

PREPARATION AND CHARACTERIZATION OF SUCROSE-LOADED VESICLES

From the Ph.D. thesis of Dr. David Hokanson.

2.7.2 Sucrose-Loaded LUVs

Large unilamellar vesicles (LUVs) with 100 nm diameter were prepared by extrusion (Hokanson and Ostap, 2006). Lipids to be used in sedimentation experiments were resuspended in 176 mM sucrose, 12 mM HEPES pH 7.0, to a total concentration of 1 or 2 mM. This solution was used to increase the density of the LUVs so they would form a pellet in our sedimentation assays while maintaining the same osmolarity as our HNa100 buffer. Five cycles of rapid freeze-thaw using ethanol/dry-ice and a 37 °C water bath, followed by a one minute incubation in a bath sonicator, were applied before passing each lipid solution through a 100-nm filter (11 times) using a mini-extruder (Avanti Polar Lipids). LUVs were dialyzed overnight versus HNa100. LUVs were stored at 4°C under N₂ gas and discarded after 3 days. PS and PIP₂ percentages reported throughout the text are the mole percentages of total PS and PIP₂ in a background of PC. Lipid concentrations are given as total lipid unless otherwise noted.

We checked that the diameter of our LUVs were uniformly 100 nm, by using dynamic light scattering (DLS) to measure the dispersion of sizes. LUVs were consistently found to be 100 nm in diameter with very little dispersion. We did find that under high divalent cation levels, such as Ca²⁺, the dispersion of LUV sizes tended to increase as well as the average size of LUVs (Figure 19) due to a known effect of Ca²⁺ to the lipid vesicle structure (Bentz and Duzgunes, 1985).

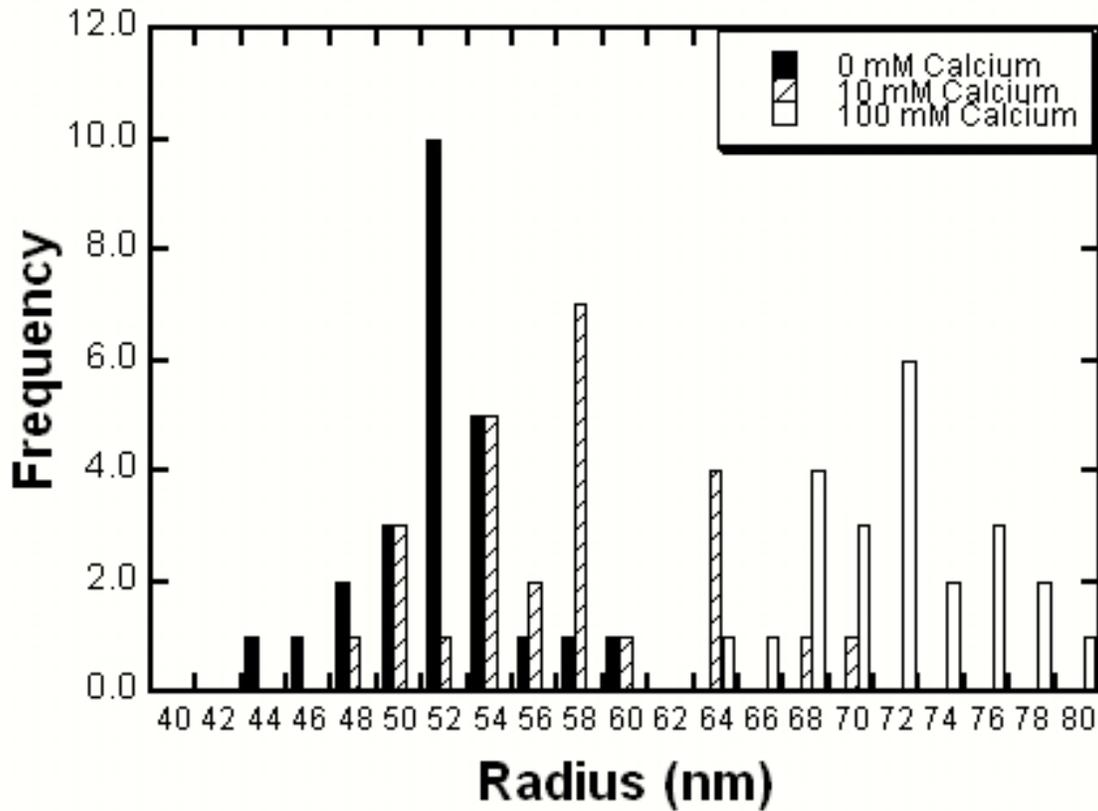


Figure 1 Ca²⁺ effect on DLS of LUVs

Figure 19: A histogram of the distribution of LUVs by DLS. Size distribution of 2% PIP₂ LUVs with 0 mM (black bars), 10 μM (hashed bars), or 100 μM Ca²⁺ (open bars) by DLS. Frequency of radii from 28 scans per each sample is shown. Note that the addition of Ca²⁺ causes the size distribution to shift to the right and become more dispersed.

