Effect of number of extrusion passes on liposome nanoparticle size

Part I: Kimberly Nguyen
Part II: Renee Parker
Part III: John Fukuyama
Part IV: Thomas Arnold

Abstract

Liposomes are lipid vesicles that may be used as nanocarriers, with applications in diagnostics and therapeutic agents. Liposomes show promise of being able to maneuver the immune system to achieve specific biomolecular targets. As nanocarriers, liposomes are favorable due to their small size, allowing intermolecular forces to dominate. In this experiment, we varied the cholesterol content of 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) solutions as well as the number of extrusion passes through a 100 nm membrane to investigate how two experimental parameters are responsible for the particle size and polydispersity index (PDI) of liposomes. Although the size of the particles and its PDI showed no distinct trend as cholesterol content increased, particle size did decrease as number of extrusion passes was increased, with no distinct trend in PDI. The ideal particle size of 120 nm was not reached; however, we suspect slower extrusion passes and lower liposome concentration could achieve liposomes closer in size to the desired particle diameter.
1 Introduction

The field of medicine has historically employed effective drug delivery systems. The development and use of drug delivery systems is not without its obstacles, however. Often times, the therapeutic agents employed in drug delivery are undermined by the intermolecular forces at play in biological environments.¹ Nanomedicine is a broad and emerging field of medicine that seeks to employ nanoscale agents to more properly navigate biological environments by controlling for the same intermolecular forces that so often undermine larger therapeutic carrier agents.² One such nanoscale carrier agent is the liposome.

Liposomes are spherical nanoscale vesicles comprised of one or more amphiphillic lipid bilayers.³ The bilayers employ hydrophobic heads connected by hydrophilic tails, constituting the backbone of the bilayer. Liposomes may be small unilamellar, large unilamellar, or multilamellar. Between 1961 and 1965, Alec Bangham and R.W. Thorne pioneered the use and characterization of liposomes as therapeutic agents for drug delivery.⁴

As nanoscale vesicles, liposomes possess a critical application in the diagnostic and therapeutic industries as nanocarriers, having been developed in the treatment of diabetes, infections, and even cancer.⁵ The small size of liposomes as nanocarriers allow for intermolecular forces to dominate and for higher specificity and sensitivity in seeking out targets.⁶ The amphiphillic properties of liposomes may be used to carry both hydrophobic and hydrophilic cargo as well as improve the solubility of poorly water-soluble drugs.⁷ The manufacture of liposomes may be designed to manipulate structural qualities such as particle size, polymerization, and coating type.⁸

⁴Danilo.
⁷L. Zhang, “Nanoparticles in medicine: therapeutic applications and developments”.
⁸Zhang L, “How to stabilize phospholipid liposomes (using nanoparticles)".
Limitations to liposomes as drug delivery agents include its structural integrity and production cost. For example, vesicles may leak active drug if their stability is not well-controlled. As well, current manufacture of liposomes are laborious, involving multiple steps and high precision. Research into improving stability of liposomes is currently taking place at the University of California, San Diego. Professors and research staff here have successfully conceived and tested a process for manufacturing liposomes that remain stable for the longest duration studied (50 days).

2 Background

As stated before, liposome nanoparticles are a method of drug delivery that has recently gotten more attention in the nanomedicine field. Liposome nanoparticles can be formed by a variety of methods including: sonication, freezing-thawing, dehydration-rehydration, and extrusion. Although all of these methods are useful in creating multi-vesicular structures, our experiment utilized a combination of the extrusion methods. The extrusion method is particularly useful when it comes to creating uniformly sized small particles, between 50 and 180 nm, which is desirable for the formation of liposome nanoparticles. These small unilamellar vesicles are favorable because they have longer lifetimes, more stability, and can deliver to target cells more readily. Extrusions work by forcing a large vessel through a filter whose pore size is approximately that of the desired liposome particle size. This process is simple but the equipment is fragile and relatively expensive. Thus, this method is limited to small scale production or well-funded research facilities.

microscopy, sedimentation techniques. Electron microscopy is useful for the examination of individual particles however it is very time consuming and expensive. Sedimentation techniques are useful for testing a wide range of sizes, but they require constant attention and are not the best for quick results. Dynamic light scattering is quick, precise and versatile; however it collects only a fraction of the full electrons scattered dependent on where the sensor is located. Despite this disadvantage, dynamic light scattering is the best option given our time constraints and its simplicity.

