Interleaflet Diffusion Coupling When Polymer Adsorbs onto One Sole Leaflet of a Supported Phospholipid Bilayer

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Received October 9, 2006
Revised Manuscript Received January 11, 2007

Introduction. Lipid mobility in fluid-phase lipid bilayers attracts much interest not just because this problem is fundamental but also because it is so closely related to biological processes such as membrane ion channel formation, ligand−receptor binding, and membrane adhesion and fusion.[1−3] Here we consider lipid lateral diffusion in bilayers that are supported on a solid substrate. Literature on this general question is extensive, including various aspects such as free Brownian motion in single- and multicomponent bilayers,[4,5] anomalous subdiffusion in heterogeneous bilayers,[6] obstructed diffusion in phase-separated bilayers,[7] and hop diffusion in compartmentalized cell membranes.[8] However, the fact that any lipid bilayer is comprised of an inner leaflet and an outer leaflet raises the question of whether lipid moves synchronously in the two leaflets—a problem of interleaflet coupling in phospholipid bilayers. Here we focus on discriminating between diffusion on the two sides of the bilayer.

Previous studies of interleaflet coupling in phospholipid bilayers did not investigate the situation where the adsorbate was located onto one sole side. Important prior studies investigated lipid raft formation in the two leaflets due to temperature decrease or incorporated cholesterol molecules.[9,10] Also, in the absence of adsorbate, lipid diffusion in the outer and inner leaflets of a planar-supported lipid bilayer was studied with the conclusion that strong coupling exists between diffusion in the inner and outer leaflets.[11] We address the basic question of what happens when adsorbate is present. As adsorbate, we allow polymers to adsorb at low surface coverage to the outer leaflet, and using iodide quenching of diffusion in the outer leaflet, we discriminate between lipid diffusion in the outer and inner leaflets.

Experimental Section. For study, phospholipid DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine, was purchased from Avanti Polar Lipids (Alabaster, AL), into which we doped at 10 ppm (0.001%) molar concentration DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine, with polar head group labeled by rhodamine B. Supported bilayers of the mixture were formed on hydrophilic quartz using known protocols based on the fusion of small unilamellar vesicles.[12] Fully quaternized poly(4-vinylpyridine), QPVP, was prepared by us from parent poly(vinylpyridine) (Polymer Source Inc., Québec, Canada) by reaction with an excess of ethyl bromide.[13] The surface coverage of QPVP on DLPC bilayers was quantified using Fourier transform infrared spectroscopy in the mode of attenuated total reflection (FTIR-ATR) using known methods.[13] All measurements were made in PBS buffer (10 mM, pH 6.0).

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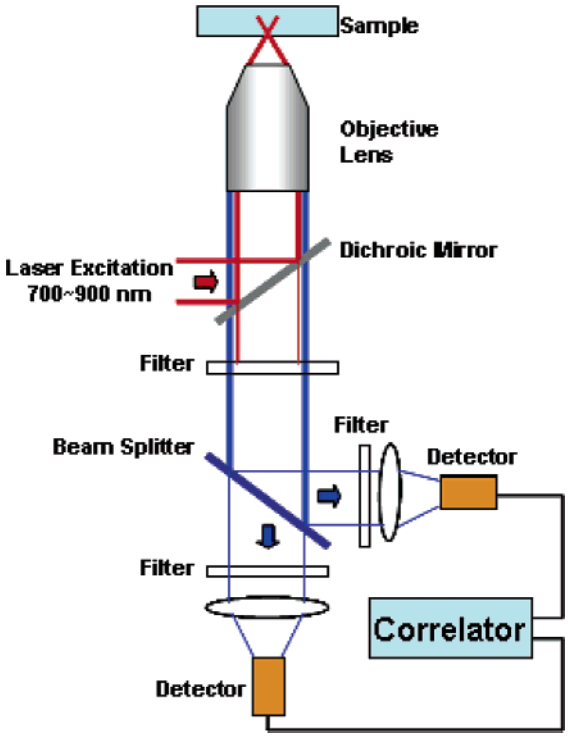

Figure 1. Experimental setup for fluorescence correlation spectroscopy (FCS) in the two-photon excitation mode. See text for detailed description.

