INDUCED ASYMMETRY IN LIPID BILAYERS DETECTED BY SUM FREQUENCY VIBRATIONAL SPECTROSCOPY

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Phospholipids, a major constituent of cell membranes, contain a hydrophobic tail and a hydrophilic head group. Cell membranes are composed of two leaflets forming a bilayer. The hydrophobic tails form the bilayer core while the hydrophilic headgroups form the exterior of the bilayer. While phospholipids have a similar structure in general, there is variety among headgroup moieties as well as tail moieties. In living cells, phospholipids are distributed asymmetrically between the two leaflets of the bilayer according to head group chemistry. However, it has been shown that phospholipids can undergo rapid flip-flop between the two leaflets, which would favor a symmetric distribution due to mixing. Sum frequency vibrational spectroscopy (SFVS), a surface specific technique, can be used to determine the amount of asymmetry in a lipid bilayer on a planar support. As such, SFVS was used to study one possible mechanism of bilayer asymmetry maintenance, namely electrostatic association. Specifically, asymmetry was induced in bilayers consisting of 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine and 1,2-distearoyl-*sn*-glycero-3-phosphatidylserine by electrostatic interaction with the positively charged polypeptide polylysine.

Lipid bilayers were created on planar silica surfaces through the Langmuir-Blodgett/Langmuir-Schaefer deposition method. To confirm the validity of using the Langmuir-Blodgett/Langmuir-Schaefer deposition method for the asymmetry study, mass

spectrometry was used to verify bilayers of a known composition that were formed on a silica substrate.

For my loving family

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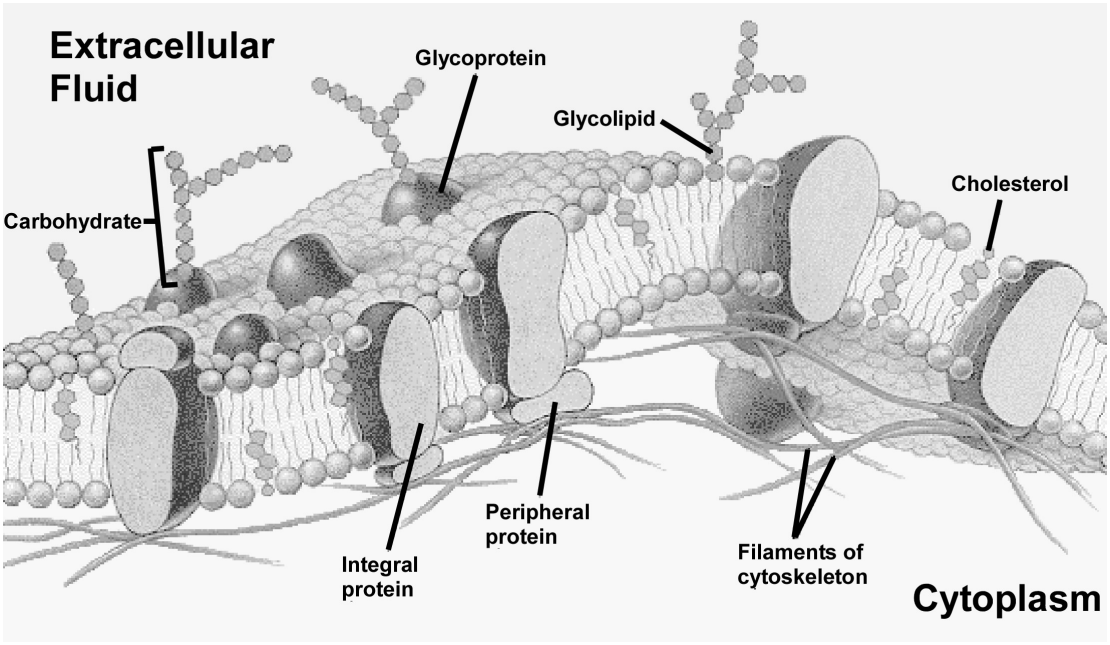
INTRODUCTION

Lipid Bilayers

Cell membranes play an important role in many biological processes. They serve as a platform for many biological interactions as well as create a barrier between intracellular and extracellular environments. Phospholipids are a major constituent of cell membranes. Phospholipids are amphiphilic molecules with a hydrophobic hydrocarbon tail and a hydrophilic polar headgroup. Amphiphilicity facilitates the formation of bilayers in which the hydrocarbon tails of each layer are in close proximity, forming a hydrophobic core, and the polar headgroups are located on the outer portion of the bilayer in contact with the polar aqueous environment, as seen in Figure 1.[1]

Phospholipid biosynthesis occurs on the cytosolic side of cell membranes. In order to accommodate cell growth, lipid translocation from the cytosolic leaflet to the outer leaflet must occur. This translocation between leaflets of the bilayer is called flip-flop. Several studies have presented information indicating that lipid flip-flop is a protein mediated process.[2-6] However, no protein has been identified to date as specifically responsible for lipid flip-flop. Several studies have also reported on a free lipid flip-flop rate, in the absence of protein.[7, 8] The rate of lipid flip-flop reported in these studies was generally slow, from hours to days. These studies, however, relied on the use of labeled lipid probes, from electron-spin labeled probes to fluorescent probes.[8, 9] Conversely, Liu and Conboy developed a method to measure the rate of lipid flip-flop without a labeled probe by utilizing the surface specificity of sum frequency vibrational spectroscopy

Figure 1. Illustration of a cell membrane.

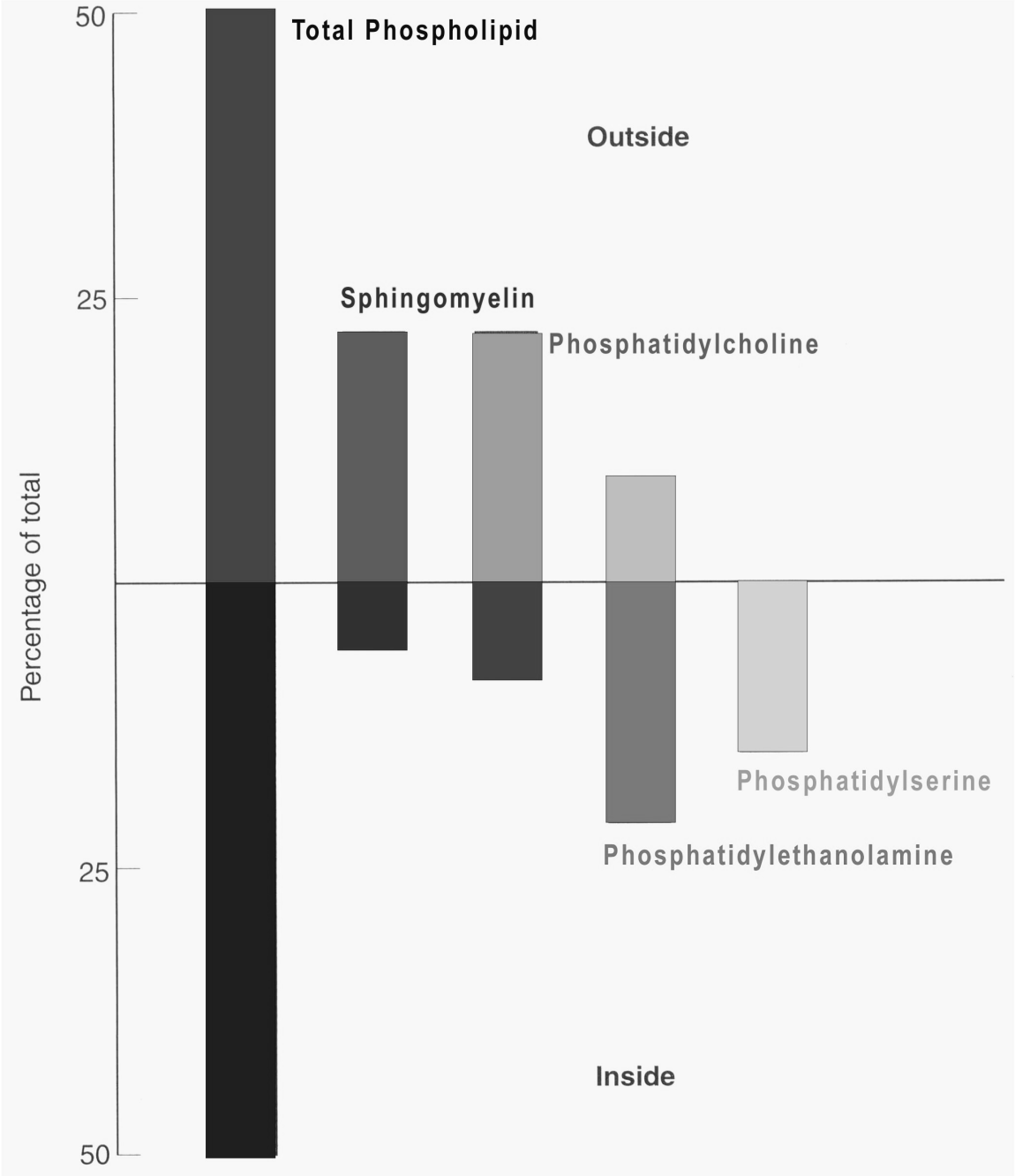

Extracellular Fluid
Glycoprotein
Glycolipid
Cholesterol
Carbohydrate
Peripheral protein
Integral protein
Filaments of cytoskeleton
Cytoplasm

(SFVS).[10] With this method, the measured flip-flop rate of phosphatidylcholine lipids was found to be minutes.

Despite apparent rapid lipid flip-flop, cell membranes in eukaryotes maintain an asymmetric distribution of phospholipids between each leaflet of the bilayer with respect to headgroup chemical structure. Lipid membrane asymmetry is essential for cell stability and viability.[11] As seen in Figure 2, phosphatidylcholine (PC) and sphingomyelin (SM) are located primarily in the exterior leaflet. Phosphatidylethanolamine (PE) is located predominantly in the cytosolic leaflet, and phosphatidylserine (PS) is located almost exclusively in the cytosolic leaflet.[12] This asymmetry is thought to be associated with various biological processes and be involved with intercellular signaling functions. PS serves as a cofactor with many enzymes. For example, PS is involved in the activation of protein kinase C,[13-17] Raf-1 protein kinase,[18-20] Na^+/K^+ ATPase,[18] dynamin-1,[21] and neutral sphingomyelinase.[22] Additionally, the loss of PS asymmetry in the bilayer is associated with various biological processes. PS becomes externalized during platelet activation and is involved in the blood coagulation cascade.[23] PS exposed on the exterior leaflet activates coagulation factor Xa in the presence of calcium and factor Va, which leads to the conversion of prothrombin to thrombin, a central molecule in the blood coagulation cascade.[24] The loss of asymmetry with respect to PS is also associated with cell death. During the early stages of apoptosis, PS is exposed to the outer leaflet. It appears that PS is a signal recognized by phagocytes marking the cell for phagocytosis.[25-27]

The need for lipid flip-flop in order to accommodate cell growth is in opposition to the need for membrane asymmetry associated with cell stability. As has been shown,

Figure 2. Distribution of phospholipids between the inner and outer leaflets of the human erythrocyte membrane.[12]


50
25
25
50
Percentage of total
Total Phospholipid
Outside
Sphingomyelin
Phosphatidylcholine
Phosphatidylserine
Phosphatidylethanolamine
Inside

flip-flop appears to be rapid. It has been hypothesized that specific membrane bound translocation proteins transfer lipids to create an asymmetric distribution at the expense of ATP. It has also been hypothesized that weak interactions between membrane lipids and cytoskeletal proteins induce and maintain membrane asymmetry.

In this study, model membrane systems are introduced to charged polypeptides as a model for weak interactions between cytosolic molecules and the cell membrane. Planar supported lipid bilayers were studied with SFVS to determine the effect of charged polypeptides on membrane asymmetry. This study will demonstrate, for the first time, that asymmetry can be induced in a lipid bilayer through interaction with charged polypeptides.

Sum Frequency Vibrational Spectroscopy

Sum frequency vibrational spectroscopy is a novel technique used to measure the degree of asymmetry or population inversion in lipid membranes. SFVS has an advantage over other techniques used to measure membrane asymmetry which require the use of labeled lipid species to track lipid motion and asymmetry, such as electron spin resonance or fluorescence. The main advantage of SFVS over other methods is the fact that it has the ability to measure asymmetry of native lipid species without the use of bulky probes that have major chemical and structural differences from the native lipids.

SFVS is a coherent nonlinear optical vibrational spectroscopy which has the chemical selectivity of IR and Raman transitions. Asymmetry is required to generate a sum frequency response. This is true on the molecular level as well as the macroscopic level. Thus, an isotropic bulk phase material, which is centrosymmetric, will not produce a sum frequency signal. An interface creates a break in symmetry allowing sum

frequency generation. These conditions allow molecules at the surface to be exclusively probed, as opposed to traditional vibrational spectroscopies. In addition to a break in inversion symmetry, a net polar orientation of the molecules at the interface is necessary for sum frequency generation.[28-31]

Sum frequency generation occurs when two pulsed laser sources, a visible (ω_{Vis}) and a tunable IR (ω_{IR}) source, are overlapped spatially and temporally at an interface. A third photon (ω_{SF}) is emitted at the sum of the two frequencies, given by:

$$\omega_{SF} = \omega_{Vis} + \omega_{IR} \tag{1}$$

The intensity of the sum frequency response is given by

$$I_{SFVS} = \left| \tilde{f}_{SF} f_{Vis} f_{IR} \chi^{(2)} \right|^2 \tag{2}$$

and is equal to the square of the Fresnel coefficients for the sum frequency ($\tilde{f}_{SF}$), visible (f_{Vis}), and IR (f_{IR}) beams, and the second order nonlinear susceptibility tensor ($\chi^{(2)}$). The Fresnel coefficients describe the magnitude of the electric field at the interface for a given polarization and incident angle of the light. The second order nonlinear susceptibility tensor describes the response of the interfacial molecules to the applied electric fields. The second order nonlinear susceptibility ($\chi^{(2)}$) tensor can be described by the resonant ($\chi_R^{(2)}$) and nonresonant ($\chi_{NR}^{(2)}$) susceptibility tensors.

$$\chi^{(2)} = \chi_R^{(2)} + \chi_{NR}^{(2)} \tag{3}$$

The nonresonant susceptibility tensor describes the nonresonant sum frequency response arising from the substrate. The non-resonant susceptibility tensor ($\chi_{NR}^{(2)}$) approaches zero for most optical substrates used to prepare planar supported lipid bilayers, such as

silica.[30] As such, the non-resonant term ($\chi_{NR}^{(2)}$) can be neglected in Equation 3 for the present system.

