THE EFFECT OF LYSOPHOSPHATIDYCHOLINE INCORPORATION ON LIPOSONAL MEMBRANE FUSION AND RUPTURE

By

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To my Mom and Dad
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I would like to thank my parents for their help and encouragement. I would also like to thank Dr. Hughes for his help and guidance as well as the rest of the Pharmaceutics Department at the University of Florida for helping me when needed.
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THE EFFECT OF LYSOPHOSPHATIDYCHOLINE INCORPORATION ON LIPOSOme FUSION AND RUPTURE

By

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The goal of nucleic acid delivery to cells has been introducing DNA or RNA into a cell via a vector leading to a successfully transfected cell. This outcome can be silencing a gene or delivering a gene which is deficient in a certain cell type. Non-viral vectors are usually taken up by the cell through endocytosis, entrapping the vector and nucleic acid complex inside of the endosome. This endosome has the unique characteristic of having an acidic environment. If the DNA or RNA does not escape the endosome, it will be degraded in this harsh environment. Here we have used the lower pH of the endosome as a trigger for this release by using N-dodecylimidazole, a pH sensitive molecule which can intercalate in the liposomal membrane. While this shows an improvement over more traditional liposomes, we investigated the effects of an added lysolipid component to the lipid bilayers. While there was an effect observed with respect to membrane fusion by incorporating 10 mol% lysolipid, an even larger effect was seen with relation to membrane leakage. Across all samples the incorporation of lysolipid improved the release of the aqueous contents of liposomes over samples containing no lysolipid. Future studies involving varying molar ratios of lysolipid could further elucidate the overall effects and determine if there is a concentration dependent effect.
CHAPTER 1
INTRODUCTION

Introduction

Nucleic acids have been the focus of many groups wishing to alter the expression of genes, both in silencing and up regulating expression (Freese, 1972). This is an attractive endpoint since genetic diseases could theoretically be cured as opposed to simply masking symptoms as current small molecule drugs do. The main two methods to deliver oligonucleotides are based on viral and non-viral carriers. While viral carriers are very efficient in transfection, they pose a safety issue due to factors such as immunological response and genome integration (Maguire-Zeiss & Federoff, 2004). On the other hand, non-viral vectors face challenges such as low transfection efficiency due to physiological barriers. These non-viral vectors often times become entrapped within the endosome after they are internalized in the cell and if they are released, diffusion through the cytoplasm to the nucleus is very slow (Lechardeur & Lukacs, 2002). Therefore, increased endosomal escape may allow more efficient transfection of cells since oligonucleotides will not be degraded inside of the harsh endosomal environment.

Objective

The primary objective of these initial studies was to determine if the addition of a lysolipid component had an effect on the membrane properties of liposomes in vitro.

Hypothesis

The hypothesis of this study was that while the pH sensitive molecule N-dodecylimidazole acts as a trigger for liposome fusion and membrane leakage at acidic pH values, the addition of a lysolipid can further increase these effects.
Liposomes

Liposomes have been investigated for quite some time for their ability to carry a multitude of drugs, both hydrophobic and hydrophilic (Papahadjopoulos et al., 1985). This is due to their unique membrane properties. The internal compartment of a liposome will contain hydrophilic solutes which were dissolved in the aqueous medium when liposome formation took place. Hydrophobic molecules may also be incorporated into the liposomes by introducing them to a dried lipid film before rehydration (Heirwegh et al., 1988). During this process these molecules will be placed into the lipid bilayer instead of in the aqueous internal compartment. This formation is based on rehydrating a dry film of lipids to spontaneously form liposomes. This is due to the hydrophobic interactions of the phospholipid tails as well as the hydrophilic head groups through hydrophobic interactions (Igimi & Murata, 1983). This effect is similar to how a long-chain oil behaves in water. This hydrophobic effect causes aggregation of like molecules in water, and in the case of phospholipids will orient and pack the molecules to form a membrane which somewhat resembles the lipid bilayer of the cell.