2.7.3 Phosphorous Assay

To check that the concentrations of LUVs we were calculating were correct, we determined the total amount of phosphorous in a typical LUV sample following a modified procedure of Fiske & Subbarow from Avanti Polar Lipids (Fiske and Subbarow, 1925). We used 0 - 0.0456 μmoles KH_2PO_4 to make phosphorous standards along with our samples of LUVs made up of various lipids. Solvent was removed with a stream of N_2 and 90 μL of 8.9 N H_2SO_4 was added to each tube. All samples were heated to 210 $^\circ\text{C}$ in an aluminum heating block for 25 minutes. Tubes were removed and left to cool for 5 minutes before adding 30 μL of H_2O_2 . Tubes were reheated at 210 $^\circ\text{C}$ for 30 minutes, then allowed to cool to ambient temperature before adding 780 μL of H_2O . We then added 100 μL of 2.5% ammonium molybdate tetrahydrate solution and again vortexed each tube five times. We next added 100 μL of 10% ascorbic acid and vortexed each tube five times. A marble was placed over each tube to minimize evaporation and tubes were heated to 100 $^\circ\text{C}$ for seven minutes. Absorbance of standards was measured at 820 nm using a UV/Visible spectrophotometer and used to generate a linear calibration curve to determine the concentration of phosphorous in each LUV sample. Predicted concentrations of LUVs were found to be accurate within 5%.

2.7.4 Sedimentation Assay

To test the binding of myo1c-tail to LUVs, we sedimented LUVs in a TLA100 rotor (Beckman), with a r_{ave} of 3.45 cm at 63,000 rpm to produce forces of 150,000 x g according to:

Equation 1

$$g_{cen} = \frac{(\omega)^2 r}{g}$$

where ω is the angular velocity in revolutions per second, r is the average radius of the rotor, and g is a gravitational constant of 980 cm/s².

2.7.4.1 Defining Pellet

We determined that sucrose loaded LUVs sedimented to the bottom 10% of a centrifuge tube when centrifuged at 150,000 x g after 20 minutes, but that they do not form a stable pellet. 200 μ L samples of LUVs made with 5% dansyl labeled PS were centrifuged at 150,000 x g for 30 minutes. Five samples at sequential depths were carefully extracted from each centrifuge tube. We checked the samples for fluorescence at 340:500-620 nm and found that no lipid was found in the top 90% of the tube, but ~100% of the expected lipid was found in the bottom 10% (Figure 2). Following removal of the supernatant, HNa100 was added to the tubes to suspend any pellet; no fluorescence was found in this sample. This experiment was repeated with LUVs that did not contain dansyl labeled lipids, but were monitored by total phosphorous as described above; the results were identical. We thus define the “pellet” as the bottom 20% of the sample in a centrifuge tube after a 30 minute spin at 150,000 x g for all of our sedimentation assays.

