SPIN: Rapid Synthesis, Purification, and Concentration of Small Drug Loaded Liposomes

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Liposomes are one of the most studied nano-delivery systems. However, only a handful of formulations have received FDA approval. Existing liposome synthesis techniques are complex and specialized, posing a major impediment in design, implementation, and mass production of liposome delivery systems as therapeutic agents. Here, we demonstrate a unique 'synthesis and purification of injectable nanocarriers' (SPIN) technology for rapid and efficient production of small drug loaded liposomes using common benchtop equipment. Unilamellar liposomes, with mean diameter of 80nm and polydispersity of 0.13 were synthesized without any secondary post-processing techniques. Encapsulation of dextrans (300-20,000Da) representing small and large molecular drug formulations was demonstrated without affecting the liposome characteristics. 99.9% of the non-encapsulated molecules were removed using a novel filter centrifugation technique, largely eliminating the need for tedious ultracentrifugation protocols. Finally, the functional efficacy of loaded liposomes as drug delivery vehicles was validated by encapsulating a fluorescent cell tracker (CMFDA) and observing the liposomal release and subsequent uptake of dye by metastatic breast cancer cells (MDA-MB-231) in vitro. The proposed simplified technique addresses the existing challenges associated with liposome preparation in resource limited settings and offers significant potential for advances in translational pharmaceutical development.

Keywords: liposomes, drug delivery, vesicles, nanoparticles, drug encapsulation

Introduction

Since their initial discovery in 1964(Bangham and Horne 1964), liposomes have been recognized for their potential as a drug delivery system (DDS). These colloidal, vesicle-like particles are amphipathic and allow for compartmentalization of both hydrophilic and hydrophobic molecules. By altering their size, affinity, and surface chemistry, the rates of biodegradation, bioavailability, and release of therapeutics at target tissues can be customized(Veronese and Mero 2008, Szebeni and Moghimi 2009). Despite their

well-documented use as the delivery vehicles for chemotherapeutics (e.g. Doxil(Barenholz 2012)), antibiotics (e.g. Abelcet(Adedoyin *et al.* 2000)), and opioids (e.g. Diprivan(Olufolabi *et al.* 2006)), only a small number of liposomal drugs have been approved by the FDA(Schilt *et al.* 2016). Clearly there is a separation between the development of liposomal delivery systems and their widespread implementation as both theranostic and therapeutic platforms.

A major roadblock in the engineering of potent and therapeutic grade drug delivery systems revolves around complement system activation and scalability of the synthesis procedure(Svenson 2013). It is widely accepted that small unilamellar liposomes (SULs) with a net neutral charge are the least reactogenic DDS's(Szebeni and Moghimi 2009, Peer 2012). However, obtaining a population of SULs with low polydispersity index (PDI) and optimal size requires specialized equipment and multiple secondary processing techniques. Thin film deposition is the most common method of producing SULs. Using this approach, larger multilamellar vesicles are synthesized and then the size is altered through separate post-synthesis processing, such as sonication, extrusion, or homogenization. While this method is able to effectively decrease the heterogeneity and number of lamellae, it damages fragile encapsulated molecules(Colletier *et al.* 2002), requires tedious and time consuming protocols(e.g. chromatography or dialysis), and necessitates use of equipment not readily available in laboratory settings (e.g. ultracentrifuge and rotary evaporators)(Akbarzadeh *et al.* 2013). Thus, this approach is very difficult to scale up for the mass production that is necessary for widespread use(Meure *et al.* 2008), and is not applicable for point-of-care applications.

A more simplistic approach towards producing SULs uses a fine tip needle to inject an alcoholic lipid solution into an aqueous medium, such as ultrapure water or

saline containing the therapeutic(Batzri and Korn 1973, Sonar *et al.* 2008). This method has been shown to reproducibly form SUL's with a narrow PDI(Domazou and Luigi Luisi 2002, Stano *et al.* 2004, Sonar *et al.* 2008), and has been recently implemented on a large scale to mass produce SUL's with a PDI of less than 0.2(Charcosset *et al.* 2015). A noted drawback of this method is the low encapsulation efficiency of hydrophilic drug molecules because of the high surface area to volume ratio, (Szoka and Papahadjopoulos 1978, Jaafar-Maalej *et al.* 2010). Thus, a secondary post-processing step to remove excess non-encapsulated molecules and concentrate the solution is necessary. Typically, this is carried out through precipitation methods similar to those previously mentioned (i.e. ultracentrifugation and dialysis), neither of which are readily available in common laboratory settings. Furthermore, precipitation via centrifugation has recently been shown to offer poor recovery due to the low density and sedimentation coefficient associated with nanoscale liposomes, substantially decreasing the overall yield(Lane *et al.* 2015). More recently, several microfluidic approaches to synthesize liposomes have also been demonstrated that provide greater control over mixing of lipid and aqueous solutions(Pradhan *et al.* 2008, Hood *et al.* 2013). These techniques also require specialized microfabrication facilities and only offer proof-of-concept strategies to investigate lipid self-assembly at small scales.

Several factors including the concentration and choice of reagents (lipids, cholesterol, and alcohols), synthesis temperature, injection method, and the choice of encapsulated drug can influence the characteristics of liposomes. To our knowledge, no existing study has comprehensively investigated these factors and demonstrated an efficient and robust technique for liposome production. Addressing these limitations, we have developed a gentle method for rapidly synthesizing, concentrating, and purifying drug encapsulated SUL's using common laboratory equipment. Initially, for proof-of-

concept, liposomes containing fluorescent molecules of known molecular weights are produced using the injection method. Non-encapsulated fluorophores are then removed via benchtop filter centrifugation, using a molecular weight cut-off filter that prevents the loss of liposomes while ensuring the removal of free small molecules. We also demonstrate the application of our approach by exposing cells to liposomes containing 5-chloromethylfluorescein diacetate (CMFDA), a marker that becomes fluorescent upon being hydrolyzed by esterases in the cytoplasm of a cell. Ultimately, this efficient and accessible synthesis approach offers the potential to facilitate the discovery of novel liposomal formulations for a wider implementation in clinical settings.

