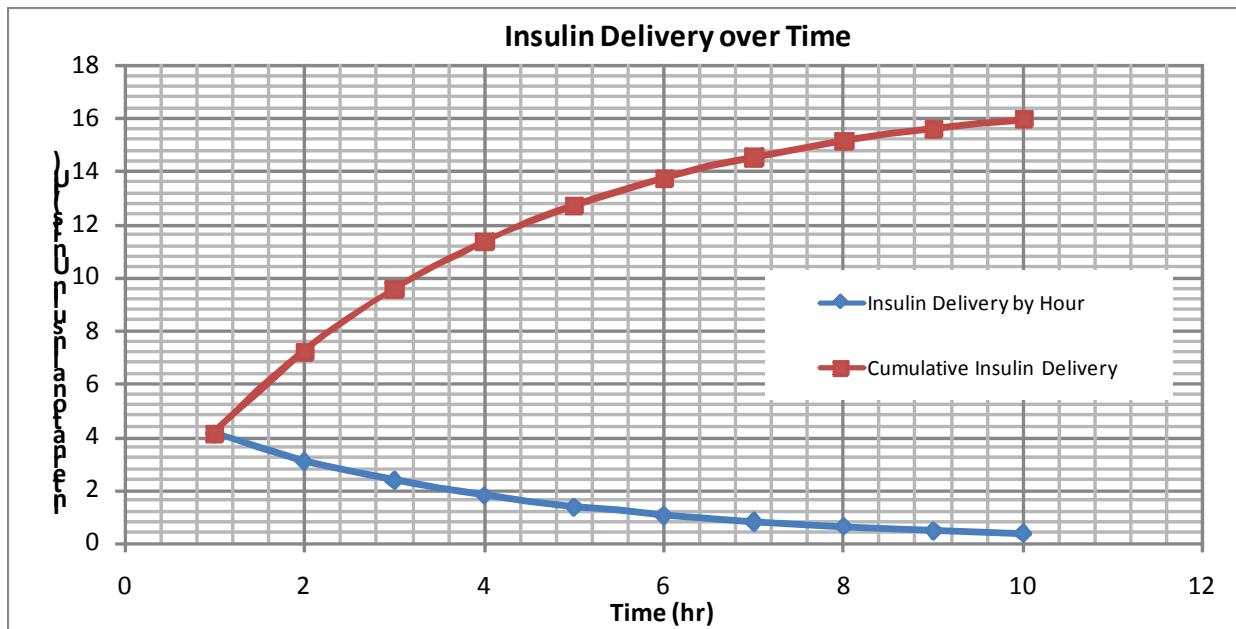
Figure 19: Alterative 3- Increase surface area to 12cm^2 and increase volume to 2cm^3.

Model Parameters	
Initial Concentration (g/cm^3)	7.77E-04
Diffusion Coefficient (cm^2/min)	1.88E-04
Barrier Thickness (cm)	0.5
Patch Volume (cm^3)	2
Patch Contact Area (cm^2)	12
Patch Thickness (cm)	0.166667

Hour	Insulin Delivered (mg)	Insulin Delivered (IIU)	Cumulative Insulin Delivery (IIU)
1	0.200	4.390	4.390
2	0.171	3.767	8.157
3	0.150	3.291	11.448
4	0.131	2.875	14.323
5	0.114	2.512	16.835
6	0.100	2.194	19.029
7	0.087	1.917	20.946
8	0.076	1.674	22.620
9	0.066	1.463	24.083
10	0.058	1.278	25.361

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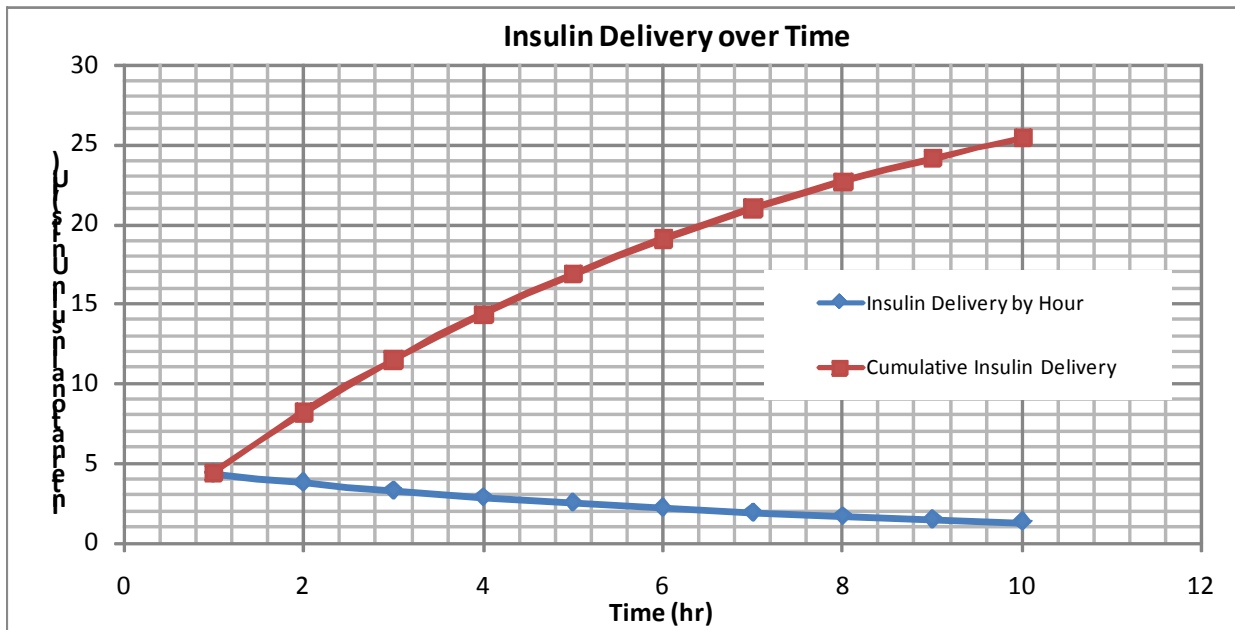
As can be seen from figures 17, 18, and 19, changing the parameters allows us to achieve a number of delivery designs with drug delivery potentials ranging from 10 IIU to 25 IIU. With further tweaking, even more combinations are possible. Different combinations of surface area and volume of the patch can control the quantity of drug delivered and the delivery profile.

