Thorough Characterization of Liposomes by Complementary Light and X-Ray Scattering Techniques

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ABSTRACT

Liposomes have been used in drug discovery and drug delivery for some time, and the biophysical characterization of these systems and their payloads is critical to understanding and optimizing their fabrication and function. This study looks at optimal conditions for extruding liposomes as well as their stability under different conditions. Our aim is to further educate the public about the intricacies of liposome formation and characterization as measured by Nanoparticle Tracking Analysis (NTA) from the NanoSight product range, Dynamic and Electrophoretic Light Scattering (DLS/ELS) from the Zetasizer product range, and Small-angle and Wide-angle X-ray scattering (SAXS/WAXS) from the X-ray analytical product range within Malvern Panalytical. A broad range of characterization information and combination of both NanoSight and Zetasizer systems helped further optimize fabrication and understand the function of liposomes as well as labeling efficiency of fluorescent lipids. NTA through NanoSight provided number-based high resolution sizing, accurate distribution profiles, concentration (particles/mL), and fluorescence measurements. DLS provided excellent reproducibility, mean size and PDI measurements over a broad range and non-invasive trend analysis. ELS provided zeta potential as a functionality and stability metric of particles. SAXS allowed to investigate the bilayer stacking in MLVs and from WAXS data the order of the alkyl chains (gel phase vs. liquid phase) could be studied as a function of sample temperature.

Keywords: nanoparticle tracking analysis, dynamic light scattering, electrophoretic light scattering, x-ray scattering, liposomes

1 INTRODUCTION

Liposomes have been attractive delivery systems for decades due to their composition of natural biological lipids and structural resemblance to cell membranes suggesting metabolic compatibility, low toxicity, overall expectations of biocompatibility and lack of a strong immune response [1]. They comprise of spherical vesicles with an aqueous core enclosed by one or more phospholipid bilayers or lamellae and are frequently classified based on their size, polydispersity, and number of bilayers [2]. Control over these parameters has remained a challenge with most preparation methods and is further accentuated when moving from a laboratory to industrial scale. The desirable size for drug delivery ranges between 50 and 200nm [3]. They are often used in studies of model biological membranes, phase transition and spacing, targeted drug delivery to specific areas of a human body, etc [4]. The extent of liposome instability in various biological fluids such as plasma depends on the relative concentrations, size and lamellarity, lipid composition and incubation temperatures [1]. Thus, biophysical characterization of these systems and their payloads is critical to understanding and optimizing their fabrication and function. This study looks at optimal conditions for extruding liposomes as well as their stability under different conditions. We highlight the limit of detection for fluorescently labeled liposomes. Malvern Panalytical affords a cost-effective suite of direct characterization of these critical parameters. Nanoparticle Tracking Analysis (NTA) from the NanoSight product range measures size, concentration and fluorescence measurements. Dynamic (DLS) and Electrophoretic (ELS) Light Scattering from the Zetasizer product range measures size, zeta potential, and payload stability. Small- and Wide-Angle X-ray scattering (SAXS/WAXS) measurements enable to determine the lamellarity, structures and dimensions of the lipid bilayers.

2 MATERIALS

DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), and cholesterol (ovine wool, >98%) lipids were obtained in powder form from Avanti Polar Lipids (Alabaster, AL). Fluorescent rhodamine-DPPE lipids were obtained in powder form from Biotium (Fremont, CA). All lipids were dissolved initially in chloroform to create stock solutions and stored at -20°C. Buffer reagents (MOPS, NaCl, NaOH) were obtained from Sigma Aldrich.

3 METHODS

3.1 Liposome Preparation and Extrusion

DOPC, DOPC-cholesterol, and Rh-DPPE incorporated lipid mixtures were initially prepared at the desired molar ratios in chloroform, and the chloroform was subsequently evaporated to a film using a gentle nitrogen stream and overnight vacuum. Lipid films were then rehydrated to the desired molar concentration (typically 2mM DOPC) using aqueous buffer (typically 100 mM NaCl and 10 mM MOPS, pH 7.4 which resulted in the formation of Multi Lamellar Vescicle (MLV) suspensions. For DPPC lipids with a transition temperature of 42°C, the MLV suspension was heated after hydration of the lipid powder (single-component and bTLE) or film (multi-component mixtures). MLV suspensions were repeatedly frozen/thawed 5x, unless otherwise noted, and stored at -20°C until extrusion and Small Unilamellar Vesicle (SUV) formation. SUVs were formed by extruding thawed MLV suspensions through hand-held NanoSizer mini-extruders housing polycarbonate track-etched membranes of various pore-sizes including 50 nm, 100 nm, 200 nm, and 400 nm. The single-use NanoSizer kits (extruders, syringes and needles) and reusable extruder heating block were obtained from T&T Scientific (Knoxville, TN). The total number of passes through the extruder was varied as part of this study and is reported for each sample in the results section.