Materials and Methods

Materials

Cholesterol, 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), and all fluorescent dextrans were purchased from Sigma Aldrich (St. Louis, MO). Isopropyl Alcohol (IPA, 99% Pure), Ethanol (EtOH, 99% pure), Trypsin-EDTA, and 5-chloromethylfluorescein (CMFDA) were purchased from Fisher Scientific (Hampton, NH). Glass syringes were purchased from Hamilton (Reno, NV) and luer lock dispensing needles were purchased from Jensen Global (Santa Barbara, CA). Amicon Ultra 100kDa filter centrifuge tubes were purchased from EMD-Millipore (Billerica, MA). A NanoJet syringe pump from Chemyx Inc. (Stafford, TX) was used for all experiments. Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), and Penicillin-Streptomycin antibiotic were purchased from VWR (Radnor, PA). MDA-MB-231 metastatic breast cancer cell line was acquired from ATCC (Manassas, Va).

Liposome Synthesis

DSPC and Cholesterol were dissolved in alcohol (either isopropyl or ethyl) at a constant 2:1 molar ratio to a final concentration of 10M and 5M, respectively. All experiments were done using these concentrations, unless otherwise noted. The solutions were heated to 50 ^{O}C while being shaken at 270 rpm for 15 minutes to ensure adequate mixing of the chemicals. The alcoholic solution was aspirated by a glass syringe and injected into a vial containing distilled water using the syringe pump set to a constant infusion rate. The aqueous solution was constantly vortexed at 600 rpm during infusion and the vortexing continued for 3 minutes following the infusion. All infusions were done at room temperature and 50 $\mu L \cdot min^{-1}$ unless otherwise noted.

Molecule Encapsulation and Filtration

Liposomes which encapsulated either a fluorescein molecule or cell tracker CMFDA were made utilizing the previously described protocol, with the molecules being first dissolved in the vortexing water prior to lipid infusion. Fluorescein (332 Da) and dextrans (4 kDa, 10 kDa, and 20 kDa) were introduced to the aqueous solution at 100uM and 100mM concentrations respectively, and filtered using a 0.2 um syringe filter to remove any undissolved crystals. Similarly, CMFDA was prepared according to the manufacturer's recommendation and syringe filtered prior to introduction to the vortexing aqueous solution, to sterilize the solution and remove any remaining insoluble crystals. Samples were stored at 4 °C until used.

Filtration was performed using 100kDa filter centrifugation tubes. Liposome colloids were inverted several times to ensure adequate mixing and added to the filter insert. The tubes were then centrifuged at 4 ^{O}C for 10 minutes at 6000 x g. Following each

filtration, the liposome solution was rediluted to the initial concentration, collected, and centrifuged again in a fresh filtration tube. The final concentrated liposome solution was stored at 4 °C until further use. Used filtration tubes were cleaned by two consecutive rounds of centrifuging 70% IPA. Tubes were stored after use in distilled water at 4 °C. Ultracentrifugation was performed using a Thermo Scientific WX Ultra Series ultracentrifuge with a Sorvall TH-660 Swinging Bucket Centrifuge Rotor. Liposomes were centrifuged for three hours at 21,000 RPM, which equates to 60k x g. The supernatant and pellet was removed and analyzed via DLS following each cycle.

Analysing Filtration and Encapsulation Efficiency and Liposome Morphology

Liposome filtration efficiency was measured collecting the liposome sample following each filtration cycle. The fluorescence of liposomes was measured using a Tecan Infinite 200 Pro (Männedorf, Switzerland) plate reader and compared to a standard curve made from fluorescent molecules of known concentration. Liposome size and PDI was determined using an N5 Submicron Particle Size Analyser (Beckman Brea, California).

Encapsulation efficiency was determined following filtration. Liposomes were lysed by incubating with 0.1% Triton X-100 for ten minutes at 55 °C. The fluorescence of the solution was analyzed using a plate reader, and compare to a standard curve. The encapsulation efficiency was calculated as the ratio of moles of fluorescein within liposomes to the total number of moles initially added to the vortexing water.

Cell Culture and Imaging

MDA-MB-231 cells were grown to confluence in DMEM containing 10% FBS and 1%

Penicillin Streptomycin. Cells were removed and collected from the culture using Trypsin-EDTA followed by centrifugation. The cells were then reintroduced to a 96 well plate at a concentration of 4000 cells per well and cultured overnight. Non-adhered cells were aspirated and fresh phenol red and serum free media was added prior to liposome introduction. Liposomes were added to the solution at a concentration of $1x10^{18}$ liposomes per well based on the volume estimation (Section 2.3). Fluorescence imaging was done on fixed cells using Zeiss LSM 800 (Oberkochen, Germany) laser scanning confocal microscope.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 5 (La Jolla, CA). For each condition, at least three samples were independently prepared and each sample was analysed three times. Statistical analysis was performed using either a student's unpaired t-test or a One-Way Analysis of Variance (ANOVA) with a Tukey post-test. Data was deemed statistically significant if p values were less than 0.05. Graphs show the mean and the standard error of mean (SEM) of sample groups.

Results and Discussion

Liposome Synthesis and Characterization

Liposomes composed of DSPC and cholesterol were synthesized using the injection method (Figure 1A). The organic solution, containing membrane components, was injected into distilled water and the resulting phase change from organic to aqueous caused the spontaneous assembly of liposomal vesicles, encapsulating the surrounding media. Liposomes that were synthesized via this method were visualized using Cryo-EM to ensure unilamellarity (Figure 1B). Dynamic light scattering (DLS) was used for

subsequent characterizations. The size of liposomes observed through Cryo-EM were slightly smaller than those measured through DLS. This is a phenomenon that has been observed previously and is expected due to both the media in which the particles are observed, and weighting of the size distributions in DLS. A thorough description of this phenomenon was described by Egelhaaf, et. al(Egelhaaf *et al.* 1996). DSPC, a synthetic lipid, was used because of its wide range of advantages when compared to other lipids, most notably the higher phase transition temperature (55 °C) due to its longer carbon tail and greater purity. This high transition temperature imparts many favourable characteristics including longer release and degradation kinetics(Li *et al.* 2015). It has also been shown to have higher encapsulation efficiencies than other similar lipids(Anderson and Omri 2004). However, these characteristics also complicate the synthesis procedure due to the strong intramolecular forces that resist vesiculation (i.e. rate of membrane closure), leading to polydisperse liposome populations. We determined several factors, described in the following sections, that influence the morphology of liposomes and we optimized the process to rapidly synthesize monodisperse DSPC SUL populations without the need of secondary processing steps.