5. Discussion

Currently, needle injections are by far the most popular method of insulin delivery for diabetics. Although needles are an efficient and reliable means for administering drugs, needle-based insulin therapies are often subject to low patient compliance, ultimately resulting in poor management of blood-glucose levels in diabetics (Cramer, 2004 and Morris et al., 1997). Issues such as needle anxiety, fear of pain associated with injections, and social embarrassment can all contribute to poor adherence to an insulin regimen (Korytkowski et al., 2005). Because the regular administration of insulin is crucial not only during acute hyperglycemic events but also for the long-term management of insulin-dependent diabetes, maintaining high patient compliance is a vital component of any successful insulin therapy.

The use of lipid vesicles, or liposomes, to deliver insulin across the skin is a potential alternative to traditional needle injections. Such vesicles have already been shown to transport insulin across intact mammalian skin, as well as to induce hypoglycemia in mice (Cevc et al., 1998). More importantly, liposomes offer the advantage of being noninvasive. Therefore, an insulin-delivery regimen utilizing a vesicle delivery system could promote better compliance in insulin-dependent diabetics.

A key determinant of whether a liposome can pass through the skin is vesicle size. Larger vesicles have greater difficulty passing through small pores on the surface of the skin, whereas smaller vesicles are much less inhibited. Thus any vesicle to be used as a drug carrier must remain stable in its small size after it is prepared. Flexibility is another important factor in vesicle penetration. Typically liposomal membranes consisting of a pure lipid bilayer are relatively rigid. The flexibility of liposomes may be enhanced by incorporation of surfactant in the lipid membrane, and the resulting increase in deformability allows for better penetration of the skin

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barrier (Cevc et al., 2002 and Van Kujik-Meuwissen et al., 1998). However, past studies have shown that surfactant-infused liposomes destabilize into other microscopic structures when kept in solution. Specifically, average vesicle radius is shown to vary inversely with surfactant concentration, and with increased surfactant levels, vesicles are shown to collapse into spherical and cylindrical micelles (Vinson et al., 1989 and Walter et al., 1991).

5.1 DLS Size and Intensity Measurements

Our data corroborates the inverse relationship between surfactant concentration and initial mean vesicle radius. In addition, consistent with our expectations, vesicles with more surfactant displayed greater variability in size over the course of two and six weeks. These fluctuations may represent the aggregation of liposomes into large vesicles which subsequently collapse into smaller particles, although further work is needed to identify the mechanisms underlying the observed trends. The marked decreases in the 50% and 75% surfactant samples in the six week trial suggest that these vesicles underwent a transition to the micelle state. This hypothesis is further supported by a change in the appearance of the fifty and seventy-five percent samples from cloudy to clear. Vinson et al. observed a similar change when their vesicles transitioned to the micelle phase (1989). Although the mean radius of both the 50 and 75% samples dropped to approximately 5 nm during the six week trial, the rapid decrease in vesicle size occurred much earlier in the 75% sample. It is therefore possible that the transition from vesicles to micelles occurs much sooner with higher levels of surfactant.

DLS intensity, which is a measure of the number of light-scattering particles in solution, shows a limited relationship with mean radius. It was our initial belief that decreases in mean radius resulted solely from the disaggregation of large liposomes into smaller vesicles, and that increases in mean radius represented the opposite phenomenon. If this were the case, we would

have observed an increase in intensity (signifying an increase in the number of light-scattering particles) concomitant with each decrease in mean radius and vice versa. However, our data showed no such correspondence between the two trends, nor does the range of intensity fluctuation seem to correlate with the amount of surfactant in the vesicle sample. The 25% surfactant liposomes in the two week trial, for instance, showed a greater range of intensity values than the 50% sample. In the six week trials, the relatively low intensity values obtained for the 50% and 75% surfactant samples seem to correspond with the steep drop in mean radius, which can be explained by a sharp rise in the number of micelles in solution. Because surfactant micelles are too small to scatter light appreciably, light scattering and intensity would expectedly decrease as a result of their formation. Furthermore, the 25%, 50%, and 75% samples in the six week trials attained much lower values of intensity than the 0% surfactant liposomes. This is again indicative of the formation of surfactant micelles in the former three samples, whereas such micelles could not have formed in the surfactant-free 0% sample.

Our data shows an association between mean particle radius and number of light-scattering particles only when the trends suggest a transition from lipid vesicles to micelles. Essentially, mean radius decreases as surfactant molecules "fall off" the liposomes and form small micelle structures, which lowers the overall intensity of the sample. When this is not the case, however, mean radius and intensity trends show very little correlation, and the mechanisms underlying these intensity trends are not fully understood.

5.2 Flexibility and Extrusion Rate

Flexibility, rather than size, is perhaps the most important determining factor in whether a lipid vesicle can carry drugs across the skin. In the case of rigid and flexible liposomes of equal radius, the flexible liposomes will be able to pass through pores of smaller sizes and more

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irregular shapes. This is due to the ability of a flexible liposome to deform and "squeeze" through pores with radii smaller than the vesicle's own.

As expected, our rigid 0% surfactant liposomes could not be extruded through pores smaller than the vesicles themselves. In contrast, all samples infused with surfactant showed some level of passage through the extruder. We believe that this demonstrates the ability of surfactant-infused liposomes to deform when passing through small openings, as described in previous studies. The 25% surfactant vesicles are the only sample for which we have valid flexibility data both before and after the six-week storage period, and our results show a marked decrease in flexibility over that time period. This may represent a "bleeding out" of surfactant molecules from the liposomes, leaving them more rigid as time progresses. When comparing the day 0 measurements for the 25% and 50% samples, we found the latter to have a greater flexibility index, corroborating the view that higher surfactant concentrations yield greater flexibility.