The Endosome

Before covering the importance of these mechanisms by which a liposome will release its contents, the pathways which nucleic acids are delivered to a cell should be explained. The predominant pathway by which a liposome will enter a cell is by endocytosis (Somlyai et al., 1985). This consists of a cell enveloping a foreign particle with its own cell membrane to form a vesicle which will be brought into the cell. This vesicle is named the endosome and has several unique characteristics as well as changes over time. The first stage of an endosome after it has entrapped the foreign material is named the ‘early endosome.’ These are mildly acidic vesicles of
about 1 micrometer in diameter. Receptors that were on the cell membrane and now on the endosome membrane will dissociate from their ligands and at times leave the membrane (Kuronita et al., 2002). The next stage of endocytosis is the ‘late endosome.’ These endosomes further resemble lysosomes with a lower pH of about 5.5 and contain more degradation enzymes (Schmid, Ellinger & Kosma, 1998). The final stage an endosome will appear in is the lysosome, with an even lower pH of less than 5 and contain many digestive enzymes such as carbohydrases, proteases, nucleases, and lipases, among others. A lysosome containing these enzymes will obviously pose a problem for any nucleic acid or liposomal material still in the endosome at this point. This is where the common problem of endosomal escape of nucleic acids becomes a concern (Kjeken et al., 2004). If a liposome can enter a cell efficiently by way of endocytosis and then take advantage of the unique endosomal environment to escape, nucleic acids could potentially be protected until reaching the cell and then be realized before being degraded in the lysosome. In addition to this target of the endosome, the added effectiveness of liposomes over naked DNA or RNA has been proven in vitro and in vivo. Naked nucleic acids have difficulty reaching the cell nucleus and is not protected from enzymes which may degrade the molecule while cationic polymers provide a larger particle size which may be more easily internalized by the cell but are more cytotoxic (Kunath et al., 2003; Taniyama et al., 2002).

Liposomes as Nucleic Acid Carriers

Liposomes present an efficient delivery method for nucleic acids in addition to these small molecule drugs (Maurer et al., 1999). A main advantage of these liposomal carriers is the ability to protect the molecule being delivered from outside sources of degradation such as nucleases in the blood (Liu & Huang, 2002). In addition to this protective effect, an oligonucleotide can be delivered more specifically since the active agent will only be effective when it is released from
the liposome. If liposomes contain targeting ligands, this can also be advantageous if specific cells are of interest. This characteristic of entrapping material can be both beneficial as well as a concern for nucleic acid delivery. As mentioned before, the liposomes can protect these nucleic acids from degradation; however they must be released inside of the cell to have their effect. This has been a major issue for drug and nucleic acid delivery since many times the nucleic acids never escape the endosome after being internalized by the cell. In these studies the two distinct lipid bilayer properties which could correlate with nucleic acid efficiency are membrane fusion and membrane leakage.

**Lysolipids**

When investigating membrane fusion and leakage we would like to observe the effects of a single chain phospholipid, also called a lysolipid. These lipids are distinctly different from diacyl phospholipids in that they have a hydroxyl group in the place of the ester bond which connects the second carbon chain on a diacyl phospholipid (Figure 2-1).

![Figure 2-1. An example of the molecular structure differences between A) 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC); B) 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (MPPC or Lyso-PPC); C) 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (MSPC or Lyso-SPC); D) 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (MOPC or Lyso-OPC).](image-url)
This lack of a second carbon tail as well as the added hydroxyl group gives the lysolipid a more hydrophilic nature while still allowing it to intercalate into a lipid bilayer. It has been shown that these lysolipids have an effect on the liposomal membrane with respect to ion permeability at the lipid transition temperature (Mills & Needham, 2005). In this study the addition of a lysolipid component significantly increased the rate at which ions were released from liposomes at increased temperatures around the phase transition of the lipids used. We have attempted to investigate these processes further by using some similar lysolipid formulations and using pH as a trigger as opposed to temperature. This effect may be due to the formation of micelles within the membrane as well as decreased phospholipid hydrophobic tail interaction due to increased volumes which are unoccupied by lipid tails. In addition to the effect a lysolipid may have on a liposome, we will also provide a pH sensitive molecule to act as a trigger in the endosomal environment.