Effect of Solvent on Liposome Formation

Traditionally, liposomes are synthesized using the injection method by dissolving lipids and cholesterol in EtOH and are injected to a final concentration of 10% (v/v)(Pons *et al.* 1993). However, it was recently reported that IPA may be a more suitable solvent for use with other synthetic and natural lipids including Dipalmitoylphosphatidylcholine (DPPC), Dipalmitoylphosphatidylglycerol (DPPG), egg phosphatidyl choline (EPC), and egg phosphatidylglycerol (EPG)(Gentine *et al.* 2012), as it is a slightly less polar

alcohol than EtOH and therefore may stabilize the membrane curvature during vesiculation, while still capable of being miscible in the aqueous buffer. Membrane components were dissolved into either IPA or EtOH with the DSPC:cholesterol ratio being held constant at 2:1(Sonar *et al.* 2008). The solution was then delivered to the aqueous buffer to a final alcohol concentration (v/v) of either 5% or 10% (Figure 2). We observed a significant decrease in diameter and PDI ($p<0.0001$) with higher IPA concentrations resulting in smaller, less disperse particles, reaching a minimum average diameter of 144nm and PDI of 0.31 with 10% IPA. We believe that this effect may be due to an increased solubility and therefore, higher stabilization of the lipid bilayer during vesicle formation. To further explore this, we synthesized liposomes using a 10% final IPA concentration and lipid concentrations ranging from 10 M to 100 μM, while maintaining a constant DSPC:Cholesterol concentration of 2:1 (Figure 3A). We found that as the concentration of lipids in solution decreases from 10 M to 100 mM, the PDI decreases ($p<0.0001$) from 0.31 to 0.20 with no significant change in mean diameter ($p>0.05$). We saw no difference in either diameter or polydispersity from 100 mM to 100 μM, indicating that further decreasing lipid concentration will have no effect on liposome characteristics. Thus, we demonstrated that solely by altering the alcohol identity (IPA) and concentration of lipids in the solution, the polydispersity and size of liposomes can be effectively decreased without any secondary processing steps such as extrusion or size exclusion filtration.

Effect of Temperature on Liposome Synthesis

Several mechanisms have been proposed to explain the self-assembly of liposomes during thin film hydration (Antonietti and Förster 2003, Sunthar and Phapal 2015), but the physics underlying the formation of liposomes using injection method is not well

understood. It is clear there are several factors that influence the size of liposomes, most notably the vesiculation rate, which can be altered by varying the temperature and the duration for which alcohol solvent stays with the lipids(Zook and Vreeland 2010). The precise influence of variations in temperature on liposome size has been minimally explored using microfluidics, and there is limited knowledge available regarding its effects on liposomes formed using the injection method(Lasic *et al.* 1991, Ulrich 2002, Zook and Vreeland 2010). To further investigate the influence of temperature during liposome formation, we incrementally increased the temperature of aqueous buffer to the liposomal phase transition temperature (55 °C). Despite seeing a 33% decrease in PDI when reducing lipid concentrations from 10 M to 100 mM at room temperature, there was no reduction in PDI for solutions made with lower lipid concentrations at high temperatures (data not shown). Therefore, to increase the total concentration of liposomes in solution, a 10 M solution of lipids was used. We observed a significant ($p<0.0001$) change in both size and PDI when temperature increases from 4 °C to 55 °C (Figure 3B). At relatively lower temperatures below the transition temperature (< 37 °C), the liposomes were highly polydisperse (0.75-0.36) with mean diameters near 200 nm. This is likely due to an increase in membrane rigidity, which resists bending of the bilayer and thus slows the closure speeds of liposomes(Zook and Vreeland 2010, Huang *et al.* 2017). As temperatures neared the phase transition of DSPC (50-55 °C), the lipids convert from a solid gel phase to a disordered liquid phase with increased vesiculation rates. This phase change decreased the rigidity of the lipid membrane, thereby increasing the closure rates and directly affecting liposome size and PDI. Near the transition temperature, liposome diameter decreases to 80 nm in diameter and 0.13 in PDI, without the aid of any secondary post processing steps. These results indicate that temperature is the most critical factor that can be exploited to control liposome size and

polydispersity, and solely by increasing the temperature of aqueous medium in which liposomes are produced to near transition temperature, we can rapidly synthesize populations of monodisperse DSPC SUL's less than 100 nm in diameter.

Effect of Flow Rate on Liposome Synthesis

Production of liposomes in an economical, efficient, and scaled manner would require large amounts of materials to undergo the synthesis process. Increasing flow rate of the alcoholic solution is a simple way to drastically increase the overall throughput. However, little is known about the effect of shear stress and turbidity on the formation of liposomes. Using microfluidic devices with hydrodynamic focusing, researchers have postulated that the size and PDI of liposomes can be fine-tuned by controlling shear of laminar flows(Zook and Vreeland 2010, Hood *et al.* 2013). However, in our platform, we believe that all shear imparted on the lipids within the syringe and at the aqueous:organic interface are negligible compared to the convective forces within the vortexing aqueous buffer. By increasing the infusion rate, we can greatly enhance the throughput of our synthesis protocol. We tested a range of flow rates of alcoholic solution into the aqueous buffer and found no significant difference ($p>0.05$) from 25 $\mu L \cdot min^{-1}$ to 400 $\mu L \cdot min^{-1}$ (Figure 3C). However, above this range, the liposome size increased to 133 and 212 nm for flow rates of 800 $\mu L \cdot min^{-1}$ and 1200 $\mu L \cdot min^{-1}$ respectively. Similarly, the PDI slightly increased to 0.3 and 0.38 ($p<0.0001$) respectively. We attribute this change to an increase in lipid concentration relative to the available water molecules at the needle tip when flow rates are higher, and not due to any fluctuations in shear, an effect observed in microfluidic based liposomal synthesis (Zook and Vreeland 2010). Additionally, we found no significant difference when decreasing the gauge of needle (data not shown), further validating that the shear rate

during injection does not affect liposome size or distribution.

