Lipid diffusion compared in outer and inner leaflets of planar supported bilayers

Liangfang Zhang and Steve Granick
Materials Research Laboratory and Department of Chemical & Biomolecular Engineering, University of Illinois, Urbana, Illinois 61801

(Received 4 August 2005; accepted 19 October 2005; published online 8 December 2005)

The translational diffusion coefficient (D) of lipids located in the outer and inner leaflets of planar supported DLPC (1,2-dilauroyl-sn-glycero-3-phosphocholine) bilayers in the fluid phase was measured using fluorescence correlation spectroscopy of dye-labeled lipids at the low concentration of 0.001% and using iodide quenching of dyes in the outer leaflet to distinguish diffusion in the inner leaflet from that in the outer leaflet. To confirm the generality of these findings, the bilayers were prepared not only by vesicle fusion but also by Langmuir–Blodgett deposition. We conclude that regardless of whether the bilayers were supported on quartz or on a polymer cushion, D in the inner and outer leaflets was the same within an experimental uncertainty of ±10% but with a small systematic tendency to be slower (by $<5\%$) within the inner leaflet. © *2005 American Institute of Physics*. [DOI: 10.1063/1.2138699]

Lipid bilayers supported on planar substrates are studied and used increasingly because they afford well-defined, biofunctionalizable surfaces with potential use in many areas; among them biosensors, protein dimerization, chromatographic separation, protein immobilization, and study of membrane-membrane interactions.[1] The presence of a thin, lubricating water layer or soft polymer cushion between bilayers and the underlying solid causes supported bilayers to maintain many of the structural properties of free-standing bilayers.[1,2] We are interested here in knowing the mobility of lipids in supported lipid bilayers—a problem that is physically fundamental and highly related to potential biological sensor functions of supported bilayers. Previous work has shown already that lipid mobility in the fluid phase is slower than in free-standing vesicles by roughly a factor of 5.[3] What about the distinction between mobility in the inner and outer leaflets?

Here we investigate this question with systematic experiments. Previous related studies left the question open, on the one hand because of the manner in which fluorescent probes were employed (more on this below) and on the other hand because in the sole probe-free study of which we are aware, the bilayers resided on (curved) colloidal surfaces rather than flat substrates. Specifically, concerning the special case of bilayers supported on a polymer cushion, earlier fluorescence-based studies already concluded that lipid diffusion is the same in both leaflets of a supported lipid bilayer,[4,5] but the generality of the conclusion was unclear because analysis was based on the assumption that lipid "flip-flop," a transbilayer movement, is so slow, on the order of hours,[6] as to be negligible during the experiment time scale. Challenging this assumption, a recent label-free study found lipid flip-flop to be considerably faster, on the order of minutes, for lipid bilayers in the fluid (rather than gel) phase state.[7] If this finding were also to hold for lipids that carry a fluorescent dye, it would require reassessment of fluorescence experiments in which diffusion in the inner and outer leaflets was not discriminated. The study described below addresses this problematic point with direct experiments.

Taking a different experimental approach, the pioneering work of Bayerl and co-workers employed a dye-free NMR technique and spherical silica beads as the substrate for the supported bilayers; they concluded that lipid diffusion in the inner leaflet was slower by a factor of 2 than that in the outer leaflet.[8] The relevance of this study to planar-supported bilayers is uncertain, however, first because bilayers that coat a colloidal substrate necessarily possess much higher curvature than planar bilayers (and perhaps also because these colloids typically are rougher than planar surfaces).

In view of these uncertainties, it seemed worthwhile to revisit this question on planar solid supports using few-molecule fluorescence methods based on fluorescence correlation spectroscopy (FCS),[9] and specifically to compare mobility in the presence and absence of a polymer cushion. The question is significant because lipid lateral diffusion in planar supported bilayers is surely related to their function.

FCS is a powerful technique that can be used to measure diffusion with single molecule sensitivity.[10–12] Compared to fluorescence recovery after photobleaching (FRAP), a more commonly used diffusion-measuring technique, the abundance of fluorescent probe is orders of magnitude lower; the typical probe content for a FRAP experiment is 0.5 -2 mol % of the matrix molecules, while 0.001% (10 ppm) is good enough for FCS measurements. Also, the FCS technique is spatially resolved and can easily characterize the homogeneity of the diffusion process at different spots within the sample, so as to more accurately give information regarding system homogeneity. However, FCS is more complicated and expensive than a typical FRAP setup.