Liposomes exhibiting pH sensitivity have been investigated in the past via several methods (Connor, Yatvin & Huang, 1984; Wen et al., 2004; Zignani et al., 2000). Here a method by which an amphiphilic pH sensitive molecule can position itself in the liposomal membrane is utilized. The aim is to elicit an effect at a lower pH of around 5, the pH of a later stage endosome. The molecule chosen is N-dodecylimidazole for its pKa of 6.3 which will give sufficient ionization in a medium resembling the pH of the endosome. While not fully understood, in this ionized state imidazole molecules gain surface activity which could disrupt membranes (Liang et al., 2000). This could lead to increased permeability of membranes needed to release the contents of a liposome. Here the combined effects of a lysolipid as well as N-dodecylimidazole were evaluated as a method to trigger efficient release of the liposomal contents as well as observe any effects on membrane fusion and rupture.
CHAPTER 3
METHODS

Materials

Unless otherwise noted, all lipids were purchased from Avanti Polar Lipids (Alabaster, AL). The main lipid component used was 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), a synthetic saturated phospholipid containing two 16-carbon tails and a neutrally charged phosphatidylcholine head group. 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (Lyso-PPC or MPPC) is the lysolipid counterpart of DPPC containing only one alkyl chain and having a hydroxyl group in place of the other. Another diacyl lipid tested was 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) which is a saturated 18-carbon phospholipid along with its lysolipid derivative 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (Lyso-SPC or MSPC) and 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (Lyso-OPC or MOPC) which has the same single carbon chain length but also contains a double bond at the 9Z position.

Buffers used for pH sensitive experiments were 5mM TES, 100mM NaCl pH 8, 5mM MES, 100mM NaCl pH 6.4, and 5mM NaOAc, 100mM NaCl pH 5. All fluorescence measurements were obtained with a Bio-Tek Synergy plate multi-well plate reader (Winooski, VT).

Liposome Formation

All liposomes were made using a standard protocol of rehydrating a dried lipid film in a round bottom flask. This consisted of transferring the appropriate amounts of each lipid component dissolved in chloroform to a 50 or 100ml round bottom flask. Initial trials using calcium as a fusion trigger were composed of 90% 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) with 10% 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) as a negatively charged phospholipid.
The amount used for pH sensitive experiments was usually 30-40mg of lipid and 7-10 mol% of DI. The volume of chloroform was around 10ml in the round bottom flask, which was then attached to a rotary evaporation apparatus (Buchi, Switzerland). To evaporate the chloroform the water bath was kept at a slightly elevated temperature (approx. 30° C) while applying a vacuum. This formed a thin lipid layer on the walls of the round bottom flask which was then placed in a high vacuum for at least 1-2 hours for removal of any residual chloroform. The dried lipid film was then rehydrated with the appropriate medium (5mM TES, 100mM NaCl pH 8 buffer for pH sensitivity experiments) at an elevated temperature and agitation. At this point liposomes had formed in the aqueous medium This resulted in a final lipid concentration of 10-20mg/ml. After this liposome suspension was sufficiently rehydrated, the entire volume was passed 8 times through a high pressure LIPEX™ extruder (Northern Lipids Inc., Burnaby, BC, Canada) containing a 600nm polycarbonate filter. Liposome suspensions were then kept at 4° C and were stable for over a month.

**Lipid Mixing Assay for Fusion**

When performing lipid mixing (membrane fusion) trials the two fluorophores utilized were 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (DPPE-NBD) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (DPPE-Rh) (Figure 3-1).

![Figure 3-1](image.png)  
A) Molecular structure of DPPE-NBD; B) Molecular structure of DPPE-Rh.
The lipid mixing assay represents membrane fusion through the use of fluorescence resonance energy transfer (FRET). This method is based on donor and acceptor fluorescent molecules to vary the detectable levels of emission. In this trial one liposomal population was labeled with both NDB conjugated lipid and Rhodamine conjugated lipid at a molar ratio of 0.8% each. A separate liposomal population is then made which is identical except the fluorescent probes are absent. These formulations require no purification steps after formulation since the probes are intercalated into the lipid bilayer and can be used to measure membrane fusion (Struck, Hoekstra & Pagano, 1981).