Filtration and Concentration of Drug Loaded Liposomes

A noted drawback of the injection method is that passive encapsulation techniques have a low efficiency for capturing drug molecules within liposomes. Unlike active encapsulation techniques where sequestration is driven via transmembrane gradients, passive techniques sample the surrounding medium, and close around the aqueous drugs during liposome self-assembly. This leads to a large concentration of non-encapsulated drug in suspension that needs to be removed prior to usage. Most studies encapsulate small drugs such as doxorubicin, and remove the non-encapsulated molecules using ultracentrifugation or dialysis(Li *et al.* 1998, López-Pinto *et al.* 2005, Jaafar-Maalej *et al.* 2010, Gentine *et al.* 2012). However, not much is known about the encapsulation and removal of larger molecule drugs (>1000 Da), which may be desirable in cases such as long term insulin release(Choudhari *et al.* 1994, Zhang *et al.* 2014), adoptive cell therapy(Zheng *et al.* 2013, 2017), or gene therapy(Balazs and Godbey 2011). Regardless, the purification techniques are lengthy and the limited availability of necessary instrumentation can be prohibitive and especially limits the point-of-care use. Chromatography and dialysis, while capable of purifying liposomes, cannot adequately concentrate the liposomes, creating dilute solutions that need further post processing techniques (e.g. lyophilization or speedvac). Ultracentrifugation has also been shown as an effective purification method, however it necessitates multiple cycles of centrifugation at >30k x g for several hours and can be damaging to fragile liposomes. Additionally, a large number of liposomes are lost after each cycle due to the low sedimentation coefficient associated with SULs(Lane *et al.* 2015). To address these limitations, we utilized filter centrifuge tubes containing pores large enough to ensure

sieving of large molecules, yet small enough to inhibit loss of liposomes in the colloidal solution.

Liposomes were synthesized using our previously optimized protocol (10M, 55 °C and 400 μL. min-1). Fluorescein molecules of varying molecular weights were then doped into the colloid and filter-centrifuged at 6000 x g for 10 minutes (for reference, standard benchtop centrifuges can achieve rotation speeds of 15,000 x g). The fluorescence of the liposome solution was analyzed using a plate reader following each filtration cycle, and used as a measure of concentration in the solution (Figure 4A and 4B). In each case, 6 filtration cycles result in a net removal of 99% of the non-encapsulated drugs, while 8 filtration cycles result in a net removal of 99.9% of the non-encapsulated drugs. DLS was used to measure the size and polydispersity of liposomes before and after filtration. A slight change in diameter and polydispersity was observed (Figure 4C), with a decrease in average diameter by 10 nm and decrease in PDI by 0.08 ($n=12$, $p<0.0001$), indicating that no agglomeration occurred as a result of centrifugation. We attribute this slight decrease in diameter to the removal of large liposomes which may not be stable, either through disintegration or by adhering to the membrane. The rotational speed of our centrifugation approach is nearly 20x slower than typical ultracentrifugation rotational speeds, and therefore the liposomes largely stay in the solution phase, unlike ultracentrifugation which require sedimentation and pelleting. During filtration, retention is mediated by pore size of the membrane, which only allows sieving of soluble molecules. As a comparison, we also ultracentrifuged liposomes at 60k x g for three hours, a combination that is typically used in literature. (Figure 4D). The mean liposome diameter increased 38% following ultracentrifugation, and polydispersity decreased 7%. This is likely due to the loss of small liposomes which do not readily pellet, shifting the mean towards a higher mean. Evidence of this was

also present in the supernatant, where we found liposomes <100 nm for each sample while no liposomes were found in the filtrate. The complete synthesis and filtration of monodisperse SULs takes less than three hours, which is remarkably faster than commonly employed protocols such as thin film deposition with ultracentrifugation that can normally take several days for the entire process. Finally, the sensitivity of DLS allows for simplistic testing of sterility of nanoparticle solutions, with the presence of multiple peaks (≥1 micron) indicating possible contaminations(Loske *et al.* 2014). However, we observe only single peaks in DLS, demonstrating the ability of our method to maintain sterility. Therefore, using sequential rounds of filter centrifugation, we can effectively deplete the concentration of non-encapsulated molecules within the solution while maintaining the integrity of synthesized liposomes.

Synthesizing Liposomes for Drug Delivery

The primary use of liposomes is delivery of therapeutics that may either be sensitive to clearance by the reticuloendothelial system(Sercombe *et al.* 2015), or may be cytotoxic to the host(Bozzuto and Molinari 2015). Therefore, for our synthesis and purification protocol to be applicable, it is critical that all non-encapsulated molecules be removed from the solution while maintaining the bioactivity of the encapsulated drugs. We first analyzed the encapsulation efficiency of our protocol. This is defined as the ratio of moles of drug within the nano-carrier versus the moles outside the nano-carrier. As previously mentioned, typical reported encapsulation efficiencies of the injection method are >1%. However, these reports may be misleading as there are no mentions of any negative control to demonstrate that all non-encapsulated molecules have been removed (e.g. a control where molecules have been added following vesiculation and processed alongside the samples to ensure all non-encapsulated drugs have been

removed.). We synthesized liposomes to encapsulate fluorescein and then filtered these samples alongside a negative control (Figure 5). Following eight filtration cycles we found the encapsulation efficiencies to be 0.10% (n=5). As a comparison, we ultracentrifuged fluorescein liposomes for a total of three times, which reduced the fluorescence of the negative control back to baseline. These liposomes only had an encapsulation efficiency of 0.012%, likely due to loss of vesicles during the centrifugation process. This value is slightly less than the values reported in literature. However, in these reports liposomes are typically only ultracentrifuged at most twice. Following the second ultracentrifugation, we still found non-encapsulated drugs retained in the negative control, which could obscure the encapsulation efficiencies in other samples.

