# Separation of Membrane-Bound Compounds by Solid-Supported Bilayer Electrophoresis

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## Supplementary Materials

## Experimental

*Materials*. The following lipids were used in these experiments: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). These were obtained from Avanti Polar Lipids (Alabaster, AL). Fluorescently labeled lipids used in the separation experiments were: Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red DHPE) and *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (BODIPY DHPE), obtained from Molecular Probes (Eugene, OR). Phosphate buffer saline (PBS) used in the preparation of vesicles was made with 10 mM $Na_2HPO4$, 5 mM $NaH_2PO_4^2$, and 150 mM NaCl (pH 7.4). Sodium chloride, sodium phosphate monobasic, and sodium phosphate dibasic were all obtained from Sigma-Aldrich (St. Louis, MO). The pH was adjusted to 7.4 using sodium hydroxide (EMD, Germany). The purified water used in the preparation of all solutions was obtained from a NANOpure Ultra Water System (Barnstead, Dubuque, IA) and had a minimum resistivity of 18 MΩ·cm. Polycarbonate filters (Whatman, Fisher Scientific) with pore diameters of 100 nm and 50 nm were used in the preparation of vesicles. Glass coverslips (VWR) were used as supports for the bilayers and were boiled in 7X solution (MP Biomedicals, Aurora, OH) that was diluted four fold by volume with purified water. Polydimethylsiloxane (PDMS; Sylgard 184) was obtained from Dow Corning. Platinum electrodes were made from platinum wire with a diameter of 0.25 mm from Alfa Aesar (Ward Hill, MA). Thin layer chromatography (TLC) was carried out using glass-backed TLC plates made of silica gel with pore sizes of 60 Å and a layer thickness of 250 μm (VWR).

## Separations Experiments

*Preparation of the supported bilayers used in the separation experiments*. Small unilamellar vesicles (SUV) were used to form solid-supported lipid bilayers (SLBs) on glass substrates by vesicle fusion.[1-5] The vesicles used to create the separation bilayer were composed of POPC and cholesterol. Vesicles containing the fluorescently labeled lipids were composed of POPC mixed with a suitable mol% of Texas Red DHPE and/or BODIPY DHPE. To make the various vesicle solutions, the appropriate components were first dissolved in chloroform and dehydrated under vacuum for three hours. The dried mixtures were rehydrated in PBS and subjected to ten freeze/thaw cycles by

alternating between liquid nitrogen and a 30 $^{o}$C water bath. The solution was then extruded several times through a polycarbonate filter to produce vesicles of uniform size. For the first extrusion, a filter with 100 nm diameter pores was used, followed by five passes through a filter with 50 nm diameter pores. The resultant SUVs were sized by dynamic light scattering using a 90Plus Particle Size Analyzer (Brookhaven Instruments Corporation) and were found to be highly uniform at each composition and had an average diameter of 80-90 nm.

Glass coverslips used as supports for the bilayers were cleaned in 7X solution following established procedures.[6] They were then annealed in an oven at 550 $^{o}$C for five hours to yield flat surfaces with a typical root mean square roughness (RMS) value of 0.13 nm over a 1 $\mu m^2$ area as determined by atomic force microscopy. A drop of vesicle solution (~200 $\mu$L) was placed on the clean hydrophilic glass coverslip. The solution was confined to a rectangular area in the center of the glass coverslip by a thin hydrophobic PDMS mold. The mold was made by cross-linking PDMS between two silanized glass microscope slides separated by a thin metal spacer between 200 to 400 $\mu$m thick. After cross-linking, a rectangular hole approximately 1 $cm^2$ was cut out of the center of the elastomeric sheet using a razor blade. The outer edges of the mold were trimmed to fit exactly over the glass coverslip.

The separation bilayer was prepared first. The vesicle solution containing cholesterol was incubated on the glass slide for ten minutes and rinsed with copious amounts of purified water to remove any excess, unfused vesicles from the surface. In the next step, a thin strip of the supported bilayer was completely removed so that vesicles containing the mixture of fluorophores could be fused in that region. We found that the edge of a coverslip wrapped with several ply of Teflon tape was an effective tool to plow out a thin line (80 $\mu$m wide) of bilayer material while not causing any damage to the underlying substrate. Immediately following this step, ~ 100-200 $\mu$L of vesicle solution containing the dye-labeled lipids were added to the PDMS well. After about five minutes, the well was thoroughly rinsed with DI water.

