Transdermal Delivery of Insulin: An Application of pH Sensitive Liposomes

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ABSTRACT OF THE THESIS

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Diabetes is a disease where the body either cannot produce insulin (type I) or the cells simply reject the insulin (type II). While type II can develop in adults, type I diabetes is more common in children and is harder to control. Since insulin is not naturally produced in type I diabetics, synthetic insulin has been developed to be administered to the body and control blood sugar levels. This administration is usually done through a pump which is not only uncomfortable but is also bulky. The aim of this study is to determine if liposomes can be used to effectively administer insulin transdermally by using the pH gradient between the stratum corneum (pH 4.0) and the bloodstream (pH 7.4). Evaluation of encapsulation efficiency and time-release of liposomes was performed using a Pierce BCA protein assay. At each time point, the released insulin was separated using size exclusion chromatography. The results of this study show that lyophilized liposomes can encapsulate about 60% of insulin. Liposomes retain their contents in acidic pH values while all of their contents are released within one hour at pH 7.4.

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SECTION 1: INTRODUCTION

Liposomal delivery of proteins and peptides is now being more widely studied. Park et al. explained that because these drugs have short half- lives and are highly unstable, the patient needs to take frequent injections, resulting in much discomfort and tenderness at the site of administration[5]. Because of this, alternative methods are being developed to deliver these peptides. To overcome this issue, others have used liposomes to increase the half-life on insulin in the body to provide less frequent injections, to deliver insulin orally[5,9] or deliver inhalable insulin[1]. Transdermal delivery of insulin is potentially advantageous because it provides a pain free alternative to injections while still providing the rapid release similar to injections. Also, there is no bioavailability issue as there is for oral delivery and therefore whatever amount of insulin is loaded into the liposomes will be released directly into the bloodstream. Because of the hydrophilic nature of proteins, passive delivery of these macromolecules alone through the skin barrier is impossible. Technology such as microneedles, electrophoresis, sonophoresis and iontophoesis could be used to deliver proteins transdermally[3]. This study focuses on providing an alternative transdermal delivery mechanism, using pH sensitive liposomes to release peptides (with insulin as a model drug) transdermally. pH sensitive liposomes were studied previously to deliver antitumor drugs using a pH gradient from 7.4 (bloodstream) to 4.0 (tumor environment). This study aims to use the reverse of this gradient, retaining at 4.0, the pH of the stratum corneum, and releasing at 7.4, the pH of the bloodstream. To provide an optimum efficacy and cost effectiveness, the liposomes need to have a few important properties which were studied: high association of insulin, high retention at low pHs, fast release at neutral pHs and fast skin permeation.

SECTION 2: MATERIALS AND METHODS

2.1: Materials

Materials: Insulin from bovine pancreas powder, sucrose for 200 mg/mL solution, citric acid, sodium phosphate, Sephadex G50 powder and HCl were purchased from Sigma Aldrich (St. Louis, MO). The Pierce BCA assay was purchased from Thermo Fischer Scientific (Grand Island, NY). The lipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG), 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DMPS), Cholesterol, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-lipid), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (16:0 Rhodamine)were purchased from Avanti Polar Lipids (Alabaster, AL) and47mm diameter Strat-M membranes were ordered from EMD Millipore (Billerica, MA).

Instruments: A Beckman Coulter DTX 880 Multimode Detector and a Long Island ScientificFluoroMax-2 were used throughout the studies.

2.2: Methods

2.2a: Preparation of Insulin Solution

A 1 mg/mL stock solution was prepared by dissolving the powdered insulin into the 10 mM HCl solution. The pH was then bought up to 4.0 ± 0.1. This is the pH at which encapsulation took place.