To confirm that sequestered liposomal molecules are released following filtration, cell tracker CMFDA was encapsulated using the previously optimized approach. This molecule allows for convenient analysis as it becomes fluorescent only upon entering the cell and being hydrolyzed by esterases. In order to deliver identical concentrations of CMFDA across samples, the number of liposomes in suspension (N_{total}) was estimated based on the volume method (Epstein *et al.* 2006):

$$N_{total} = \frac{V_{phospholipids}}{V_{lipids}} \qquad (1)$$

where $V_{phopsholipids}$ is the volume of the phospholipids in the alcohol solution and V_{lipids} is the volume of the lipids within the shell of the liposome. $V_{phopsholipids}$ can be found as the quotient of the lipid mass in the alcohol (M_{lipid}, here 7.9015 mg $\cdot$ mL^{-1}) divided by the density of the lipid (ρ, here 1.03 g $\cdot$ mL^{-1} at 55 °C)(Nagle and Wilkinson 1978):

$$V_{phospholipids} = \frac{M_{lipids}}{\rho} \qquad (2)$$

V_{lipids} can be estimated by subtracting the void volume (V_{void}) of the liposome from the total volume (V_{total}) of the liposome:

$$V_{lipids} = V_{total} - V_{void} \quad (3)$$

$$V_{total} = \frac{4}{3}\pi r^3 \quad (4)$$

$$V_{void} = \frac{4}{3}\pi(r - w)^3 \quad (5)$$

where r is the radius (determined by DLS), and w is the membrane thickness (determined to be 3 nm by atomic force microscopy). The variables for this calculation are illustrated in figure 6A.

As a control, blank liposomes were also synthesized and CMFDA was doped into the colloidal solution. All samples were filtered to remove non-encapsulated molecules, therefore depleting the control of all CMFDA. MDA-MB-231 cells were cultured and exposed to identical concentrations of liposomes ($1x10^{18}$ liposomes/well). The fluorescence of the cells was recorded at regular intervals for 48 hours (Figure 6B). Liposomes with CMFDA encapsulated in the core progressively increased the mean fluorescence intensity (MFI) of the solution 6.5 fold within 48 hours (blue curve in Figure 6B). Although we assumed that the control samples would have no increase in fluorescence, there was a 2.9 fold increase in the MFI. We attribute this increase to advantageous binding of hydrolyzed CMFDA molecules to the shell of the liposomes and not to remaining non-encapsulated molecules in the solution, as the increase in fluorescence occurred several hours after introduction of the liposomes (CMFDA enters the cell and becomes fluorescent within 30 minutes) and no increase was observed after

8 hours. Following the 48 hour incubation, cells were fixed using paraformaldehyde and imaged using a confocal microscope (Figure 6C). Green fluorescence was present in cells that were incubated with filtered CMFDA encapsulated liposomes, but not with the cells that were incubated with liposomes doped with CMFDA and subsequently filtered. The disparity in intensity between cells incubated with CMFDA loaded liposomes and those incubated with control samples validates that drug loaded liposomes synthesized by the simplified approach proposed here can be used as potent drug carriers for therapeutic delivery.

Conclusions

During the process of drug development, extensive pre-clinical studies are performed to help understand the molecular level interactions between the therapeutic and the patient. However, even in the best scenarios, the translation from a pre-clinical to a clinical study is time consuming, expensive, and often fails due to low efficacy, specificity, or therapeutic index. This barrier has led to a decline in novel pharmaceutical agents which have entered the market, and an increase in efforts to develop systems which increase the control over drug delivery(Ranade and Cannon 2011). DDS's have been engineered to alter the solubility, biodistribution, and targeting capabilities of the drug while eliminating many adverse effects that occur as a result of off- targeting(Allen and Cullisz 2003). However, development and implementation of novel DDS's require tedious protocols and equipment not readily available in most clinical or laboratory settings.

Here, we have developed and characterized a novel, cost effective, and convenient strategy to rapidly produce a monodisperse population of drug loaded SULs using benchtop equipment commonly found in most laboratories. There have been

many reports on the effect of lipid composition, environmental factors, and external stimuli (e.g. heat or ultrasound) to affect drug release from liposomes(Lindner and Hossann 2010). By altering the synthesis conditions, small unilamellar DSPC liposomes in the range of 100 nm can be reliably produced. Despite the higher cost and challenges associated with synthesizing SULs from gel phase phospholipids like DSPC, these synthetic and semisynthetic phospholipids offer several advantages compared to natural phospholipids. Aside from the previously mentioned advantages of higher encapsulation efficiencies and transition temperature achievable with DSPC phospholipids, many of the PEGylated phospholipids used for targeting and stealth capabilities are synthesized from DSPC precursors, facilitating insertion of these molecules into the bilayer(Immordino *et al.* 2006). Furthermore, liposomal formulations prepared with DSPC exhibit longer plasma circulation lifetimes(Dos Santos *et al.* 2007).

Following vesiculation, it is necessary to filter out non-encapsulated molecules from the solution. Here, we have shown that our method is capable of filtering molecules up to 20 kDa with 8 ten-minute centrifugation cycles, which efficiently increased the purity of our samples up to 99.9%. This also illustrates the necessity for multiple filtration cycles, as many established protocols only utilize a single filtration or ultracentrifugation cycle without a reported control to ensure complete sieving(Xu *et al.* 2012, Zheng *et al.* 2017). We found the filtration inserts would lose their sieving ability following the second consecutive use. This is likely due to the high amounts of excess dye resulting in concentration polarization across the filtration membrane(Kosto and Deen 2005, Roberts *et al.* 2016). However, we were easily able to restore sieving efficiency by washing the filters twice with 70% IPA and once with deionized water to remove any residual IPA. By following this approach, we were able to restore the

sieving efficiency of the units. Each unit was used for at least ten wash cycles (20 total filtrations).