The lateral diffusion of dye-labeled lipids was measured by fluorescence correlation spectroscopy (FCS) in the two-photon excitation mode using a home-built apparatus.[14] As illustrated in Figure 1, near-infrared femtosecond pulses from a Ti:sapphire laser (800 nm, 82 MHz, pulse width ∼100 fs) were directed into a water immersion objective lens (Zeiss Axiovert 135 TV, 63x, NA = 1.2) via a dichroic mirror and focused onto the sample, giving an excitation spot whose diffraction-limited diameter was ∼0.35 μm, thus affording measurements that were spatially resolved depending on where the laser beam was focused. Fluorescence from the sample was collected by the same objective and split into two channels after passing through the dichroic mirror and the emission filter. Each channel was connected to a single photon counting module (Hamamatsu). The photon counting output was recorded by an integrated FCS data acquisition board (ISS, Champaign, IL) and was analyzed to give an autocorrelation function curve, $G(\tau)$, which one can fit to standard equations to give the translational diffusion coefficient (D).[15,16] For the planar-supported phospholipid bilayer system studied here, it is appropriate to fit data of this kind to a 2-dimensional Gaussian model with two-photon excitation using the following equation[17]

$$G(\tau) = G(0)\left(1 + \frac{8D\tau}{\omega_0^2}\right)^{-1} \quad (1)$$

where τ is the time lag of the measurement, ω_0 is excitation beam waist, and $G(0)$ is amplitude of the autocorrelation curve, which is inversely proportional to sample concentration. When τ is in the range of τ_c, defined as the average lateral diffusion time for a molecule through the effective excitation area, $\pi\omega_0^2/4$, eq 1 is easily transformed to the following relation, in which

10.1021/ma062335s CCC: $37.00
Published on Web 02/01/2007

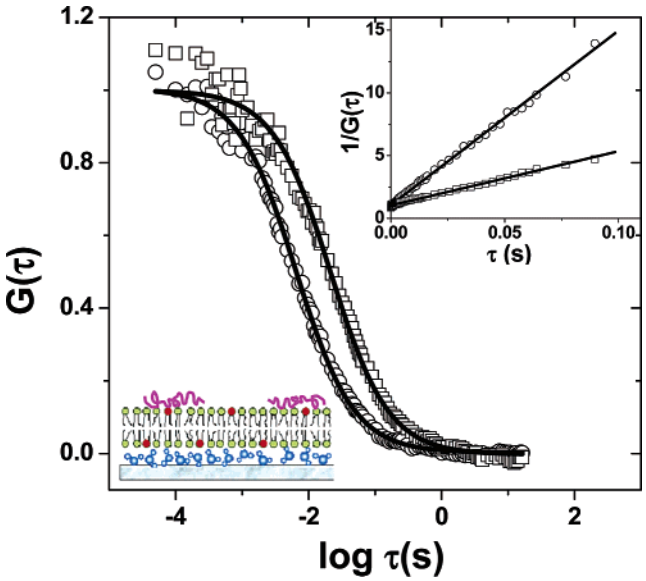

Figure 2. Autocorrelation curves of single measurements plotted as a function of logarithmic time lag τ for a DLPC supported lipid bilayer carrying quaternized poly(4-vinylpyridine) at fractional surface coverage 20%. The QPVP, 100% quaternized, possessed weight-averaged molecular weight, $M_w = 81\,500$, and a narrow molecular weight distribution. Note that in the presence of this adsorbate lipid translational diffusion splits into two populations (fast and slow diffusion modes) depending on where the interrogatory laser spot was focused. Inset: $1/G(\tau)$ plotted as a function of time lag τ. This shows the coexistence of two diffusion modes. The slopes of these linear fits are proportional to lipid diffusion coefficient (D). The mean D of the fast mode is $2.62 \pm 0.18\ \mu m^2/s$. The mean D of the slow mode is $0.50 \pm 0.12\ \mu m^2/s$.