3.2 Analysis

Samples were analyzed by a NanoSight NS300 equipped with a high sensitivity Hamamatsu sCMOS camera, 20x objective lens, and a 50 mW green 532 nm laser. Samples were analyzed using NTA 3.2 Build 16 software. NanoSight technology (Malvern Panalytical) calculates size based on the relationship between Brownian motion and hydrodynamic diameter through the Stokes-Einstein equation. Concentration is calculated by particle observation on a frame-by-frame basis by the sCMOS camera (25 frames per second). When recording the video it averages the concentration across all the frames giving an absolute number average. Samples were processed across 3x 60 sec videos.

Samples were further analyzed by a Zetasizer Nano ZSP at 25 °C. Measurements were made with a disposable cuvette (DTS0012) and disposable folded capillary cell (DTS1070) for sizing and zeta potential respectively. The Nano ZSP incorporates non-invasive backscatter (NIBS™) optics for sizing measurements. The detection angle of 173° enables size measurements of concentrated, turbid samples to be made.

Small-and wide-angle X-ray scattering (SAXS/WAXS) data were measured using the Empyrean Nano edition (Malvern Panalytical), a versatile X-ray scattering platform. The experimental setup uses the line focus of a Cu X-ray tube, a focusing X-ray mirror, an evacuated beam path and a hybrid pixel array detector. The samples were filled in a 1 mm quartz capillary and measured at a liposome concentration of 25 mg/ml. DPPC and DOPC liposomes, before and after extrusion, were investigated with the SAXS/WAXS technique under variation of sample temperature. Extrusion was done by repeatedly passing the liposome samples through an extruder with a pore size of 100 nm.

4 DISCUSSION

NTA, DLS, and ELS provided a comprehensive characterization of liposomes across various conditions. Both NanoSight and Zetasizer are similar because they both rely on the Brownian motion of and light scattering from the particle. Both utilize the Stokes-Einstein equation and relate diffusion to size (hydrodynamic diameter). NTA produces a number-based size distribution from a particle-by-particle measurement, while DLS produces an intensity-based distribution from an ensemble measurement. This is further exemplified in the set of experiments shown here.

NTA and DLS confirmed that approximately 11 passes were needed to reach the target pore size. During the initial passes, DLS reflected the larger particles that were present because of their higher scattering intensity. Once the larger particles were removed, passes quickly reflected the pore size of the extruder. NTA displayed an initially larger standard deviation in the data because the particle sizes varied more by number. Neither original lipid concentration nor freeze-thaw cycles had a discernable effect on extruded sizes through different pore sizes (Figure 2). However, DOPC liposomes subjected to freeze-thaw cycles did have noticeably higher initial concentration when increasing the number of extrusion passes (Figure 2).

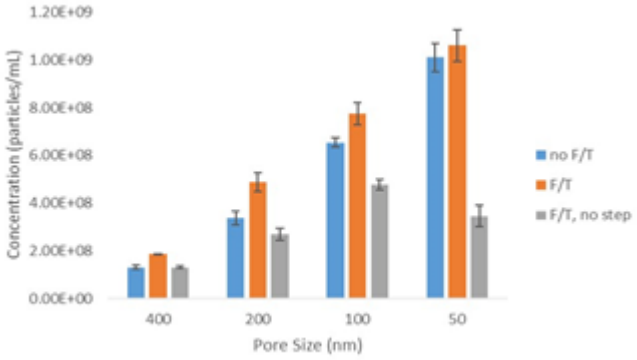

Figure 1. Effect of both freeze/thaw cycles and step-down extrusion on concentration (11x passes/pore size) – NTA.