2.2b.1: Preparation of liposomes- Dehydration-Rehydration

This method was developed by combining the methods from papers by Mugabe et al, Karve et al, and Muppidi et al.[4,7,8] A 10 mM solution of liposomes was prepared. The compositions prepared for experimentation contained DMPC:DPPG:14 PEG:Cholesterol:Rhodamine at 6.65:2.85:0.2:0.3:0.004 mole ratios and DMPC:DMPS:14 PEG:Cholesterol:Rhodamine at the same mole ratios. The rhodamine-lipid was added to enable visual observation of fraction of liposomes eluting from the G50 size exclusion chromatography column when doing association and release runs, and to measure concentrations in skin penetration experiments. Two mixtures of the same composition were prepared: one for the background of the assay and one for the sample reading. After the lipids were mixed in this composition, the chloroform was evaporated using a rotovap and the dried lipid films were then hydrated with 1 mL double distilled water and a mass of sucrose equal to that of the lipids. The solutions were then left to anneal for two hours at 60°C. After annealing, the liposomes were kept in a -80ºC freezer for 15 minutes until the solutions were frozen, and then was left to lyophilize overnight. The next day, one set of liposomes was rehydrated with 1mL buffer solution of pH 4.0 and the other was hydrated with 0.5 mL of the stock solution and 0.5 mL of the pH 4.0 buffer (resulting in a total insulin concentration of 500 µg/mL). The liposomes were then left to sit for 30 minutes and then extruded 21 times through two 100 nm membranes. After extrusion, the liposomes were separated from the unassociated insulin using a Sephadex G50 column at pH 4.0. A 1 mL fraction was collected of both the background and the sample. A volume of 0.33 mL of the background liposomes was mixed with 0.67 mL of pH 4.0 buffer. A

volume of 0.33 mL of the insulin sample was also mixed with 0.67 mL of buffers of pH 4.0, 5.0 and 7.4 respectively.

2.2b.2: Preparation of Liposomes- Passive Encapsulation

Two mixtures of 10 mM liposomes were prepared: one for the background of the assay and one for the sample reading. After the lipids were mixed in this composition, the chloroform was evaporated using a rotovap and the dried lipid film was then hydrated, the background liposomes with 1 mL pH 4.0 buffer, and the sample with 1 mL of the insulin solution. The solutions were then left to anneal for two hours at 50°C. After annealing, the liposomes were extruded 21 times through two 100 nm membranes and then separated from the unassociated insulin using a Sephadex G50 column at pH 4.0 for both the background and the sample. A 1 mL fraction was collected of both the background and the sample. A volume of 0.33 mL of the background liposomes were mixed with 0.67 mL of pH 4.0 buffer. A volume of 0.33 mL of the insulin sample was also mixed with 0.67 mL of buffers of pH 4.0, 5.0 and 7.4 respectively.

2.2b.3: Preparation of Liposomes- Electrostatic Association

Two mixtures of 10 mM liposomes were prepared: one for the background of the assay and one for the sample reading. After the lipids were mixed in this composition, the chloroform was evaporated using a rotovap and the dried lipid film was then hydrated with 1 mL pH 4.0 buffer. The solutions were then left to anneal for two hours at 50°C. After annealing, the liposomes were extruded 21 times through two 100 nm membranes and then separated from the unassociated insulin using a Sephadex G50 column at pH 4.0 for both the

background and the sample. A 1 mL fraction was collected of both the background and the sample. Enough liposomes were then added drop wise (vortexing after each drop) to 1 mL an insulin solution to neutralize the effective charge of the protein and an equal volume was added to 1 mL of the pH 4.0 buffer. The liposomes were then separated once more through the G50 and then a volume of 0.33 mL of the background liposomes were mixed with 0.67 mL of pH 4.0 buffer. A volume of 0.33 mL of the insulin sample were also mixed with 0.67 mL of buffers of pH 4.0, 5.0 and 7.4 respectively.

2.2c: Preparation of G50 Column

Four G50 columns were prepared, including: two columns at pH 4.0 (one for the background and one for the sample), one column at pH 5.0 and one column at pH 7.4. The buffer at the respective pH was used to make the column. In each column, 1.86 g of the Sephadex G50 powder was added to 35 mL of buffer. The solution was then run through an empty column until the gel settled.

2.2d: Preparation of BCA Assay

A 1:50 reagent B: reagent A solution was made. Six standard solutions were prepared by first diluting 1mL of the insulin stock solution by 2 and then diluting each resulting solution by 2 until six standards were made. The pH 4.0 buffer was used as a blank for the assay. Two wells of a 96 well plate were used for each standard and sample reading. In each of these wells, 200 µL of the reagent mix was pipetted along with 100 µL of each standard and sample. The plate was left in a 37ºC incubator for 3 hours. The reading was then taken using a plate reader using a 462 nm emission wavelength.

2.2e: Preparation of Buffer

A 2L stock solution of pH 4.0 was made of the buffer. The buffer consisted of 0.1 M citric acid and 0.2 M sodium phosphate. Also added was 1 mM EDTA, a chelating agent. The pH of the buffer was adjusted to 4.0 ± 0.1 and the osmolarity was brought to 300 ± 20 mOsm.

