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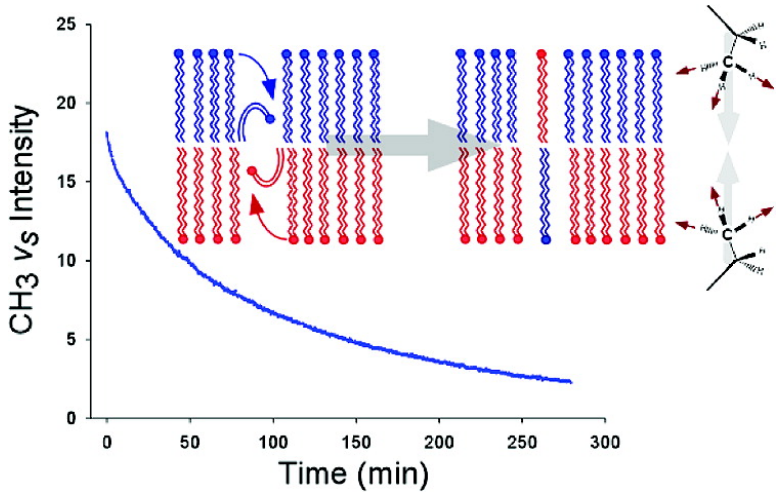

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Direct Measurement of the Transbilayer Movement of Phospholipids by Sum-Frequency Vibrational Spectroscopy

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The transbilayer movement of phospholipids in cellular membranes, also known as lipid flip−flop or translocation, is an area of significant biological importance. As the biosynthesis of lipids occurs on the cytosolic side of a lipid bilayer, the translocation of lipids is crucial for the functioning of cellular membranes.[1] It has long been believed that the exchange of lipids between the inner and outer leaflets of cellular membranes could only occur by a protein-mediated process.[2] Several researchers have tried, with limited results, to identify a putative "flipase" or "flopase" which might be responsible for lipid migration.[2] Studies employing NMR, fluorescence, and capacitance measurements have used chemically modified lipids to show that the transbilayer movement of lipids is possible in the absence of protein-mediated processes, but the measured rates of flip−flop are usually very slow, on the order of hours.[2−4] However, these studies only detected the movement of the labeled lipid species, with significant chemical and structural differences compared to those of the native lipid species found in biological membranes. The inability to directly measure the transbilayer movement of lipids has led to a lack of thermodynamic information on the energetic barrier of lipid flip−flop and has given rise to considerable speculation about the mechanism.

In the studies presented here, we report the first direct measurement of the transbilayer moment of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) in a planar supported lipid bilayer (PSLB) at the fused silica/D_2O interface obtained with sum-frequency generation (SFG) vibrational spectroscopy. The intrinsic sensitivity of SFG to the symmetry of an interface provided a direct measurement of the asymmetric distribution of DSPC and perdeuterated DSPC (DSPC-d_{83}) lipids in asymmetrically prepared DSPC/DSPC-d_{83} PSLBs. The activation energy for transverse motion was determined directly from intensity decay measurements at several temperatures and indicates that lipid translocation is a facile process at temperatures above the main phase-transition temperature (T_m) of DSPC.

The theory of SFG has been described previously in detail.[5] SFG is a coherent vibrational spectroscopic technique which couples the selectivity of IR and Raman with the surface specificity of a coherent second-order nonlinear optical process. Experimentally, SFG is performed by spatially and temporally overlapping a visible and IR laser source (ω_{vis} and ω_{IR})) on a surface. The subsequent nonlinear polarization induced at the surface results in the coherent emission of light at the sum of the frequencies ($\omega_{sum} = \omega_{vis} + \omega_{IR}$). When the IR source is on resonance with a vibrational transition, an increase in the intensity of light at ω_{sum} is observed. The symmetry constraints on SFG restrict this process to the interface where the inversion symmetry of the bulk phases is broken.

These same symmetry constraints also provide a highly sensitive method for determining the structure of lipid bilayers. This ability has been used previously to measure the phase-transition temperature of a single lipid bilayer.[6] In addition, the symmetric nature of the bilayer can also be used to measure the rate of transbilayer movement. For a lipid bilayer composed of identical lipids in the inner and outer leaflets, near complete destructive interference of the terminal fatty acid methyl symmetric stretch (CH_3 υ_s) at 2875 cm^{-1} is observed due to the antiparallel orientation of the transition dipole moments. By creating an artificially asymmetric structure in which a DSPC lipid monolayer is placed in contact with a lipid monolayer of DSPC-d_{83}, changes in the membrane lipid composition due to exchange between leaflets can be followed directly by measuring the decay in the CH_3 υ_s intensity with time.