A 100 V potential was applied laterally across the bilayer by placing a platinum wire electrode on each side of the PDMS well using a standard regulated power supply (Lambda Electronics Corp., Long Island, NY) while monitoring the current through the system with a digital multimeter (Keithly). DI water was used to minimize Joule heating. We maintained currents of only a few microamps or less during all experiments so Joule heating was negligible. Time lapse images were taken of the slide every five minutes to monitor the movement of the fluorescent bands and subsequent separations. A 4X microscope objective was used to maximize the field of view in our set up. Imaging was performed with a Nikon Eclipse TE2000-U equipped with a Sensys CCD camera (Photomatrics, Roper Scientific) working in conjunction with MetaMorph software (Universal Imaging).

It should be noted that the electric field lines run parallel to the bilayer and solid support. When an electric field is applied parallel to the bilayer, then charged species move by electrophoretic forces. If these species protrude far enough outside of the bilayer, then electroosmotic forces are also important.[7,8] It should also be noted that the technique presently separates a few femtomoles of material. It might be possible, however, to scale this up significantly in future designs.

**The Effect of Cholesterol on Bilayer Diffusivity**

*Preparation of supported bilayers with various amounts of cholesterol*. The effect of cholesterol on the fluidity of solid supported lipid bilayers was examined using fluorescent recovery after photobleaching (FRAP). Bilayers of POPC, DOPC, and DLPC with varying mole percentages of cholesterol and 0.2 mol% Texas Red DHPE were studied. Briefly, the phospholipids, cholesterol, and Texas Red DHPE (all in chloroform) were mixed in proper proportions and dried first under a stream of nitrogen gas and then desiccated for three hours. PBS (pH 7.2) was added to each sample for rehydration. Each was then frozen with liquid nitrogen and thawed a total of five times with vortexing in between the freeze-thaw cycles. These vesicles were extruded through a polycarbonate filter (50 nm pore size) a total of seven times. The effective diameters of these unilamellar vesicles were determined via light scattering and found to be within the diameter range of 80-110 nm.

Bilayers were then formed from these vesicles by vesicle fusion on previously cleaned and annealed glass, using a PDMS slab with a hole cut in the middle to contain the bilayer and bulk buffer above it. Bilayers formed in this manner appeared to be uniform down to the diffraction limit under all conditions investigated.

*Diffusion measurements*. Diffusion coefficients and mobile fractions of Texas Red DHPE were measured by fluorescence recovery after photobleaching (FRAP).[9] The dye-labeled lipids were bleached with the 568.2 nm line from a mixed gas $Ar^+/Kr^+$ laser beam (Stabilite 2018, Spectra Physics). 100 mW of power was directed onto the sample for less than 1 second. The beam, which was sent through a 10X objective, had a full-width at half-maximum of ~11 μm at the sample plane. The recovery of the photobleached spot was followed with the same microscope system described above. The fluorescence intensity of the bleached spot was determined after background subtraction and normalization for each image. The diffusion coefficient of the dye-labeled lipids was determined as follows. First, the fluorescence recovery as a function of time was fit to a single exponential equation, from which we obtained the mobile fraction of the dye-labeled proteins and the half-time of recovery ($t_{1/2}$). The diffusion coefficient, D, was then calculated from the following equation: $D = \frac{w^2}{4t_{1/2}}\gamma$, where *w* is the full width at half-maximum of the Gaussian profile of the focused beam and γ is a correction factor (= 1.1) that depends on the bleach time and geometry of the laser beam.

Figure S1 summarizes the effect of cholesterol on the diffusion coefficients for three different lipids, DLPC, POPC, and DOPC, as the cholesterol content was varied. As has been well studied,[10,11] cholesterol is a hydrophobic molecule that is incorporated in the tail region of the bilayer and influences both the tail ordering and fluidity of the bilayer. We found that at 25 mol% cholesterol in POPC, the diffusion coefficient drops by over a factor of three. Because we wanted to depress diffusive mixing with only a modest amount of cholesterol, we chose to employ this mixture in all electrophoresis separations experiments.