The resonant nonlinear susceptibility tensor can be expanded in terms of the IR (A_l) and Raman (M_{jk}) transition probabilities, given by

$$\chi_R^{(2)} = \sum_{\nu} \frac{N \left\langle A_l M_{jk} \right\rangle}{\omega_\nu - \omega_{IR} - i\Gamma_\nu} \tag{4}$$

N is the number of molecules at the interface, ω_ν is the frequency of the ν^{th} normal mode vibration, ω_{IR} is the IR input frequency, and Γ_ν is the linewidth of the ν^{th} transition. As the IR input frequency approaches a normal mode vibration of interfacial molecules that are both IR and Raman active, a resonant enhancement of the SFVS signal results. A SFVS spectrum is obtained by scanning the IR laser source over the range of molecular vibrational modes. For example, the C-H vibrational modes found in the 2750 cm^{-1} to 3050 cm^{-1} range were probed in the present study.

Because a net polar orientation of molecules at the interface being probed is necessary for sum frequency generation, the relative orientation of lipids in each leaflet of a lipid bilayer can have an effect on the sum frequency signal generated. The symmetric stretching vibrational mode of the terminal methyl group (CH_3 ν_s) in gel phase lipids is nearly normal to the surface.[32] Because SFVS is a coherent process, destructive interference will result between the methyl symmetric stretch transitions of oppositely oriented terminal methyl groups in each leaflet of the bilayer. The sum frequency signal from one leaflet will have the opposite phase as that from the other leaflet, leading to cancellation of the net sum frequency signal because SFVS is a coherent process.

This relationship between each leaflet of the bilayer can be described with $\chi^{(2)}$ expressed in terms of the orientational average of the molecular hyperpolarizabilities (β) for the terminal methyl symmetric stretching vibration.

$$\chi^{(2)} = \frac{N_{distal}}{\varepsilon_0} < \beta^{CH_3 \nu_s} > - \frac{N_{proximal}}{\varepsilon_0} < \beta^{CH_3 \nu_s} > \quad (5)$$

N_{distal} refers to the fraction of lipids in the distal leaflet of the bilayer while $N_{proximal}$ refers to the fraction of the lipid molecules in the proximal leaflet and ε_o is the vacuum permittivity constant.[30] By combining Equations 2 and 5, the sum frequency signal intensity can be related to the distribution of lipids within the bilayer.[10]

$$I_{CH_3}(t) \propto (N_{distal} - N_{proximal})^2 \quad (6)$$

Deuterated lipid analogs are SFVS inactive at the resonance frequencies of proteated lipids. If proteated lipids comprise one leaflet of the bilayer while deuterated analogs comprise the other leaflet, dipole cancellation will not occur, and the sum frequency signal will increase at the C-H resonance frequencies.

In order to determine the degree to which charged polypeptides could induce an asymmetric distribution of phospholipids, mixed PSLBs are made in which 1,2-distearoyl-d70-*sn*-glycero-3-phosphocholine (DSPC) is deuterated and 1,2-distearoyl-*sn*-glycero-3-[phospho-L-serine] (DSPS) is not. Thus, the SFVS signal in the probed frequencies arises from DSPS asymmetry. If charged polypeptides induce DSPS to distribute predominantly in one leaflet, a rise in SFVS signal will result as compared to a symmetric distribution of DSPS. The same principle will apply when DSPS is deuterated and DSPC is not.

Planar Supported Lipid Bilayers

The use of in vivo membrane systems would be ideal for the study of membrane asymmetry as well as flip-flop, but the complexity of these systems makes it currently impossible to isolate and monitor a particular response. Hence, in vitro model systems are used to gain insight to the larger system. Solution phase vesicles have been the most widely used model to study lipid flip-flop. As with any model system, there are limitations to solution phase vesicles. The radius of curvature of small unilamellar vesicles is much smaller than that of cell membranes.[33] This subjects the bilayer to forces not seen in natural cell membranes. Also, labeled lipid probes are necessary when using solution phase vesicles. As has already been mentioned, the use of labeled lipid probes has drawbacks. Namely, structural and chemical changes from the native lipid species dramatically affect kinetic and dynamic measurements.[10]

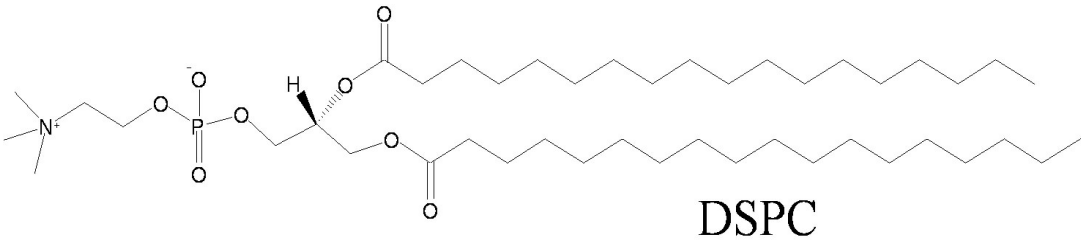
For the present study, planar supported lipid bilayers (PSLBs) are utilized. PSLBs are bilayers formed on a solid support. This results in a bilayer with an infinite radius of curvature, which more closely mimics an actual cell membrane. PSLBs can be formed through vesicle fusion or by the Langmuir-Blodgett / Langmuir-Schaeffer (LB/LS) method. Vesicle fusion does not allow for preparation of asymmetric bilayers. However, the LB/LS method allows for control of surface pressure as well as leaflet composition by depositing each leaflet of the bilayer separately. Thus, the LB/LS method is ideally suited to prepare PSLBs for study with SFVS.

EXPERIMENTAL

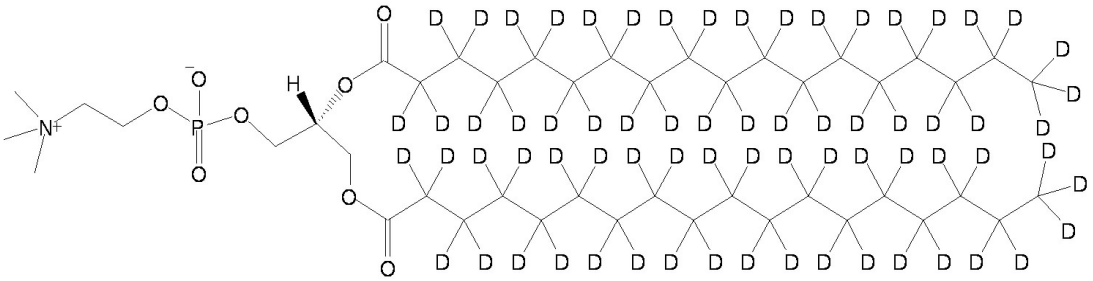
Materials

Deuterium oxide (D_2O) was purchased from Cambridge Isotope Laboratories (Andover, MA) and used after filtration through a 0.45µm Whatman puradisc^{TM} 25mm (Florham Park, NJ). Spectroscopic grade chloroform ($CHCl_3$) and methanol (CH_3OH) were supplied by Mallinckrodt (Mallinckrodt Baker Bioscience, Phillipsburg, NJ) and Sigma-Aldrich Corp. (St. Louis, MO), respectively. 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE), 1,2-distearoyl-d70-*sn*-glycero-3-phosphocholine (DSPC-d_{70}), DSPC, 1,2-distearoyl-d70-*sn*-glycero-3-[phospho-L-serine] (sodium salt) (DSPS-d_{70}), and DSPS (sodium salt) were purchased from Avanti Polar Lipids (Alabaster, AL) as either lyophilized powders or dissolved in $CHCl_3$, and were used without further purification. All lipids were stored in the freezer (-20 °C) until use. Structures of the lipids used are found in Figure 3. Poly-L-lysine hydrobromide (M.W. 24,000) was used as supplied by Sigma-Aldrich Corp. (St. Louis, MO). All the water used was purified by a Nanopure Infinity Ultrapure water purifying system (Barnstead/Thermolyne, Dubuque, IA) with a resistivity of 18.0-18.3 MΩ-cm. The substrates used to support the planar lipid bilayers were hemicylindrical fused silica prisms from Almaz Optics (Marlton, NJ) and fused silica slides from Chemglass (Vineland, NJ). The substrates were immersed in a solution of 70%-concentrated sulfuric acid / 30%-30% hydrogen peroxide (both from Mallinckrodt) to remove organic material.

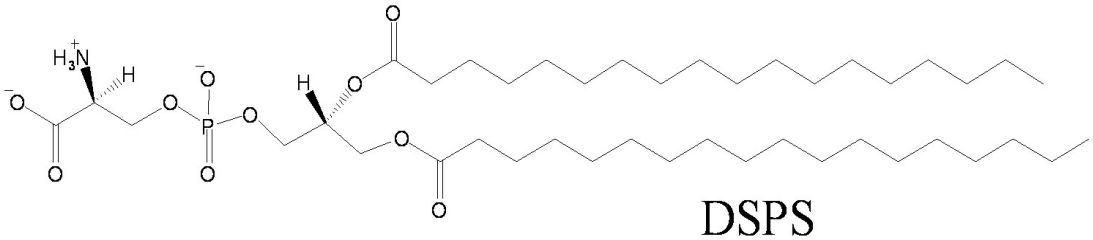
Figure 3. Chemical structure of phospholipids used in the study: DSPC, DSPC-d_{70}, DSPS, DSPS-d_{70}, and DSPE.



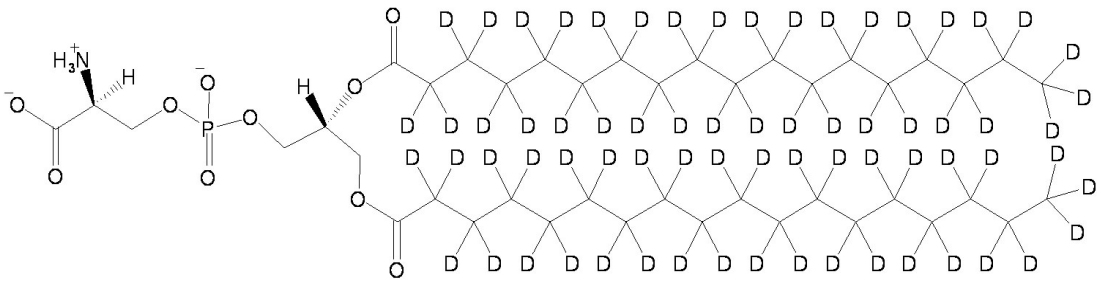
DSPC



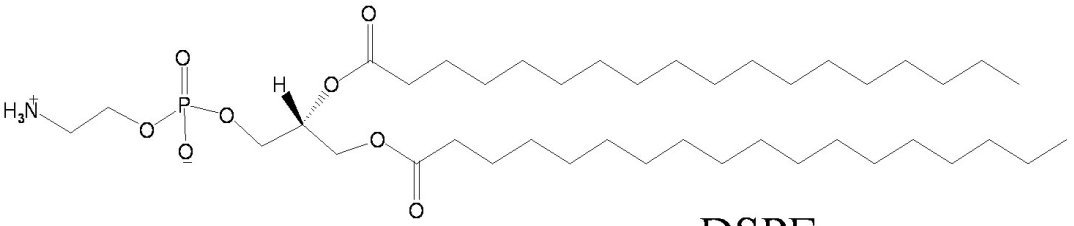
DSPC-d70



DSPS



DSPS-d70



DSPE

Planar Supported Lipid Bilayer Formation

After rinsing in copious amount of Nanopure water, the silica substrates were cleaned with an argon plasma cleaner (Harrick Scientific Plasma Cleaner/Sterilizer, Ossinining, NY) for 2 minutes. PSLBs were prepared by the LB/LS method. PSLBs were used for induced asymmetry experiments. Monolayers prepared by the LB method were used in mass spectrometry experiments.

Care was taken in choosing the lipid combinations for the induced asymmetry experiments. For the first lipid layer, a 1 mg/ml solution of lipid or lipid mixture dissolved in 65:35:8 $CHCl_3$:MeOH:H_2O was spread at the air-water interface on a LB trough (KSV Instruments, Helsinki, Finland). The monolayer was compressed on the LB trough until a surface pressure of 30 mN/m was reached. The monolayer was allowed to equilibrate for 15 min at 30 mN/m. The first (proximal) layer was then deposited on the silica substrate via a vertical pull from the aqueous subphase into air (LB transfer). For the second layer, the same procedure was followed to deposit a lipid monolayer at the air-water interface on the LB trough. To deposit the second (distal) layer and complete the bilayer, the prism was pushed through the air-water interface horizontally (LS transfer). Table 1 is a list of the different lipid combinations used for the induced asymmetry experiments. A 1:1 ratio indicates a 1:1 mole ratio of phospholipids.

Table 1. List of SFVS Lipid Mixtures

LB	LS
DSPC	1:1 DSPC:DSPS-d_{70}
DSPC-d_{70}	1:1 DSPC-d_{70}:DSPS
1:1 DSPC:DSPS-d_{70}	DSPC

All lipid bilayers were maintained in an aqueous environment after deposition. The silica prism was assembled into a custom-made Teflon flow cell. The flow cell was flushed with D_2O in order to avoid spectral interference from water. A schematic of the cell can be seen in Figure 4. Temperature control was attained with a feedback loop between a thermistor and Peltier—thermoelectric cooler module (both from TE Technology, Inc., Traverse City, MI) as well as a circulating water jacket connected to a water bath (from Thermo Fisher Scientific, Inc., Walthman, MA).