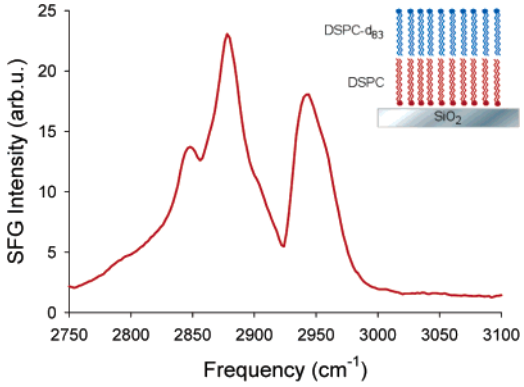

Figure 1. SFG spectrum of a DSPC/DSPC-d_{83} bilayer recorded at 24 °C with *s*-polarized sum-frequency, *s*-polarized visible, and *p*-polarized IR. Insert: schematic of asymmetric lipid bilayer structure.

For the studies described here, an asymmetric lipid bilayer was prepared by the Langmuir−Blodgett-Schaeffer method[7] on the surface of an IR-grade fused silica substrate by first depositing a monolayer of DSPC, followed by the deposition of a monolayer of DSPC-d_{83}, shown schematically in the insert of Figure 1. The DSPC and DSPC-d_{83} lipid films were deposited at a surface pressure of 30 mN/m, which corresponds to an area per molecule of 46 ± 1.3 Å^2. The samples were transferred to a Teflon flow cell equipped with a type K thermocouple with an accuracy of 0.2 °C and a circulating water jacket for temperature control. The SFG experiments were performed by overlapping 3 mJ/pulse of 532-nm light from a Nd:YAG laser with ∼3 mJ/pulse of IR light (tunable from 2000 to 4600 cm^{-1}) from an OPO/OPA (LaserVision) which was pumped with the fundamental (1064 nm) output from a nanosecond Nd:YAG laser with a repetition rate of 10 Hz. The total sample area illuminated was ∼4 mm^2, corresponding to ∼15 pmol of DSPC.

Shown in Figure 1 is the SFG spectrum of a DSPC/DSPC-d_{83} bilayer in the CH stretching region (2750−3100 cm^{-1}) recorded at 24 °C. The peaks at 2848 and 2875 cm^{-1} are identified as the CH_2 υ_s and CH_3 υ_s, respectively, with the shoulder at 2905 cm^{-1} assigned as the CH_2 Fermi resonance (FR). The peak centered at 2950 cm^{-1} is a combination of the CH_3 FR (2938 cm^{-1}) and the CH_3 antisymmetric stretch (υ_{as}) (2960 cm^{-1}). All resonances are from the fatty acid chains of DSPC.[8,9]

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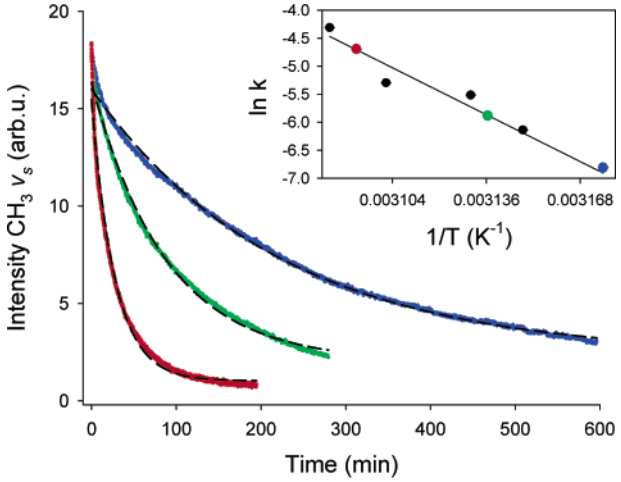

Figure 2. CH_3 vs intensity decay for DSPC/DSPC-d_{83} bilayer at various temperatures; the blue line was recorded at 41.7 °C, green at 45.7 °C, and red at 50.3 °C. The dashed lines are the fits to the data using eq 3.

