Application Note

Poseidon™

Imaging Liposomes in Liquid with Transmission Electron Microscopy

Introduction

Soft materials, such as liposomes and micelles, are of considerable interest to the pharmaceutical community due to their role in drug transport and delivery. With a growing interest in the precise delivery of drug therapies, there is a critical need to assess these microemulsions in a liquid environment like that in a living system. However, due to their fragile structures and susceptibility to beam damage, liposome delivery vehicles can be challenging to image with a transmission electron microscope (TEM). The difference in density between lipid-based species and their aqueous environment is small, causing these samples to be particularly challenging to image without contrast reagents.

Despite these inherent challenges, groups at both Sandia National Laboratory and Virginia Tech have demonstrated the ability to image functional liposome nanoparticles *in situ* within liquid using the Protochips' Poseidon system for TEM.

Khalid Hattar's group at Sandia National Laboratory in Albuquerque, NM, imaged 1-palmitoyl-2-oleoyl-sn-glycero-3- phosphocholine (POPC) liposomes, such as those shown in Figure 1A. The group observed the POPC liposomes as they flowed through the holder tip, and studied the effect of surface treatment and lipid additives on liposome structure. Deborah Kelly's group at the Virginia Tech Carilion Research Institute in Roanoke, VA successfully imaged pegylated liposomal control vehicles, shown in Figure 1B, under low dose conditions.

Experiments

A pair of Poseidon E-chips with 50 nm thick, amorphous silicon nitride windows were used for all experiments. The top and bottom E-chips were positioned in the tip of the Poseidon holder and secured using a lid and three brass screws to form a hermetic seal to protect the hydrated sample from the vacuum of the microscope. A solution of liposomes was continuously flowed during the course of the experiment using an external syringe pump via microfluidic tubing integrated through the shaft of the holder and into the tip.

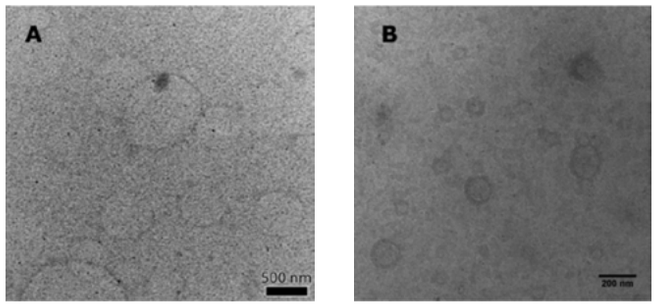

Figure 1: Liposomes imaged in liquid with TEM (A) POPC lipids imaged under continuous flow at 200 KV acceleration voltage. (B) Pegylated liposomes imaged in static liquid conditions using a 120 KV acceleration voltage.

Preparation of POPC Liposomes in Liquid

POPC liposomes were prepared by extruding 1 - palmitoyl - 2 - oleoyl - sn - glycero - 3 - phosphocholine, purchased from Avanti Polar Lipids, through a series of two 100 nm polycarbonate pore filters. Prior to assembling the sample chamber the E-chips were made hydrophilic by soaking them in a solution of alcian blue stain, which imparts a positive charge to the E-chip surface. A dilute solution of POPC liposomes flowed into the sample holder tip at a rate of 100 µL/hour. The sample was imaged using a JEOL 2100 TEM operating at 200 kV, and real time images were recorded at a frame rate of 12 frames per second with a 500 ms exposure time. Images were taken

over the course of two hours, while fresh sample was continuously delivered to the tip of the holder.

Preparation of Pegylated Liposome Suspensions

Pegylated liposomes, with a nominal diameter of 80-90 nm (product #300103S) were purchased from Avanti Polar Lipids and diluted to ~0.3 mg/ml with Milli-Q water prior to use. A sample volume of 0.5 µL was deposited onto an E-chip with a static spacer thickness of 150 nm. Samples were imaged using an FEI Spirit Biotwin operating at 120 kV. Images were acquired with a defocus value of -2 µm using low dose conditions.

Imaging Structural Effects of Lipid Additives to POPC Liposomes

POPC liposomes spiked with distearylglycero triethylglycyl iminodiacetic acid (DSIDA) were prepared to study the effect of lipid additives on the vesicle structure. A solution of 10% DSIDA/POPC liposomes flowed through the holder at a rate of 100 µL per hour over alcian blue-treated E-chips. The same TEM conditions were used to image the DSIDA/POPC liposomes as the POPC liposomes described above.