Sum Frequency Vibrational Spectroscopy

A custom made spectrometer was used to perform the SFVS experiments for the analysis of induced lipid membrane asymmetry due to charged polypeptide interactions. A schematic of the laser instrument can be found in Figure 5. The spectrometer contained an optical parametric oscillator (OPO) / optical parametric amplifier (OPA) pair from Laser-Vision (Bellevue, WA) to achieve a tunable IR laser source. The fundamental output of a Q-switched Nd:YAG laser (Continuum Surelite II, Excel Technology Continuum Lasers, Santa Clara, CA) at 1064 nm and 10 Hz with a pulse duration of 7 ns was used to pump the spectrometer. This beam was frequency doubled through a potassium titanyl phosphate (KTP) crystal to produce 532 nm light which was used to pump the OPO. The idler output of the OPO was then coupled with the fundamental 1064 nm output in the OPA to achieve a tunable IR laser source in the mid-IR range from about 2700 cm^{-1} to 4000 cm^{-1}. A 532 nm visible laser source was obtained by frequency doubling the remaining fundamental 1064 nm Nd:YAG output. The visible 532 nm laser source and the tunable IR laser source were overlapped temporally and

Figure 4. Schematic of assembled Teflon flow cell flushed with 1 µM Poly-L-lysine in D_2O.

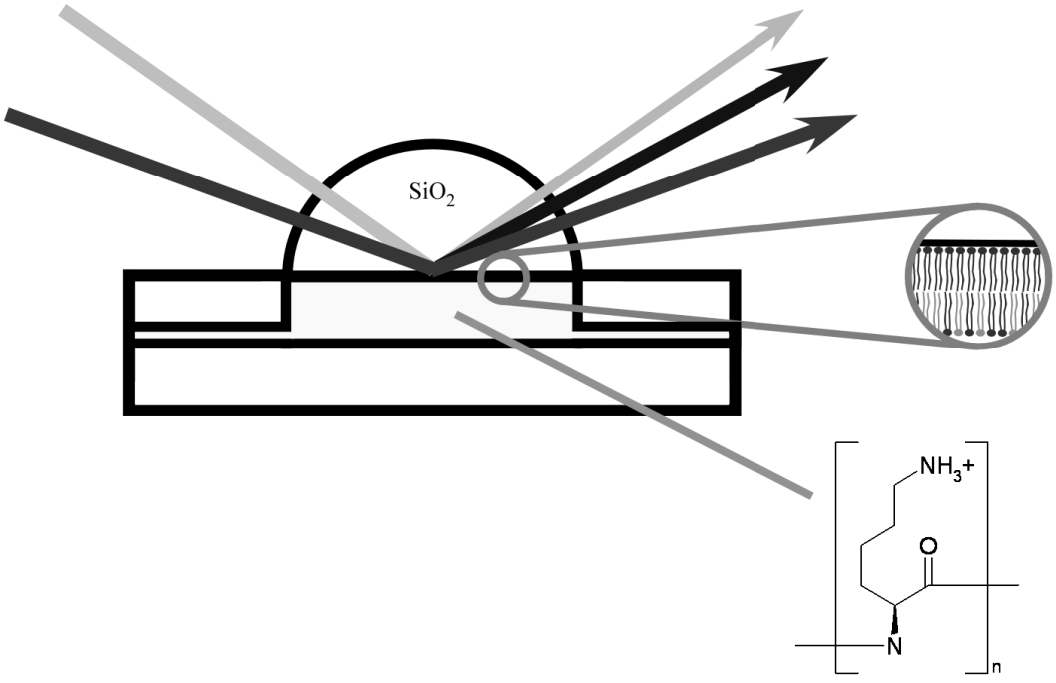
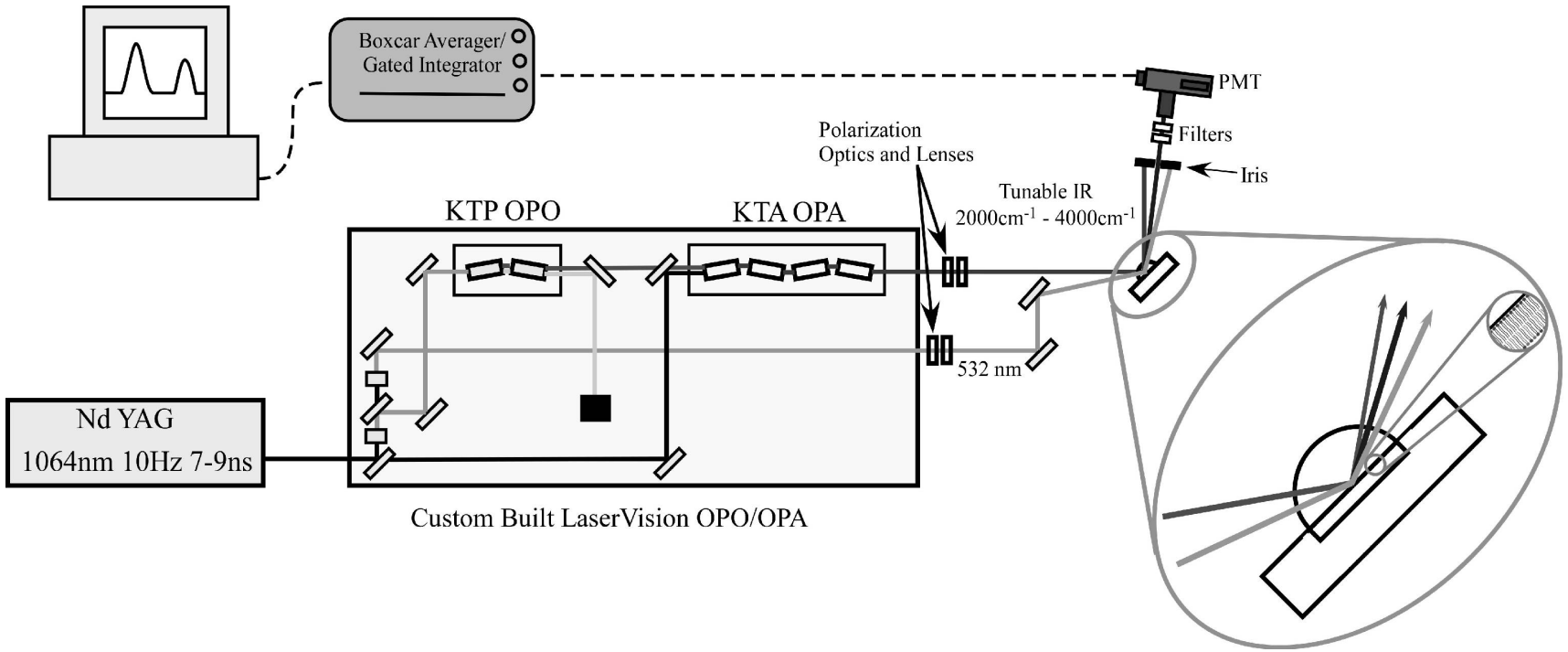

SiO2
NH3+
O
N
n

Figure 5. Schematic of sum frequency spectrometer.



Custom Built LaserVision OPO/OPA

spatially in a total internal reflection geometry at the silica prism/D_2O interface. The sum-frequency signal was filtered with a holographic notch filter (Kaiser Optical, Ann Arbor, MI) and several color filters to remove the residual 532 nm light. The sum frequency signal was then detected with a photomultiplier tube. The signal from the photomultiplier tube was collected with a boxcar integrator (Stanford Research Systems, Sunnyvale, CA). SFVS spectra were obtained by recording the intensity of the sum-frequency response from the surface as a function of the IR frequency. Spectra were acquired by scanning the IR at 2 cm^{-1} intervals.

Mass Spectrometry

The chemical composition of monolayers created by the Langmuir-Blodgett deposition method were compared to the chemical composition of the parent lipid mixtures used in the preparation of the monolayers by electrospray ionization mass spectrometry (ESI-MS). Monolayers of known lipid mixtures were prepared on fused silica slides by the Langmuir-Blodgett method, described above. DSPC, DSPS, and DSPE were used in different combinations for direct comparison with standard mixtures. The lipids were removed from the slides by washing with a 2:1 $CHCl_3$:MeOH mixture. The wash solution was collected and evaporated under Ar gas. The remaining lipids were then reconstituted in 500 µL of 2:1 $CHCl_3$:MeOH in order to create a more concentrated solution. A standard solution was created for comparison by diluting 100 µL of 1 mg/mL parent lipid solution with approximately 5 mL of 2:1 $CHCl_3$:MeOH. This was done to mimic the washing step of the LB monolayers. The standard solution was evaporated under Ar gas and then reconstituted in 1 mL of 2:1 $CHCl_3$:MeOH. The mass spectrum of each solution was obtained with negative ion mode ESI-MS by direct

infusion of the sample solution. A Fisons VG Quattro II (Waltham, MA) quadrupole mass spectrometer was used in the present study. The capillary was set at 3.50 kV and the cone was set to 35 V while the source block temperature was 85 °C and the desolvation temperature was 100 °C.

RESULTS AND DISCUSSION

Sum Frequency Vibrational Spectra

SFVS spectra were obtained for selectively deuterated bilayers. DSPS was probed by combining it in the bilayer with DSPC-d_{70}. Figure 6 shows the spectrum of a bilayer consisting of DSPC-d_{70} in the proximal leaflet and a 1:1 mixture of DSPC-d_{70} and DSPS in the distal leaflet with the corresponding individual peaks used in fitting the spectra. The spectrum was obtained with *s*-polarized sum frequency, *s*-polarized visible, and *p*-polarized IR. Transitions in the direction of the surface normal are probed in the *ssp* polarization combination. The peaks for this bilayer are located at 2848 cm^{-1} for the CH_2 symmetric stretch (ν_s), at 2874 cm^{-1} for the CH_3 ν_s, and at 2905 cm^{-1} for the CH_2 Fermi resonance. The CH_3 Fermi resonance is located at 2938 cm^{-1}, and the CH_3 asymmetric stretch (ν_{as}) is located at 2960 cm^{-1}. These peak assignments were previously made for the SFVS spectra based on the IR and Raman transitions of phospholipids and related material.[32, 34] The individual peaks shown in Figure 6 correspond to the sum-frequency intensity contribution from each individual normal resonance mode according to Equations 2, 3, and 4. For example, the intensity of the CH_3 ν_s fitting peak is proportional to the square of Equation 4 with respect to just the CH_3 ν_s resonance. Figure 7 is a simple illustration of distribution of lipids in a bilayer with DSPC-d_{70} in the proximal leaflet and a 1:1 DSPC-d_{70}:DSPS mixture in the distal leaflet. Figure 7 illustrates the orientation of the transition dipole moments of the CH_3 ν_s and CD_3 ν_s. The

Figure 6. SFVS spectrum of a DSPC-d_{70} (proximal) / 1:1 DSPC-d_{70}:DSPS (distal) bilayer with the corresponding peak fit.

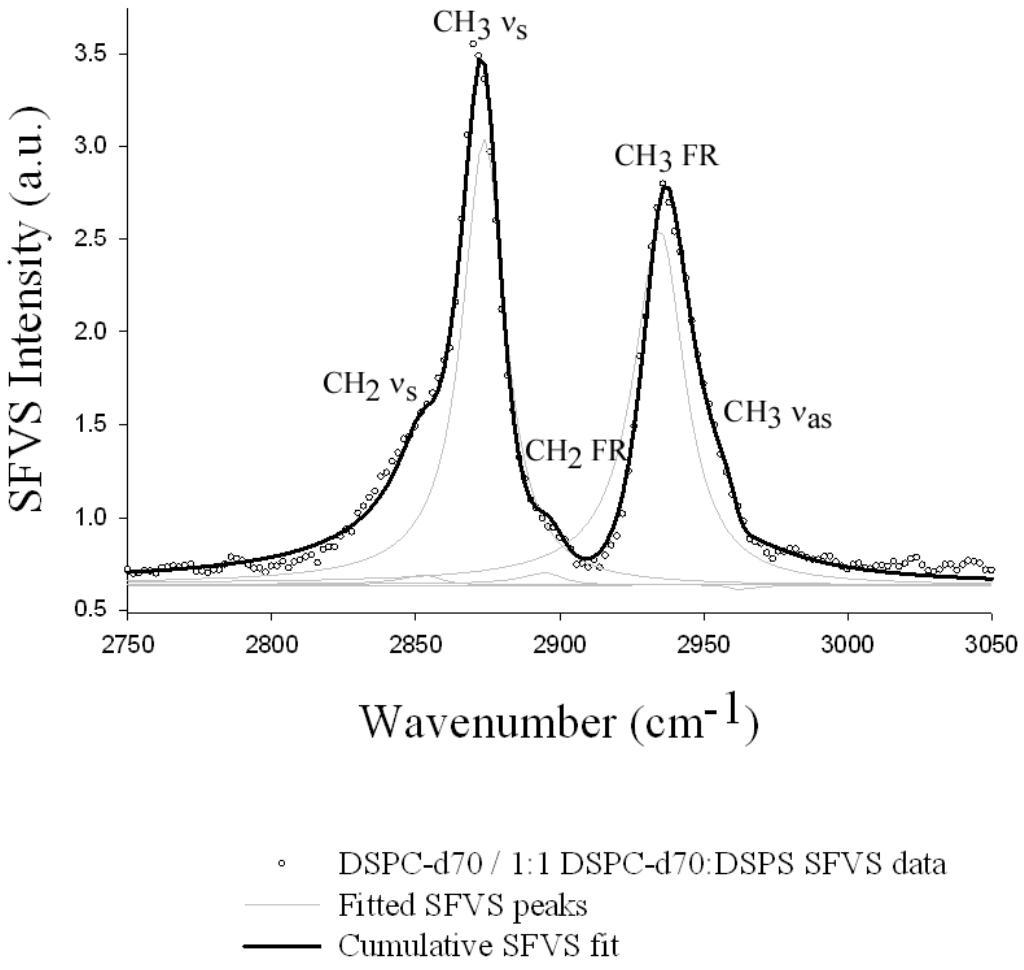
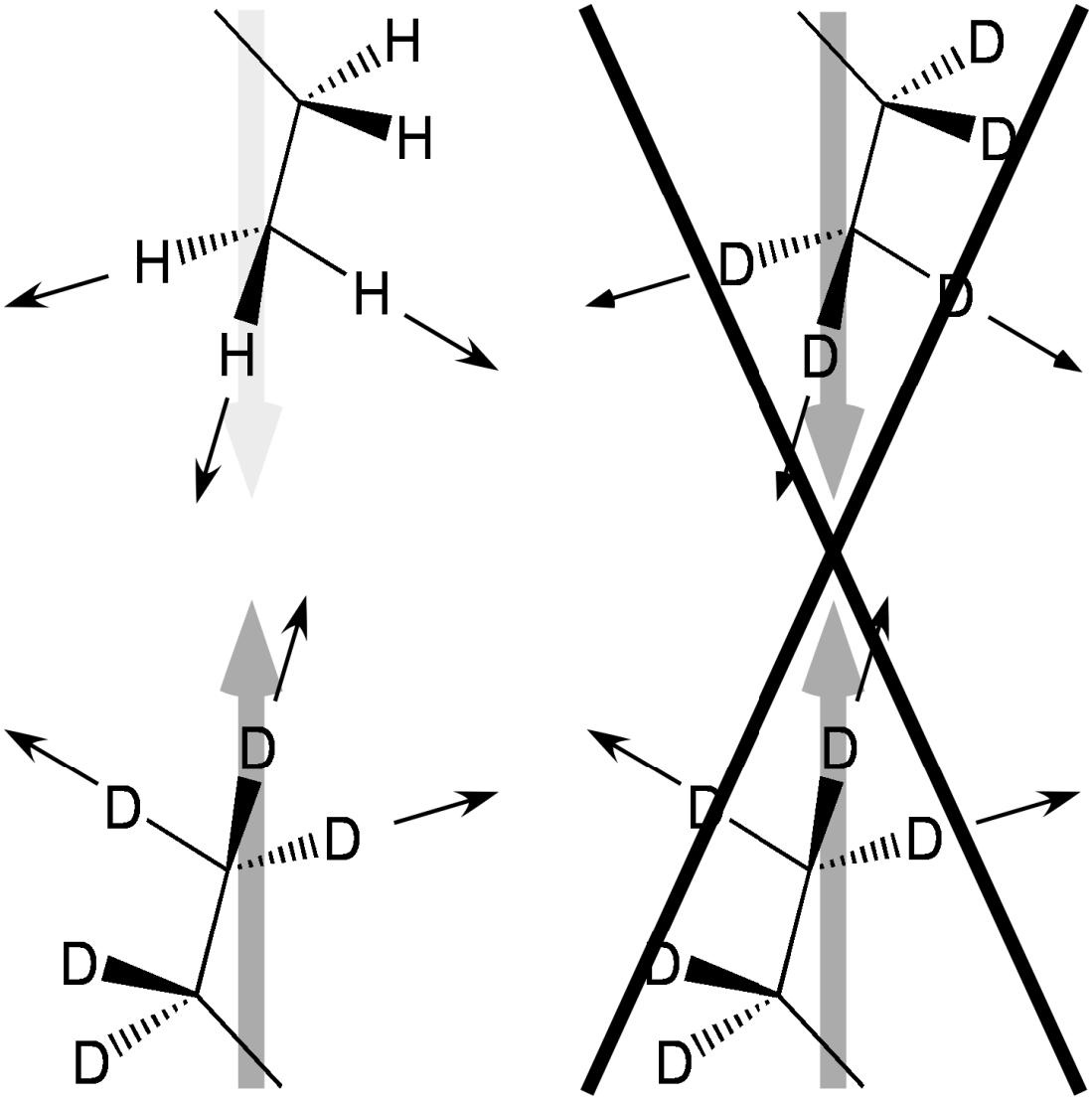