The terminal CH_3 υ_s intensity from the fatty acid chains was measured continuously as a function of time and at various temperatures, to determine the rate of lipid flip−flop, Figure 2. The CH_3 υ_s intensity decreases with time, and the decay rate is highly dependent upon temperature. Since SFG is sensitive to the net population inversion of DSPC in the bilayer, the intensity of the CH_3 υ_s (I_{CH_3}) can be expressed as:

$$I_{CH_3} \propto (N_T - N_B)^2 \quad (1)$$

where N_T and N_B are the fractions of DSPC molecules in the top and bottom layers respectively, such that $N_T + N_B = 1$. Initially the bilayer is completely asymmetric with $N_T = 0$ and $N_B = 1$. With time, the concentration of DSPC in the upper leaflet increases at the expense of the lower leaflet, thereby reducing the asymmetry in the bilayer and reducing the measured CH_3 υ_s intensity. A minimum in the SFG intensity is reached when the bilayer is at equilibrium (50% DSPC in both layers). This intensity is the same intensity measured for a premixed 1:1 DSPC:DSPC-d_{83} bilayer, see Supporting Information.

The kinetics of DSPC transbilayer movement were determined from the experimental data in Figure 2, assuming a unimolecular process:

$$N_T \underset{k_-}{\overset{k_+}{\rightleftharpoons}} N_B \quad (2)$$

with the forward and reverse rate constants given by k_+ and k_- respectively. If the forward and reverse rates are identical (which has been proven experimentally[10]), the integrated rate expression in terms of the measured CH_3 υ_s intensity is given by the following:

$$I_{CH_3} = I_{max} \exp(-4kt) + I_{nr} \quad (3)$$

where I_{max} is the maximum intensity, k is the rate of DSPC transbilayer movement, and I_{nr} is the nonresonant SFG background. The extremely good fit of the experimental data to this kinetic model, Figure 2, indicates a possible unimolecular mechanism for the transbilayer movement of DSPC in a PSLB.

For comparison, the measured temperature-dependent flip−flop rates obtained from the data are summarized in Table 1. At 25 °C the rate of transbilayer movement is extremely slow with a calculated $t_{1/2}$ of 18 days. ($t_{1/2}$ is the time required for the population inversion of DSPC to reach one-half of the initial value.) As the temperature is increased to 51.3 °C, just below the phase-transition temperature (T_m = 58 °C) of DSPC,[11] the $t_{1/2}$ decreases to 25.9 min. Above the T_m of DSPC, the rate of interconversion was too fast to measure experimentally.

Table 1. Temperature-Dependent Rate Constants and $t_{1/2}$ for the Exchange for a DSPC Bilayer

temp (°C)	rate k ($\times 10^{-3}$ min^{-1})[a]	$t_{1/2}$ (min)
41.7 ± 0.3	1.11	312
44.5 ± 0.3	2.16	160
45.7 ± 0.3	2.80	124
46.3 ± 0.4	4.03	86.0
49.2 ± 0.2	5.01	69.2
50.3 ± 0.1	9.10	38.1
51.3 ± 0.2	13.4	25.9

[a] Error is estimated at less than 5%.

The rate of translocation of a DSPC molecule between leaflets exhibits Arrhenius behavior over the temperature range examined, insert of Figure 2. An activation energy of 206 ± 18 kJ/mol was calculated from the data in Figure 2. The large activation barrier measured is presumably due to the high energetic cost of moving the hydrophilic headgroup across the hydrophobic membrane core and the energy cost associated with the reorganization of the lipid chains.[2] Based on the Arrhenius behavior observed in Figure 2, the rate of lipid transport at 68 °C, only 10 °C above the main phase transition, increases substantially with a calculated $t_{1/2}$ of 43 s. These results have important implications for understanding the transbilayer movement of lipids in biological membranes. Cellular membranes are composed of a vast array of lipid species with a broad range of transition temperatures. Based on our preliminary findings, the migration of certain lipid species in living cells could be quite facile under physiological conditions. Research is underway to determine the effect of the T_m and lipid composition on lipid transbilayer movement. Efforts are also directed at measuring the influence of other membrane constituents, such as cholesterol and proteins, on this dynamic process.

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Supporting Information Available: Detailed experimental description and kinetic model. This material is available free of charge via the Internet at http://pubs.acs.org.

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