The basis for choosing NBD and Rh are that NBD has an excitation and emission of 460nm/534nm, while Rh has an excitation and emission of 557nm/571nm. The overlap in these spectra causes the quenching of NBD fluorescence when the probes are within a close proximity of one another. When the labeled liposome population fuses with the blank population, the probes will be distanced from each other enough so the NBD will no longer take place in the FRET phenomenon, therefore increasing detectable fluorescence. At this point Triton X-100 (Sigma-Aldrich, St. Louis MI) is added to the medium which will solubilize liposomes. This will cause the distance between the NBD/Rh fluorophores to be increased to a point where neither molecule is within the effective FRET range. Therefore this addition of Triton X-100 increases fluorescence to a theoretical maximum which is then used to determine a fusion percentage achieved by the assay.

In initial calcium based fusion experiments, varying concentrations of Ca+2 was added to the external medium to determine the extent of fusion caused by the attractive force between the negatively charged 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) component of the lipid membrane and a divalent cation (Ingolia & Koshland, 1978). An experimental sample containing
10% lyso-OPC was utilized to determine if a lysolipid could have an effect on fusion when utilizing calcium as a catalyst.

DI was incorporated into all liposomal formulations at a 7-10 mol% to serve as a pH trigger for fusion in later experiments. pH triggered liposomes were mainly composed of DPPC with 10 mol% lyso-MPC serving as the complementary lysolipid. In certain cases cholesterol was incorporated to see if it had a bearing on liposome fusion in these trials.

**Calcein Leakage**

Calcein (Figure 3-2) (Sigma-Aldrich, St. Louis, MI) was used to perform any leakage assays from liposomal membranes. This molecule was chosen due to its ability to self quench fluorescence at high concentrations (over 50mM). This characteristic is important since a high concentration is retained within the internal aqueous compartment after liposome formation, therefore exhibiting little fluorescence. However, once the calcein leaks from the interior of the liposome it will be diluted in the external medium, leading to an increase in fluorescence signal. This detectable signal can be used to determine the percentage of calcein which has been released from the liposome. This is accomplished by solubilizing the liposomal membrane using Triton X-100. The addition of Triton X-100 will release all calcein from the liposomes which is then used to calculate the amount of calcein released in the experiment. Again the liposomes had DI incorporated to facilitate a pH dependent release of contents as well as one population containing 10 mol % lysolipid.

In addition to examining the effects of lysolipid incorporated into the liposome membrane, lysolipid presence in the external medium was examined to further determine the effect on liposome leakage. In this method the lysolipid may act as a surfactant on the lipid bilayer while dissolved in the aqueous medium. This would further explain how this component affects membranes.
Aqueous Content Mixing

Aqueous content mixing is also a measure of liposome fusion and can lend further insight into the extent at which liposomes fuse under certain conditions as well as support data from other assays. This assay employs the two compounds, p-xylene-bis-pyridium bromide (DPX) and 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (ANTS) (Figure 3-3). ANTS is a fluorescent molecule which is detectable via a fluorescence reader, however when DPX comes within close proximity, the level is significantly decreased (Duzgunes et al., 1985).
Figure 3-3. Molecular structures of A) ANTS and B) DPX. ANTS fluorescence is quenched in the presence of DPX which may be used as a measure of liposome fusion.

This assay which measures the internal aqueous content mixing between liposomes is another measure of liposome fusion. In this assay ANTS is the fluorescent dye which can be detected and measured. This was entrapped within one population of liposomes. This initial fluorescence reading can be viewed as no fusion between liposomes, then after this the second population containing DPX is added fusion can be measured. When the two liposome populations undergo a fusion event, the DPX will come into close proximity with the fluorescent ANTS. This will causes quenching of the signal, indicating fusion. This method is also helpful since a leakage of the contents in either population will not have an effect on the outcome.

