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Electrostatic Induction of Lipid Asymmetry

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Supporting Information

ABSTRACT: The asymmetric arrangement of phospholipids between the two leaflets of the plasma membrane of eukaryotic cells is an integral part of cellular function. ATP-dependent translocases capable of selective lipid transport across the membrane are believed to play a role in this lipid asymmetry, but our understanding of this process is incomplete. Here we show the first direct and quantitative experiments demonstrating the induction of phosphatidylserine asymmetry in a membrane by electrostatic association of poly-L-lysine in an attempt to elucidate the complex factors which govern the establishment and maintenance of lipid compositional asymmetry in the plasma membrane on a fundamental level. The attractive electrostatic interactions between the charged surface-associated polylysine and phosphatidylserine are sufficient to both induce and maintain an asymmetric arrangement of phosphatidylserine in a planar supported membrane, as measured by sum-frequency vibrational spectroscopy. These studies provide a glimpse of the physical and chemical underpinnings of lipid asymmetry in the eukaryotic plasma membrane.

Eukaryotic cellular membranes contain a nonrandom and unequal distribution of phospholipids in the two leaflets of the plasma membrane (PM) which is crucial to cellular function.[1−6] In the PM of eukaryotic cells the aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE), are predominately located on the cytosolic leaflet while sphingolipids, phosphatidylcholine (PC) and sphingomeylin, are localized predominantly on the extracellular leaflet.[5,7−9] Changes to this asymmetric lipid arrangement cause functional cell responses such as cell budding, signaling, and endo/exocytosis.[2−6,9−14] Furthermore, loss of this asymmetric arrangement, in particular the expression of PS on the extracellular leaflet of the membrane, triggers phagocyte recognition and eventually cell death.[12−15]

The unambiguous biological significance of the compositional asymmetry in the PM has prompted researchers to investigate the cellular mechanisms responsible for the creation and regulation of lipid asymmetry. Research by Devaux,[16,17] Daleke,[18,19] and van Meer,[20,21] among others, report the involvement of ATP-dependent proteins capable of selective lipid translocation. These proteins, dubbed "flippases" for movement to the inner leaflet and "floppases" for movement to the outer leaflet, are believed to be ATP-dependent lipid transporter proteins.[2,5,13] Such proteins are thought to identify lipids according to their headgroup chemistry and selectively and directionally transport them across the membrane to achieve the lipid distribution necessary for biological processes.[1−3,5,11] The movement of the negatively charged PS lipids has been of particular interest because the extent of PS expression on the extracellular leaflet of the bilayer has a dramatic effect on cell function.[6,8−11] For example, a small amount of PS lipids on the exterior leaflet cause blood coagulation while a larger amount trigger apoptosis.[3−5,11] The ability of a cell to manage the location of its membrane lipids is of extreme importance, and understanding this process can lead to new insights into cellular function.

In addition to protein mediated PS asymmetry in the PM, direct physical controllers of lipid compositional asymmetry may also play an important role. Other possible mechanisms include pH or potential gradients across the membrane[22] or electrostatic interactions with surface associate proteins.[5,9] There have also been several reports on the preparation of asymmetric planar supported lipid bilayers (PSLBs) by vesicle fusion, where the underlying substrate charge is used to create a nonrandom lipid distribution between the proximal and distal leaflets of the bilayer.[23−25] However, these studies could not directly quantify the lipid distribution in the membrane nor could asymmetry be created from a formally symmetric membrane. The present study investigates for the first time the specific induction of PS asymmetry in an initially symmetric PSLB by electrostatic interaction with a surface associated polypeptide, poly-L-lysine (PLL).

The nonlinear spectroscopy sum-frequency vibrational spectroscopy (SFVS) can be used for the direct and noninvasive measurement of membrane asymmetry, as demonstrated by Liu et al.[26−28] A comprehensive explanation of SFVS can be found elsewhere.[26,29−31] Briefly, SFVS is a coherent, nonlinear optical technique that involves the spatial and temporal overlap of fixed visible and tunable infrared (IR) laser sources resulting in the emission of light at a frequency which is the sum of the two incident light sources (S1). By tuning the frequency of the incident IR radiation, a vibrational spectrum is obtained. The coherent nature of SFVS prevents emission of the sum-frequency light for media which possess inversion symmetry, i.e. bulk isotropic materials. This property has led to the use of SFVS for the characterization of a variety of interfacial phenomena due to the lack of inversion symmetry at the boundary between two dissimilar media.[29,31]

Because symmetric lipid bilayers contain a plane of inversion between the two leaflets, the normal components of the IR transition dipoles, in particular the symmetric stretch (ν_s) of the terminal methyl groups (CH_3) on the hydrocarbon tails of the lipids, will negate each other due to phase cancellation resulting in no SFVS response as illustrated in Figure 1A. However, deuterating the lipids in one leaflet alters their transition dipole