In order to form liposome nanoparticles we used a two syringe, one filter extrusion process. Following the extrusion, the samples were analyzed through a DLS system. This data was analyzed and agreed with literature published previously. This extrusion process proved to create liposome nanoparticles within 100 and 180 nm. We can therefore conclude that this process is sufficient for creating a smaller vessels and a bimodal distribution of sizes. The correlation between number of extrusion and particle size, and the percentage of cholesterol and particle size was determined in this experiment.

3 Theory

Liposomes have unique properties that make them ideal candidates for drug delivery. They are composed of phospholipid bilayers that allow them to encapsulate hydrophilic agents in the core and hydrophobic agents within the lamellae. Phospholipids are comprised of a phosphorous molecule surrounded by two long carbon chains, and a hydroxyl chain. The long carbon chains cause hydrophobic interactions while the hydroxyl chain has a hydrophilic tendency, thus allowing for the creation of an amphipathic spherical vessel. The addition of DPLC and cholesterol is primarily for the stabilization of the spherical shape. DPLC and cholesterol are amphipathic and thus when agitated with water can form the liposomes tested in this experiment.

Dynamic light scattering (DLS) is used to determine the average diameter and size distribution for the liposomes created in the extrusion process. DLS utilizes the beam of a laser on a given set


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of particles and measures the intensity of the light reflected. The intensity of the light reflected is
dependent on the size of the particles getting lit. This allows us to classify the different sizes of the
particles. From this information, one can find the time dependence and therefore the diffusivity of
the molecule based on the Stokes-Einstein equation

\[ D = \frac{kT}{6\pi \eta \alpha} \]  

(1)

Where \( k \) is the amount of energy per molecule per degree Kelvin, \( T \) is the temperature, and \( 6(\pi \eta \alpha) \)
is the force vector in laminar flow. As seen by the Stokes-Einstein equation, the smaller the vessel
the higher the diffusivity. Thus if a molecule moves faster, we lose track of it quicker and the graph
decays quickly. The opposite is true for large molecules. Given this information, the mean particle
size and polydispersity index, PDI, can be estimated. PDI measures the width of the particle size
distribution on a 0 to 1 scale. A low number indicates a thin, monodisperse solution.

4 Methods

The procedure in this experiment for producing liposome vesicles from DLPC and cholesterol
involved micropipettes for preparing solutions, a laminar flow fume hood to avoid contamination,
glass vials, 100 nm twelve filters, twenty four filter supports, two syringes and syringe needle, two
extruder dies, and an extruder set involving housing, gasket and tightening nut.

4.1 Preparation

First a 10% and 30% sample set was prepared by combining Cholesterol, DLPC, and Chloroform
in a glass vial with the amounts listed in Tab. 1. The resulting solutions were dried by placing the
glass vial underneath a tube emitting compressed air for about 10 minutes each. Once the glass vial
appeared to only contain solids, the samples were hydrated with about 2 mL of deionized water.
The set of solutions were each placed in the vortex for 3 minutes and sonicated for 8 minutes.
### Table 1: Volumes of reagents added to pre-extrusion solution.

<table>
<thead>
<tr>
<th>% Cholesterol</th>
<th>Cholesterol (µL)</th>
<th>DLPC (µL)</th>
<th>Chloroform (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>50</td>
<td>450</td>
<td>1.5</td>
</tr>
<tr>
<td>30%</td>
<td>150</td>
<td>350</td>
<td>1.5</td>
</tr>
</tbody>
</table>

#### 4.2 Extrusion

The extruder set was assembled by placing wetted filter supports on either sides of a filter membrane in between the two extruder gaskets of the two extruder dies. One die was placed in a housing while the other was encompassed in the tightening nut. The housing and the tightening nut were fastened together. The syringes were then connected on either side, with one filled with the sample and the other emptied.

A single pass through the extruder was conducted by pushing the syringe with the sample and simultaneously pulling the empty syringe to force the sample through the filter membrane.

#### 4.3 Experiment

In the first part of this experiment, three sets of 10% and 30% volume cholesterol in DLPC samples were produced in a laminar flow hood. The first set was extruded 5 times, the second at 11 times, and the third 15 times. The resultant solutions were placed in a clean glass vial in a fridge to be collected by a DLS operator for size distribution analysis.