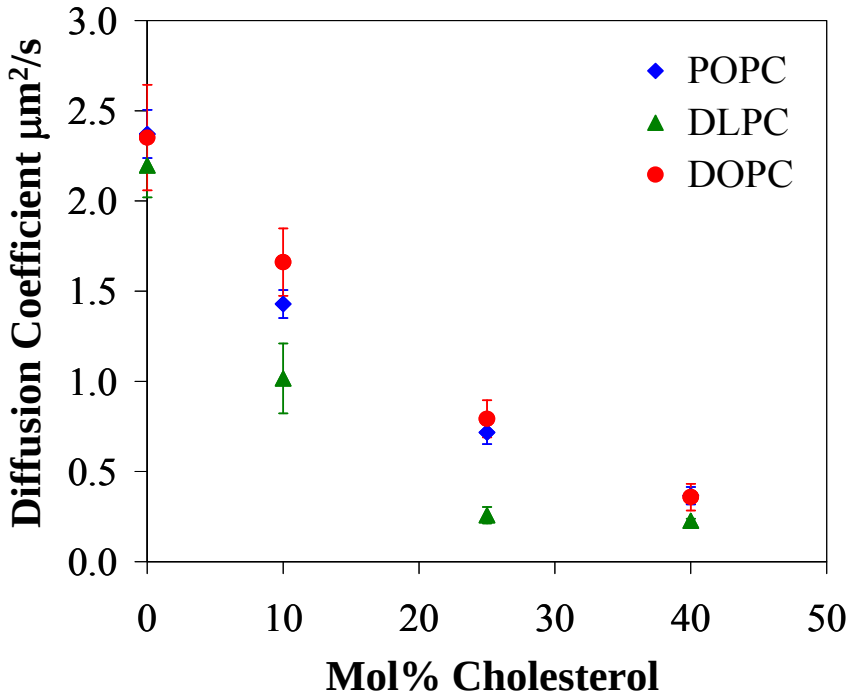


***Figure S1.*** The diffusion coefficient of Texas Red labeled probe lipids as a function of the mol % of cholesterol in membranes composed of DLPC, DOPC, or POPC.

**Thin Layer Chromatography Separation of Texas Red DHPE Lipids**

In order to determine whether the Texas Red bands we observed were due to isomers, we purified our lipids using thin layer chromatography (TLC). Several small spots of Texas Red DHPE in chloroform were formed on a TLC plate using a glass pipette. The spotted plate was then placed into a development jar and eluted with ethanol. Figure S2 shows the TLC plate after separation of the bands from six individually placed spots. Texas Red labeled phospholipids were recovered from the TLC plate by carefully scraping the separated bands with a razor blade and re-suspending in ethanol to extract the lipids from the silica beads. The mixture was centrifuged at 13,500 RPM (5415, Eppendorf) for 5 minutes and the supernatant was collected. This procedure was repeated (typically 5 times) until no Texas Red phospholipids were detected in the pellet by observing the color. To remove the ethanol, the samples were dried with nitrogen followed by desiccation under vacuum for 1 hour.

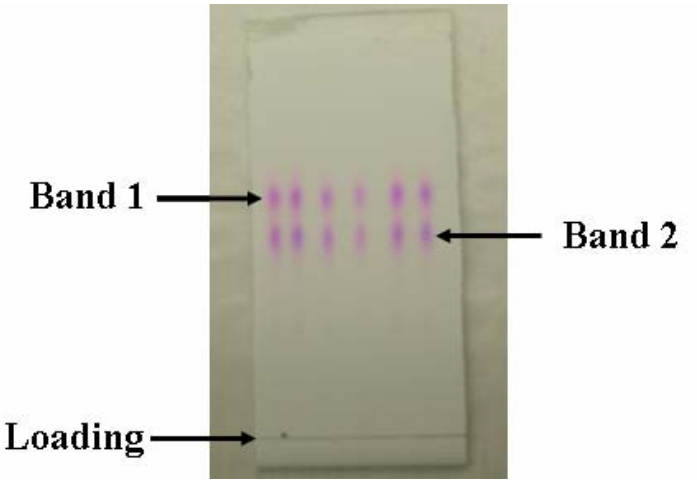


***Figure S2.*** Image of a TLC plate after Texas Red DHPE separation. Six individual spots of Texas Red DHPE were placed at the bottom of the plate (horizontal loading line) and eluted with ethanol for approximately 20 minutes.

To prepare small unilamellar vesicles containing the purified Texas Red lipids, each fraction was mixed with POPC in chloroform and allowed to dry under a stream of nitrogen and then desiccated under vacuum for 4 hours. After evaporation of the solvent, the lipids were reconstituted in PBS buffer and subjected to ten freeze-thaw cycles by alternating between liquid nitrogen and a 30 ºC water bath. The vesicle solutions were then extruded five times through a polycarbonate filter (50 nm pore size) to produce small unilamellar vesicles of uniform size. Small unilamellar vesicles prepared by this method were approximately 80 nm in diameter as determined by dynamic light scattering.