2.2f: Skin Penetration Evaluation

Due to budget constraints, the following setup was used to evaluate the diffusion coefficient of liposomes through a skin substitute. A static Franz cell-like setup was used to evaluate skin penetration. The Millipore Strat-M^{TM} membrane has a similar diffusivity and physical characteristics to the skin and therefore this membrane was chosen for this experiment. A membrane was cut into 4 pieces and one piece was put onto a 12 well plate cell crown and then fitted by an o-ring. The effective area of diffusion was 1.54 cm^2. In a 12 well plate, 2 mL of buffer at pH 7.4 was placed as the receptor fluid. The cell crown setup was suspended above the bottom of the plate. At time points of every half hour up to three hours, 40 µL of receptor fluid and 20 µL of the donor fluid were taken to take a fluorescence reading to quantify the percentage of liposomes that went through the membrane. Fresh buffer was replaced at each time point. The diffusion coefficient was measured by plotting the liposome flux against the ratio of the difference in lipid mass in the top and bottom compartments to the thickness of the membrane and taking the negative slope of the linear trend line.

SECTION 3: RESULTS

For the delivery of insulin to be effective, three aspects need to be considered: extent of insulin association by liposomes, rate of release of insulin from liposomes, and penetration of liposomes and of insulin through the skin barrier. In this study, the extent of association and the rate of release of insulin from liposomes were studied.

3.1 Extent of Association

The insulin association for different compositions of liposomes was compared. This comparison was performed with the aim to determine the maximum amount of insulin that could be obtained within liposomes. Figure 1 summarizes the results of this comparison.

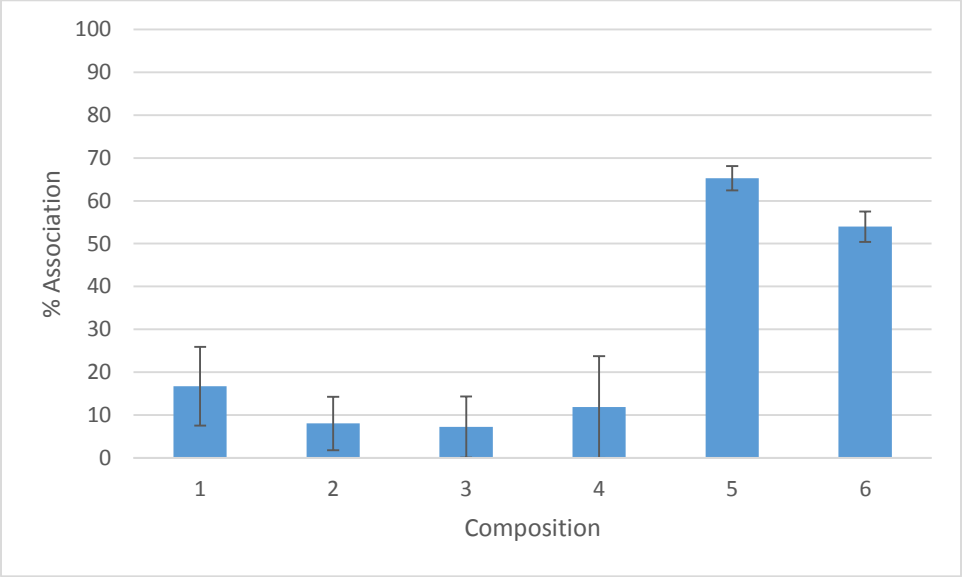

Figure 1: Comparison of Extents of Encapsulation of Insulin for Different Compositions. Errors correspond to standard deviations of N measurements (which varies for each preparation). Compositions corresponding to preparations numbered 1 to 6 are shown on Table 1.

Table 1: Extent of Insulin Loading in Liposomes (Insulin Loading= 500 µg) (N is number of preparations)

Number	Composition	Mechanism of Encapsulation	% Association	N
1	7:3 Egg PC: Cholesterol + 2% PEG + 5% Cholesterol	Passive Encapsulation	16.70 ± 9.20 %	3
2	7:1.5:1.5 DMPC:DLPA:14 TAP +2% 14 PEG + 5% Cholesterol	Passive Encapsulation	8.00 ± 6.22 %	8
3	7:3 DMPC:DPPG + 2% 14 PEG + 5% Cholesterol	Electrostatic Association	7.20 ± 7.10 %	3
4	7:3 DMPC:DPPG + 2% 14 PEG + 3% Cholesterol	Passive Encapsulation	11.85 ± 11.89 %	6
5	7:3 DMPC:DPPG + 2% 14 PEG + 3% Cholesterol	Dehydration-Rehydration	65.27 ± 2.86 %	6
6	7:3 DMPC:DMPS + 2% 14 PEG + 3% Cholesterol	Dehydration-Rehydration	53.96 ± 3.54 %	2