In the second part of this experiment, a single set of 10% and 30% samples were prepared in a laminar flow hood using the chemicals and amounts listed in Tab. 1, as mentioned earlier. To evaluate repeatability of the study, the samples were dried and hydrated with 6 mL of deionized water instead of 2 mL. The 6 mL solutions were placed in the vortex for 3 minutes and sonicated for 8 minutes, as before. This dilution allowed for three distinct extrusion experiments from a single set of source samples. All solutions in the second part of this experiment were extruded 5 times each. The resultant solutions were placed in a clean glass vial in a fridge to be collected by a DLS operator for size distribution analysis.
5 Results

Phospholipids of 10 and 30 mol % cholesterol were formed and passed through an extruder containing a membrane made up of 100nm diameter pores. The resulting solutions were submitted for DLS analysis in order to determine their size distributions, seen in Fig. 1.

Particle size decreased with more extrusions. For a 30 mol % cholesterol solution, z-average decreased from 163.7nm (5 extrusions) to 134.7nm (15 extrusions). For a 10 mol% cholesterol solution, z-average decreased from 175.6nm (5 extrusions) to 138.6nm (15 extrusions). More extrusions resulted in smaller particle size, as seen in Fig. 2.

The desired polydispersity index (PDI) was a value less than 0.2, which would indicate that particles of a certain size had formed over a narrow range. Although a decrease in PDI was expected with more extrusions, no trend was observed. PDI for 30% and 10% cholesterol solutions ranged from 0.074 to 0.166, and showed no relationship with mol % of cholesterol or number of extrusions, as seen in Fig. 3.

6 Discussion

The particles obtained produced a desirable PDI of less than 0.2 across all samples, indicating good uniformity throughout the solution. The smallest particle diameter was observed for 15 extrusions in both the 30% and 10% cholesterol solutions (134.7nm and 138.6nm respectively), which puts the average particle diameter for each solution within 15% of the desired value of 120nm.\textsuperscript{16} Particles with smaller diameter are able to more readily diffuse through biological tissue. The effort to make the particles as small as possible is therefore born out of a practical consideration with regard to drug delivery methods.

Large PDI indicates poor uniformity throughout the solution and would suggest improper technique or faulty equipment in this experiment. Small PDI, which was achieved here, is an indication that the particles formed displayed homogeneity in the solution and exist within a narrow distri-

Figure 1: Particle size distributions for 5, 11, and 15 extrusions

PDI was expected to decrease with more extrusions, however this was not observed. This could be attributed to dead volume that was not passed through the extruder for each sample (1-2 microliters).
Figure 2: Average particle size decreased with more extrusions for both 30% and 10% cholesterol solutions

Figure 3: PDI showed no relationship with number of extrusions for both 30% and 10% cholesterol solutions
There was an observable trend with respect to the number of extrusions: more extrusions reduced the average diameter of the nanoparticles. This was to be expected since more passes through the extruder would mean that a larger volume of solution had the opportunity to be pushed through the membrane.

In order to produce more desirable results, which would include smaller average particle diameter and PDI, a method of ensuring that all of the solution passes through the membrane with each extrusion should be developed. A dead volume of 1-2 microliters was observed for each sample, which accounted for 10-20% of the entire solution being extruded. This problem arose primarily from an incomplete vacuum applied across the membrane. A more complete vacuum might be achievable with sealing surfaces and gaskets constructed to tighter tolerances and an electric pump that could apply a more consistent pressure drop across the membrane.

7 Conclusions

The experiment produced liposome nanoparticles with sizes of between 134.7 nm to 175.6 nm. These particle sizes are larger than the intended 120 nm expected from using a 100 nm filter membrane. With respect to PDI, experimental results did produce desirable PDI values, all of which were low and encompassed in the range of 0.074 and 0.180.

The desirable PDI but slightly larger particle size could be an indication of moderate membrane tearing. The membrane perhaps tore because the extrusions were not conducted not slow enough and the concentration of liposomes was too high. Such circumstances may allow the vesicles to clump together and force open the pores of the filter membrane. If this is the case, slower extrusion at lower liposome concentrations could produce liposomes of smaller particle diameter. Overall, more repeated experiments would provide more confidence in the trends detected and detail regarding the potential membrane failure.