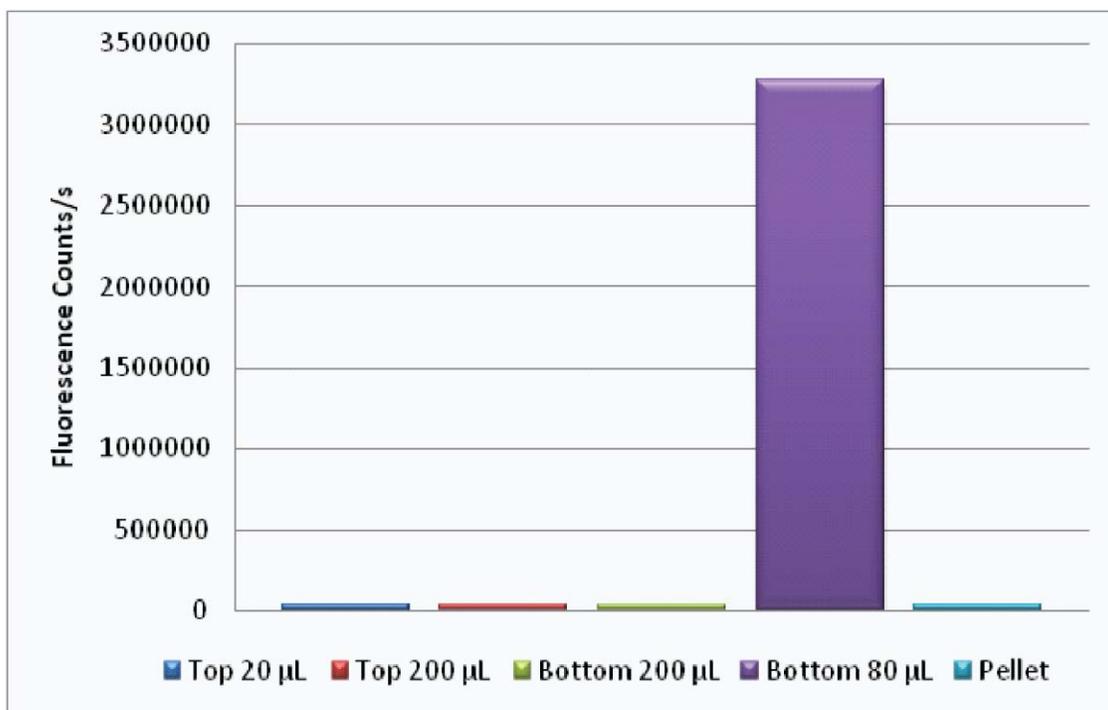


Figure 2 Sedimentation assay pellet

Figure 2: Quantification of the fluorescence of the lipid gradient of sucrose-loaded LUVs after centrifugation. A 500 µL sample of LUVs containing 5% dansyl-labeled PS was centrifuged at 150,000 x g for 30 min. Successive aliquots of the supernatant were removed and analyzed for fluorescence at 340:500-620 nm. After removal of supernatant, 40 µL HNa100 was added to centrifuge tube and analyzed as pellet.

2.7.4.2 Nonspecific Interactions

Nonspecific interactions between our protein and the walls of the polycarbonate centrifuge tubes caused a significant loss of protein, which was a concern since our experiments used protein concentrations of 40 nM to stay well below the K_d of binding. Nonspecific binding is thought to be due to the highly basic charge interaction with the acidic polycarbonate. Without protection, the myo1c-tail would practically disappear, as assessed by SDS-PAGE gel.

Two different strategies were combined to solve this problem. All tubes were coated with a bath sonicated solution of 40 μ M PC hydrated in HNa100. This formed a thin film on the polycarbonate, which prevented myo1c from binding. The addition of non-specific blocking proteins to the samples was also used to help resolve this issue. Three different proteins were tested; CaM, fibrinogen, and GST (Figure 3). CaM showed no increase in recovery of myo1c from polycarbonate tubes; fibrinogen and GST both showed close to 100 % recovery. We selected GST because of its low molecular weight and availability, and added 0.25 mg/mL to all centrifugation samples.