DSPC-d70 (Proximal) / 1:1 DSPC-d70:DSPS (Distal)
SFVS Intensity (a.u.)
Wavenumber (cm-1)
CH3 νs
CH3 FR
CH2 νs
CH2 FR
CH3 νas
3.5
3.0
2.5
2.0
1.5
1.0
0.5
2750
2800
2850
2900
2950
3000
3050
DSPC-d70 / 1:1 DSPC-d70:DSPS SFVS data
Fitted SFVS peaks
Cumulative SFVS fit

Figure 7. Illustration of dipole orientation for a bilayer of DSPC-d_{70} in the proximal leaflet and 1:1 DSPC-d_{70}:DSPS in the distal leaflet.


H
H
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D

resonance frequency of the CD_3 ν_s lies outside the scanned region. Absence of dipole cancellation of the CH_3 ν_s indicates that the SFVS signal arises from the asymmetry of DSPS in a DSPC-d_{70} / 1:1 DSPC-d_{70}:DSPS bilayer.

The spectrum of a bilayer consisting of DSPC in the proximal leaflet and a 1:1 mixture of DSPC and DSPS-d_{70} in the distal leaflet is shown in Figure 8. Dipole cancelation occurs between all of the DSPC lipids in the distal leaflet with half of the DSPC in the proximal leaflet, as shown in Figure 9. The SFVS signal arises from the remaining DSPC molecules in the proximal leaflet. Regardless of whether DSPS or DSPC is the deuterated species, the SFVS signal arises from 25% of the total lipids in the bilayer when DSPS has a mole fraction of 0.25 and is located exclusively in one leaflet. Thus, assuming mass-balance between the leaflets, the asymmetric distribution of lipids in the bilayer can be monitored by both DSPC and DSPS.

Induced Asymmetry

The spectra seen in Figures 6 and 8 indicate the formation of an asymmetric bilayer by the LB/LS deposition method. The CH_3 ν_s vibrational mode provides a useful measure of bilayer asymmetry due to the terminal methyl group dipole orientation. Equation 6 indicates that the SFVS intensity is proportional to the square of the population inversion in the bilayer. From Equation 6, $N_{proximal}$ and N_{distal} are the fraction of the probed molecules in each leaflet where

$$N_{proximal} + N_{distal} = 1. \tag{7}$$

Because the bilayers in this study were prepared as completely asymmetric bilayers, N_{distal} is equal to 1, and $N_{proximal}$ is equal to 0, leading to a maximum in the SFVS CH_3 ν_s signal, $I_{\max}^{CH_3\nu_s}$. As described by Equation 8, the percent asymmetry (%AS) present in a

Figure 8. SFVS spectrum of a DSPC (proximal) / 1:1 DSPC:DSPS-d_{70} (distal) bilayer with the corresponding peak fit.

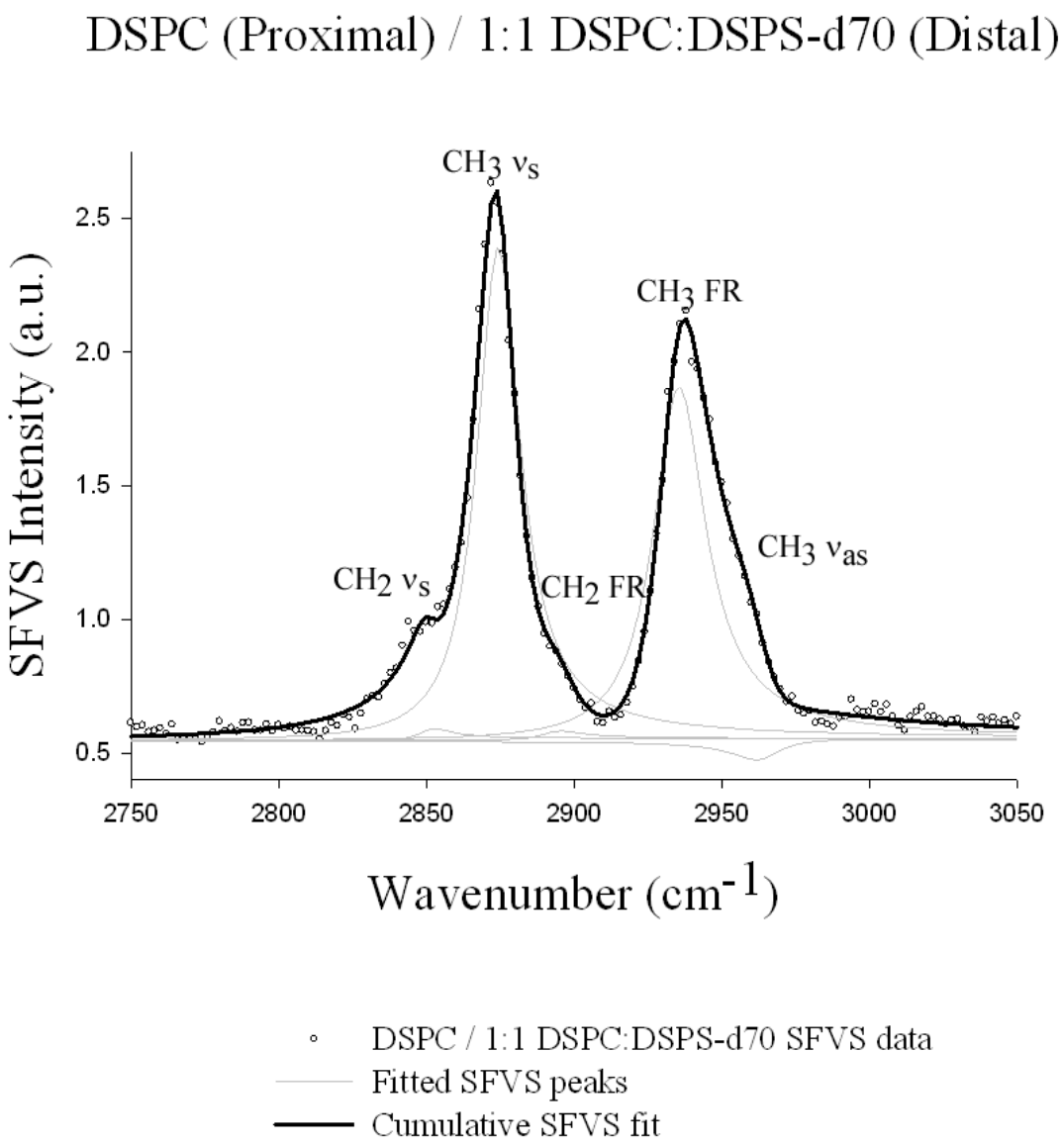
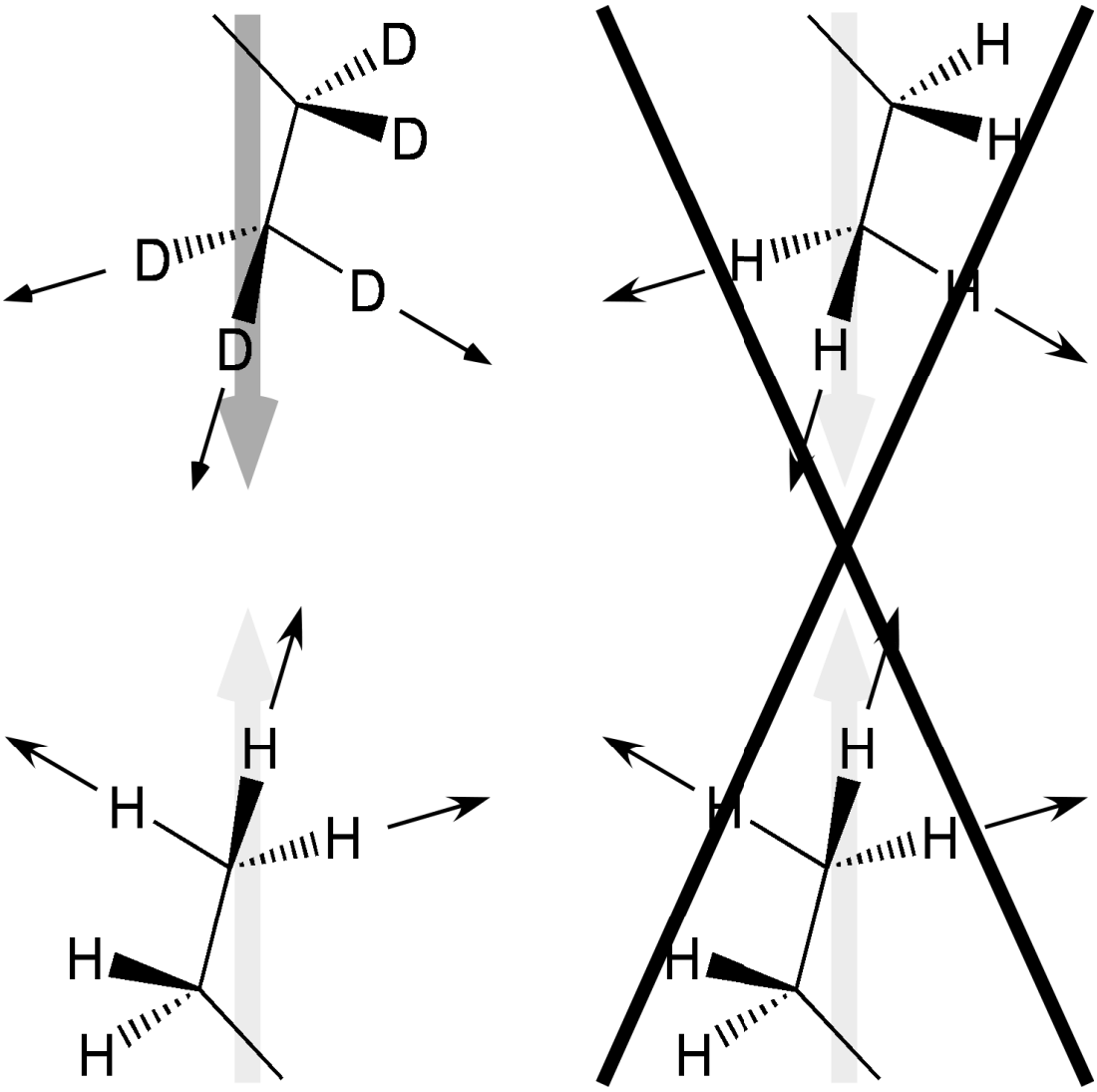

DSPC (Proximal) / 1:1 DSPC:DSPS-d70 (Distal)
SFVS Intensity (a.u.)
2.5
2.0
1.5
1.0
0.5
2750
2800
2850
2900
2950
3000
3050
Wavenumber (cm-1)
CH3 νs
CH3 FR
CH3 νas
CH2 νs
CH2 FR
DSPC / 1:1 DSPC:DSPS-d70 SFVS data
Fitted SFVS peaks
Cumulative SFVS fit

Figure 9. Illustration of dipole orientation for a bilayer of DSPC in the proximal leaflet and 1:1 DSPC:DSPS-d_{70} in the distal leaflet.