Extrusion rate, when compared to flexibility index, is a more direct gauge of a sample's absolute capacity to traverse the extruder membranes. Both the 25% and 50% samples showed a smaller rate of extrusion after the storage period of six weeks, implying some loss of pore-traversing capacity after storage. The 75% samples, however, showed very little change in extrusion rate after six weeks for reasons that are currently unclear, although we must note that these liposomes were stored for a shorter period of time than the 25% and 50% samples (26 days as opposed to 41 and 42 days, respectively).

5.3 Gel Diffusion Experiment

The continuous decrease in diffusivity values over our ten minute for each gel penetration trial was unexpected. Since diffusivity here theoretically represents a single vesicle's efficacy at

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diffusing through agarose, our values should have been constant if neither the vesicles nor gel changed during the data collection. The decrease in diffusivity during each measurement period was fitted to a power law. However, the power law regression for each trial was not a continuation of the regressions from previous days. The marked drop in diffusivity over the course of the ten minute trial can be attributed to factors associated with liposome diffusion through agarose gel and the measurements thereof; the power law regression does not describe the changes in diffusivity that result from storage of the vesicles. We offer several explanations for the inconstant diffusivities within each measurement period. First, structural changes in the vesicles and micelles induced by their passage through the agarose may have altered their diffusion properties. Second, structural changes in the agarose gel itself may have affected the passage of vesicles and micelles. Third, the concentration of fluorescent marker at any point on the slide may not have had a direct linear relationship with fluorescent intensity. Fourth, the fluorescent intensity of the visualized gel may have saturated to the point that changes in intensity could no longer be detected by our imaging system. Fifth, any dissociation of fluorophores from the vesicles and micelles as they passed through the gel would have decreased the apparent extent of diffusion, as the DiI markers we used fluoresce strongly only when embedded in a membrane. Due to the fact that the single-day diffusivity trends for all samples followed a power law, we believe that these drops in diffusivity resulted from a systematic phenomenon which we cannot definitively explain at this time.

For both the 0% surfactant liposomes and surfactant micelles, diffusivity trends did not change in one direction over the course of a week; diffusivity values for the 0% sample increased on the first two days and then decreased on the seventh day, while values for the micelles decreased over the first three days and increased on the seventh day. The 50% sample, however,

increased in diffusivity consistently between days 0 and 7. This contrasts with our extrusion rate data, in which extrusion rate decreased for the 50% sample. However, the 50% sample from the extrusion experiment was stored for a period of six weeks rather than one. During all measurement days, the 0% sample, consisting of rigid, surfactant-free liposomes, had the lowest diffusivity, most likely due to their inability to deform as they passed through the agarose. This supports our findings from the flexibility tests in which the 0% sample was the only one that could not pass through the extruder. This confirmed our hypothesis that rigid liposomes were the most ineffective at traversing both the polycarbonate membranes in the extruder as well as the agarose gel.

We initially suspected that the penetration efficacy of flexible liposomes was due to the formation of micelles in solution. Over time, surfactant molecules can become dislodged from lipid vesicle membranes, forming small, surfactant micelles and leaving more rigid, surfactant-deprived vesicles. If this was the case, then the apparent passage of a flexible liposome solution through a barrier would actually represent the movement of micelles only. However, our data shows that the diffusivity of the 50% sample approaches that of the pure micelles at seven days. In addition, based on our DLS studies, the vesicle to micelle transition in our 50% sample does not occur until after a seven day period, suggesting that surfactant-infused vesicles in solution contributes to diffusion of the 50% sample.

5.4 Insulin Patch Modeling

Based on our findings, we can reason that while liposomes are a viable drug delivery vehicle, they must be stabilized in a medium that will not allow them to change in size over time. We have proposed that the best method of delivery is through a patch device that contains a stabilizing matrix to hold the liposomes at their desired size. When necessary, the matrix may be

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heated or chemically activated to release the liposomes onto the skin. This matrix/patch device combines the stabilizing matrix that is necessary for the liposomes with the convenience of patch technology. We propose that the matrix be composed of a hydrogel, which has been demonstrated to both stabilize liposomes and release them onto an agarose skin-like barrier. A hydrogel is simply a network of synthetic polymers that can absorb a significant amount of water. Both hydrogels and liposomes can be stored for long periods of time as dry material, and reconstituted with the addition of water. We propose that a hydrogel/liposome mixture can also be submitted to the same treatment. However, little research has been performed on the viability of using hydrogels to stabilize liposomes. Recently, Mourtas et al. demonstrated that some liposome compositions such as propyleneglycol and transcutol CG are more amenable to stabilization by hydrogels, while the integrity of other liposome compositions are compromised upon integration with an aqueous gel. Four major factors control the diffusion characteristics of the model. They are diffusion coefficient, patch skin-contact surface area, volume, and initial concentration of drug in the patch.

The diffusion coefficient describes the ability of the drug molecule to travel through the skin model and is a value that is difficult to change because it is inherent to the way the insulin molecule interacts with the skin barrier. A larger diffusion coefficient tends to produce steeper curves on graphs depicting drug concentration and delivery because drugs are delivered fast initially, exhausting the supply. A smaller diffusion coefficient tends to produce steadier, more linear delivery characteristics.

In addition to the diffusion coefficient, the surface area of the patch in contact with the skin is also a huge factor in determining the delivery rates of the drug. Increased surface area would be analogous to adding lanes onto a highway. While the speed limit (diffusion coefficient)

would still remain the same, the increased lanes allow more traffic to pass through. Increasing surface area tends to increase the curvature of a delivery graph in a similar manner to the diffusion coefficient. Decreasing the surface area also tends to produce more stabilized, linear delivery at the cost of delivering less of the drug.