**Phospholipase A<sub>2</sub> Assay**

Phospholipase A<sub>2</sub> (PLA2) was chosen due to its catalytic activity which targets the sn-2 acyl bond which connects the carbon tails to the phosphatidylcholine head group in the phospholipids being investigated here. The resulting products of PLA2 activity are primarily arachidonic acid and lysophosphatidylcholine. In this method we introduced pure DPPC liposomes containing calcein to an environment containing varying concentrations of PLA2 to determine the effect on membrane stability. This ability of PLA2 to catalyze the formation of lysolipid products from diacyl lipids could lead to leakage of the membrane, therefore releasing the entrapped calcein. Here Triton X-100 was used to release 100% of the calcein to determine a percent leakage for each trial.
CHAPTER 4
RESULTS

Lipid Bilayer Mixing

Lipid bilayer mixing was a measure of fusion used in these trials. As fluorescence values increased due to the FRET phenomenon, it was concluded that fusion had occurred. Trials based on pH fusion were more conclusive with respect to fusion than were calcium triggered experiments.

Calcium Induced Fusion

Initial results of the calcium fusion experiments mainly provided insight into how the fusion trials would proceed and if we could discern an effect from the lysolipid. First, trials were conducted to determine if the method was viable, using only DOPC:DOPS liposomes (9:1 molar ratio). After establishing that fusion could be measured using the FRET method (Figure 4-1), later trials were conducted with 10% lysolipid incorporated to determine if a difference would be seen.

Figure 4-1. Liposome fusion over time at varying calcium concentrations. As liposomes mix an increase in fluorescence is seen as relative fluorescence units (RFU). Samples contain no lysolipid.
The liposomes examined for calcium based fusion with lysolipid incorporated were identical to the previous trial except for the incorporation of 10 mol% lysolipid in one population. 5mM and 10mM calcium concentrations were employed to determine if an effect could be observed between groups. Initially, only a small and insignificant difference was seen between the lysolipid groups and the standard liposomes when 5mM calcium was used as a trigger (Figure 4-2). However, with the 10mM concentration of calcium and increasing the labeled to unlabeled population (L:UL) concentrations a difference was observed (Figure 4-3). The overall levels of fluorescence were also increased throughout the experiment when lysolipid was present in the bilayer.

Figure 4-2. Time course fusion assays consisting utilizing 5mM calcium as a trigger. An increase in relative fluorescence units (RFU) indicates an increase in fusion. The ratios indicate the amount of labeled liposomes (L) to unlabeled liposomes (UL) in the trial. A) 2:10 ratio of L:UL. B) 10:50 L:UL. Note the difference in scale.
Figure 4-3. Time course fusion assays utilizing 10mM calcium as a trigger. An increase in relative fluorescence units (RFU) indicates an increase in fusion. The ratios indicate the amount of labeled liposomes (L) to unlabeled liposomes (UL) in the trial. A) 2:10 ratio of L:UL. B) 10:50 L:UL. Note the difference in scale.

**pH Triggered Fusion**

While the calcium based fusion method provided a trigger for fusion, we were interested more in the pH sensitivity of a formulation since the target is the acidic environment of the endosome. Here an initial formulation using DPPC:DMPC:Cholesterol:DI (6:1:8 :0.4 molar
ratio) was employed to somewhat resemble the cell membrane. The lipid mixing assay was employed and therefore 0.8% NBD and Rh labeled lipids were also incorporated into the lipid bilayer. This demonstrated a higher detectable level of fluorescence when lipid mixing was increased. Overall, an increased signal was seen with the incorporation of 10% lyso-PPC, even at the initial pH of 7.4. However, a large difference was not seen with respect to pH in this trial (Figure 4-4).

![Fluorescence over time with respect to the FRET lipid mixing assay.](image)

Figure 4-4. Fluorescence over time with respect to the FRET lipid mixing assay. Higher fluorescence indicates a potentially higher level of fusion. pH 7.4 and 4 were examined with “Lyso” indicating the presence of 10% lyso-PPC in the liposome population.

Other formulations were examined to determine if an effect could be seen with respect to pH as well as incorporation of lysolipid. Therefore the liposomes were changed to a single phospholipid component, egg phosphatidylcholine (EPC), while increasing the ratio of cholesterol. The concentration of DI was also increased to have a final liposome composition of
EPC:Cholesterol:DI (7:8:3.2) with 10 mol% lyso-PPC in one experimental sample. With this formulation a significant (p<0.05) difference was seen between pH 7 and 4 as well as between samples which do and do not contain lysolipid (Figure 4-5). 100% fusion was established by adding Triton X-100 to the sample wells, using this method the overall percentage of fusion was determined. The overall fluorescence readings for this fusion experiment are shown in figure 4-6 displaying an increase in fluorescence with respect to pH as well as lysolipid incorporation.