For study, the phospholipid DLPC (1,2-dilauroyl-sn-glycero-3-phosphocholine) was selected because its gel-to-fluid phase transition of -1 °C was well below the experi-

0021-9606/2005/123(21)/211104/4/$22.50 © 2005 American Institute of Physics

Downloaded 08 Dec 2005 to 130.126.103.95. Redistribution subject to AIP license or copyright, see http://jcp.aip.org/jcp/copyright.jsp

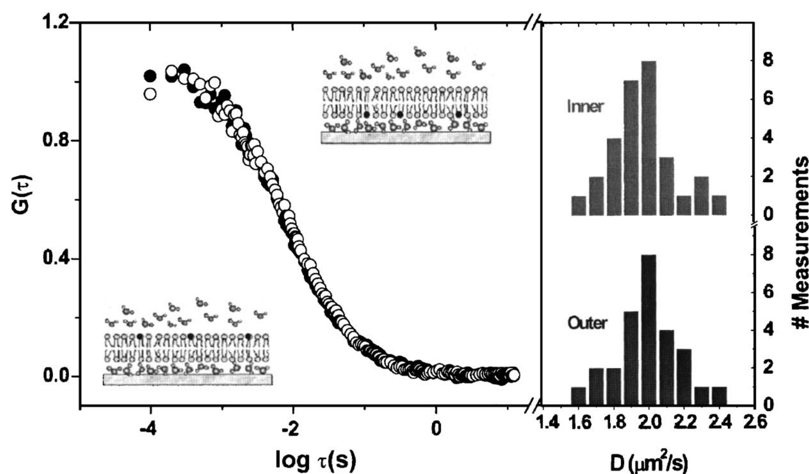

FIG. 1. Left: Autocorrelation curves of lipid diffusion in the outer leaflet (solid circles) and the inner leaflet (open circles) of solid-supported DLPC bilayers formed by LB deposition as described in the text. The D in the outer and inner leaflets was 2.0 ± 0.2 and 1.9 ± 0.2 $\mu m^2/s$, respectively. Right: Histogram of diffusion coefficient of lipids in the inner and outer leaflets obtained from measurements on a number of samples.

mental temperature, 23 °C. Lipid DMPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine) with polar head group labeled by rhodamine B (DMPE-RhB) was used as a fluorescent probe. Both lipids were obtained from Avanti Polar Lipids, Inc. Two techniques, Langmuir–Blodgett (LB) film deposition (NIMA 311D, England) and the fusion of unilamellar vesicles,[13] were used to form the supported bilayers. They were formed either on hydrophilic quartz or on a thin polymer cushion previously deposited as described below.

FCS measurements were carried out in the two-photon excitation mode using a home-built apparatus. Near-infrared femtosecond pulses from a Ti:sapphire laser (800 nm, 82 MHz, pulse width ~100 fs) were focused onto the sample through a water immersion objective lens (Zeiss Axiovert 135 TV, 63×, NA=1.2), giving an excitation spot whose diffraction-limited diameter was ~0.35 μm. This focal area was calibrated using the standard method described elsewhere.[14] Fluorescence was collected by the same objective and detected by a single-photon counting module (Hamamatsu). The fluorescence intensity-intensity autocorrelation functions were fitted to standard equations[9] whose one free parameter was identified with the lateral translational diffusion coefficient (D). In the inferred diffusion coefficients presented below, each is the average of 10–20 experiments performed at different locations on the surface. All measurements were performed in PBS buffer (10 mM, pH =6.0).

To discriminate between lipid diffusion in the outer and inner leaflets, first we employed LB deposition, as this technique allows one to dope fluorescent probes solely into the target leaflet. The 10 ppm concentration of fluorescent probe was selected so that on average one sole probe molecule would reside within the FCS measurement spot. Bilayers of good quality could be formed in the range of surface pressure $\Pi=37$ to 44 mN/m, with minimal dependence on pressure; 40 mN/m was selected as the standard deposition pressure. Figure 1 shows illustrative fluorescence autocorrelation functions. The physical meaning of the autocorrelation function is to quantify the time for Fickian diffusion through the spot of known dimension illuminated by the focused laser beam. Here D of lipid in the outer leaflet was 2.0 ± 0.2 $\mu m^2/s$, while D of lipid in the inner leaflet was 1.9 ± 0.2 $\mu m^2/s$, a difference less than the experimental uncertainty. Figure 1 includes the histogram of findings in different measurements on a number of samples.