Volume and initial concentration are related and are important in determining both the speed of drug delivery and the quantity of drug delivered. Because the rate of diffusion-controlled delivery is ultimately determined by the concentration of the drug, the volume of a delivery system has a large effect on delivery rate. Increasing the volume (while keeping other factors the same) increases the potential amount of drug capable of being delivered by the patch and also reduces the curvature of the delivery profile. This is because a greater volume would decrease the concentration drop per unit time, and, as a result, would maintain more stable concentrations. Since delivery rate is directly related to concentrations, a higher, more stable concentration would result in higher, more linear delivery rates. The highway analogy would still work in this case; the volume would represent the size of the city that the traffic is originating from – all cities would produce the same initial traffic during rush hour (assuming every city has the same density of cars during the work week and the same size highways exiting the city) but larger cities will be able to sustain the flow of rush hour traffic much longer than a smaller city.

Based on the results of the model and our assumptions, our theoretical patch could potentially be the only device needed to sustain the insulin needs of both type-1 and type-2 diabetics. Instead of separating basal and bolus insulin doses using injections, diabetics could use our patch to obtain the initial bolus dose after meals (while patch delivery rates are high) and then continue to use our patch to sustain basal insulin needs (as the patch delivery rates decline). One patch could be used before every meal as a feasible glucose-management plan for diabetics.

An online provider of diabetic information for medical practitioners, Family Practice Notebook, LLC, offers general dosing information per kilogram for diabetics. In general, these numbers range from 0.1 to 0.7 units of insulin per kilogram per day (Moses 2008). For a 75 kilogram man, those numbers translate to 7.5 to 52.5 units a day; an amount which could feasibly be delivered by our model transdermal patch. By modifying the surface area and volume, a patch could be made to satisfy any dosing requirement needed by the majority of diabetics.

6. Conclusions

Our studies of liposome size, flexibility, and gel penetration have provided several key insights into the viability of liposomes for transdermal insulin delivery. In accordance with our initial hypothesis, size fluctuations were greater in samples of liposomes with a higher concentration of surfactant. Furthermore, after a period of two weeks, the mean radius of the 50% sample underwent a drastic decrease, while the 75% sample experienced a similar decrease after only three days. These decreases were accompanied by a change in the appearance of our liposome solutions from cloudy to clear. All together, these data suggest an extensive transition of liposomes to surfactant micelles. We therefore conclude that our 50% and 75% flexible liposomes are not suited for storage periods of over two weeks and three days, respectively, as there are considerable structural changes that occur over the course of these time periods. In contrast, our 0% and 25% samples remained relatively stable over both two weeks and six weeks. A past study by Verma et al. (2003) found that the optimal vesicle size for drug delivery into skin is 60-70 nm, which was just above the mean radius for our 0% and 25% samples. Because the initial mean radius of our liposome samples can be adjusted based on the size of extrusion pores used in their preparation, we conclude that our 0% and 25% surfactant liposomes can be stored successfully from a size-stability standpoint.

As expected, our data on vesicle flexibility suggests that surfactant-infused liposomes lose their flexible properties over time. In the case of our 25% sample, apparent flexibility decreased by over 80% over a period of six weeks. Data from this sample shows that vesicle flexibility can undergo drastic changes even if vesicle size remains relatively constant. Our flexibility data also indicate the effects of surfactant concentration on flexibility, as our surfactant-free rigid liposomes (0% sample) could not be passed through the extruder at all, and

the initial flexibility of the 50% surfactant sample was greater than that of the 25% sample. Because both vesicle size and flexibility are crucial factors for transdermal drug delivery, we conclude that of our four samples, the 25% and 50% vesicles are best suited for drug delivery after storage, although the storage period can be no longer than two weeks. In contrast, the 75% sample underwent a presumable vesicle to micelle transition after only three days, while our 0% sample was unable be extruded during the flexibility tests, indicating that it had the lowest pore-traversing capacity.

The diffusivity data of our liposome samples largely confirms the results seen in the flexibility experiment. The 0% surfactant liposomes, which were the most rigid, had the lowest diffusivity, while micelles (100% surfactant) had the highest diffusivity. Initially, the 50% surfactant liposomes had diffusivity values in between that of micelles and the 0% sample. Contrary to expectations, however, the diffusivity of the 50% sample increased and approached that of the micelles after seven days. Our data suggest that this rise in diffusivity of the 50% sample was not accomplished solely by the formation of new micelles since, during the size stability experiments, vesicles remained the dominant species in solution after one week. Although further work is needed to characterize the exact mechanism of the increase in diffusivity, our results show that at least some elements of our flexible liposomes are able to retain their ability to diffuse through a barrier after a week-long storage period.

Our research confirmed our first two hypotheses, that liposomes with more surfactant exhibit more size fluctuation over storage, and that vesicle flexibility decreases during the storage period. Although our 0% surfactant liposomes showed the least variation in vesicle size, their limited flexibility and diffusivity make them unsuitable for transdermal drug delivery. Flexible liposomes, on the other hand, have a more limited storage capacity, but are able to

diffuse through a barrier more effectively than their rigid counterparts. Furthermore, our 50% surfactant sample showed an increase in diffusivity over one week after formation, suggesting that these liposomes could potentially be an effective needleless insulin delivery agent within that timeframe. A patch delivery system could potentially be used to stabilize insulin-loaded flexible liposomes for an even longer period of time. Our model of a theoretical insulin patch shows that, according to our diffusivity data, such a system could deliver sufficient therapeutic amounts of insulin to adult diabetics.

Limitations

Interpretation of our data is limited by several factors, some of which are listed below:

1) Liposome and micelle concentrations were reported indirectly in terms of fluorescence, so all values are relative rather than absolute. In our interpretation of the data, many assumptions were made in correlating fluorescence intensity to concentration of liposome.

2) The extent to which the fluorescent marker is being incorporated into the liposome is unknown. It is possible that the marker is not being incorporated at all, and the fluorescence we have visualized is simply of the marker traveling down the agarose gel. This is unlikely as DiI is known to fluoresce only when incorporated in a phospholipid membrane.