From Figure 1, it is clear that lyophilization greatly increases the association of liposomes with insulin. In a 99% confidence interval, the association means of the lyophilized PCPG and PCPS liposomes were statistically similar to each other and higher than the means of the other compositions. Therefore, PCPG and PCPS compositions were chosen for further release studies because of the hypothesized electrostatic mechanism of release. To prove this mechanism, a composition of PC-TAP liposomes, which are cationic throughout the pH range, was made and encapsulation rate was tested. The results of this experiment, summarized in Table 2, showed that there was no insulin encapsulated in this composition and this implies the electrostatic mechanism. Within a 99% confidence, the association of insulin throughout the three pHs for this composition was the same. This is because the buffer in which insulin would be associated was pH 4.0 and this sample after being

separated from the unassociated insulin was split into the three pH buffer solutions. Therefore if insulin didn't bind initially, no association would be shown at any of the three pHs. The charge of the insulin has been monitored and summarized in Table 3.

Table 2: Insulin Association of 7:3 PC:14 TAP Liposomes

	% Insulin Association (n=3) p>0.01
pH 4.0	-5.63±2.87
pH 5.0	-6.13±0.04
pH 7.4	-6.98±1.54

Table 3: Charge of Insulin (pI= 5.3) Throughout the pH ranges

Amino Acid	pKa of Side Chain	Number of Residues/ Insulin Molecule	Total Charge* at pH 7.0	Total Charge at pH 4.0	Total Charge at pH 5.0	Total Charge at pH 7.4
Alanine	-	1	0	0	0	0
Arginine	12.5	1	1	1	1	1
Aspargenine	-	3	0	0	0	0
Cysteine	8.3	6	0	0	0	0
Glutamine	-	3	0	0	0	0
Glutamic Acid	4.3	4	-4	0	-4	-4
Histidine	6.0	2	0	2	2	0
Isoleucine	-	2	0	0	0	0
Leucine	-	6	0	0	0	0
Lysine	10.8	1	1	1	1	1
Phenylalanine	-	3	0	0	0	0
Proline	-	1	0	0	0	0
Serine	13	3	0	0	0	0
Threonine	13	3	0	0	0	0
Tyrosine	10.1	4	0	0	0	0
Valine	-	4	0	0	0	0
Glycine	-	4	0	0	0	0
Total/Net Charge	-	51	-2	4	0	-2

Total charge = (the charge of the amino acid at the specific pH)(the number of residues in the insulin molecule).

Also, to see how the amount of insulin associated with the liposomes changed with the amount of insulin in the rehydration solution, a 1 mL containing 1 mg insulin was used to rehydrate a PCPG liposomes composition. It was found that percent of insulin associated dropped to 29.7% of the amount of insulin in the rehydration solution. The mass of insulin that associated with the liposomes though was 296.4 µg which is similar to the mass of insulin associated with loading 500 µg of insulin in the rehydration solution (326.3±14.3 µg for the PCPG composition and 269.8±17.7 µg for the PCPS composition). From this result, it is hypothesized that roughly 300 µg of Insulin will occupy all the binding sites of the liposomes and so no more Insulin would be able to bind.

3.2 Release Studies

Figure 2 shows the release profile of a PCPG liposome composition.

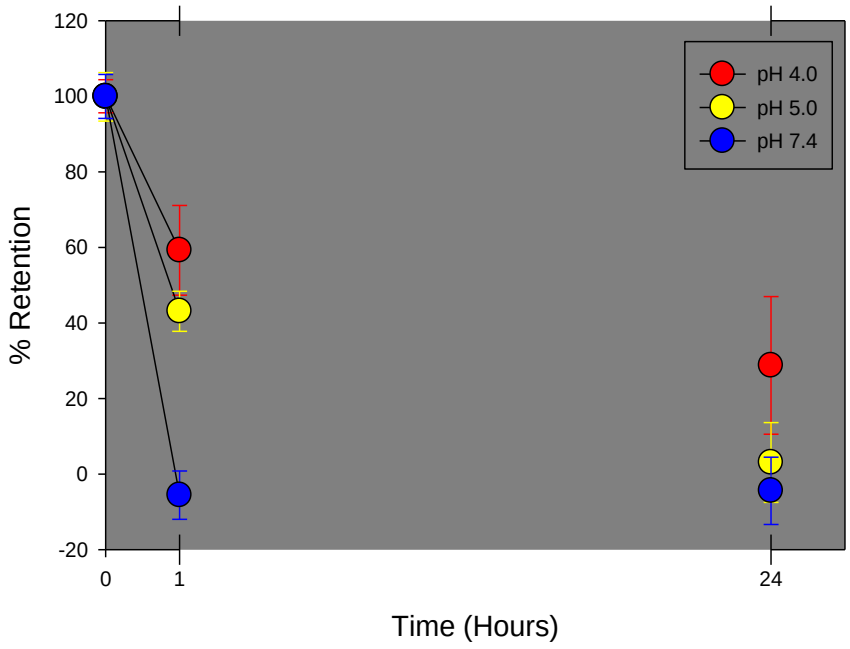

Figure 2: Retention profile of Lyophilized PCPG Liposomes. Errors Correspond to standard deviations of repeated measurements (n=6)