D
D
D
D
D
H
H
H
H
H
H
H
H
H
H
H
H

bilayer can be determined from the measured SFVS intensity of some unknown distribution, $I_{AS}^{CH_3\nu_s}$.

$$\%AS = \frac{\sqrt{I_{AS}^{CH_3\nu_s}}}{\sqrt{I_{\max}^{CH_3\nu_s}}} \times 100\% \quad (8)$$

Equation 8 assumes that the minimum SFVS intensity, corresponding to a completely symmetric bilayer, is equal to zero. However, in practice, a completely symmetric bilayer has some residual SFVS intensity. Therefore, in addition to determining the maximum SFVS intensity of the CH_3 ν_s, it is also necessary to determine the minimum SFVS intensity correlated with a completely symmetric bilayer, $I_{\min}^{CH_3\nu_s}$. $I_{\min}^{CH_3\nu_s}$ is needed in order to normalize the measured SFVS intensity, $I_{AS}^{CH_3\nu_s}$. Normalization accounts for fluctuations in SFVS intensity due to factors such as laser power and alignment. To calculate the percent asymmetry of a bilayer, the normalized SFVS intensities are used. Equation 9 is used to calculate the normalized SFVS intensities for X: max, min, AS.

$$I_{X\,(normalized)}^{CH_3\nu_s} = \frac{I_X^{CH_3\nu_s} - I_{\min}^{CH_3\nu_s}}{I_{\max}^{CH_3\nu_s} - I_{\min}^{CH_3\nu_s}} \quad (9)$$

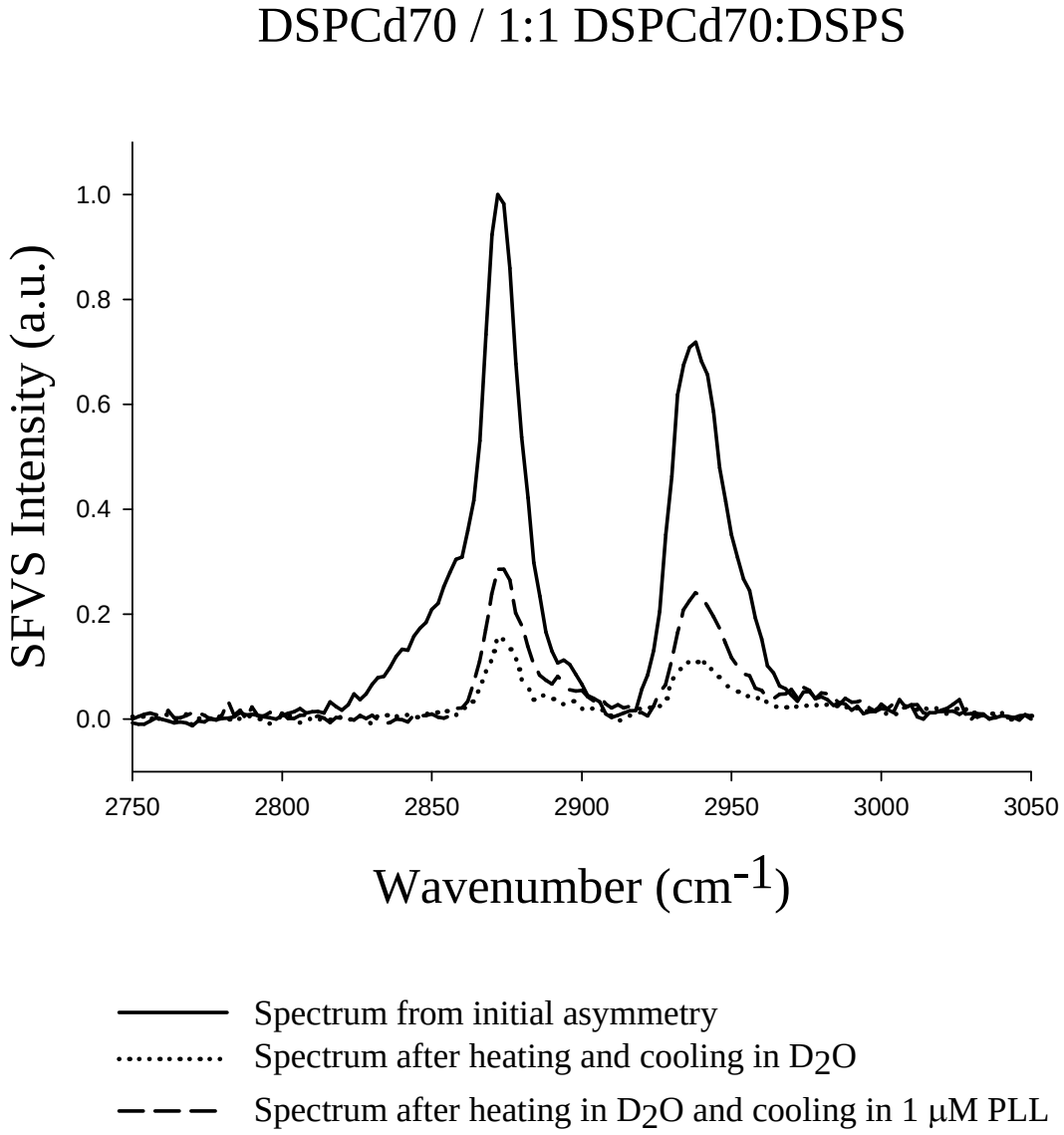
The minimum SFVS CH_3 ν_s intensity was measured after heating the bilayer from 25 °C to approximately 70 °C, above the phase transition temperatures of DSPC (55 °C) and DSPS (68 °C), and then back to 25 °C.[35] Heating above the phase transition temperature dramatically increases the rate of lipid flip-flop. In fact, flip-flop becomes so rapid, on the order of seconds, that it is experimentally impossible to measure the flip-flop rate.[10] Therefore, heating above the phase transition temperature induces complete flip-flop, creating a symmetric bilayer.

After cooling the bilayer sample, a spectrum was recorded. There is still some residual intensity measured. This result is consistent with previous SFVS studies of symmetric lipid bilayers.[36, 37] Because the bottom leaflet is supported on a fused silica substrate and the top leaflet is in contact with D_2O, a dielectric disparity exists between the top and bottom leaflets, giving rise to the small residual SFVS intensity measured for a symmetric bilayer.

After obtaining the spectrum of the symmetric bilayer at 25 °C, the sample is reheated to approximately 70 °C. At this point, the flow cell was flushed with 1 µM poly-L-lysine (PLL) in D_2O. The elevated temperature was maintained while flushing the cell with PLL. The cell was again cooled to 25 °C, at which point another spectrum was obtained. During the heating and cooling processes, the SFVS intensity of the CH_3 ν_s was continuously monitored.

The nomenclature for bilayers lists the proximal leaflet composition first followed by the distal leaflet composition, listed after a slash. For example, in Figures 10 and 11, the prepared bilayer consisted of DSPC-d_{70} in the proximal leaflet and 1:1 DSPC-d_{70}:DSPS in the distal leaflet (DSPC-d_{70} / 1:1 DSPC-d_{70}:DSPS). Figure 10 shows SFVS spectra of the bilayer at different stages. With this bilayer composition, the SFVS signal arises from the hydrogenated DSPS. The solid line spectrum corresponds to a completely asymmetric bilayer, with all DSPS in the distal leaflet. The dotted line spectrum in Figure 10 corresponds to the residual intensity of a completely symmetric bilayer arising from the dielectric disparity. After flushing the flow cell with 1 µM PLL, the cell was cooled and another spectrum was obtained, shown as the long dash line spectrum in Figure 10. The increase in SFVS signal after flushing with 1µM PLL as

Figure 10. Induced asymmetry SFVS spectrum of DSPC-d_{70} / 1:1 DSPC-d_{70}:DSPS.


DSPCd70 / 1:1 DSPCd70:DSPS
SFVS Intensity (a.u.)
1.0
0.8
0.6
0.4
0.2
0.0
2750
2800
2850
2900
2950
3000
3050
Wavenumber (cm-1)
Spectrum from initial asymmetry
Spectrum after heating and cooling in D2O
Spectrum after heating in D2O and cooling in 1 μM PLL

compared to the symmetric spectrum results from induced asymmetry due to electrostatic interaction between PLL and DSPS. In other words, PLL is a positively charged polypeptide that appears to associate electrostaticly with the negatively charged PS. The 1 μM PLL solution only comes in contact with the distal leaflet of the bilayer. The electrostatic forces between PLL and DSPS appear to induce DSPS to localize in the distal leaflet. This creates an asymmetric distribution of DSPS between each leaflet with DSPS favoring the distal leaflet.

Analysis of the heating curves shown in Figure 11 leads to the same conclusion. Figure 11 is a plot of the SFVS intensity measured at the CH_3 ν_s resonance frequency (2874 cm^{-1}) as a function of temperature. The black plot in Figure 11 tracks $I_{AS}^{CH_3\nu_s}$ as it begins at the maximum intensity. DSPS undergoes flip-flop as temperature increases until it reaches a minimum after the temperature exceeds the phase transition temperatures of DSPC (55 °C) and DSPS (68 °C). Upon cooling, a small increase is seen as the black plot returns to lower temperatures. As previously mentioned, this may be due to the dielectric disparity of the two leaflets. Also, some imperfections in the bilayer may have formed from the heating process. The light gray plot indicates the process of reheating the bilayer to above the phase transition temperatures of DSPC and DSPS, and tracks the black cooling curve. The medium gray plot indicates the cooling process in the presence of 1 μM PLL. As can be seen, the medium gray curve increases in intensity as compared to the light gray curve, indicating an increase in bilayer asymmetry with respect to DSPS. From this figure, a conclusion can be made about PLL, namely, that it induces DSPS to locate predominantly in the distal leaflet.

Figure 11. Induced asymmetry heating curves of DSPC-d_{70} / 1:1 DSPC-d_{70}:DSPS.

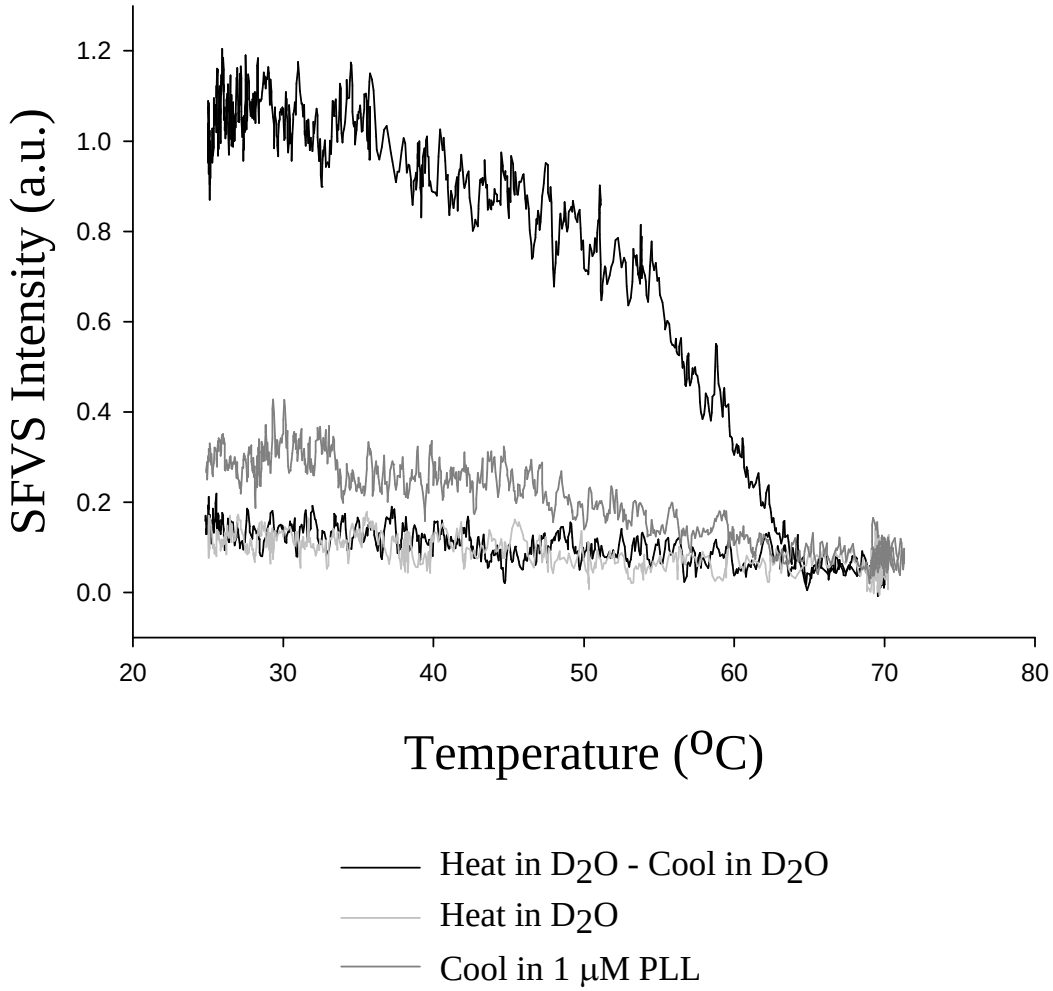

DSPC-d70 / 1:1 DSPC-d70:DSPS
SFVS Intensity (a.u.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
20
30
40
50
60
70
80
Temperature (°C)
Heat in D2O - Cool in D2O
Heat in D2O
Cool in 1 μM PLL

Figure 12 shows the SFVS spectra for a completely asymmetric DSPC / 1:1 DSPC:DSPS-d_{70} bilayer prior to heating (solid), a completely mixed (symmetric) bilayer after heating and cooling (dotted), and a partially asymmetric bilayer after cooling in the presence of 1 μM PLL (long dash). This lipid composition allows SFVS to track the location and movement of DSPC. In a completely asymmetric bilayer, the SFVS signal arises from 25% of the total lipid composition due to dipole cancellation of the remaining DSPC (see Figure 9). This is equivalent to the case of deuterated DSPC and hydrogenated DSPS where DSPS gives rise to the SFVS signal and is 25% of the total lipid composition. It should be noted that the increase in SFVS intensity after the introduction of PLL is still a function of electrostatic association between PLL and DSPS. As DSPS is induced by PLL to congregate in the distal leaflet, DSPC congregates in the proximal leaflet to the same degree due to mass balance transfer. As DSPS flips to the distal leaflet, DSPC is crowded out and flops to the proximal leaflet.