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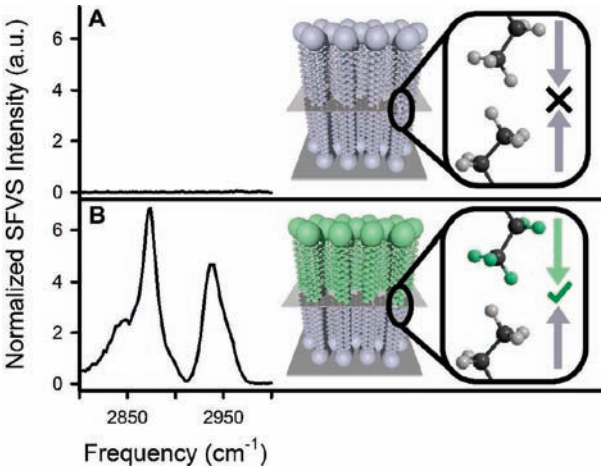

Figure 1. Normalized SFVS spectra for (A) a DSPC bilayer with both leaflets composed of proteated lipids (gray) and (B) one leaflet composed of proteated lipids and one composed of deuterated DSPC-D70 (green). Shown in the magnified views of the bilayer are illustrations of the normal components of the CH_3 ν_s and CD_3 ν_s transition dipole moments and the effect of phase cancelation on the SFVS signal.

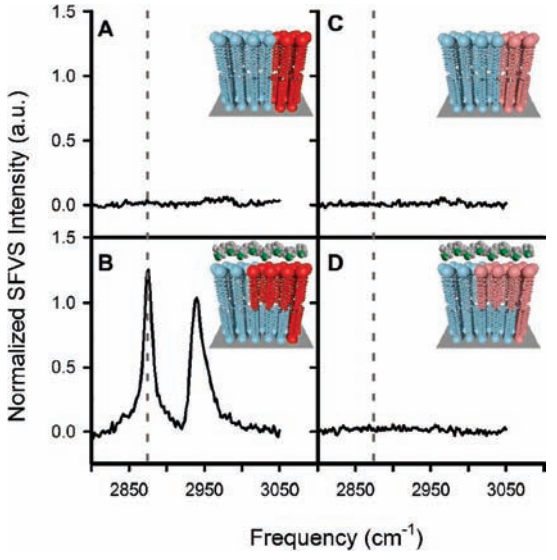

Figure 2. Normalized and averaged SFVS spectra of a gel phase bilayer composed of 65 mol % DSPC-D70 (blue) and 35 mol % DPPS (red) ($n = 4$) for (A) a symmetric bilayer, $N_D - N_P = 0$, and (B) PS asymmetry induced by the presence of PLL at 50 °C. Also shown for comparison are the SFVS spectra of a gel phase bilayer composed of 65 mol % DSPC-D70 and 35 mol % DPPS-D62 (pink) ($n = 2$) for (C) a completely symmetric bilayer and (D) DPPS-D62 asymmetry induced by PLL at 50 °C. The illustrations graphically represent the distribution of each lipid component in the distal and proximal leaflets of the membrane.

moments and breaks the inversion symmetry resulting in the spectrum shown in Figure 1B which shows pronounced resonances for the CH_3 ν_s at 2875 cm^{-1} and the Fermi resonance (ν_{FR}) at 2940 cm^{-1} from the terminal methyl groups on the lipid acyl chains. The substitution of deuterium for hydrogen does not significantly alter the size or chemical composition of the lipids thereby allowing SFVS to detect natural lipid behavior.[26−28] Quantitatively, the SFVS intensity (I_{SF}) is proportional to the population difference of the proteated lipid species in the two leaflets of the membrane:[26−28]

$$I_{SF} \propto (N_D - N_P)^2 \quad (1)$$

where N_D and N_P represent the fraction of proteated lipids in the distal and proximal leaflets of the bilayer, respectively. This dependence of I_{SF} on the population difference provides a direct means to quantify the degree of lipid compositional asymmetry in a lipid membrane.[28]

The Langmuir−Blodgett/Langmuir−Schaeffer (LB/LS) deposition method was used to generate PSLBs on hemicylindrical fused silica substrates as described in detail elsewhere[26,27] and summarized in the Supporting Information. The lipids used were 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-L-serine (DPPS) and its deuterated counterpart 1,2-dihexadecanoyl-D62-*sn*-glycero-3-phospho-L-serine (DPPS-D62) as well as 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and its deuterated counterpart 1,2-distearoyl-D70-*sn*-glycero-3-phosphocholine (DSPC-D70). The transition temperatures (T_m) of these lipids are nearly identical (~54 °C)[32] which minimizes phase segregation. Unless otherwise specified, all bilayers used in this study were composed of ~35% DPPS and ~65% DSPC to roughly mimic the biological distribution of aminophospholipids and sphingophospholipids in the PM of eukaryotic cells.[2−9]