3) It is not completely known that the formation of micelles is causing the increase in rigidity of the liposomes over time. We have only assumed this based on previously reported results by other groups.

4) While we can use mathematical models to predict the feasibility of our liposomes in carrying insulin across the skin, testing these models by experimentation is imperative for future development of this drug delivery design.

5) Many different materials for liposomes and surfactant exist, both in the literature and on the market. Our choice of egg PC and Tween 80 limits our ability to extrapolate our results to all liposome compositions.

<u>Future Work</u>

In order to extend the significance of our work, we would like to perform a series of follow-up experiments. First, we would like to develop a method of quantifying the ratio of liposomes to micelles in any given mixture. To do this, we propose using size-exclusion chromatography, a common method used in chemistry to separate a heterogeneous mixture based on the size differences of the different species. Since micelles are much smaller than liposomes, they would have a different elution time on the column; we could determine the relative ratio of micelles to liposomes from the elution profile. In order to perform this experiment, we would have to label the liposomes with DiI before subjecting the mixture to the column. Once this assay is developed, we can use the data collected to confirm if formation of micelles is the cause of the decrease in liposome rigidity and size over time.

We would also like to perform a series of experiments that will directly link our results to transdermal insulin delivery using liposomes. We plan to repeat the gel permeation experiment with these insulin-encapsulated liposomes, visualize their penetration, and repeat the same measurements and calculations that we performed with the normal liposomes. It would be interesting to observe if there are any changes in diffusivity between the normal liposomes and the insulin-encapsulated liposomes. If these penetration experiments are deemed successful, we would like to test the ability of our liposomes to pass through pig skin.

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Appendix A: Business Proposal

Opportunity

We believe that a patch technology developed to replace needle-based insulin delivery would be viable to develop and market due to the perceived need for an alternative to needles amongst the diabetic population. Specifically, we plan to target children and the elderly, two demographic groups who are the most needle-averse. Our insulin patch regimen would replace an insulin injection regimen for these populations. We will reach these groups through a push strategy aimed at endocrinologists and other medical professionals. Our main challenge will be persuading these professionals of the superiority of our product. However, because our core benefit proposition is improved patient compliance due to ease of use and effectiveness of the regimen, we do not foresee this being an issue.

Team

Our 10-person team possesses unique strengths that we believe will make our business successful. We are a close-knit group that has known and worked with each other successfully for almost four years. As a result, we know each others' strengths and weaknesses, we have established group norms and group dynamics, and we have honed our collective work ethic. Additionally, we have a wide range of skill sets -- members have backgrounds in chemical engineering, biology, business, and finance. Our team was created through the Gemstone Program at the University of Maryland, College Park. The Gemstone Program is a four-year, multidisciplinary research program that fosters leadership, teamwork, and the creation of original research. Students picked for this program are academically successful, passionate, and ambitious people. This combination of forces has contributed to the well-oiled machine that is our team.

The Market

The estimated global prevalence of diabetes by age is: 0.22% of people 20 years old and younger, 9.6% of people between the ages of 21 and 59, and 20.9% of people 60 years and older. According to the American Diabetes Association (ADA), there are over 20.8 million children and adults in the United States who have diabetes, or approximately 7% of the population. There are also an estimated 40.1 million Americans with pre-diabetes conditions. Based on projections by the ADA, the total number of diagnosed diabetics could reach 29 million by 2050. Of these individuals, we expect 50-65% to require insulin. The 6 million people with Type I diabetes will need insulin administered to their bodies every day for the rest of their lives. Eventually, many patients with type II diabetes, especially as they age, will also require an insulin regimen. The blog DiabetesMonitor.com asserts that "The proportion of Non-Insulin-Dependent Diabetes Mellitus (NIDDM) patients treated with insulin increased with longer duration of diabetes, from 22% at 0-4 years to 58% at more than 20 years." These numbers indicate that the total projected number of diabetics who will use insulin is estimated to be 8.8 million in 2015, 10.5 million in 2025, and close to 20 million by 2050. Those are growth rates of 19% and 90%, respectively – a huge increase. The rapid widening of the insulin market demands innovation of the system of administering insulin.

Current Technology

Hypodermic needles, the current most popular method for insulin administration, have been used by diabetics for more than 90 years. Though widely used, they insufficiently meet our team's standards for glucose-control and patient compliance. Hypodermic needles are dangerous, costly to dispose of, uncomfortable to use, and a significant reason why many diabetics do not

adhere to their insulin treatment. Mintel reports that 40% of diabetics between the ages of 18-44 and 25% of diabetics between the ages of 45-64 do not follow their insulin regimen closely.

Insulin pumps are the second most widely used method of insulin administration. While the pump removes the discomfort of needles, it is unwieldy and must be secured to the body via a catheter.

Our vision is a technology that regulates blood-glucose levels steadily, as an insulin pump would, but does not compromise the integrity of the dermis to environmental factors. Additionally, our technology must support high levels of patient compliance in that it is easy to use and easy to remember to use.

Our Product

Thus far, our team has completed preliminary research exploring the viability of using deformable liposome vesicles to transport insulin through the dermis and into the bloodstream. Results have been promising. We hope to use vesicles loaded with insulin to form easy dose patches ranging from slow-release for use all day, to fast-acting, for use after meals. Each patch would contain a set amount of the drug, so overdosing would not be a factor. The patch would be left on for a minimum of a set amount of time, after which it could be removed. Patches would be color-coded by dose type, and a monitor included with the system would keep track of time and blood-sugar levels. We hope to develop a gel-matrix delivery system that would be activated by body heat, for use with the insulin-loaded vesicles. This delivery system is what we hope to patent. Hurdles to securing intellectual property rights include developing the system, applying for a patent, and getting approved.