Figure 13 shows the heating curves of a DSPC / 1:1 DSPC:DSPS-d_{70} bilayer. As with the heating curves found in Figure 11 for DSPS, the induction of asymmetry can be seen as the bilayer is cooled in the presence of 1 μM PLL. However, there is one distinguishing factor between Figure 11 and Figure 13. Figure 13 shows an increase and then decrease in SFVS intensity as the bilayer cooled from 60 °C to about 50 °C. This peak results from disorder in the bilayer as DSPC passes through a phase transition at 55 °C.[35] No corresponding peak is seen for DSPS in Figure 11 because DSPS has a phase transition around 68 °C. By the time the bilayer begins to cool, DSPS has already crystallized into the gel phase, and thus no increase is seen in intensity due to phase segregation.

Figure 12. Induced asymmetry SFVS spectrum of DSPC / 1:1 DSPC:DSPS-d_{70}.

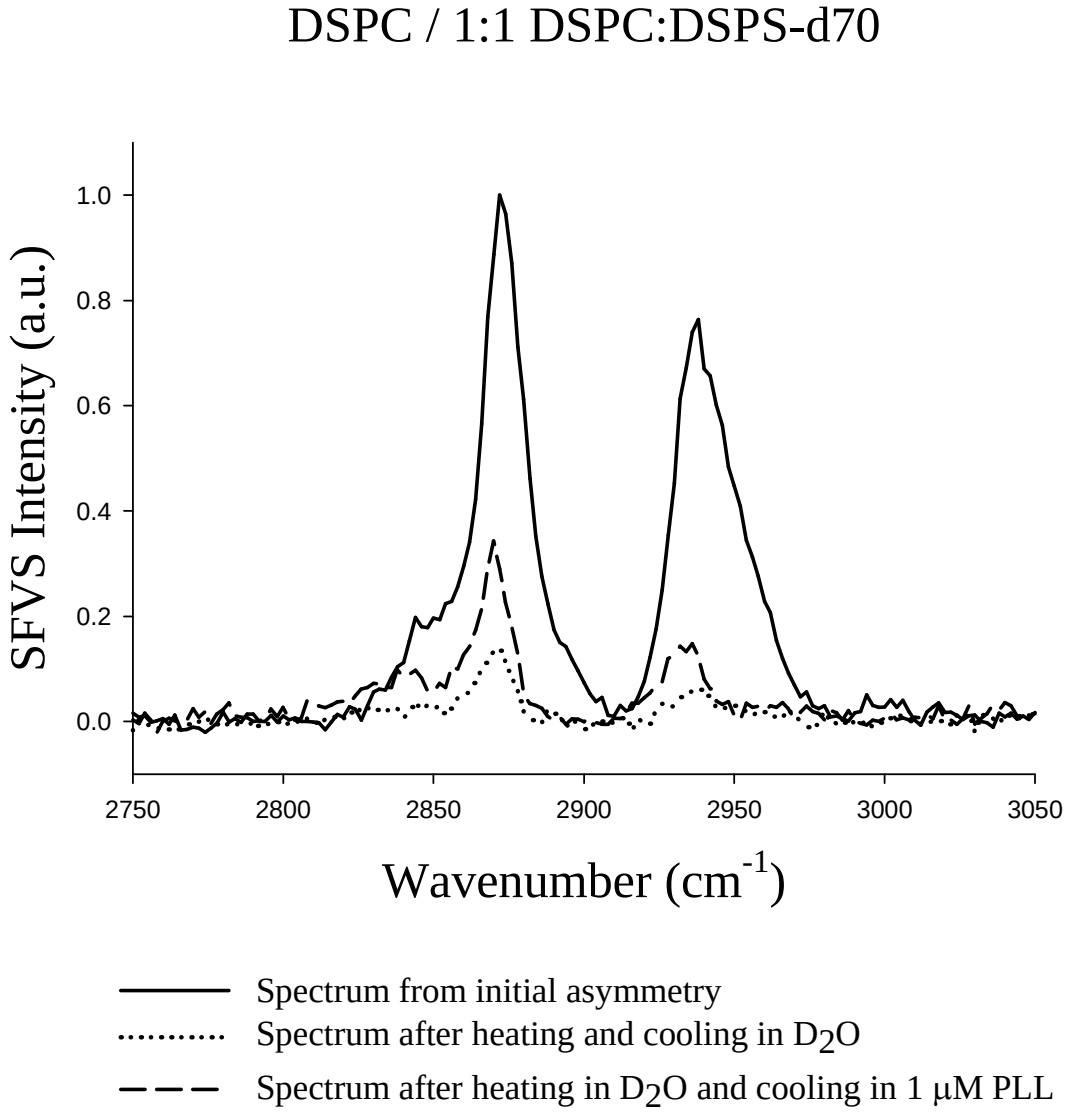
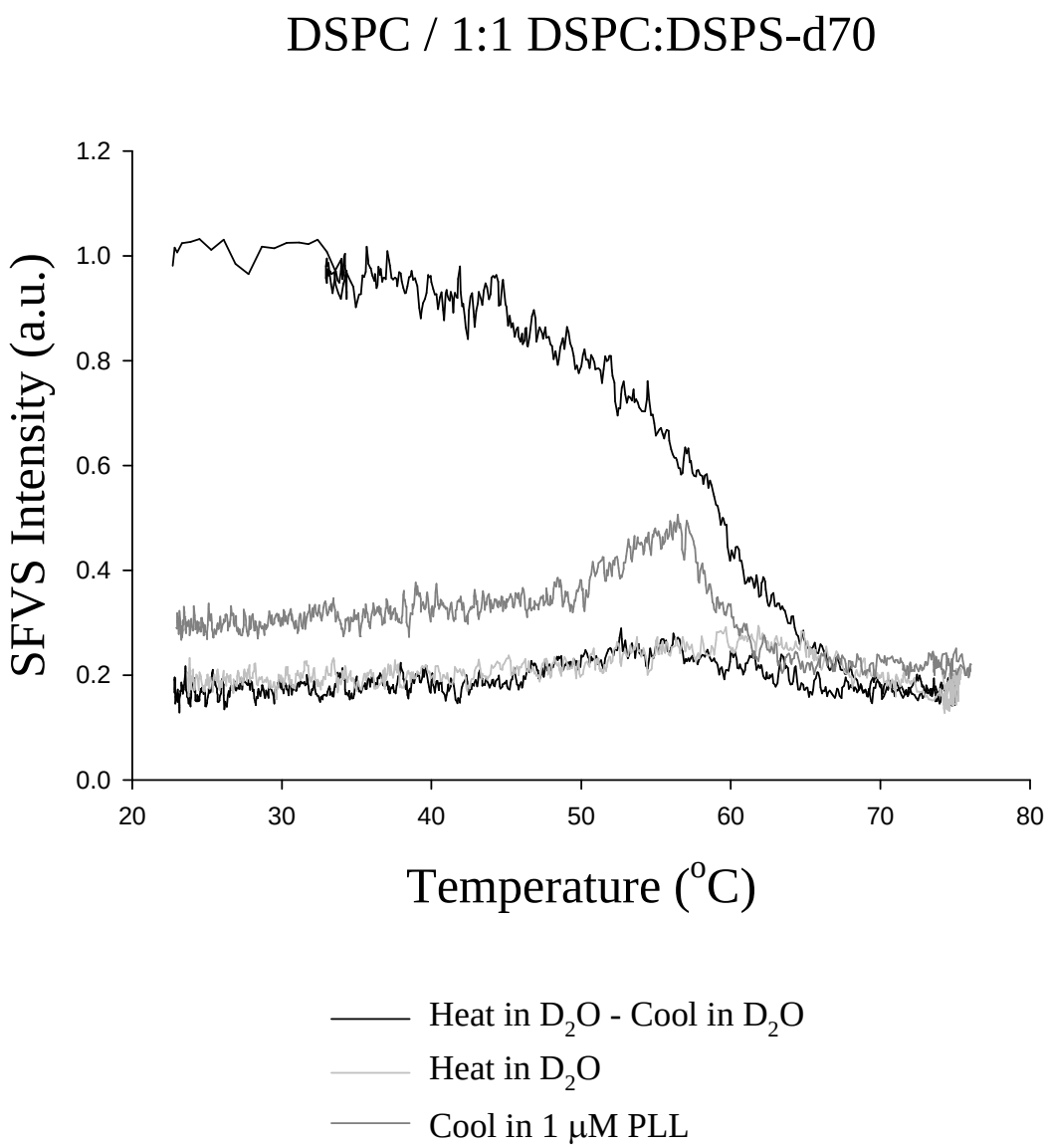

DSPC / 1:1 DSPC:DSPS-d70
SFVS Intensity (a.u.)
1.0
0.8
0.6
0.4
0.2
0.0
2750
2800
2850
2900
2950
3000
3050
Wavenumber (cm-1)
Spectrum from initial asymmetry
Spectrum after heating and cooling in D2O
Spectrum after heating in D2O and cooling in 1 μM PLL

Figure 13. Induced asymmetry heating curves of DSPC / 1:1 DSPC:DSPS-d_{70}.


DSPC / 1:1 DSPC:DSPS-d70
SFVS Intensity (a.u.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
20
30
40
50
60
70
80
Temperature (°C)
Heat in D2O - Cool in D2O
Heat in D2O
Cool in 1 μM PLL

Figures 14 and 15 show the induced asymmetry SFVS spectra and the heating curves, respectively, for a bilayer composed of 1:1 DSPC:DSPS-d_{70} / DSPC. As with the previous examples, asymmetry is induced in the presence of 1 μM PLL. The heating curves for 1:1 DSPC:DSPS-d_{70} / DSPC display a similar response when the bilayer is cooled in the presence of PLL as the reverse bilayer with DSPC in the proximal leaflet. The slight dip in the initial heating curve can be attributed to the phase transition of DSPC as well as the dielectric disparity of the bilayer, as previously mentioned. Because the leaflets have been reversed, the dielectric disparity affects the bilayer differently. The measured SFVS intensity resulting from the dielectric disparity is coherent, as is all SFVS generated signal. However, when the actual asymmetry of the bilayer is out of phase with the measured intensity from the dielectric disparity, there is a cancelation.

The amount of asymmetry induced due to the presence of PLL can be quantified. Equation 8 describes the manner in which the percent asymmetry is calculated. Figures 10-15 are representative spectra and heating curves for the respective lipid compositions. Multiple experiments were performed, and the mean percent asymmetry is represented in Table 2. The percent asymmetry was calculated with the SFVS intensity from the spectra as well as the average of the intensity from the heating curves between 25 °C and 30 °C. Because $I_{\max}^{CH_3\nu_s}$ equals unity when normalized, %AS is dependent only on $\sqrt{I_{AS}^{CH_3\nu_s}}$ from Equation 8. According to Equation 6, $\sqrt{I_{AS}^{CH_3\nu_s}}$ is proportional to the population distribution of DSPS, $(N_{distal} - N_{proximal})$. Combining Equation 6 and Equation 8 gives,

$$\%AS = (N_{distal} - N_{proximal}) \times 100\% \qquad (10)$$

Figure 14. Induced asymmetry SFVS spectrum of 1:1 DSPC:DSPS-d_{70} / DSPC.

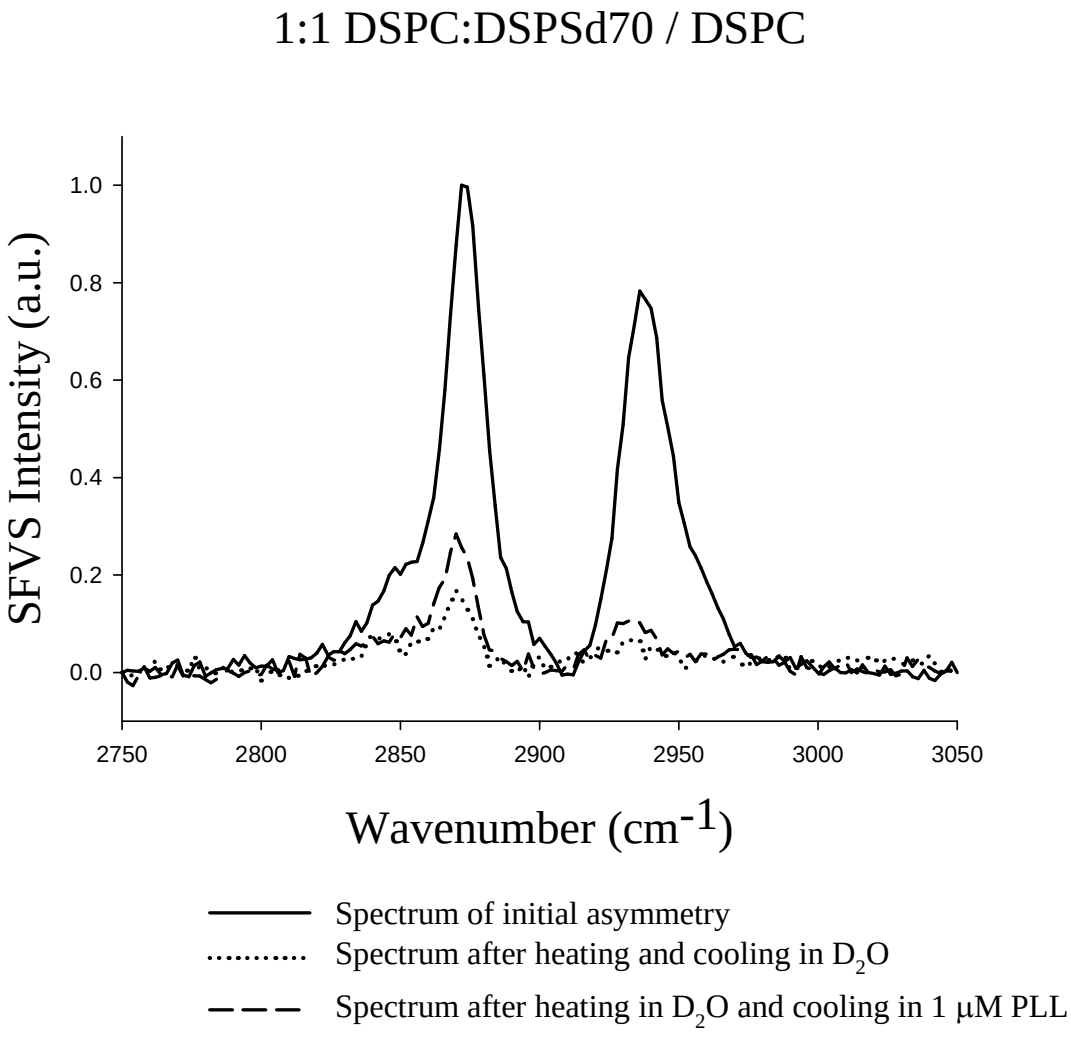

1:1 DSPC:DSPSd70 / DSPC
SFVS Intensity (a.u.)
1.0
0.8
0.6
0.4
0.2
0.0
2750
2800
2850
2900
2950
3000
3050
Wavenumber (cm-1)
Spectrum of initial asymmetry
Spectrum after heating and cooling in D2O
Spectrum after heating in D2O and cooling in 1 μM PLL

Figure 15. Induced asymmetry heating curves of 1:1 DSPC:DSPS-d_{70} / DSPC.