The amount of PS lipid asymmetry induced by the electrostatic interaction with PLL is represented by the population difference of PS lipids, $|N_D - N_P|$, in bilayers composed of proteated DPPS and deuterated DSPC (DSPC-D70). Since the population difference is related to the intensity of the methyl symmetric stretch (I_{CH_3}) according to eq 1, the PS asymmetry can be calculated directly from the SFVS signal from the bilayer.[28] Initially, symmetric bilayers were prepared ($|N_D - N_P| = 0$) in which only DPPS is proteated (Figure 2A). The absence of any measurable SFVS spectral response in Figure 2A verifies the symmetric nature of the bilayer and ensures that any signal measured in the presence of PLL will represent the compositional asymmetry of PS lipids. As SFVS is incapable of detecting a symmetric bilayer, ATR-FTIR spectroscopy was used to ensure the formation of the bilayer (see Figure S2A).

PS lipid asymmetry was induced by the association of positively charged PLL to the membrane. In order for the attractive electrostatic interactions between PS and PLL to influence asymmetry, the lipids must be able to move between the bilayer leaflets; however, the lipids used in this study are in the gel phase and have extremely slow rates of flip-flop ($t_{1/2} \approx$ days) at room temperature.[26,27] We have previously shown that lipid membranes heated near or above the fluid phase transition, T_m, undergo rapid flip-flop ($t_{1/2} \approx$ seconds).[27] In order to facilitate rapid lipid translocation, the bilayers in this study were heated to 50 °C to allow the lipids to remain in the well-ordered gel phase while also having reasonably fast rates of flip-flop ($t_{1/2}$ = minutes).[26,27] SFVS spectra taken at this temperature prior to the introduction of PLL (S3) show no CH resonances from either PLL or the lipid membrane, confirming that the bilayer has remained symmetric and that the negligible charge at the silica surface does not induce PS asymmetry. A 1 μM solution of PLL (MW = 21500) was introduced over the distal leaflet of the bilayer at 50 °C and allowed to incubate until the I_{CH_3} measured by SFVS reached a maximum. ATR-FTIR spectroscopy was also used to confirm the presence of PLL at the bilayer surface (S2B). A dramatic induction of PS lipid asymmetry is evident by the marked increase in the CH_3 ν_s and ν_{FR} in the SFVS spectrum in

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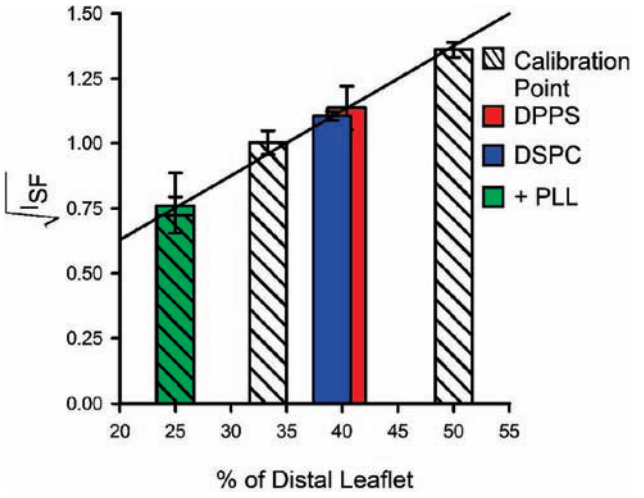

Figure 3. The SFVS response for bilayers containing 25 mol % ($n = 4$), 33.3 mol % ($n = 4$), and 50 mol % ($n = 3$) DPPS in the distal leaflet of a PSLB is shown by the hatched bars with the corresponding calibration curve shown ($R^2 = 0.97$). The induced asymmetries for DPPS ($n = 4$) and DSPC ($n = 2$) are denoted by the solid red and blue bars, respectively. The SFVS response for an asymmetric bilayer containing 25 mol % DPPS upon the addition of PLL is shown by the solid green bar.

Figure 2B. All data presented here have been normalized as discussed in the Supporting Information.