As seen from figure 2, the release of insulin from the liposomes is very fast. Within an hour, all of the associated insulin is released in a pH 7.4 buffer solution. An unfavorable result is that the amount of insulin retained at the acidic pHs is about half of what was encapsulated. To try and increase retention, the cholesterol level was increased from 3% to 10% since cholesterol increases the rigidity of the liposomes and therefore would possibly help retention. The release of insulin from this composition after one hour is shown in figure 3.

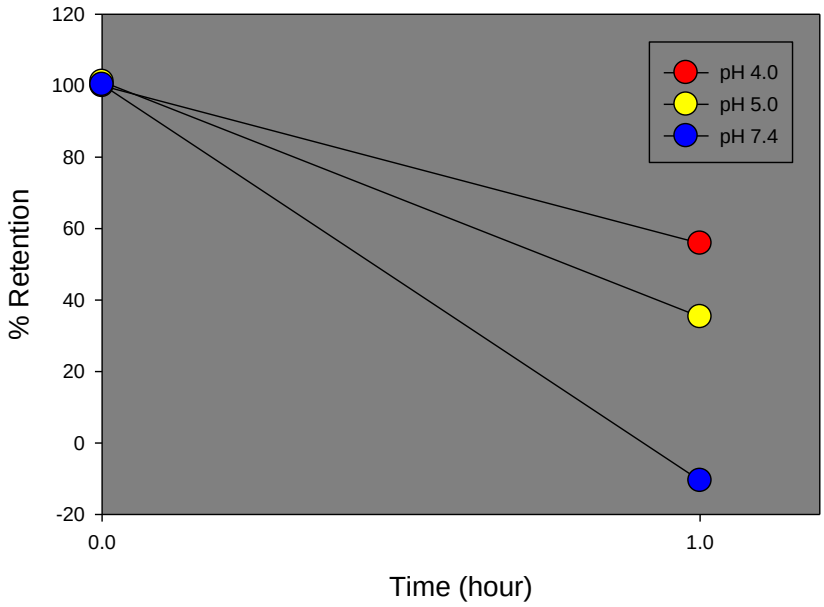

Figure 3: Retention of Insulin PCPG Liposomes with Increased Cholesterol (n=1)

Figure 3 shows that as the rigidity of the liposomes is increased, the retention after one hour is not affected. The association of insulin also dropped to 45% and so increasing the cholesterol decreased the association of insulin. Since it seemed that cholesterol didn't affect the retention profile of the PCPG composition, a PCPS composition was used to compare against the PCPG composition. The retention profile is shown in Figure 4.

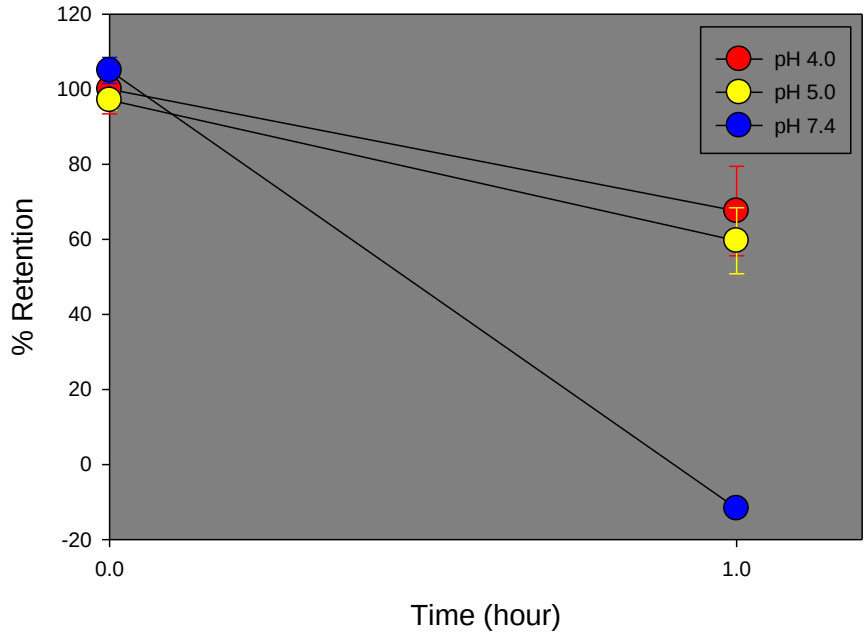

Figure 4: Retention of insulin by Lyophilized PCPS Liposomes. Errors Correspond to standard deviations of repeated measurements (n=2)