Finally, we demonstrate that liposomes synthesized using our method, release small molecules as anticipated. This was accomplished by encapsulating a fluorescent molecule, CMFDA, which is cell permeable in its inactive form, and becomes fluorescent only after being activated within the cytoplasm of the cell. Our synthesis and filtration approach efficiently produces sterile populations of liposomes containing the CMFDA molecule within three hours. This method is remarkably faster than the current methods (reduces processing time from days to a few hours), while utilizing commonly available benchtop equipment.

The passive encapsulation approach used here is a popular technique because it is capable of encapsulating nearly any type of small molecule. While it is limited in its encapsulation efficiency (Figure 5), it allows for the sequestration of hydrophobic and hydrophilic molecules alike, regardless of their ability to be ionized. This is incredibly beneficial compared to alternative methods, such as active loading(Gubernator 2011), which are limited to weak acidic or basic molecules capable of diffusion across the lipid membrane. Furthermore, it is extremely gentle on encapsulated molecules, a drawback of techniques such as sonication or freeze-thaw processing. While active loading, and other encapsulation techniques, may be a possible extension of the SPIN approach, the work here demonstrates the convenient and rapid production of SUL's for drug delivery. Future work will be focused on increasing encapsulation efficiencies, an area in liposome research that has been critically underdeveloped and understudied. We believe that the unique SPIN strategy proposed here will ultimately reduce the common limitations in DDS research and lead to further translational innovations and novel clinical discoveries for therapeutic delivery.

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Figure 1. Synthesis of Small Unilamellar Liposomes.

(A) Schematic detailing the synthesis of SULs. (1) The lipids are dissolved in an alcoholic buffer and (2) injected into an aqueous solvent containing the drug of choice. (3) Once exposed to the phase change, lipids spontaneously arrange to form SULs, encapsulating the surrounding media in the process. (4) Non-encapsulated drug is gently removed via filter centrifugation. (B) Cryo-TEM images of liposomes synthesized via the injection method at 12,000x (top) and 25,000x (bottom) magnification.

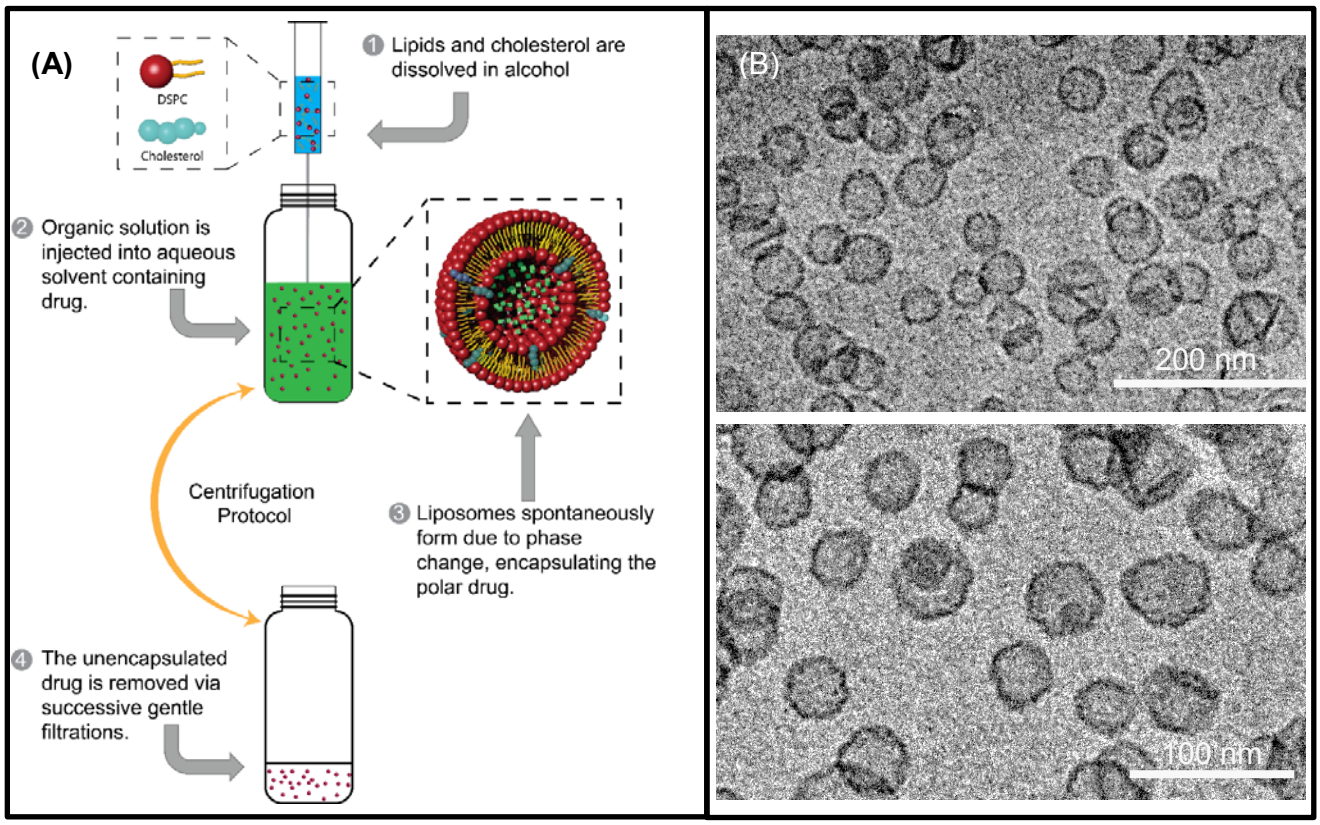


Figure 2: Effect of Solvent on Liposome Formation.