This result would be trivial if the lipids sustained flip-flop on the experimental time scale. Flip-flop is believed to occur on the time scale of hours in the absence of protein-mediated processes when fluorescence labels are involved.[6] However, a recent label-free study found lipid flip-flop to be faster, on the order of minutes,[7] so potentially this was a serious problem in these experiments where data in each

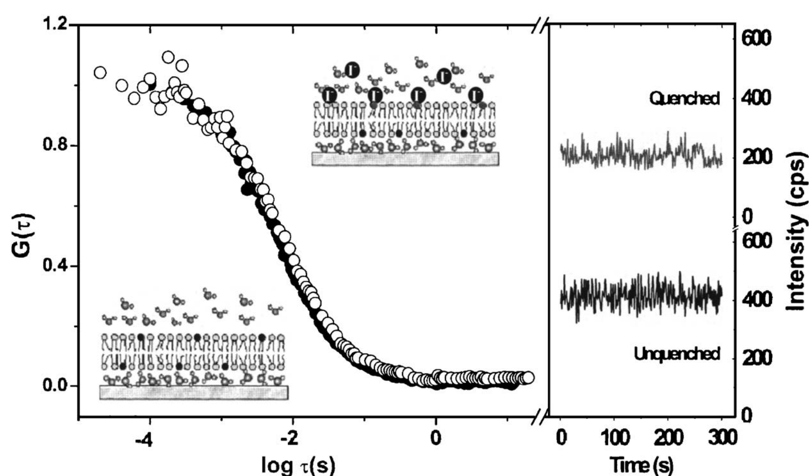

FIG. 2. Left: Autocorrelation curves of lipid diffusion in both leaflets (solid circles) and the inner leaflet (open circles) of solid-supported DLPC bilayers formed by vesicle fusion as described in the text. The D were 2.6 ± 0.2 and 2.5 ± 0.2 $\mu m^2/s$, respectively. Right: Fluorescence intensity (counts per second) is plotted against a snapshot of time to demonstrate photostability and iodide quenching efficiency. One observes that the fluorescence intensity was reduced by a factor of nearly 2 in the presence of iodide.

Downloaded 08 Dec 2005 to 130.126.103.95. Redistribution subject to AIP license or copyright, see http://jcp.aip.org/jcp/copyright.jsp

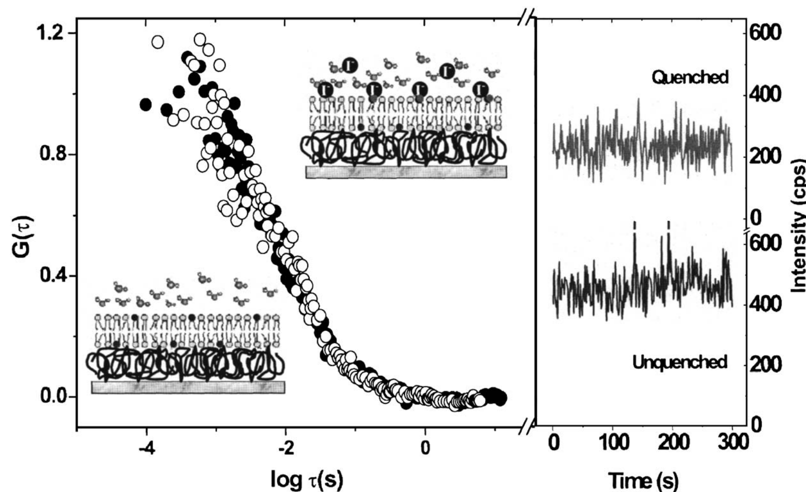

FIG. 3. Left: Autocorrelation curves of lipid diffusion in both leaflets (solid circles) and the inner leaflet (open circles) of DLPC bilayers formed on a polymer cushion by vesicle fusion as described in the text. The D were 2.1 ± 0.2 and 2.1 ± 0.2 $\mu m^2/s$, respectively. Right: Fluorescence intensity (counts per second) is plotted against a snapshot of time to demonstrate photostability and iodide quenching efficiency. One observes that the fluorescence intensity was reduced by a factor of nearly 2 in the presence of iodide.

FCS experiment were averaged for 5 min and repeated measurements were performed over a period of several hours. To rule out the possibility of a false conclusion, DLPC bilayers containing 10 ppm fluorescent probe were next formed by fusion of unilamellar vesicles, using methods described elsewhere (formation of bilayers were examined by *in situ* antomic force microscopy),[13] and iodide ions were introduced in order to quench fluorescence from probes within the outer leaflet. Iodide is an efficient quencher of fluorescence for this dye via the heavy-atom effect yet does not penetrate lipid bilayers in fluid phase.[15]

For vesicles in solution, experiments as a function of iodide concentration (not shown) demonstrated that at 50 mM KI, 90% of the fluorescence from the outer leaflet was quenched. As potentially the quenching efficiency at supported bilayers might be different, the fluorescence intensity of the supported bilayers, with and without quencher, was also investigated with the same conclusion. As shown in Fig. 2 (curve on the right), 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 probes located in the outer leaflet were almost completely quenched by iodide.