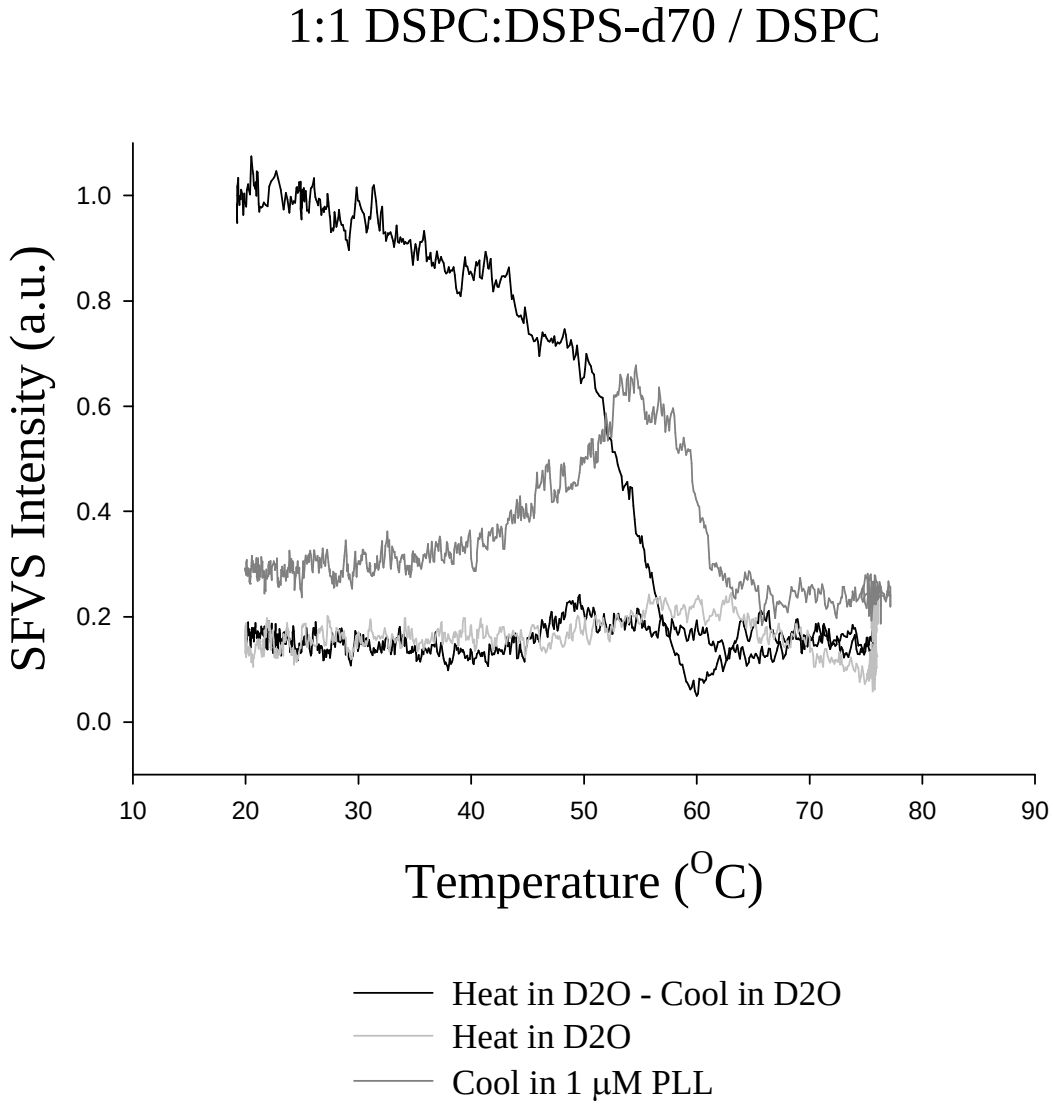

1:1 DSPC:DSPS-d70 / DSPC
SFVS Intensity (a.u.)
1.0
0.8
0.6
0.4
0.2
0.0
10
20
30
40
50
60
70
80
90
Temperature (OC)
Heat in D2O - Cool in D2O
Heat in D2O
Cool in 1 μM PLL

Table 2. Induced asymmetry results.

	Percent Asymmetry	Standard Deviation
From Spectral Calculation	42.8 %	±9.4 %
From Heating Curve Calculation	41.7 %	±8.4 %

Additionally, the standard deviation of percent asymmetry must be propagated from the standard deviation of SFVS intensity.

$$\sigma_{\%AS}^2 = \left(\frac{\partial \sqrt{I_{AS}^{CH_3\nu_s}}}{\partial \%AS} \right)^2 \sigma_{I_{AS}^{CH3\nu_s}}^2 \quad (11)$$

The data indicate that the interaction between PLL and DSPS can induce a bilayer of 25% DSPS and 75% DSPC to become about 42% asymmetric in DSPS. A percent asymmetry of 42% indicates that an excess of DSPS is located in the distal leaflet compared to the proximal leaflet equal to a mole fraction of DSPS of 0.42. In other words, the proximal leaflet retains a mole fraction of DSPS of 0.29 while the distal leaflet has a mole fraction of DSPS of 0.71. These results indicate that electrostatic interactions between PLL and DSPS induce an asymmetric distribution of lipids across the bilayer in this model. This lends credence to the hypothesis that electrostatic interactions may play a part in the maintenance of cell membrane asymmetry.

The limit of detection of SFVS can be calculated for percent asymmetry. The standard deviation of each point in a spectrum is determined as the standard deviation of the points from 2750 cm^{-1} to 2780 cm^{-1}, where there are not any resonance peaks. The standard deviation is 0.012 a.u. Accordingly, the limit of detection at the 95% confidence level is 0.039 a.u. This corresponds to a percent asymmetry of 19.7%. The intensity is consistent between the heated bilayer SFVS intensity and the SFVS intensity after being cooled in D_2O, confirming these results.

Silica has a fixed negative charge located at the surface in the presence of water due to the dissociation of surface silanol groups.[38, 39] It was hypothesized that this negative charge could interact with the bilayer and induce asymmetry through

electrostatic association in the same manner hypothesized with charged polypeptides. However, upon cooling back to 25 °C in the presence of D_2O, the CH_3 ν_s SFVS intensity remains essentially constant, indicating that the bilayer remains symmetric. In fact, the negative charge on the silica surface may induce phosphatidylserine asymmetry, but the amount of asymmetry induced is below the limit of detection of the SFVS method.

The amount of induced asymmetry due to the charge on the silica surface can be calculated as a comparison to the experimental results. This is accomplished by calculating the surface charge density at the silica interface. The silica surface charge density can be related to the charge density in the proximal leaflet of the bilayer, assuming a symmetric distribution of PS lipids, in order to calculate the population inversion expected to be caused by the charged silica surface. Each PS has a negative charge, and thus $1.602\times10^{-19}\ {}^{\mathrm{C}}\!/_{\text{PS molecule}}$. Each bilayer prepared in the current study contained 25% DSPS or DSPS-d_{70}. This corresponds to an average area of 220 $Å^2$/PS molecule for a bilayer prepared at a surface pressure of 30 mN/m. This results in a charge density in the proximal leaflet of -0.07283 C/m^2. Behrens and Grier describe a method for calculating the surface charge density on a silica surface.[39] In this method, the Basic Stern model is used to describe the environment at the silica interface. Equation 12 is the diffuse layer potential in the Basic Stern model and is a function of surface charge density. Equation 13 is the Grahame equation describing the surface charge density, and is a function of the diffuse layer potential. Equations 12 and 13 need to be solved simultaneously.

$$\psi_d(\sigma) = \frac{1}{\beta e}\ln\frac{-\sigma}{e\Gamma+\sigma} - (pH - pK)\frac{\ln 10}{\beta e} - \frac{\sigma}{C} \qquad (12)$$

$$\sigma(\psi_d) = \frac{2\varepsilon\varepsilon_0\kappa}{\beta e}\sinh\left(\frac{\beta e\psi_d}{2}\right) \quad (13)$$

ψ_d is the diffuse layer potential, $\beta = \frac{1}{k_B T}$, e is the charge of an electron, Γ is the concentration of dissociated silanol groups and equals 8 nm^{-2}.[40] σ is the silica surface charge density, and *pH* is the *pH* of D_2O, measured as 6.25. *pK* refers to the silica prism, and is 7.5 based on the surface complexation model.[41] C refers to the Stern layer capacity with a value of 2.9 F/m^2. ε is the permittivity of the solution and ε_0 is the permittivity of a vacuum. κ^{-1} is the Debye screening length, and is related to the concentration of ions, *n*, calculated to be the concentration of D_3O^+ based on the pH.

$$\kappa^2 = \frac{\beta e^2 n}{\varepsilon\varepsilon_o} \quad (14)$$

The surface charge density on the silica surface ($\sigma(\psi_d)$) was determined to be $-0.000515\ C/m^2$. This value equals the amount of charge from PS molecules in the proximal leaflet that should be pushed through the bilayer to establish equilibrium. The silica surface charge density divided by the charge density in the proximal monolayer is equal to the population inversion induced by the silica surface charge. Under these circumstances, $N_{distal} - N_{proximal} = 0.007$. This would result in a SFVS intensity of 0.000049. The silica surface should induce less than one percent asymmetry, which is far less than the noise of the measurement, and thus undetectable.

These same principles regarding surface charge density can be used as a check on whether it is logical that PLL could induce an asymmetric bilayer of the magnitude described above. For a 42 % asymmetric bilayer, the distal leaflet contains a 0.71 mole fraction of all DSPS molecules. However, DSPS only constitutes 25% of the lipids in the

bilayer. Therefore, DSPS constitutes 35.5% and DSPC constitutes 64.5% of the lipids in the distal leaflet. DSPS has a mean area per molecule in a pure DSPS monolayer at 30 mN/m of 55 $Å^2$/PS molecule. In a 42% asymmetric bilayer, DSPS has a mean molecular area of about 155 $Å^2$/PS molecule. Following the above calculation, the asymmetric bilayer has a charge density of -0.10337 C/m^2. The percent asymmetry is equal to the PLL surface charge density divided by the surface charge density of the distal leaflet arising from PS. Therefore, the surface charge density of PLL equals -0.04341 C/m^2. This correlates to 4.50 $\mu mol/m^2$ surface concentration assuming, *arguendo*, that each PLL has only one positive charge. However, there is approximately one positive charge for every 146 MW units of PLL, or 164 positive charges per PLL molecule having an average molecular weight of 24,000 MW units. If each of the 164 positive charges on a PLL molecule could be localized at the surface, the required surface charge density to induce a 42% asymmetric bilayer with respect to DSPS would be associated with a PLL surface concentration of 0.03 $\mu mol/m^2$. A 1 μM solution of PLL was used in this experiment. Many charges on PLL will be separated from the distal leaflet due to steric hindrances. Still, a 1μM PLL solution should be sufficient to induce the amount DSPS asymmetry seen experimentally, further supporting the role PLL plays to induce an asymmetric bilayer.

Bilayer Lipid Composition Analysis

PSLBs prepared by the LB/LS method are ideally suited to SFVS due to compositional control between each leaflet of the bilayer. As has already been discussed, careful selection of lipid mixtures allows for SFVS signal generation as a result of a specific known lipid. Insight is gained by probing different molecules in similar systems.

However, in order to give more confidence to the SFVS results, the efficacy of the LB/LS method must be determined. At question is whether the lipid composition prepared at the air/water interface is maintained during the LB/LS deposition process.

Electrospray ionization mass spectrometry was used to compare standard lipid mixtures with monolayers prepared by the Langmuir-Blodgett method. This was done to confirm that the LB monolayers consisted of the same lipids as the parent mixture. The procedure and instrumentation have been described in the experimental section. Preliminary data has been collected in which the integrated areas of mass peaks for each lipid present in a mixture are compared. The ratio of the areas in the standard mixtures is compared to the ratio of the areas in the monolayer samples. The results are shown in Table 3. Figures 16-18 show the mass spectra of the standard lipid mixtures compared to deposited monolayers. It should be noted that the abundance of particles in the case of the washed monolayers is nearly an order of magnitude smaller than the standard mixtures. Also, as the absolute abundance decreases in number, the degree of error increases. As can be seen, the lipids that are deposited onto the Langmuir trough for deposition onto a silica surface appear to transfer to the silica support.

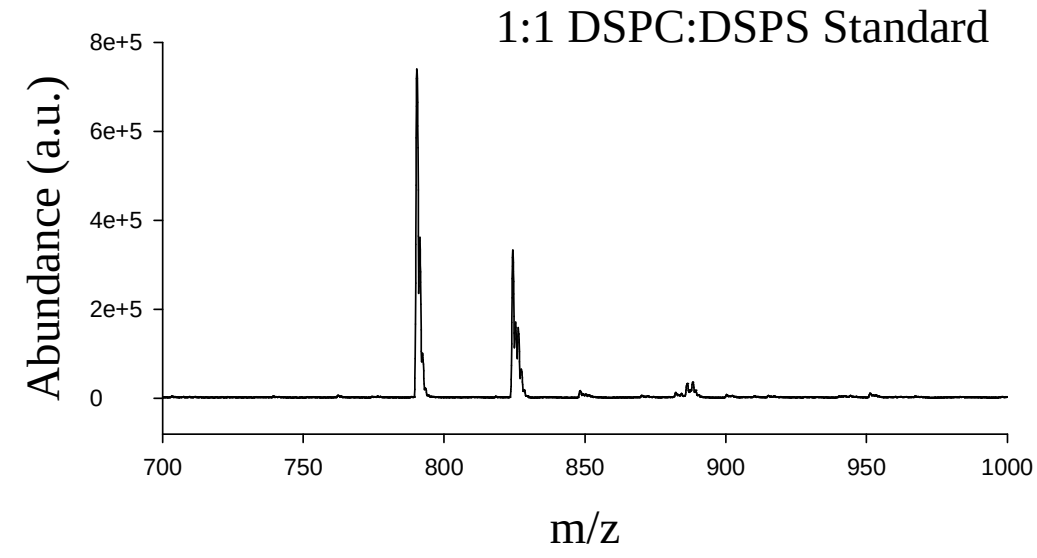
Conclusions

The results of this study give insight into the mechanism by which asymmetry is maintained in cell membranes. Electrostatic interaction appears to be an effective method for inducing and maintaining asymmetry in planar supported lipid bilayers of simple systems. The mechanism of membrane asymmetry maintenance is most likely a combination of many factors one of which has been shown to be electrostatic interactions. This study has also demonstrated the efficacy of sum frequency vibrational

Table 3. Results of mass spectrometry comparison between LB monolayers and standard lipid mixtures.