Effect of Bovine Serum Albumin on POPC Liposome Stability

Bovine Serum Albumin (BSA) was used to passivate the surface of the E-chips prior to introduction of the liposomes into the liquid chamber. A solution of BSA dissolved in deionized water was likewise flowed through the holder for 1 hour to ensure the E-chip surfaces were thoroughly passivated. A solution of POPC liposomes was then flowed into the imaging chamber at a rate of 100 µL/hour and TEM images were acquired as described above.

Discussion

Under continuous liquid flow, POPC liposomes, shown in Figure 1A, were observed after flowing for 30 minutes. As liposomes entered the liquid chamber between the E-chips, they were likely attracted to the positively charged E-chip surface and immobilized upon contact. Thus, liposomes introduced early during the flow may not have reached the area of the chamber containing the transparent windows of the E-chips. Though in this case, immobilization is advantageous because it reduces overall Brownian motion, leading to less blurring in the recorded images and better resolution. The average diameter of the liposomes measured in TEM was 497 nm. The large diameter was likely a result of liposomes fusing together over the course of the experiment. Structural changes caused by altering the liposome composition were studied by preparing liposomes composed of a mixture of POPC and DSIDA lipids. DSIDA are more rigid POPC, and are less likely to form curved structures. At room temperature, the DSIDA lipids are known to separate from the POPC, forming DSIDA enriched domains within the liposome, causing localized regions of increased rigidity.

The altered, non-circular, liposome-like structures are visible in the TEM images, as shown in Figure 2A. The average size of the DSIDA/POPC liposomes was 183 nm, indicating that the DSIDA/ POPC liposomes were less stable at larger sizes than the POPC liposomes. The effect of surface chemistry on the liposome structure and stability was studied by passivating with BSA, which is known to destabilize and degrade the liposome membrane in the absence of cholesterol. The denatured liposome material, which is too small to form stable liposomes (the radius of curvature

requires that liposomes have a diameter greater than 30 nm) is shown in Figure 2B. Commercially available pegylated liposomes, such as those currently used in the clinic to deliver the chemotherapeutic doxorubicin to tumor cells, were imaged with Poseidon without loaded drug molecules as shown in Figure 1B. A heterogeneous mixture of liposomes, which varied in diameter from 50 nm to up to 125 nm, was observed and is consistent with the predicted average diameters of 80 – 90 nm. Multiple sequential images of liposomes in solution were recorded using a dose of 0.5 electrons per square Angstrom. Images in solution revealed a continuous blurring in the outer boundaries of the liposomes, likely due to beam damage. This observation demonstrates how susceptible the liposomes are to the electron beam. Optimizing the concentration of the liposome sample was also important for obtaining good quality images. Samples that were too concentrated resulted in crowding of the liquid chamber with liposomes, and consequently individual liposome structures could not be adequately resolved, as shown in Figure 2C.

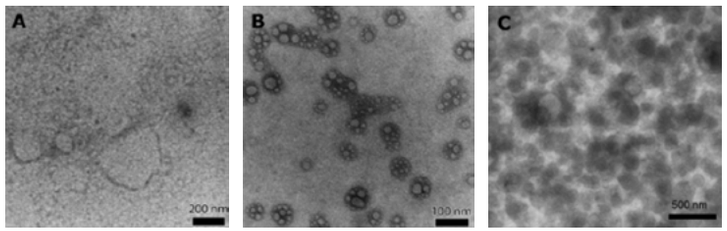

Figure 2: Effects of the liquid environment on liposomal structure and packing.
(A) DSIDA/POPC liposomes imaged under continuous flow with a 200 KV acceleration voltage. DSIDA/POPC liposomes exhibit distorted structural features due to the increased bending rigidity of DSIDA.
(B) Denatured POPC liposomes. The liposomes denatured on contact with the BSA treated window surface.
(C) Closely packed liposomes. The high concentrations of liposomes in the fluid cell make it difficult to distinguish the structure of individual liposomes.

The results show the potential for imaging functional liposomes in liquid using TEM, demonstrating the feasibility of studying dynamic processes, such as liposome formation, interaction, degradation and drug delivery in real time at high resolution.

Applications

Micelle and liposome suspensions play a key role in modern pharmaceutical applications. These nanomaterials are used in the clinic to encapsulate and transport chemotherapeutics and other drugs to specific targets within the body. In addition to their role in drug delivery, micelles are a model system for studying membrane behavior and cellular interactions. Thus, there is a critical need to assess these systems in a physiologically relevant, dynamic liquid environment. Contact us to discuss the full range of capabilities of Poseidon. We can be reached at (919) 377-0800 or at contact@protochips.com.

References
Langmuir 2013, 29, 9958-9961. *Microscopy and Microanalysis*: http://dx.doi.org/10.1017/S1431927613013858

Protochips