Competition

Other companies pursuing non-invasive insulin administration include Phosphagenics Limited, located in Melbourne, Australia, and Encapsulation Systems, Inc, located in Pennsylvania. Phosphagenics has developed a proprietary gel that works with the skin's natural mechanisms to foster drug absorption, while Encapsulation Systems Inc, has developed an ultrasonic patch that dilates pores, allowing insulin molecules to penetrate the dermis and enter the blood stream. Both of these systems are currently undergoing phase 2 clinical trials. While they are similar to our proposed technology in that they are non-invasive, we feel that our patch has a competitive edge. Topical gels may get brushed or washed off or not rubbed in adequately, and the ultrasonic patch must be used with an insulin-pump like system, kept on the body at all times. The clean and compact aspects of our proposed patch technology give it an advantage over these technologies.

Future Works

Our team is currently entering the initial stages of development. We have proven the viability of our liposome formulation to cross a skin-like membrane (agar gel). Now, we need to test the viability of the liposomes loaded with insulin through a more realistic membrane such as pig skin or human cadaver skin. This should take about five months. Over the five months after that, we will formulate a gel matrix to stabilize the liposomes. Ten months from now, we should be able to proceed to developing a complete prototype for the patch.

Following the development of our prototype, we will seek FDA approval to begin clinical trials. Extensive data collection will be necessary to pass FDA approval, therefore we will need to secure superior materials, equipment and lab space. Our current funding needs are $28,000 broken down as follows:

$5,000 - Purchase new extruder

$700 - Materials for liposome formation

$700 - Insulin

$1,200 - Pig skin and human cadaver skin

$2,000 per month for 10 months - Rental of lab space with access to appropriate equipment

$400 - other materials -- filters, glass slides, etc.

Total: $28,000

Appendix B: Future Research Proposal

Proposal: Elastic Nano-Vesicles for Transdermal Delivery

Statement of Need

What is the Need?

Today, if you find out that you were diabetic, you better hope that you were not afraid of needles. Today, if you have to take your young child to the doctor's for a vaccination, you better be prepared to fight through the thrashing, crying, screaming, puffy eyes, and tear-induced stuffy noses as you watch nervously as the nurse walks into the room with that sharp needle. Today, in the places less fortunate than the United States, millions of diabetics and millions of children are dying because they don't have the luxury of the medical attention required to treat diabetes or to receive vaccinations.

Fortunately, scientists and engineers are working hard to produce novel nano-scale drug delivery methods that will simplify the diabetics life, put to ease the frantic mother and her screaming child, and give a chance to the possibly billions of people who need medical treatment that today is too complex to adequately serve them. Three companies have announced novel drug carriers that can transport life-saving molecules across undamaged skin, and they have received a considerable amount of attention for the potential impact their technology could have. Shockingly, not one of these companies operates in the United States.

Need is causation in many different forms: a man dying of thirst needs water, a former champion needs redemption, a less fortunate human being needs aid from a more fortunate one. The need for the development of these novel drug carriers in the United States is the need of the relatively well-off nation, who was first in the race to the moon, the automobile, the computer,

the internet, to help out both her own people and her less fortunate neighbors and to regain the edge in a field that she should, for any reason, not be number one.

What's Wrong with the Technology Today?

The impermeability of the skins provides the greatest challenge in developing transdermal delivery technology. Traditionally, it is stated that drugs over 500 Daltons cannot cross the skin barrier unassisted (*1*). In navigating the barrier function of the skin, the stratum corneum (SC) is the primary obstacle. This layer of the skin is on average 20 cells thick and consists of overlapping layers of terminally-differentiated corneocytes bound by extra-cellular lipids. The composite of these materials creates a "brick-and-mortar" feel to the SC, which creates difficult boundary to penetrate for the majority of molecules (*2*). Currently only small, hydrophobic molecules are able to diffuse through the lipid layers of the SC and be delivered into the body (*3*).

Despite their successes, the three drug carriers mentioned before – Transfersomes®, Ethosomes, and TPM-02, are still not relatively well understood. These novel drug carriers have been studied enough to show that they are effective in delivering certain category of drugs. Transfersomes® are being tested for topical analgesics (*4*); Ethosomes® have been shown to deliver the hormone testosterone better than previous technologies (*5*); TPM-02 is finished with phase I trials as a method of delivering insulin to type 1 diabetics (Phosphagenics press release). These products are being developed in spite of the fact that a true understanding of the mechanism of function is not well understood. While this in itself is not an issue, it is important to have a true understanding of the mechanism of function in order to fully realize the potential of transdermal delivery. The first generation of products, with perhaps the exception of TPM-02 Insulin if it is successful, generally focuses on improving products already delivered via

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transdermal methods rather than breaking ground on previously undeliverable drugs (*4*). While their research has shown that delivery of these drugs is possible, inconsistency in the reproducibility of the results often hinder progress.

What Do We Want to Do?

Of interest to us is a report from Cevc et al. which reported a novel highly deformable liposome formulation (called Transfersomes®) are capable of permeating undamaged skin intact and deliver drug agents like insulin at therapeutically significant levels (*6*). Unlike the transport of drugs through a chemical activity gradient (concentration), Cevc states that ultra-deformable Transfersomes® penetrate the skin due to vesicle hydration across the water gradient across the stratum corneum, and the rate of penetration is dependent on the rigidity of the penetrating liposome. Cevc further claims that the rate of delivery is independent of the liposome concentration (*7*).

Although Cevc has reported outstanding results for Transfersomes® in delivering therapeutic levels of large drug molecules such as insulin, his results were repeated with mixed levels of success (*6, 8*). El Maghraby et al. reported only one to three percent of drug applied topically was delivered in 30 minutes compared to the near 50% statistic reported by Cevc. Later on, three separate *in vivo* studies involving tape-stripping of human skin reported that surfactant vesicles showed deep penetration into the stratum corneum within an hour (*9*).