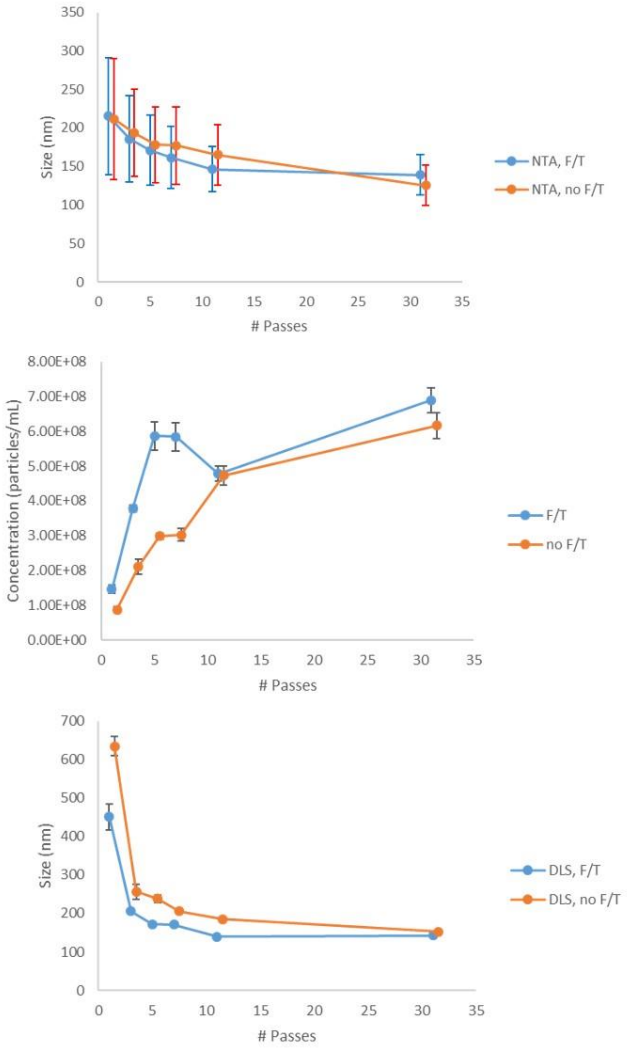

Figure 2. Effect of Freeze / Thaw cycles (5x) on particle size and concentration as extruded through 100nm pores.

Robustness of the Nanosizer Extruder by T&T Scientific was demonstrated by the stable size and concentration over 20 mL (11 passes/mL) of extruded DOPC liposomes. There was a noticeable difference in resulting concentrations of liposomes subjected to step-down extrusion vs extrusion directly at different pore sizes (Figure 1). This provided milder conditions and higher retention of liposomes. Increasing the percentage of cholesterol in DOPC further displaced head groups in the lipid bilayer causing an increase in the zeta potential.

Fluorescence measurements on Rh-DPPE incorporated into DOPC showed that lower percentages could be further measured to determine the true limit of detection. The number of fluorescent molecules per vesicle was estimated from geometrical considerations, assuming a liposome size of 130 nm and a lipid headgroup area of 0.6nm^2. Rhodamine dye conjugated to DOPC vesicles gave a photo-stable and bright fluorescence signal when excited with the 532 nm laser and using a 565 nm long-pass filter before the sCMOS camera of the NanoSight NS300. Comparison was made between the concentration measured under fluorescence mode and under scatter mode. Since the sample was pure, all particles present and measured in scatter mode were assumed to be liposomes that should be labeled with the dye. This efficiency % is the ratio of the two results. As expected, with increasing amounts of dye, the fluorescence efficiency increases. Scatter and fluorescence size distribution profiles match well. 100% efficiency is seen with as little as 0.25% dye which is equivalent to 113 fluorophores/particle. More than 0.25% dye in a sample resulted in more than 100% efficiency. This indicated some measurement difficulties that could be optimized with further work, including investigation of dye aggregation or difficulties in scatter mode measurements as potential causes. Decreasing % labeling efficiency with higher mol% Rh-DPPE indicates potential fluorophore quenching.

The SAXS data from the liposomes prior to extrusion show several equidistant Bragg peaks, as a consequence of the lipid bilayer stacking in these multilamellar vesicles. The repeat distances in the lamellar stacks, as deduced from the peak positions, were 6.4 nm for DPPC and 6.5 nm for DPPC. The peak widths are similar for the two samples, which suggests that the number of lamellae in the stacks is comparable. However, more higher order peaks are observed for DPPC, which indicates a better long-range order in the lamellar stack. In the WAXS data a distinct diffraction peak at the scattering angle 2θ of 20.9° is seen in case of DPPC, whereas no such peak is present in the data measured from DOPC. The peak is associated with the ordering of the alkyl chains and indicates that DPPC is in the ordered gel phase with a Bragg spacing of 0.42 nm, whereas DOPC is in the disordered fluid phase. This finding is consistent with the tabulated gel-to-fluid transition temperatures, which are +41°C for DPPC but -17°C for DOPC [5].

SAXS and WAXS measurements on DDPC at different temperatures ranging between 5 and 70°C was performed and the deduced Bragg spacings are given in Table 1.