$1/G(\tau)$ is linearly proportional to time lag τ and the slope is proportional to the translational diffusion coefficient (D):

$$\frac{1}{G(\tau)} = \frac{1}{G(0)} + \frac{8D}{G(0)\omega_0^2}\tau \qquad (2)$$

Note the advantage of using eq 2 to fit data of the kind considered here. Mathematically it is equivalent to eq 1, but for systems that possess strong fluorescence triplet noise at short times or that possess unusual dynamical behavior at the longest autocorrelation times, quantification using eq 2 will give the more accurate D value for single-component diffusion because it deemphasizes both the very shortest times and those times that exceed τ_c. In the present experiments, $\tau_c \approx 10$ ms for the naked lipid bilayer and ≈50 ms for lipids in the presence of adsorbed polymer.

Results and Discussion. First, we illustrate findings that did not discriminate between diffusion in the inner and outer leaflets. In-plane diffusion within both leaflets of planar-supported DLPC lipid bilayer was measured in the situation where only the outer leaflet contained adsorbed polymer at low surface coverage. It is natural to wonder whether the adsorbed polymer might migrate underneath the bilayer and reside at the bottom leaflet, rather than at the top leaflet as we suppose here. Earlier studies from this laboratory investigated this systematically, comparing the behavior of cationic and anionic polymers in proximity to the same anionic surface, and concluded by excluding this possibility.[14,19]

As shown in Figure 2, spatially resolved measurements using FCS find that the mobility of lipids located within this bilayers splits into two populations depending on where the laser beam is focused. There is a fast mode, characteristic of lipids without adsorbate, and a slower mode, characteristic of lipids exposed to adsorbate. An earlier communication from this laboratory summarized experiments of this kind.[14]

The lines through data in Figure 2 are fits to eq 1 with one sole fitting parameter, the diffusion coefficient D. Lipid translational diffusion took place over the time span of milliseconds to hundreds of milliseconds. In the inset, the reciprocal of $G(\tau)$ is plotted equivalently against time lag τ. In this representation, the slopes of a linear fit to eq 2 is proportional to the translational diffusion coefficient (D). Two values of D were observed, fast and slow, depending on where the laser beam was focused. The mean D of the fast mode was $2.62 \pm 0.18\ \mu m^2/s$, characteristic of naked lipid diffusion, while the mean D of the slow mode was $0.50 \pm 0.12\ \mu m^2/s$. Elsewhere, it was shown that the slow mode was slower, in proportion to the molar mass of adsorbed polymer.[14] The data illustrated in Figure 2 show that polymer adsorption induced dynamical heterogeneity in lipid bilayers, according to spatial location of the probe molecule. The mobility of lipids containing a dilute surface coverage of adsorbed polymer was slowed, but the mobility of lipids in spatial locations not containing adsorbed polymer was not affected. Further study found that the slow diffusion coefficient varied in inverse proportion to the degree of polymerization of the adsorbed polymer.[14]

Now we address the question whether diffusion was perturbed in just one leaflet or in both. In order to discriminate between diffusion in the inner and outer leaflets of the bilayer, iodide ions were introduced into the aqueous solution above the supported lipid bilayer, quenching fluorescence from dyes located within the outer leaflet. Iodide is known to be an efficient quencher of fluorescence for rhodamine B dye via the heavy-atom effect yet does not penetrate lipid bilayers in the fluid phase.[11,18] In addition, as these bilayers were formed first and iodide ions were added afterward, at an ionic strength where the Debye length was 3 nm, the iodide ions did not reside in the nanometer thick region between the (negatively charged) solid surface and the inner bilayer leaflet above it.

As it is known that polymers adsorbed at low surface coverage take a flat, "pancake" conformation, the polymer adsorbing to the outer leaflet of the bilayer,[19] it follows that for this case of adsorbed QPVP that was fully quaternized (one charge per segment) with molecular weight $M_w = 81\,500$ the persistence length (ξ) of the 2D polymer was roughly 1.4 nm.[20] We verified that the fluorescence intensity of bilayers in contact with 50 mM iodide was reduced to roughly half the level without iodide. In other words, those fluorescent dyes located within the outer leaflet were almost completely quenched by iodide, leaving measurable fluorescence signal only from lipids located within the inner leaflet.