The individual Texas Red DHPE lipid fractions in POPC were assayed by FRAP. A diffusion constant value of 4.1 (± 0.6) × $10^{-8}$ $cm^2$/sec was obtained from band 1. For membranes composed of POPC and Texas Red from band 2, a diffusion constant of 4.4 (± 1.0) × $10^{-8}$ $cm^2$/sec was obtained. Both fractions had immobile fractions of less than 2%.

TLC purification was also carried out on BODIPY DHPE to verify that this compound was pure. We did not detect any band splitting using this method in agreement with the electrophoresis studies.

**Mass Spectroscopy**

Matrix assisted laser desorption ionization (MALDI) mass spectroscopy was performed on the isolated fractions of Texas Red DHPE obtained from TLC. The spectrum for each band is shown in figure S3. Comparing the spectra, both fractions have nearly identical masses, indicating that they arise from two different isomers of the same compound. The mass of Texas Red DHPE is 1381.85 g/mol. The compound is a salt and consists of the negatively charged dye-conjugated lipid, 1279.65 g/mol, as well as triethylammonium cations, 102.2 g/mol. The low mass peak in each spectrum corresponding to *m/z* 1302.7 represents the negatively charged dye-conjugated lipid with one sodium ion and one proton. The peak at *m/z* 1324.7 represents the dye-conjugated lipid with two sodium ions. The peak at *m/z* 1340.7 corresponds to the dye-conjugated

lipid with one sodium ion and one potassium ion. The peak at *m/z* 1354.7 represents the dye conjugated lipid with two potassium ions. The peak at *m/z* 1382.7 is attributed to the Texas Red salt with an additional proton.

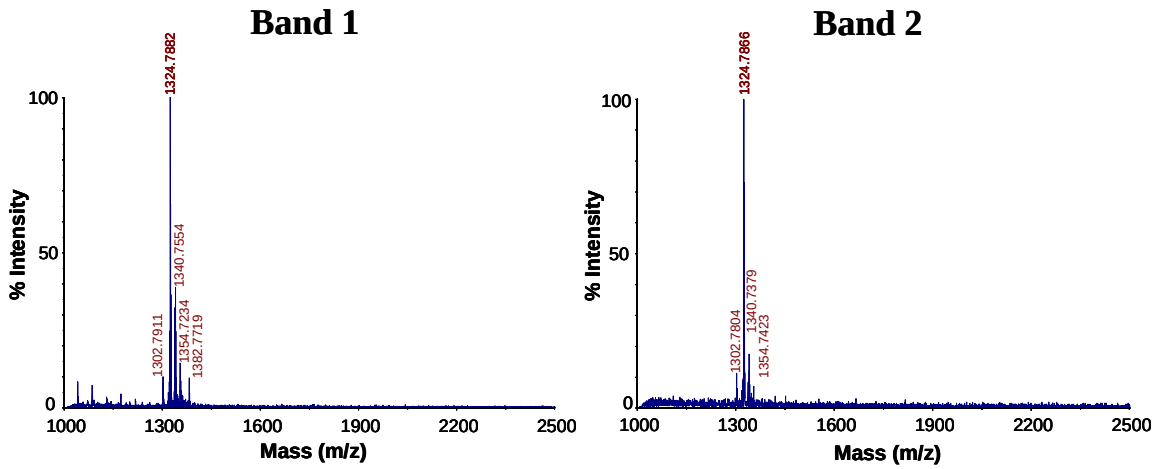


**Figure S3.** Mass spectra of each fraction of the TLC purified Texas Red DHPE isomers.

## UV-Visible Spectroscopy

In order to determine the relative intensity ratio of Texas Red bands purified by TLC we used UV-visible spectroscopy. The measurements were carried out using a *Lambda* 35 UV/VIS Spectrometer (Perkin Elmer Instruments). Texas Red DHPE shows an absorption maximum at 596 nm. Vesicles prepared using band 1 from the TLC showed an absorption maximum of $A_{596}$ = 0.0710, while vesicles prepared with band 2 showed an absorption maximum of $A_{596}$ = 0.0390. These results yield a 65:35 ratio for the two Texas Red DHPE isomers. It should be noted, however, that different batches of Texas Red DHPE obtained from Molecular Probes showed varying ratios of the two isomers. In fact, the ratio varied from 65:35 to about 75:25.

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