The choice of alcohol solvent can affect the vesiculation of the liposomes. Liposomes were synthesized using either EtOH or IPA with final concentrations of 5% or 10%. The resulting solutions were analyzed using DLS. Liposomes synthesized under 10%IPA demonstrated both lower diameters and polydispersities. All DLS data was obtained by triplicate measurements of at least 3 independently synthesized suspensions ($n \geq 3$). An ANOVA with a Tukey post-test was used to determine the statistical significance between samples (*** = $p < 0.0001$).

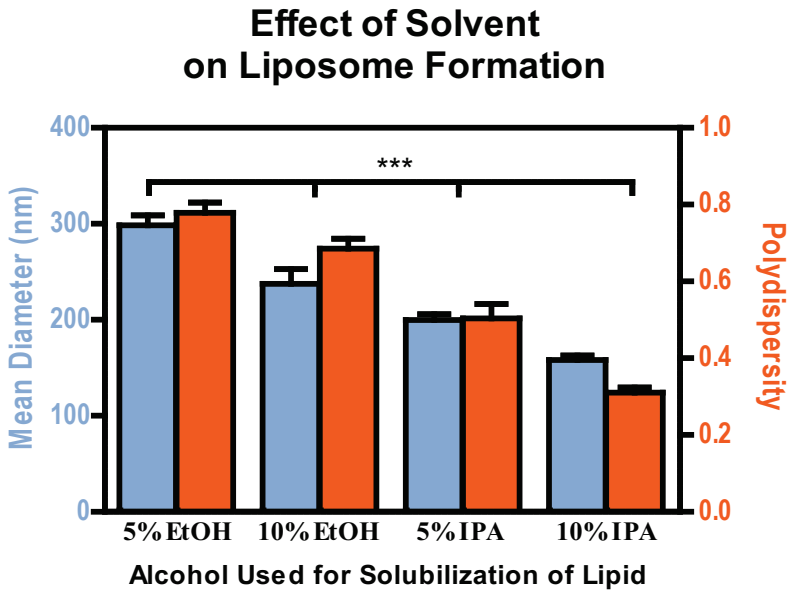


Figure 3: Characterization of Liposome Synthesis.

(A) Altering concentration of the lipid, (B) altering temperature of the aqueous buffer, and (C) altering the injection flow rate of DSPC solution into the aqueous buffer. 10% IPA was identified as the optimal alcohol and concentration and was used in all subsequent experiments. Unless otherwise noted, all experiments were conducted at room temperature with a 50 $\mu L \cdot min^{-1}$ infusion rate. All DLS data was obtained by triplicate measurements of at least 3 independently synthesized suspensions ($n \geq 3$). An ANOVA with a Tukey post-test was used to determine the statistical significance between samples (*** = $p<0.0001$).

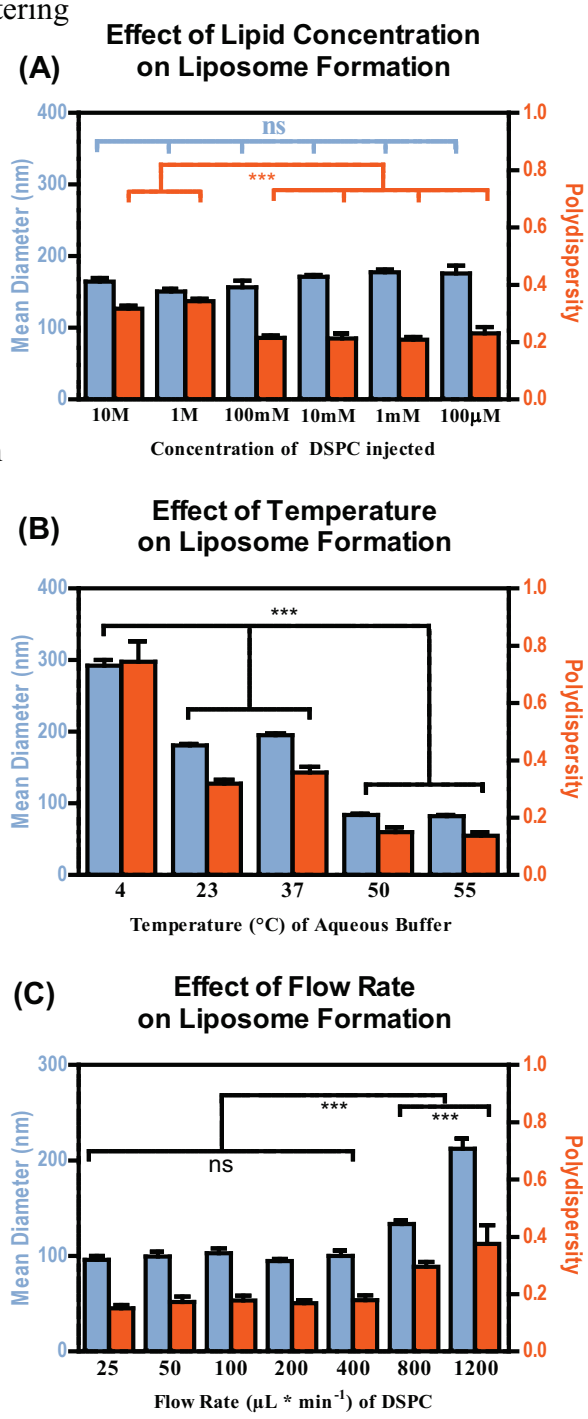
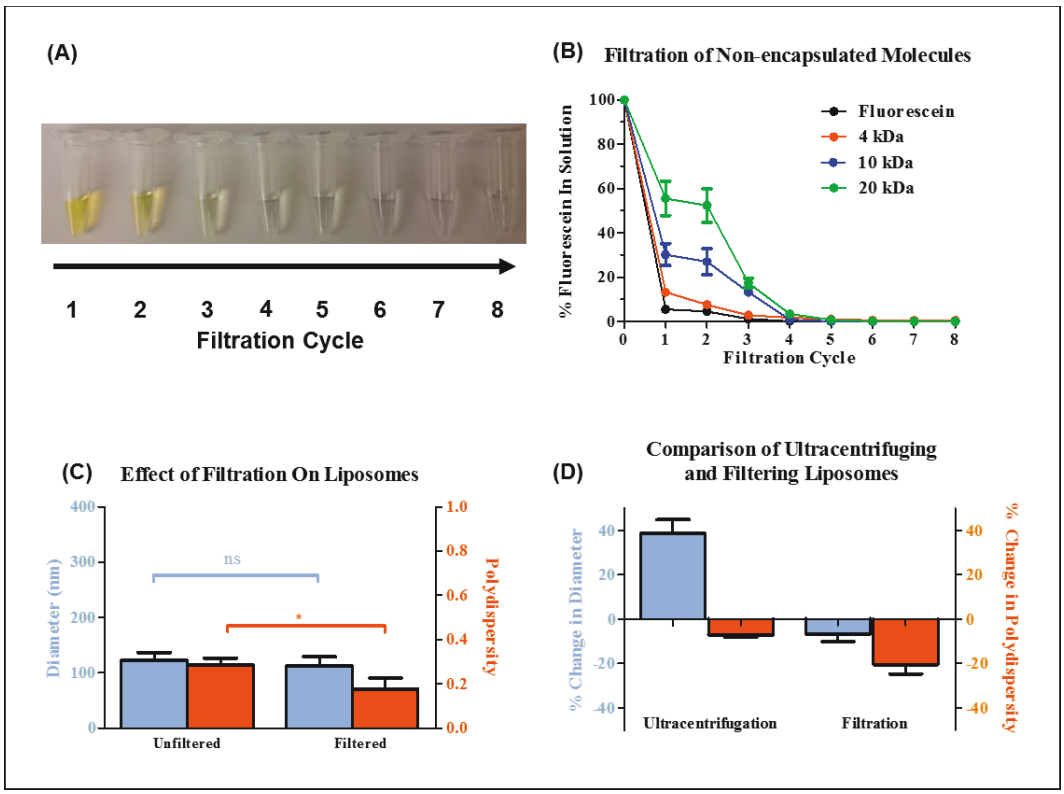