Figure 3 shows FCS measurements of lipid diffusion within the inner leaflet of lipid bilayers, when polymers adsorbed onto the outer leaflet at fractional surface coverage 20%. Lipid diffusion splits into two populations according to spatial location at which the measurement was made. A fast mode and a slow mode coexist in the inner leaflet. Fitting this data using eq 2 gives the mean D of the fast and slow modes, 2.53 ± 0.21 and $0.54 \pm 0.08\ \mu m^2/s$, respectively.

Strikingly, this matches well with what was observed without discriminating between diffusion in the outer and inner leaflets. It follows that lipid diffusion in the inner leaflet, in the presence of dilute adsorbate (reported here for the first time), coincides with measures of lipid diffusion in both leaflets, outer and inner, averaged together.[14] One might have expected, upon adsorption solely to the outer leaflet, to find slow diffusion within the outer leaflet and faster diffusion within the inner leaflet, but this was

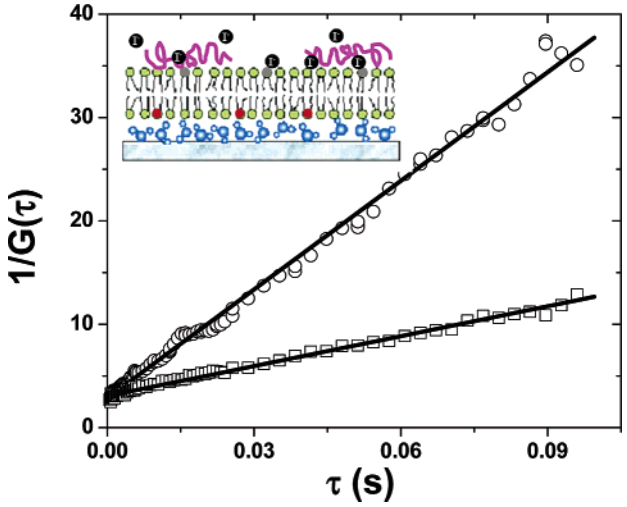

Figure 3. Same system as for Figure 2, except that iodide quenching of the fluorescent dyes located in the outer leaflet caused only motions of lipids located within the inner leaflet to be measured. The $1/G(\tau)$ of the inner leaflet lipids is plotted as a function of time lag τ. One observes that lipid diffusion within the inner leaflet splits into the same two populations shown in Figure 2, where inner and outer leaflet diffusion were not resolved. The fast mode is 2.53 ± 0.21 $\mu m^2/s$, and the slow mode is 0.54 ± 0.08 $\mu m^2/s$.

not observed. Instead, we found that lipids in both leaflets, top and bottom, presented the same mobility. In the future it will be desirable to seek structural information about how adsorbed polyelectrolyte may have modified lipid packing within the bilayer, but this lies beyond the scope of the present Communication.

Looking to the future, we mention speculative possibilities. While the mechanism by which lipid diffusion couples between the two leaflet of a bilayer is not yet known definitively, recent theory points toward a speculative explanation.[21] If one accepts for the sake of argument that the findings presented here might also generalize to the common biologically relevant situation where proteins adsorb to one sole side of a cell membrane, these findings may have a bearing in explaining why stimulus to the outer surface of the membrane may help to activate proteins located on its inner surface. The nature of this strong coupling remains speculative but may stem from strong hydrophobic attraction between the two leaflets of the bilayer, from slight interdigitation of lipids between the two bilayer leaflets, or from adsorption-induced changes in area per headgroup.[21]

Acknowledgment. This work was supported by the U.S. Department of Energy, Division of Materials Science, under Award DEFG02-02ER46019. Partial support from equipment purchased using Grants NSF-DMR 0605947, NSF-CMS-0555820, and NSF (NIRT) CBET 060978 is also acknowledged.

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MA062335S