Figure 4-5. Percent fusion between samples based on lysolipid presence in the formulation. pH 7 and 4 were used as a basis for simulating physiological pH and endosomal pH. Between ‘no lysolipid’ and ‘10% lysolipid’ groups p<0.05.
Figure 4-6. Fusion with respect to pH. pH is indicated under the sample bars. “Lyso” indicates incorporation of 10% lysolipid. Data displayed as relative fluorescent units (RFU) which increases with respect to fusion.

This trial was then converted to display a percentage fusion and to determine if there was a significant difference with respect to a percentage of the maximum after adding Triton X-100. A near significant difference in percent fusion was seen at pH 8 (p=0.07) and a significant difference at pH 5 (p<0.05) (Figure 4-7). This trial led to the utilization of a similar formulation
while still manipulating certain aspects to further determine if lysolipid has an effect in later experiments.

Figure 4-7. Fluorescent values converted to percent fusion based on pH of EPC:Cholesterol:DI samples. Samples containing lysolipid showed a significant increase in fusion at pH 5. * p=0.07, ** p<0.05.

Next a separate formulation using the synthetic lipid DPPC was used to add further insight. This formulation should be slightly more stable since the DPPC lipids contain a longer carbon chain and therefore will have a higher transition temperature. Again cholesterol was used in a ratio of DPPC:Cholesterol:DI (7:2:1) for stability. These liposomes exhibited an even greater difference in fusion levels with respect to the incorporation of 10% lyso-PPC (Figure 4-8).
Further manipulation of the formulation was done to determine if the effect of the lysolipid incorporation would be seen throughout the samples. With this formulation DMPC was used as opposed to DPPC as the main phospholipid component while still utilizing cholesterol. The formulation employed for this trial was DMPC:Cholesterol:DI in a 10:4:1 molar ratio with the experimental sample containing 10 mol% lysolipid. The results show that again there is a significant difference when lysolipid is present in the liposome bilayer (Figure 4-9).
A similar formulation was tested which contained dodecanol in the place of N-dodecylimidazole. Interestingly, this formulation also showed a significant pH dependence as well even without the ionizable imidazole head group of the DI. However the addition of lyso-PPC to the formulation again had a significant difference over samples containing no lyso-PPC (Figure 4-10).
Figure 4-10. pH based fusion of samples containing dodecanol instead of N-dodecylimidazole. Samples still show a difference based on lysolipid incorporation, however overall fusion levels are lower.

**Calcein Leakage**

Calcein leakage was a valuable tool to determine the effectiveness of lysolipid incorporated vesicles. Results from these studies were positive with respect to increasing triggered release of the entrapped liposomal contents. In most studies a large and significant increase in release was observed over liposomes without lysolipid incorporated.
Calcein Leakage with Lysolipid Incorporated Membranes

Calcein loaded liposomes were tested by measuring the amount released and related back to the presence of lysolipid incorporation in the liposomal membrane. The formulation tested included 30mg DPPC (27mg when lysolipid was incorporated), 1.5mg DI, and 3mg lyso-PPC (in the case of the experimental formulation) which was rehydrated with 1.5ml pH 8 buffer to a concentration of about 20mg/ml lipid. The results of the first trial show that lysolipid has a significant effect on membrane leakage when triggered by pH (Figure 4-11).

![Graph showing percent calcein release based on pH value and lysolipid incorporation]

Figure 4-11. Percent calcein release based on pH value as well as incorporation of 10 mol% lyso-PPC. p<0.05 between all groups lacking and containing lyso-PC.
The next step in determining the overall effect of the lysolipid with respect to calcein release was to examine samples which did not contain DI but did contain lysolipid. These combinations were tested and it was observed that the lysolipid still had a significant effect on calcein leakage even without DI incorporation (Figure 4-12).