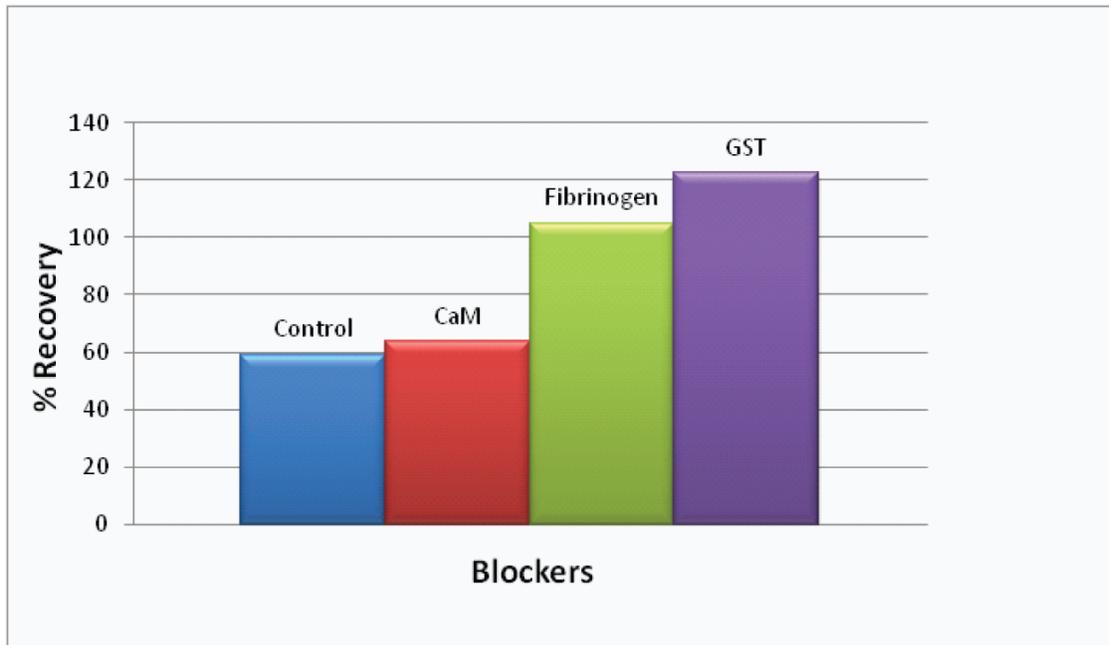


Figure 3 Nonspecific blockers

Figure 3: Quantification of the recovery of myo1c-tail after sedimentation in the presence of proteins to block nonspecific interactions to the polycarbonate tubes. CaM, Fibrinogen, or GST (5 mg/mL) were added to 40 nM myo1c-tail and centrifuged at 150,000 x g for 30 minutes. Samples were analyzed for the percentage of myo1c-tail recovered from supernatant after centrifugation.

2.7.4.3 Sedimentation

Protein binding to LUVs was determined by sedimentation assays conducted with 200 μ l samples in an ultracentrifuge with a TLA-100 rotor (Beckman). Polycarbonate centrifuge tubes were incubated for an hour in 50 μ M solution of PC in HNa100. GST (0.25 mg/ml) was added to every sample. Sucrose-loaded LUVs were sedimented at 150,000 x g for 30 min at 25 °C. The top 160 μ L of each sample was removed and analyzed as supernatant. We called the remaining 40 μ L the “pellet,” although it contains approximately 20 % of the supernatant. The pellets were re-suspended with 10 μ L SDS-PAGE sample buffer and boiled for three minutes. Bound proteins were resolved by SDS-PAGE and stained with SYPRO-red (Invitrogen) for quantization as described below. A sample gel with standards is shown (Figure 4). Supernatants containing GFP-proteins were assayed for GFP fluorescence in a fluorimeter. Gels were scanned using a gel scanner, and data were analyzed with MetaMorph (Universal Imaging).

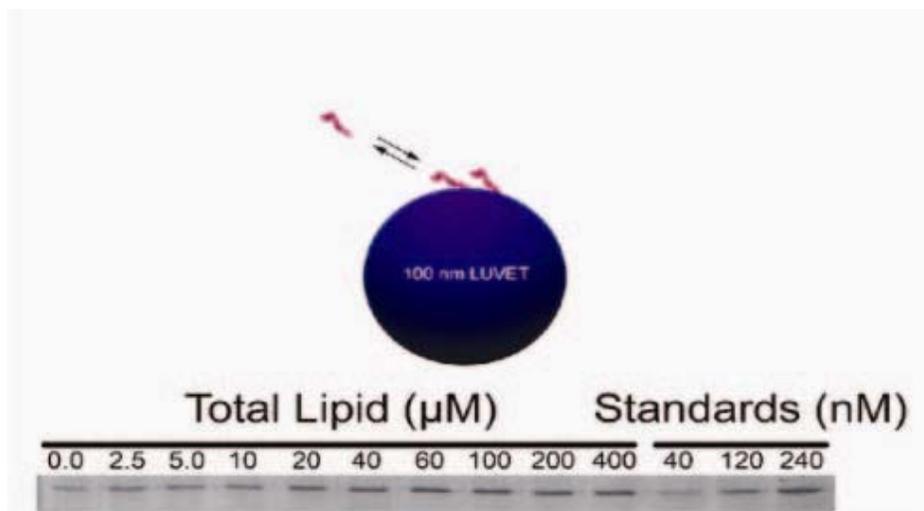


Figure 4 Sedimentation assay

Figure 4: A representative SYPRO red stained SDS-PAGE gel from a sedimentation assay. Top) Approximate scale representation of a 100 nm LUV and a full length myo1c binding. Bottom) SYPRO-red stained SDS-polyacrylamide gel of myo1c-tail from the pellets of a sedimentation assay with 0 - 400 μM LUVs (total lipid) containing 2% PIP_2 . The last 3 lanes are myo1c-tail standards used for normalization. Bottom figure originally published in (Hokanson and Ostap, 2006)

Bentz, J., and N. Duzgunes. 1985. Fusogenic capacities of divalent cations and effect of liposome size. *Biochemistry*. 24:5436-43.

Fiske, and Subbarow. 1925. Phosphorous assay. *J. Biol. Chem.* 66:374-389.

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