T [°C]	**d1 [nm] from SAXS data**	**d2 [nm] from WAXS data**
5	6.3	0.43 (gel phase)
20	6.4	0.42 (gel phase)
30	6.4	0.43 (gel phase)
39	7.3	0.43 (transient ripple phase)
43	6.7	- (liquid phase)
50	6.6	- (liquid phase)
70	6.3	- (liquid phase)

Table 1: Structural parameters of DPPC liposomes, as a function of sample temperature. d1: Bragg spacing due to lamellar repeat distance; d2: Bragg spacing due to alkyl chain ordering in the gel phase

The lamellar repeat distances and the alkyl chain ordering did not change significantly between 5°C and 30°C. At 39°C, which is just slightly below the gel-to-fluid phase transition temperature of DPPC, pronounced changes were observed in the SAXS data: the diffraction peaks are shifted to smaller scattering angles and become significantly broader. This indicates an increased spacing and a reduced order in the lamellar stack. At 43 °C, just above the phase transition temperature, the WAXS peak disappears, confirming the presence of the fluid phase. Furthermore, with increasing temperature the peaks in the SAXS region shift back to the higher angles, indicating a reduction of the lamellar repeat distance. The interesting behavior that was observed just below the transition temperature, indicates the onset of some restructuring in the multi-lamellar vesicles, which points to the occurrence of a transient 'ripple phase'.

The background-corrected SAXS data (see fig. 3) of the extruded DPPC liposomes, measured at 20°C, show the characteristic features from the scattering of single lipid bilayers: a broad hump, followed by some higher oscillations. The sharp Bragg peaks, as observed on the same sample prior to extrusion, are no more present. This confirms that upon extrusion the multi-lamellar vesicles were transformed to (almost) unilamellar vesicles. Two very broad Bragg peaks that are still visible (arrows in fig. 10) on top of the broad hump due to the lipid bilayer structure, indicate that the vesicles are not strictly unilamellar, but still contain a small number (around 2-4) of lipid bilayers. A similar behavior was observed for the extruded DOPC vesicles (not shown).

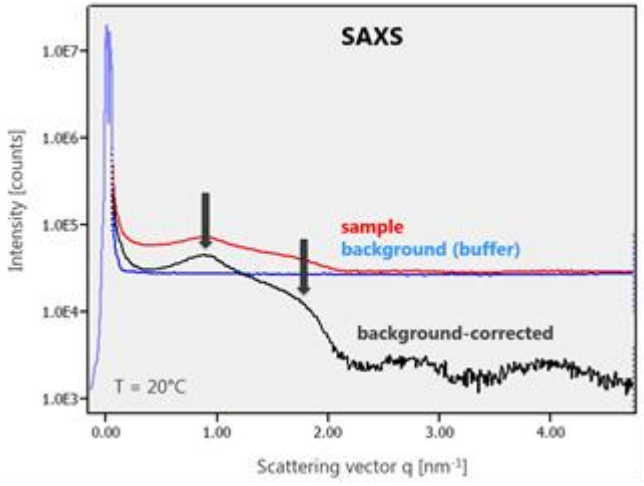

Figure 3. SAXS results on DPPC liposomes, after extrusion, measured at 20°C.

From the extruded DPPC vesicles a WAXS peak, though significantly broadened (see fig. 4), could still be observed at T = 20°C, which again points to the formation of the gel phase. The absence of such peak in case of the extruded DOPC vesicles confirms that these are in the fluid phase.

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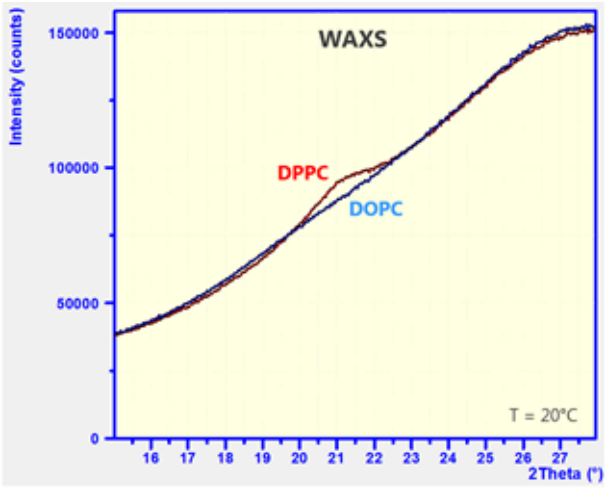

Figure 4. WAXS results on DPPC and DOPC liposomes, after extrusion, measured at 20°C.

The modeling of the electron density profile across the lipid bilayer as well as a more detailed study of the interesting behavior of DPPC close to the gel-fluid phase transition temperature are still pending.

5 CONCLUSION

A deeper understanding of liposome formation and staiblity can be obtained through the use of multiple characterization techniques. Using both NTA and DLS techniques helped optimize fabrication and understand the function of liposomes. NTA provided high resolution size distributions, number concentration, and fluorescence measurements. DLS provided highly reproducibile mean size and PDI measurements. ELS provided zeta potential as a stability metric. SAXS data gave insight in the lipid bilayer stacking as a function of sample temperature, and the WAXS data investigates the alkyl chain packing.

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