From figure 4, it can be concluded statistically, in a 99% confidence, that the PCPS composition retained the same amount of insulin as the PCPG composition at acidic pHs and still released all of the insulin at pH 7.4. The amount of insulin that associated with the PCPS composition was also not that much lower than the PCPG composition.

3.3: Skin Penetration Studies

Skin penetration studies using a franz cell-like setup (shown in figure 5) were attempted though the results were inconclusive. Figures 6 and 7 show the results of plotting the flux against the concentration gradient of the PCPG and PCPS samples using the fluorescence data collected at the time points. The diffusion coefficient found for the PCPG composition from this data was $-7.55*10^{-4} \pm 6.85*10^{-4}$ cm^2/hr and the diffusion coefficient for the PCPS composition was found to be $2.07*10^{-3} \pm 3.73*10^{-3}$ cm^2/hr. Because the two experiments

were carried out at different temperatures to attempt to minimize evaporative loss in one of the experiments, the diffusion coefficients for the compositions cannot be accurately compared though with the numbers obtained, in a 99% confidence, the diffusion coefficients are the same.

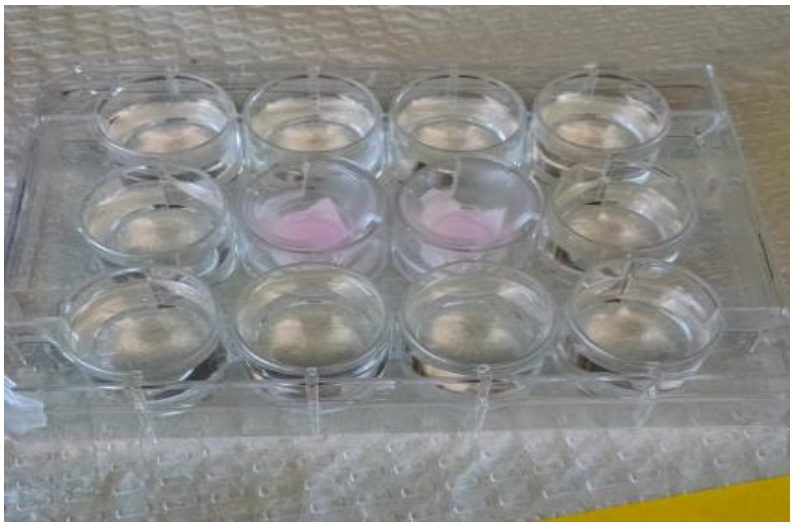

Figure 5: Setup Used for Skin Permeation Experiments

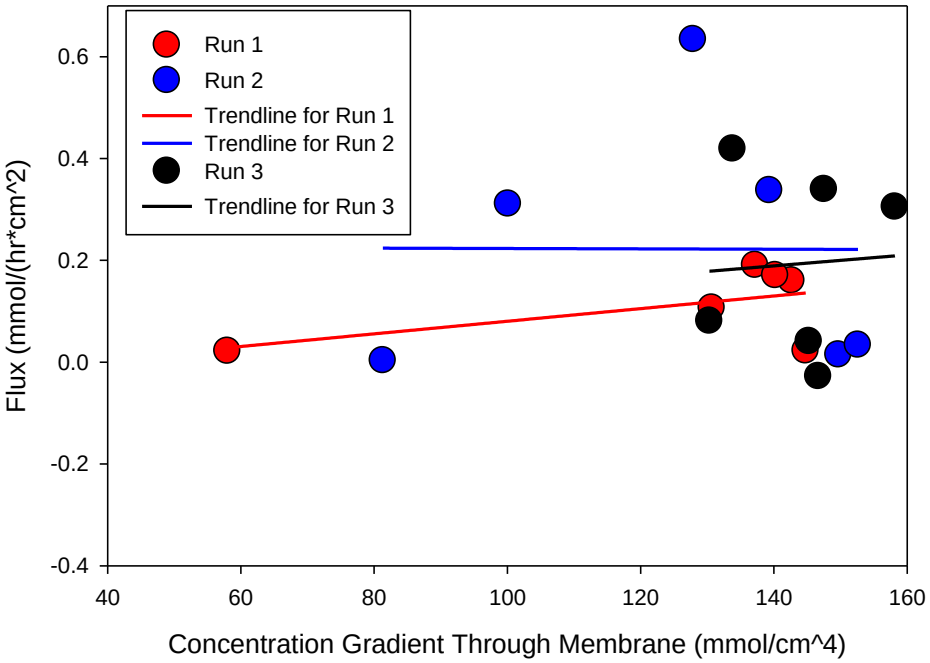

Figure 6: Skin Penetration of DMPC:DPPG Liposomes at 37°C (n=3)

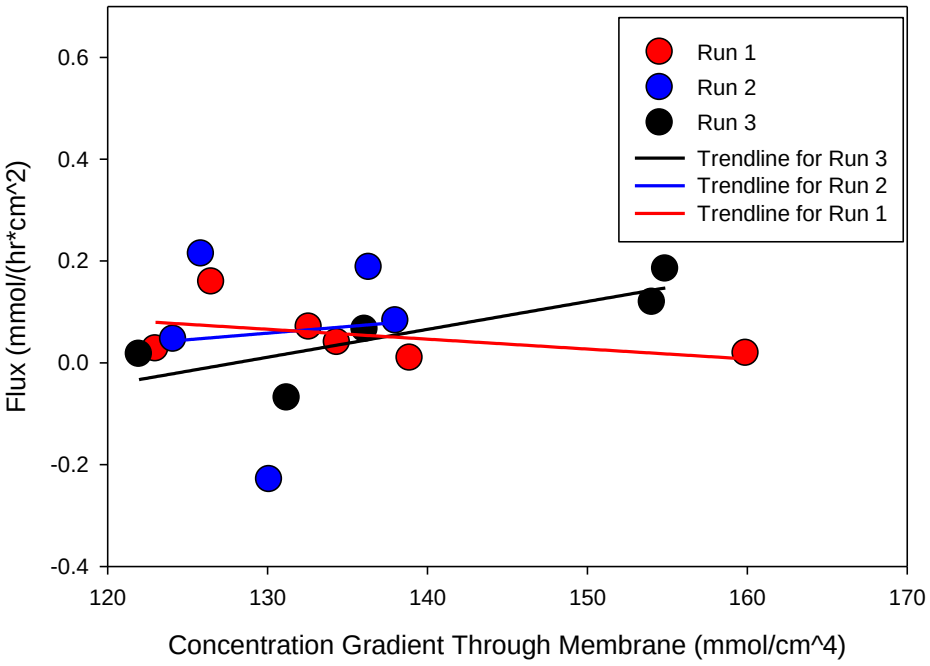

Figure 7: Skin Permeation of DMPC:DMPS Liposomes at Room Temperature (n=3)

SECTION 4: DISCUSSION

4.1: Formulation Challenges

Some incompatibilities with insulin were observed throughout this process. The first is that though lyophilization helped insulin association in the PCPG case, when the DPPG was replaced by DLPA, the lyophilized liposomes became incompatible with the insulin and the insulin solution had a gel-like consistency that would not elute through the G50 column. This effect was not noticed in the PCPA liposomes rehydrated with only the buffer which leads to the theory that these liposomes may have aggregated upon rehydration of the insulin solution. Further incompatibilities with insulin arose when the salt concentration of the insulin stock was raised to 300 ± 20 mOsM using sodium chloride. This resulted in a precipitate crashing out of the solution and so experiments were carried out with the insulin stock solution having an osmolarity of roughly 30 ± 5 mOsM. This would result in an osmotic pressure gradient when the liposomes went through the size exclusion column but the osmolarity of the liposome fraction that came out of the column was 300 mOsM. Conducting a BCA assay on the supernatant of the high salt insulin solution showed that less than half of the insulin was still in the supernatant and so the precipitate may contain the balance of the insulin complexing with the salt.