Figure 4-11. Calcein leakage shown with and without lysolipid or DI and varying combinations. Lysolipid had an effect even without the ionizable DI to facilitate pH triggered leakage.
Calcein Release with Lysolipid in External Medium

The next calcein release experiments consisted of adding liposomes to an aqueous medium which contained varying concentrations of lysolipid. Here the lipid bilayer did not have the lysolipid incorporated. Again, release was measured by the amount of calcein released and a percentage was calculated based on the complete solubilization of liposomes after the addition of Triton X-100. The presence of lysolipid in the external medium had a pronounced effect on leakage from intact DPPC liposomes in all trials tested. Three lysolipids were tested in the process: lyso-SPC, lyso-OPC, and lyso-PPC. The oleoyl based lysolipid had the most pronounced effect, reaching almost 100% leakage, with lyso-OPC being lower at around 50% for the highest concentration tested. Lyso-PPC had the lowest effect on leakage and was the lysolipid used for all previously described studies (Figure 4-12).

Figure 4-12. The varying effects of the presence of lysolipid in the external medium on calcein leakage from intact DPPC liposomes. MOPC = 18:1, MSPC = 18:0, MPPC = 16:0.
Aqueous Content Mixing

To further explore the idea of membrane fusion, studies were conducted to determine if the internal contents of the liposomes would mix more or less, based on the presence of lysolipid. In this trial a decrease in fluorescence indicates the fluorophore ANTS is mixing with DPX. Therefore the internal contents must mix to facilitate this decrease. It was observed that the lysolipid had a slight effect at pH 6.4, however it was not significant. At pH 5 there was a larger difference which was nearly significant (p=0.056). The data here is explained through a decrease in fluorescence from the initial recorded value, indicating liposome fusion (Figure 4-13).

![Figure 4-13](image-url)

Figure 4-13. Measured fluorescence decreases with respect to pH for samples containing no lysolipid and 10 mol% lysolipid. (pH 5 p=0.056).

Phospholipase A<sub>2</sub> Assay

With increasing concentrations of PLA<sub>2</sub> an increase in calcein leakage was seen. Amounts of enzyme added to samples ranged from 0 to 40 enzyme units to intact DPPC liposomes
containing entrapped calcein. Samples showed a concentration dependent release percentage in all trials as well as time dependence (Figure 4-14).

Figure 4-14. Increasing amounts of phospholipase A2 showing an increase in calcein leakage over time. A significant concentration dependence existed between groups.
CHAPTER 5
DISCUSSION

Introduction

Reports have been published pertaining to the influences lysolipids have on certain membrane properties with relation to transition temperature (Sandstrom et al., 2005). Reported here are preliminary studies on the effects \textit{in vitro} with an attempt to determine the feasibility for gene delivery by taking advantage of an acidic environment. This study aimed to take advantage of the unique pH value of the endosome to trigger enhanced fusion and in addition to this, use the lysolipid to further facilitate fusion and rupture of the membrane.

Calcium Based Fusion

Initial trials aimed to use calcium as a trigger for liposomes containing phosphatidylserine head groups. Here the liposomes are actively brought into close proximity by the opposing charges. Therefore the effect of this attractive force may be so large that any effect the lysolipid has may be undetectable. Also calcium induced liposome fusion can cause visible aggregation (Miller & Dahl, 1982) and possibly convolute data. However, averages in all cases with lysolipid incorporation were higher than the samples lacking lysolipid. The formulations were then moved to a pH sensitive formulation since while the calcium based fusion was an established method, we were more interested in utilizing pH as a trigger. This allowed the study of a more suitable model which would be closer to the final formulation used to transfect cells in culture.

N-Dodecylimidazole Based Fusion

The incorporation of N-dodecylimidazole was chosen due to past literature showing it had an effect on liposomal membrane properties when subjected to varying pH levels. This also allowed the study of lysolipid incorporation in different ways, such as observing fusion and rupture without the actual physical attraction the calcium assay utilizes. This may further
resemble fusion processes which are occurring in actual cell transfections as opposed to an artificially induced fusion as seen in the calcium based method.