In Fig. 2 (curve on the left), it is obvious that the autocorrelation function in phosphate buffer solution containing 50 mM KI nearly overlaps with data taken in the absence of this quencher. Quantitative analysis shows that the D of lipids diffusing in the inner leaflet was 2.5 ± 0.2 $\mu m^2/s$, and that 2.6 ± 0.2 $\mu m^2/s$ was the average value of the inner and outer leaflets. Note that these numbers are slightly higher than in bilayers formed from the LB method, possibly because the vesicle formation method produces bilayers of lower surface pressure than can be produced by the LB method, but the conclusion is the same. By considering equal fluorescence contribution from inner and outer leaflets, we conclude that the difference between D in the two leaflets was the same within experimental uncertainty.

The generality of these conclusions was next tested using polymer-supported bilayers. A cationic polyelectrolyte, quaternized poly-4-vinylpyridine, was allowed to adsorb from dilute solution onto quartz, dried in air, exposed to unilamellar vesicle solution in order to form bilayers,[13] and finally fluorescence in the outer leaflet was quenched using iodide as described above. In Fig. 3 (left), one observes fluidity similar to that in the solid-supported bilayers. Specifically, D in the outer and inner leaflets was 2.2 ± 0.2 $\mu m^2/s$ and 2.1 ± 0.2 $\mu m^2/s$, respectively. Tentatively, we attribute the noisier data to swelling of the polymer layer resulting in an uneven surface.

In summary, this study shows that lipid diffusion in the inner and outer leaflets of solid-supported bilayers is sensibly the same. It is true that the three systems studied all show a systematic tendency for D in the inner leaflet to be 5–10% slower than in the outer leaflet, but this difference is within the experimental uncertainty. The observed molecular optical form anisotropy of lipid bilayers supports independently our conclusion of strong coupling. Theoretical arguments have interpreted such behavior to indicate that leaflet-leaflet dynamical coupling is stronger than leaflet-substrate coupling across an intervening thin water film or polymer cushion,[17] but the precise nature of this strong coupling remains unclear.

We thank Sung Chul Bae for incisive comments. This work was supported by the U.S. Department of Energy, Division of Materials Science, under Award Contract No. DEFG02-02ER46019 through the Frederick Seitz Materials Research Laboratory at the University of Illinois at Urbana-Champaign. The FCS equipment was supported under Award No. DEFG02-91ER45439. Some support was also provided by NSF-DMR-0071761, the NSF Nanoscience Engineering Initiative.

[1] E. Sackmann, Science **271**, 43 (1996).
[2] L. K. Tamm and H. M. McConnell, Biophys. J. **47**, 105 (1985).
[3] A. Sonnleitner, G. J. Schütz, and Th. Schmidt, Biophys. J. **77**, 2638 (1999).
[4] M. L. Wagner and L. K. Tamm, Biophys. J. **79**, 1400 (2000).
[5] C. A. Naumann, O. Prucker, T. Lehmann, J. Rühe, and C. W. Frank, Biomacromolecules **3**, 27 (2002).

Downloaded 08 Dec 2005 to 130.126.103.95. Redistribution subject to AIP license or copyright, see http://jcp.aip.org/jcp/copyright.jsp

[6] M. A. Kol, A. I. P. M. de Kroon, J. A. Killian, and B. de Kruijff, Biochemistry **43**, 2673 (2004).
[7] J. Liu and J. C. Conboy, J. Am. Chem. Soc. **126**, 8376 (2004).
[8] M. Hetzer, S. Heinz, S. Grage, and T. M. Bayerl, Langmuir **14**, 982 (1998).
[9] N. L. Thompson, A. M. Lieto, and N. W. Allen, Curr. Opin. Struct. Biol. **12**, 634 (2002).
[10] E. Haustein and P. Schwille, Methods **29**, 153 (2003).
[11] A. Benda, M. Beneš, V. Mareček, A. Lhotský, W. Th. Hermens, and M. Hof, Langmuir **19**, 4120 (2003).
[12] L. Zhang and S. Granick, Proc. Natl. Acad. Sci. U.S.A. **102**, 9118 (2005).
[13] A. F. Xie, R. Yamada, A. A. Gewirth, and S. Granick, Phys. Rev. Lett. **89**, 246103 (2002).
[14] H. Zettl, W. Hafner, A. Boker, H. Schmalz, M. Lanzendorfer, A. H. E. Muller, and G. Krausch, Macromolecules **37**, 1917 (2004).
[15] M. Langner and S. W. Hui, Chem. Phys. Lipids **60**, 127 (1991).
[16] J. J. Ramsden, Philos. Mag. B **79**, 381 (1999).
[17] E. Evans and E. Sackmann, J. Fluid Mech. **194**, 553 (1988).

Downloaded 08 Dec 2005 to 130.126.103.95. Redistribution subject to AIP license or copyright, see http://jcp.aip.org/jcp/copyright.jsp