Lipid Mixture	Standard Ratio	Monolayer Ratio
1:1 DSPC:DSPS	0.585	0.519
3:1 DSPC:DSPE	2.900	3.459
9:1 DSPC:DSPE	9.282	9.128

Figure 16. Mass spectrometry comparison of 1:1 DSPC:DSPS.


1:1 DSPC:DSPS Standard
Abundance (a.u.)
8e+5
6e+5
4e+5
2e+5
0
700
750
800
850
900
950
1000
m/z

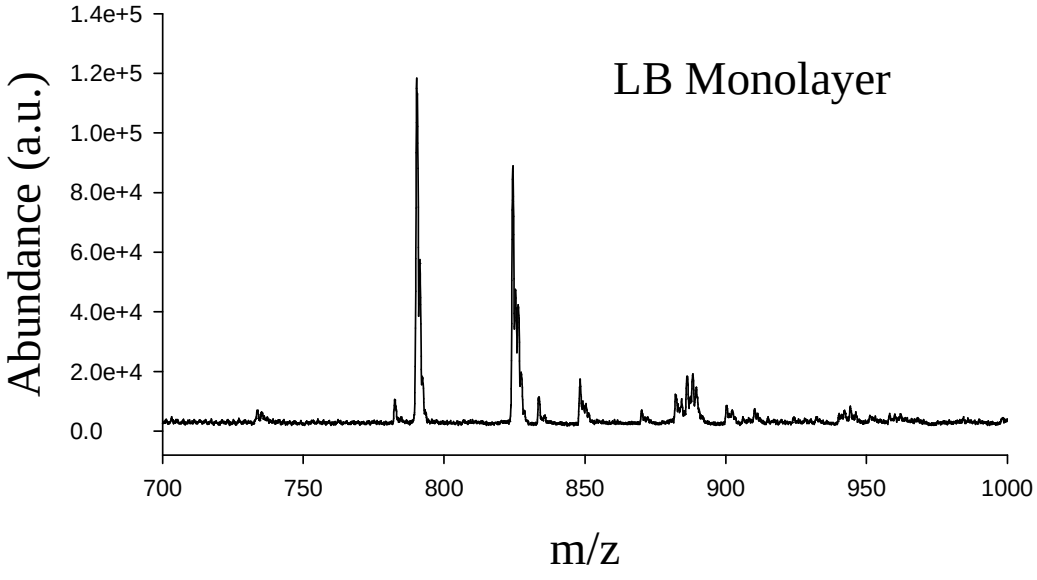
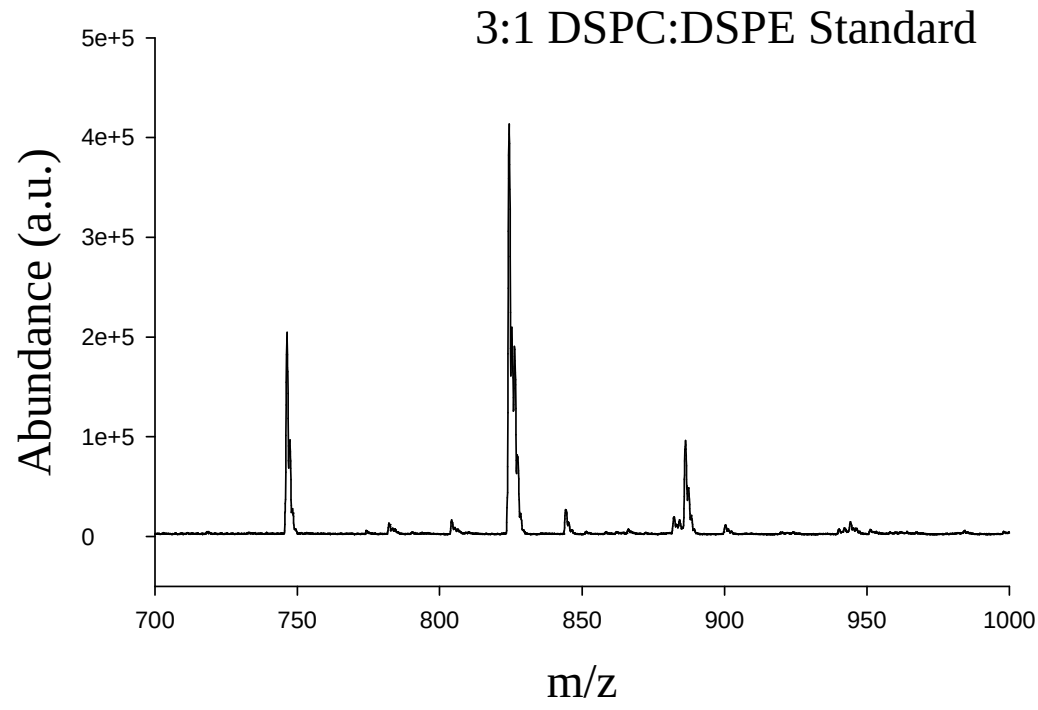

LB Monolayer
Abundance (a.u.)
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
700
750
800
850
900
950
1000
m/z

Figure 17. Mass spectrometry comparison of 3:1 DSPC:DSPE.


3:1 DSPC:DSPE Standard
Abundance (a.u.)
5e+5
4e+5
3e+5
2e+5
1e+5
0
700
750
800
850
900
950
1000
m/z

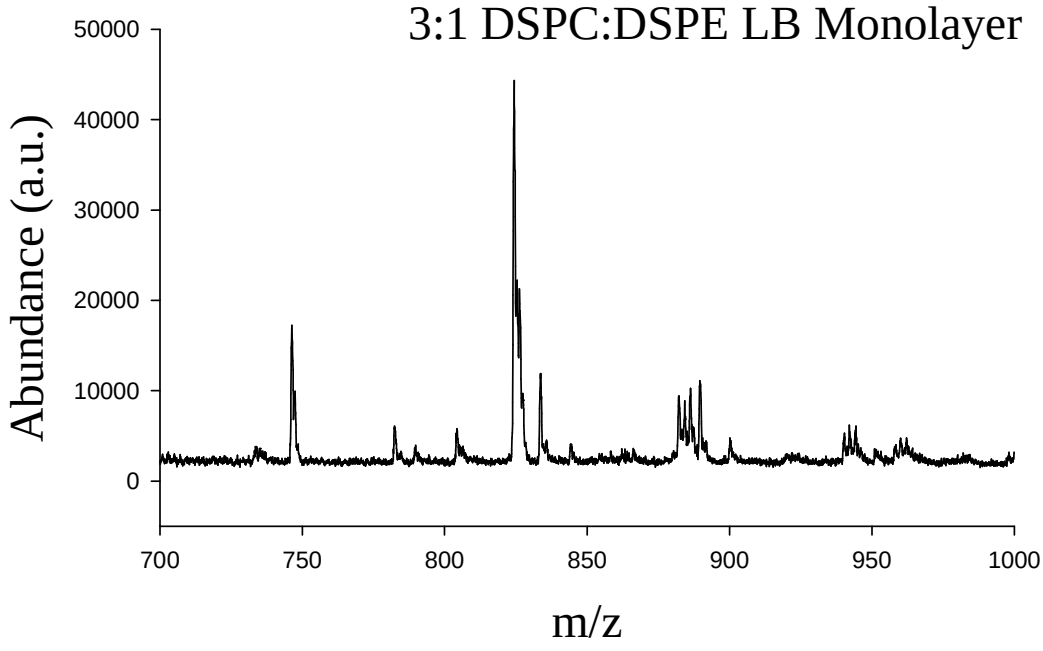
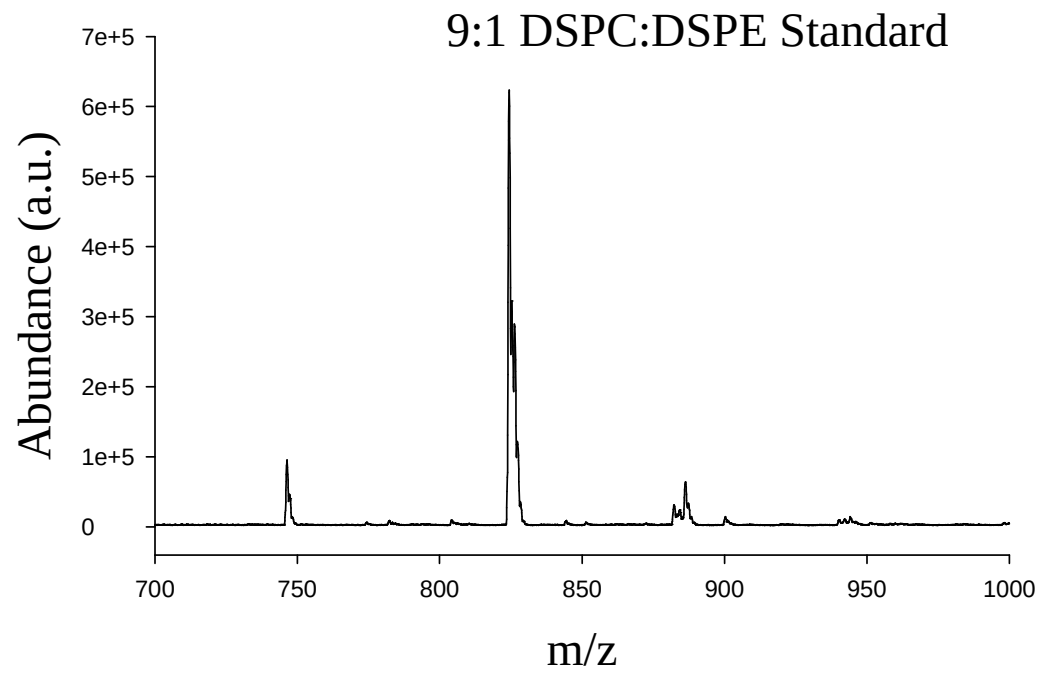
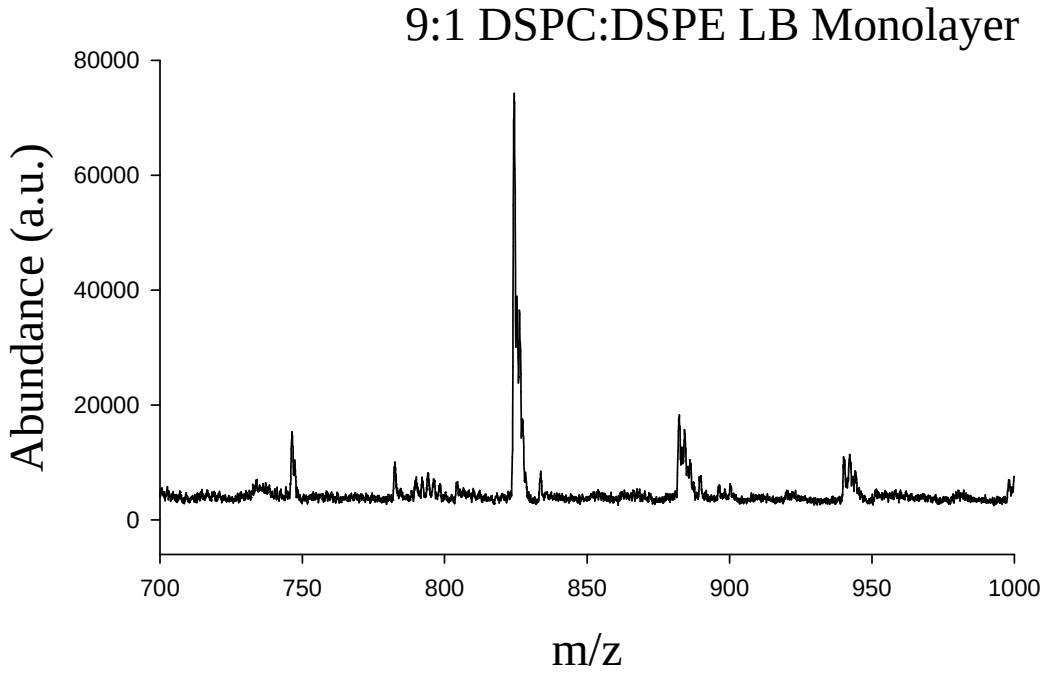

3:1 DSPC:DSPE LB Monolayer
Abundance (a.u.)
50000
40000
30000
20000
10000
0
700
750
800
850
900
950
1000
m/z

Figure 18. Mass spectrometry comparison of 9:1 DSPC:DSPE.


9:1 DSPC:DSPE Standard
Abundance (a.u.)
7e+5
6e+5
5e+5
4e+5
3e+5
2e+5
1e+5
0
700
750
800
850
900
950
1000
m/z


9:1 DSPC:DSPE LB Monolayer
Abundance (a.u.)
80000
60000
40000
20000
0
700
750
800
850
900
950
1000
m/z

spectroscopy to study membrane asymmetry maintenance. SFVS can thus also be used to investigate other possible mechanisms of bilayer asymmetry maintenance.

The results of this study also indicate that the Langmuir-Blodgett / Langmuir-Schaeffer method of preparing planar supported lipid bilayers allows for compositional control. This suggests that the LB/LS method is an effective method to prepare asymmetric lipid bilayers in which compositional control is essential.

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