It was also observed that increasing amounts of DI were required to achieve a higher amount of fusion in certain trials. DI incorporation was 7-10% for studies comparing the different values. It would be of interest to increase the amount of DI in liposomes while keeping the molar ratio of lysolipid constant and vice versa. The amounts chosen here were based on some previously reported values of ionizable surfactants (Liang & Hughes, 1998). While the method of membrane disruption is not clear, at an acidic pH the surface activity of DI will increase. In this case the DI can be a part of liposomal membranes at neutral pH values and act as a surfactant at the lower pH. This is due to the ionizable imidazole head group and the long carbon tail attached which can intercalate into the hydrophobic area of the liposomal membrane. Overall the effect of the DI as a pH based trigger served its purpose in these studies and is explained in greater detail in the previously mentioned publication.

**Phospholipase A₂**

This assay is another approach to determine the effect that lysolipids have on the membrane properties of liposomes. As intact liposomes were incubated with phospholipase A₂, the enzyme catalyzed the production of lysophosphatidylcholine from diacyl lipids (Whalen et al., 1999). Therefore, as incubation time or concentration increases, more lysolipid will be produced. In this assay it was clear that as intact DPPC liposomes were exposed to phospholipase A₂ the amount of calcein leakage increased. This is most likely due to this production of lyso-PPC from DPPC lipids. It would be interesting to evaluate other liposome compositions such as the longer tail lipid DSPC or shorter tail DMPC.
Relation to the Endosome

The main goal of this study was to evaluate membrane properties in an acidic environment and initial data has shown that formulations tested here may have improved efficacy when content release is measured in cell culture after liposomes are internalized by endocytosis. Since previous studies utilizing N-dodecylimidazole increased transfection efficiency in cell culture, it may be reasonable that since the addition of lysolipid increased release even further that the formulation would be more effective with added lysolipid. However this must be studied in actual cell culture to make this assumption.

Concerns, Limitations, and Future Direction

An obvious concern with the formulation is the stability of the liposomes. With the addition of a lysolipid component, the membrane will most likely be less stable and therefore storage concerns may arise. A longer term stability study is of interest when changing membrane properties of liposomes to determine if the incorporation of lysolipids will limit feasibility. In addition to overall stability, utilizing other buffers would be of interest since many times significant changes in storage stability can be seen with relation to the buffer used (D'Silva & Notari, 1982). However, basic use of liposomes in studies conducted here showed that most formulations were stable over a period of two months or more. However, even with liposomes retaining their effectiveness in membrane fusion and rupture studies, a more controlled study simply observing shelf life should be considered.

Another concern is liposomal effectiveness when carrying nucleic acids and transfecting cells in culture. Small formulation changes must be made such as the addition of a cationic lipid to facilitate liposome-nucleic acid interaction and therefore other lipids need to be incorporated which have not been studied here. In addition to the cationic lipids needed to complex nucleic acids, an actual DNA or RNA molecule is physically and chemically different from fluorescent
markers utilized. Calcein has a low molar mass of 622.55 g/mol when compared to nucleic acids, making it more permeable to liposome membranes. Therefore, it may be released more easily when compared to larger nucleic acids. Also the electronic attraction between positively charged lipids and negatively charged nucleic acids may hinder affect release of the entrapped molecules. Many of these concerns should be addressed by carrying out cell culture transfections which is the next logical step in a study such as this.

An aspect of this formulation that was focused on here was the integration of a lysolipid component. In all trials 10 mol% was used to determine the effects. While this amount did elicit a significant effect, increasing amounts would certainly provide further insight into the overall effect of a lysolipid. This would be especially interesting during cell transfection to determine if a significant difference exists and if a concentration dependence exists with respect to transfection efficiency.

CHAPTER 6
CONCLUSION

While the trials completed are not entirely representative of actual biological membranes, it frames some groundwork for actual studies involving transfection with nucleic acids. This study also shows that lysolipids do have an effect on liposomal membranes and whether this will have an impact on cell transfection efficiency has yet to be determined. The lysolipid was seen to have a differing effect when measuring the different parameters we observed in vitro. In the case of calcein release a more distinct and larger difference was seen when compared to the lipid mixing assay. Therefore further study into how lysolipids affect membrane properties is certainly a viable option.


Mills, J.K., Needham, D. 2005. Lysolipid incorporation in dipalmitoylphosphatidylcholine bilayer membranes enhances the ion permeability and drug release rates at the membrane phase transition. Biochim Biophys Acta 1716:77-96


BIOGRAPHICAL SKETCH

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