We do not doubt the effectiveness of Cevc's Transfersome® formulation nor we deny that vesicle elasticity and the hydration gradient has an impact on delivery. We have also noticed, however, that Ethosomes and TPM-02 also deliver based on claims of elasticity despite the three all having claims to separate intellectual property. From our review of literature and from some initial experiments we have conducted, we believe that there is a different mechanism

at play (at least partially different) and we will examine the various elements of these elastic vesicle systems to discover components of the true mechanism of function, one that we believe can tie together all three of the novel drug carries we have mentioned. With a better understanding of the mechanism, we believe we can build a more robust product capable of delivering a greater variety of drugs with greater efficiency.

Due to our unique location at the University of Maryland, we can potentially take advantage of the hundreds of peptide drugs in development, some of which may hold the cure to cancers, HIV vaccination, and some of the other biggest challenges in medical treatment today. Even with a technology of equal effectiveness, our unique proximity to the heart of biotechnology research will allow us to compete successfully against foreign technologies.

Regardless of future product and intellectual property possibilities, our research will at least contribute to the fundamental understanding of these novel vesicle systems. While potential future market shares and revenue streams are uncertain, what is certain is that any contribution we make to a breakthrough transdermal technology will undoubtedly affect everyone whose lives would be greatly simplified, or even saved, by this new technology.

Project Description:

General Strategy:

When attempting to make improvements to a piece of technology, whether it's upgrading a computer, modifying a car, updating computer code, or redefining vesicle transdermal delivery mechanisms, the first priority be to have a solid general understanding of how the entire system operates. Understanding and being able to predict how a certain modification will impact the

rest of the system serves as a critical prerequisite for any attempts to modify the system for a certain benefit.

As such, we will partition our research into two major phases to reflect the steps necessary to accomplish our goal of making improvements to Transfersome® and other elastic vesicle technology. The first phase will consist of experiments associated with familiarization and tabulation of different vesicle properties and their effect on transdermal delivery. We will identify the variables with the greatest impact on the vesicle system performance and document how variations in these variables cause changes in penetration characteristics of skin or skin-like barriers.

Once we understand the components of the elastic vesicle system and their relative impact on the ultimate goal of efficient delivery, we enter the second phase of our research. During this phase, we will take an analytical approach in hypothesizing and verifying the driving forces behind the relationships we discovered in the first phase. Instead of just figuring out what happens, we will focus on why it happens and how each phenomenon pieces together to formulate a cohesive mechanism for drug delivery at the skin.

The benefit of taking a systematic, step-by-step approach is that we can easily backtrack to familiar territory and re-adjust in the likely case that our research produces an unexpected result. In addition, through our systematic approach each negative result will provide as much information as positive results in directing future research.

Previous Work

Over the past two years, we have performed preliminary experiments to investigate the effect of different lipid compositions on the stability and flexibility of the liposome over a six-week period in normal storage conditions. We also used fluorescently labeled vesicles of various

lipid compositions (i.e., to study their rate of diffusion across a skin-like model membrane. Although this was only a preliminary investigation, we were able to conclude that lipid composition greatly affects the stability in size and flexibility of the liposome during long term storage. Our membrane permeation studies showed that while liposomes with greater surfactant concentration seemed to diffuse through the skin at a higher rate, they were also the least stable in terms of size, and are therefore not viable for manufacture. We were unable to make concrete justifications for our observations, and there were little or no comprehensive studies characterization of liposomes in the literature. Our experience with liposome research has led us to propose this systematic approach to liposome characterization, which would aid not only our own research, but all liposome research aimed at drug delivery. .

Specific Aims:

Milestone I:

Familiarization with Elastic Vesicles and Tabulation of Cause and Effect Relationships

We expect to spend about nine months in phase one of our research. We justify spending a significant amount of time familiarizing and exploring our vesicle system by drawing an analogy to driving around Washington D.C. for the first time. Ten minutes of simple preparation before heading out the driveway could potentially save hours of time circling unfamiliar streets. There is a small chance that our research will miraculously turn out the exact results we want, but the far greater, almost infinitely greater, possibility is that something unexpected will happen. In these cases, we will be prepared and first phase of our research will be both the map to guide us back to familiar territory and the framework to make connections we otherwise would not have seen.

In general, we are looking to replicate the spectrum of results (as opposed to the best result) published in literature and to isolate trends associated with modifying certain variables. In addition, we want to visualize as many of the processes and phenomena we encounter as possible.

The ultimate goal of all these efforts is to evaluate the components of the current mechanism and label each part as incorrect, incomplete, or correct. We hope to have charts tabulating the performance of the spectrum of possible variable combinations. From these, we can analyze and visualize trends in a systematic fashion. We hope to be able to draw information from these trends for phase two of our research.

In particular, there are several quantitative and qualitative measurements that we are interested in:

- *Vesicle Size:* Vesicle size fluctuations and equilibrium will be tabulated for each composition of vesicle using dynamic light scattering (DLS). Vesicle size, especially at equilibrium, is interesting because it provides information on the natural stability of that formulation. Vesicle size fluctuations may be indicative of structural changes in the vesicle that may be an important part of the delivery mechanism

- *Vesicle Rigidity:* Vesicle rigidity will be tabulated for all vesicle compositions. These measurements will be made by extrusion (using a pressure-driven extruder) through pore sizes smaller than the vesicle itself. Rigidity is related to the proportion of the pore size and the diameter of the vesicle. Vesicle rigidity is stated as one of the two biggest factors in the delivery of Transfersomes® across the skin barrier according to Cevc (*7*).

- *Vesicle Penetration through Skin or Skin Model:* The penetration of vesicles into and through skin or skin models will be made by fluorescently labeling our vesicles with DiI

fluorescent lipid dyes. DiI is incorporated into the lipid structure of the liposome and will fluoresce noticeably less when not incorporated into a liposome. Skin will be prepared for microscopy by sectioning into thin slices.

- *Occlusive Vs Non-occlusive Delivery:* The importance of preventing moisture loss will be monitored for the delivery of all vesicle compositions. Occlusive delivery prevents water loss from the delivery medium. A custom diffusion cell, described by Cevc et al., where one end is in contact with an aqueous solution and the other side open to a controlled humidity environment, will be used to control the moisture loss of the delivery medium (*7*). Delivery of Transferomes® consisting of sodium cholate is dependent on non-occlusion during delivery in order to penetrate the skin barrier. We want to see if all vesicle compositions require the same condition.