Figure 4: Removal of non-encapsulated molecules by filter centrifugation.

Liposomes encapsulating fluorescent molecules of different molecular weights (300-20,000 Da) were synthesized and filter centrifuged at 6,000 x g for 10 minutes using 100 kDa filters. (A) Image representing typical change in color of lipsosomal solutions following each filtration cycle. (B) Graph depicting the fluorescence of samples following each filtration cycle. Samples were doped with fluorescent molecules of various sizes. The concentration of the non-encapsulated molecules was reduced by 99% through 6 filtration cycles, and 99.9% through 8 filtration cycles, irrespective of the molecular weight. All measurements were done in triplicates of at least six replicates (n≥6) (C) DLS measurements were performed before and after filtration to ensure filtration has no effect on liposome characteristics and does not lead to aggregation of liposomes. Triplicate measurements were conducted on 12 samples (n=12). A t-test was conducted to determine statistical significance of differences between samples. (D) DLS measurements were conducted to compare the change in mean size and polydispersity of liposomal solutions following ultracentrifugation and filtration. The liposomes' average diameter increased 38% and PDI decreased 7.8% with ultracentrifugation, while filtration decreased the diameter by 6.5% and PDI by 20%.



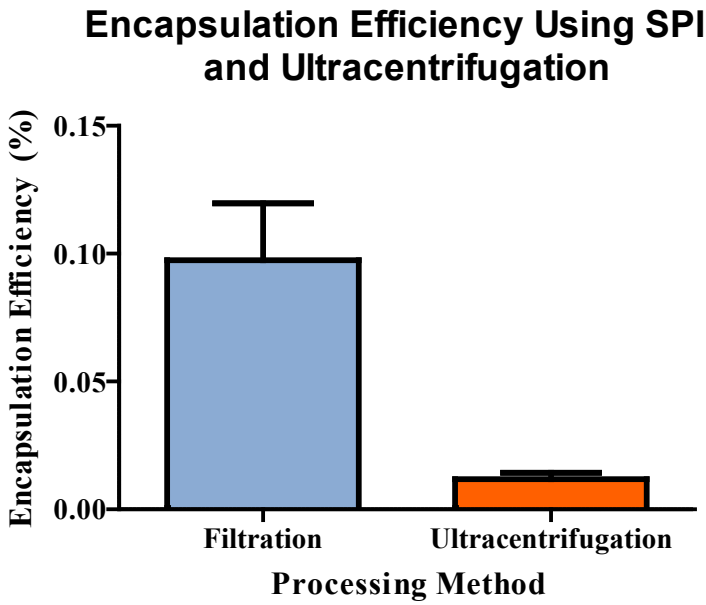


Figure 5: Encapsulation Efficiency Using SPIN. Liposomes were synthesized to encapsulate fluorescein using SPIN. The liposomes were then either filtered eight total times, as previously described, or ultracentrifuged for 3 hours three separate times at 60k x g. Liposomes were then lysed using Triton x-100 and analyzed for fluorescence. All samples were compared to a negative control where fluorescein was doped into a blank liposome suspension following vesiculation, and then processed according to their respective methods. Filtration maintained an encapsulation efficiency of 0.1%, whereas ultracentrifuge was remarkably lower at 0.012%. All samples were run in replicates of at least 4.

Figure 6: Encapsulation and delivery of CMFDA. (A) Schematic depicting the variables used for estimating the number of liposomes in solution. (B) The fluorescence intensity of the cells was analyzed over several hours to measure uptake of CMFDA from filtered solutions of liposomes either encapsulating CMFDA (blue curve) or empty liposomes doped with CMFDA (orange curve). Measurements were conducted in triplicate on at least 4 independently synthesized and filtered samples. Curves represent the mean fluorescence intensity (MFI) in respect to time, determined by using a plate reader. (C) Confocal images following 48 hours of incubation with filtered suspensions containing CMFDA (green) loaded liposomes or empty liposomes with CMFDA doped into the suspension. MDA-MB-231 Cells were counterstained with DAPI (blue) and outlines of cells are traced in white. Scale bars represent 20 μm.