In order to quantify the amount of PS lipid asymmetry induced by PLL, it is first necessary to quantify the relationship between the bilayer composition and I_{CH_3}. Using the LB/LS deposition method to independently control the lipid composition of each bilayer leaflet, asymmetric bilayers were prepared with 25, 33.3, and 50 mol % PS in the distal leaflet of the PSLBs and the SFVS spectra measured. The resulting calibration curve, shown in Figure 3, demonstrates the linear relationship between $(I_{CH_3})^{1/2}$ and the mole percent composition of PS in the distal leaflet of the membrane. Based on the calibration curve shown in Figure 3, we can establish that the distal leaflet of the bilayers contain 40 ± 3% (s.d.) DPPS in the presence of PLL (Figure 3). Movement of the negatively charged PS lipids is facilitated by the positively charged PLL in the absence of a specific lipid transporter. Due to the nature of SFVS, this 40% represents the amount of PS that is not subject to phase cancelation. Based on this fact and the known amount of PS initially present in the bilayer, ca. 79% of the DPPS in the bilayer is in the distal leaflet. This observation marks the first direct measurement and quantification of the induction of lipid compositional asymmetry by electrostatic interactions via SFVS. It should be noted that the location of PS in the bilayer is not directly described by the data but is inferred based on the presence of PLL adjacent to the distal leaflet.

It has long been believed that zwitterionic lipids, such as PC, act as a mass balance to the asymmetrically distributed aminophospholipids, such as PS, in eukaryotic membranes in order to preserve the membrane shape and stability.[2,8,10] Although this process has been indirectly inferred, the nature of our study allows us to directly test this hypothesis by measuring the induced asymmetry in bilayers containing proteated DSPC and perdeuterated DPPS. As DPPS is actively "held" in the distal leaflet by PLL, equal amounts of DSPC should respond by moving to the proximal leaflet. If this occurs, then I_{CH_3} measured for bilayers containing proteated DSPC should be identical to bilayers with proteated DPPS. By using selectively deuterated DPPS (DPPS-D62) in conjunction with DSPC, the asymmetry in DSPC composition between the leaflets was measured and summarized in Figure 3. The calculated mol % of the PS and PC track each other equally. Specifically, the average induced asymmetry in bilayers with proteated DSPC is 39.1 ± 1% (s.d.), which is statistically identical to bilayers containing proteated DPPS. To the authors' knowledge, this is the first direct evidence that zwitterionic lipids act as a mass balance for actively transported aminophospholipids upon lipid translocation.

The lipid asymmetry reported here is calculated using the I_{CH_3} at 2875 cm^{-1}; however, this resonance can only be used if there are no contributions, either direct or indirect, from PLL in the spectral region of interest.[25] In order to rule out a direct spectral contribution from the terminal CH_3 groups on PLL, the experiment described above was performed on bilayers composed of all deuterated lipids, shown in Figures 2C and 2D before and after PLL is added under the same experimental conditions as the proteated bilayers in Figures 2A and 2B, respectively. The absence of a spectral response in Figure 2C is consistent with both a symmetric lipid distribution and the lack of proteated lipids. The spectral response after the addition of PLL in Figure 2D is identical to Figure 2C, showing that there are no spectral signatures from PLL. This lack of SFVS intensity confirms that PLL does not contribute to the measured SFVS spectra of DPPS and DSPC.

An indirect influence of PLL on the measured SFVS spectra could arise if the electrostatic potential at the bilayer surface gives rise to a third-order $\chi^{(3)}$ process, which would contribute to the measured SFVS intensity.[29] While PLL certainly introduces an electrostatic potential at the surface, it is unknown whether it is significant enough to manifest a $\chi^{(3)}$ effect. This possibility was investigated by adding PLL to an asymmetric bilayer containing 25 mol % DPPS in the distal leaflet, the results of which are summarized in Figure 3. The data in Figure 3 show that the SFVS response before and after the addition of PLL is identical within error, demonstrating that the additional surface charge from PLL does not cause a measurable $\chi^{(3)}$ effect and confirms that the I_{CH_3} at 2875 cm^{-1} is an accurate representation of compositional lipid asymmetry.

The ability of electrostatic forces to induce an asymmetric lipid arrangement in PSLBs was directly demonstrated and quantified here for the first time using SFVS. The attractive electrostatic interactions between PS lipids and a charged polyelectrolyte, PLL, were capable of producing an asymmetric PS lipid arrangement. Selective proteation of either PS or PC lipids allowed for a separate evaluation of the lipid response to PLL. This has revealed that electrostatic interactions between PS and PLL are able to hold about 79% of the PS lipids in the distal leaflet of a gel phase bilayer. Furthermore, our examination of PC lipid asymmetry provides direct evidence that PC lipids move in equal amounts to offset the active transport of PS. Overall, this study has shown that the fundamental electrostatic interactions between PS headgroup lipids and a surface-associated polypeptide are sufficient to influence the compositional lipid asymmetry of a membrane.

■ ASSOCIATED CONTENT

Supporting Information. Figures S1, S2, S3; Materials and Methods; Normalization Procedure; and descriptions of the

ATR-FTIR and SiO_2 surface charge control experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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