- *Effect of Vesicle Ethanol Composition on Delivery*: Vesicles with varying amounts of ethanol in their structure will be delivered non-occlusively through porcine skin in the custom diffusion cell mentioned above. Particularly with Transfersomes®, the effect of the composition of ethanol in the vesicle on delivery is not very well understood. Ethanol is a shared component in the three major drug carriers discussed in the statement of need.

- *Effect of Pre-treatment on Lipid Structure of Skin:* We will measure the difference in transport of fluorescently-tagged vesicles through porcine skin pre-treated with surfactant, ethanol, and surfactant-ethanol combinations. We will use the custom diffusion cell described above. We will also take electron microscopy images of the porcine skin surface before and after pre-treatment in order to visualize the interaction of surfactant, ethanol, and combination of the two on skin lipids. While we do not believe pre-treatment will improve delivery characteristics, we believe that surfactant/ethanol

interactions on the surface of the skin will reflect interaction that happen while vesicles are traveling through lipid channels in the skin.

Milestone II:

Formation and Validation of New Hypotheses on Elastic Vesicle Delivery Mechanism

If the journey to the first milestone were a collection of traffic reports on which route is the best way to travel, then the second phase of our journey would be analogous to investigations on why certain routes are better than others. Vesicle formulations would be the equivalent of traffic routes and we are attempting to figure out why certain routes provide better results than others. Is it because of the vesicle equivalents of traffic lights? Tolls? H-O-V Lanes? With this information in hand we can design a new traffic pattern, or vesicle, using the best of what was before and adding new features that fixes problems with the previous version.

In general, we are looking for signs of vesicle deterioration during transport through skin and the modification of lipid channels by earlier vesicles to prevent deterioration of later vesicles as they traverse. Most of our experiments will attempt to provide indirect evidence through testing in analogous in vitro settings.

The structure of experimentation in this phase will consist of hypothesis testing based on trends seen in the first phase. For example, if we noticed in the first phase that there was an optimum 15% ethanol concentration for Transfersome® delivery and we noticed from our electron microscopy images that ethanol/surfactant pre-treatments caused integration of surfactants into the lipid structure, we would hypothesize that vesicles change the structure of the surrounding lipid channel by "bleeding" surfactant and integrating them into the channel, somehow facilitating drug delivery as a result. From this hypothesis, we then follow the branching experimentation structure where rejection of the hypothesis will return us back to our

trends to formulate a new hypothesis and failure to reject our hypothesis will result in the next

logical progression in testing the hypothesis. With this method, we can always take a step or two

back at potential dead-ends to re-evaluate and adjust.

One of our major challenges in the second is to ensure that our detection methods are

accurate. It is almost impossible to detect vesicles directly, and they are usually measured

through an indirect method such as fluorescent markers and dynamic light scattering. In phase I,

we used methods previously published to be accurate. Because we are attempting to detect signs

of novel mechanisms that may not have necessarily been explored before, we will spend

significant efforts to cross-check and thoroughly understand our detection methods before we

rely on them. In many cases we expect the usual detection methods to be sufficient, but because

they are being used in a new situation we still have to be careful.

In phase II, we will be exploring these general concepts in order to extract a mechanism

from our data:

- *Vesicles Compositional Changes:* Using equipment like extruders, DLS and other
  analytical tools (ex. Differential scanning calorimetry), we want to study the changes in
  vesicle composition after being stressed through small pores through indirect
  measurements like flexibility, size distribution, and membrane phase behavior. We will
  deliver these "stressed" vesicles through porcine skin and compare them with the
  performance of fresh vesicles. These measurements will provide some indirect evidence
  to potential changes in the vesicle as it traverses the pores of the skin. Continued rounds
  of extrusion can reveal if these vesicles will "expire" after a certain number of stresses.
  These experiments will help address the deterioration aspect of our mechanism.

- *Vesicle Interactions with polar/non-polar interfaces:* We will use extrusion, DLS, and other analytic tools to examine characteristics of aqueous vesicles before and after interaction with lipids and other non-polar materials in liquid and gel forms. While information is known about vesicles stability in polar and non-polar solvents, we want to how vesicles originally stable in the aqueous phase behave when exposed to non-polar environments like lipid channels in the skin, particularly in the presence of lipid-solubilizing ethanol. We believe some vesicle degradation is inevitable when exposed to the lipid environment but we also believe that the destabilizing of vesicles initially in the channel provides stability to future vesicles in that channel. These experiments will address the deterioration aspect of our mechanism.

- *Lipid Channel Modification:* We will use the custom diffusion cell described in phase I to transport Transfersomes® through a hydrophobic gel. Then, we will see if the gel permeation to polar compounds like water has changed. We will use standard fluorescent marker techniques to detect water transport. These series of experiments will address both the deterioration and the transport-facilitating effect of previous vesicles on the transport of future vesicles portion of our mechanism.

- *Surfactant Channel Facilitation of Transport:* Using the custom diffusion cell described above, we will see if transporting Transfersomes® through a hydrophobic gel incorporated with surfactant improves the transport rate. We will use DiI incorporated into the vesicle structure to track vesicle progress. These experiments will address the transport-facilitation aspect of our mechanism.

Potential Impact of Proposed Research on the Development of Liposome Drug Delivery

Nanotechnology and Future Prospects

Once a mechanism for transdermal liposome delivery of peptides is confirmed, it will be possible

to use this information in designing a product for drug delivery. For example, our previous

research has confirmed that the flexible liposomes required for delivery of large macromolecules

is also quite unstable when stored for long periods of time. Thus, in order for these vesicles to be

manufactured into a viable product, the liposomes must somehow be stabilized in a gel-like

media. New information we learn in the 24 months of experimenting with the effect vesicle

properties on delivery characteristics will allow us to formulate similar designs.

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