4.2: Advantages and Disadvantages

PCPG and PCPS liposomes are anionic throughout the 4.0-7.4 pH range. The insulin on the other hand is cationic at pH 4.0 (when it is being encapsulated) and turns anionic at pH 7.4 (when it should be released). At pH 4.0, the cationic insulin would be attracted to the anionic liposomes and this would promote insulin association. The amount of insulin

associated in these liposomes are comparable to other liposomes that have been formulated for oral delivery. Park et al found that their liposomes that were hydrated with only PBS buffer had an encapsulation efficiency of 50.3 ± 2.2%[5]. Zhang et al found that biotinylated liposomes had an encapsulation efficiency between 35% and 42%[9]. The liposomes made for this study had an encapsulation efficiency of about 60%, similar to that found by Bi et al, who used a spray freeze drying method to prepare insulin loaded liposomes[1] for pulmonary delivery. The advantage for these liposomes is that the release of insulin is a lot faster. In the Park paper, the PEGylated liposomes took about 72 hours to achieve 100% release[5]. In this study, all of the insulin was released within one hour, which is a preferable result for transdermal insulin. This could be due to the strong electrostatic repulsion releasing the insulin quickly or it could also be that the insulin is not only on the inside of the liposomes, but also is bound to the surface of the liposome. The retention of these liposomes needs some work but on a mass basis, both the liposomes retained about the same amount on insulin after one hour. The authors of the Bi paper found that increasing the lipid: sucrose ratio to 1:6 helped with retention and this may be help retention in these liposomes at acidic pHs[1].

4.3: Skin Permeation Experimental Error

The only result from the skin permeation experiments that was conclusive was that the liposomes were able to diffuse through the membrane. By Fick's first law, plotting the liposome flux against the concentration gradient across the membrane at each time point should have produced a linear trend line but this was not the case at all. Though the concentration in the bottom compartment increased, the concentration in the top did not

change much at any time point. The conservation of mass also did not hold according to the fluorescence measurements. One main source of this error was that evaporation in the top compartment was not accounted for. To try and minimize evaporative loss, the wells surrounding the wells with the samples were filled with distilled water, the incubator was kept humid, and the sides of the well plate were tightly sealed with parafilm. Experiments were also carried out at room temperature. These adjustments reduced evaporative loss slightly but not enough to produce a linear trend. Another error could be water going through the membrane. Since the cell crown was suspended in the well, if the liquid levels weren't equivalent, the water in the top compartment could have been driven down through the membrane due to the osmotic pressure difference. The latter effect probably better explains why there is so much error in the room temperature experiments. Because throughout the process the volumes in both compartments are not constant, the concentration throughout the process is constantly getting more concentrated and this would cause a lot of fluctuations in the diffusion coefficient. Further studies would need to be conducted with conclusive data to see of the liposomes could really penetrate the stratum corneum. The 500 Dalton rule for skin penetration states that molecules weighing more than 500 Daltons would not be able to pass through the stratum corneum[2]. Liposomes have a molecular weight well above 500 Daltons but were able to slowly penetrate through the Strat-M membrane. This could be because the liposomes like the stratum corneum have a lipid bilayer structure, allowing for easy incorporation into the skin barrier. The Strat-M^{TM} membrane is impregnated with synthetic lipids and so this effect may be seen when using this membrane. Another explanation to this phenomenon is that the liposomes are not very rigid. The liposomes gel-like properties may allow them to squeeze through

the pores in the skin and pass through. Though the error associated with this experiment was very high, it did show a low diffusion coefficient. This may have been because the conditions in the bottom compartment were static and so only passive transport was occurring. Simulating a flow environment in the bottom chamber may increase the diffusivity of the liposomes through the membrane due to the added convention and the changing concentration gradient and this would also provide a more realistic model to what happens in the body. If this still results in a low diffusivity, one method that may work to effectively deliver the liposomes through the skin barrier would be to combine the use of liposomes with ultrasound. This would "disorganize the lipid bilayers in the stratum corneum"[6] and provide easier transport of the liposomes through the skin barrier.

SECTION 5: CONCLUSION

Transdermal insulin delivery is a promising alternative for patients with type one diabetes as it is an effective pain-free alternative that rapidly releases the insulin in the pH environment of the bloodstream. PCPG and PCPS liposome compositions were tested for insulin association, retention, and skin permeation. It was found that lyophilization helped insulin association and the retention of the liposomes at acidic pHs was fairly high and the liposomes released all the insulin within the first hour. Skin permeation experiments were inconclusive because of the setup of the experiment but moving forward, a few adjustment could be made. Some recommendations for improvements to this experiment are using a Franz cell that allows for flow in the bottom compartment and jacketing the bottom compartment so that the temperature of the bottom compartment alone can be 37°C while the top compartment is kept at room temperature. Because of the head space in the well plate setup, water may have been allowed to move from the top to the bottom compartment. In a Franz cell, the bottom compartment is completely filled and so only the liposomes would be diffusing through the membrane. All in all, this study provided promising results for using liposomes for transdermal delivery of insulin and this could be used for other proteins using a similar method.

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