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Letters

Single Lipid Diffusion in Langmuir Monolayers

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Individual lipid movement in a monolayer is studied over long time intervals (500 s) by darkfield microscopy of single lipids labeled with gold colloids (30 or 100 nm in diameter). Dimyristoyl phosphatidylcholine in the fluid phase shows normal diffusion, with a diffusion coefficient of $(1.1 \pm 0.2) \times 10^{-8}$ cm^2/s. Since this is consistent with values derived from the diffusive transport of many lipids, the analysis of gold-tagged lipids in a monolayer provides a reliable picture of lipid diffusion on the level of single molecules.

Introduction

Diffusive transport of lipids and membrane proteins is of fundamental interest to physics, chemistry, and cell biology, because it typifies two-dimensional diffusion[1] and plays a key role in intracellular signaling.[2] For these reasons, a number of experiments have investigated diffusion in cell and model membrane systems.[3−6] However, existing ensemble methods that measure the average transport of many lipids (such as fluorescence recovery after photobleaching, FRAP, or electron spin resonance) implicitly rely on assumptions, such as the individual lipid motion is Brownian,[7,8] and thus cannot detect complex diffusive behaviors, including subdiffusion. Recently, a method called single particle tracking (SPT) has been developed to optically visualize individual protein or lipid motion in cell membranes and supported bilayers by following fluorescent or highly scattering gold tags attached to a single molecule under investigation.[9−12] Studies by SPT have reported subdiffusive motion of proteins in the plasma membrane.[13] However, the causes of subdiffusion in cells remain unclear;[14] moreover, cells are so small that the particle of interest can only be tracked for tens of seconds,[15] resulting in inconclusive statistics.

We have developed a technique to follow the motion of single lipids on a Langmuir monolayer via SPT. Single

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(1) Pershan, P. S. *J. Phys.* (*Paris*) **1979**, *40*, 423.
(2) Janmey, P. A.; Stossel, T. P. *J. Biol. Chem.* **1989**, *264* (9), 4825.
(3) Groves, J. T.; Boxer, S. G. *Biophys. J.* **1995**, *69* (5), 1972.
(4) Axelrod, D. *J. Membr. Biol.* **1983**, *75*, 1.
(5) Jacobson, K.; Ishihara, A.; Inman, R. *Annu. Rev. Physiol.* **1987**, *49*, 163.
(6) Tamada, K.; Kim, S.; Yu, H. *Langmuir* **1993**, *9*, 1545.
(7) Gaub, H.; Sackmann, E.; Buschl, R.; Ringsdorf, H. *Biophys. J.* **1984**, *45* (4), 725.
(8) Rubenstein, J. L. R.; Smith, B. A.; McConnell, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 15.
(9) Saxton, M. J.; Jacobson, K. *Annu. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 373.
(10) De Brabander, M.; Geuens, G.; Nuydens, R.; Moeremans, M.; De Mey, J. *Cytobios* **1985**, *43* (174S), 273.
(11) Sheetz, M. P.; Elson E. L. In *Optical Microscopy*: *Emerging Methods and Applications*; Herman, B., Lemasters, J. J., Eds.; Academic Press: San Diego, 1993; p 285.
(12) Lee, G. M.; Ishihara, A.; Jacobson, K. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88* (14), 6274.
(13) Sheets, E. D.; Lee, G. M.; Simson, R., Jacobson, K. *Biochemistry* **1997**, *36* (41), 12449.
(14) Jacobson, K.; Sheets, E. D.; Simson, R. *Science* **1995**, *268* (5216), 1441.
(15) Kusumi, A.; Sako, Y.; Yamamoto, M. *Biophys. J.* **1993**, *65*, 2021.

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particle tracking in conjunction with the Langmuir technique has two significant advantages: long diffusive traces of lipids can be obtained; and the phase state of the lipid monolayer can be chosen. Darkfield microscopy is used to visualize the diffusion of gold-tagged lipids in DMPC (dimyristoyl phosphatidylcholine) monolayers, a simple, fluid phase system for which theoretical models (e.g., the free volume model[16] or Saffman and Delbruck[17]) predict normal diffusion. We track the lipids for hundreds of seconds, resulting in errors an order of magnitude smaller than those for 10-s tracks.

Method

Langmuir Trough. A film balance trough (Teflon, 15 cm × 6 cm) equipped with a Wilhelmy surface pressure sensor (Nima PS4) has been built. The monolayer is spread out of the aqueous subphase from an ultrasonified vesicle suspension in a side trough, which is heated for more rapid spreading, and is separate from the main trough to avoid stray optical signals from subphase vesicles. A wet bridge made from a silica gel TLC plate (Whatman) connects the trough surfaces.[18] The monolayer is then compressed to the desired surface pressure across a second wet bridge into a small observation trough, which can be isolated from disrupting airflows, and in which the surface tension is subsequently measured. In addition, the observation trough has a very low depth to minimize thermal convection and has a silicon wafer at the bottom to act as a mirror, thus improving the darkfield optics.

Microscopy. We conjugate a small gold colloid (30 or 100 nm diameter, Goldmark Biologicals), using immunogold labeling,[19] with an antibody (Molecular Probes) that binds to the fluorescent tag of Texas Red-X labeled DPPE (dipalmitoyl phosphoethanolamine) (Molecular Probes) to form an antibody−gold complex (AGC). The vesicles from which the monolayer is spread contain DMPC and labeled DPPE in a molar ratio of 2000:1. The ratio of gold-tagged lipid (DPPE) to untagged lipid (DMPC with DPPE) is about $1:10^9-10^{10}$. We then observe the gold particle with a SIT camera (Dage-MTI SIT-68, field of view approximately 300 μm × 200 μm) via darkfield microscopy (Olympus BX-FLA, 50 × 0.8 NA darkfield objective), resulting in a dark background with the gold particles' primary diffraction peaks appearing as bright points (see Figure 1a). Stepping motors position the microscope to avoid vibrations due to manual contact. In addition to being the antigen for the AGC, Texas Red labeled DPPE allows us to fluorescently visualize the monolayer.

To test if multiple lipids are bound to a single colloid, we prepared the AGC using an inert antibody in competition with the anti-Texas Red as described by Lee et al.[12] Our adaptation used a ratio of 20:1 of inert to active antibody in order to create AGC with approximately 1 anti-Texas Red antibody per particle. Lipids bound to AGC prepared in this manner exhibit similar scaling and diffusion coefficients to lipids bound to normal AGC, showing that we observe the diffusion of a single lipid bound to a single gold.

Data Analysis. We digitize the darkfield pictures at 30 frames/s and use the method described in Crocker et al.[20] to track the particles, with an accuracy of 100 nm (see Figure 1a superimposed). There are generally about three to five particles tracked at once for up to 500 s, a limit set by the time it takes particles to drift out of the field of view.

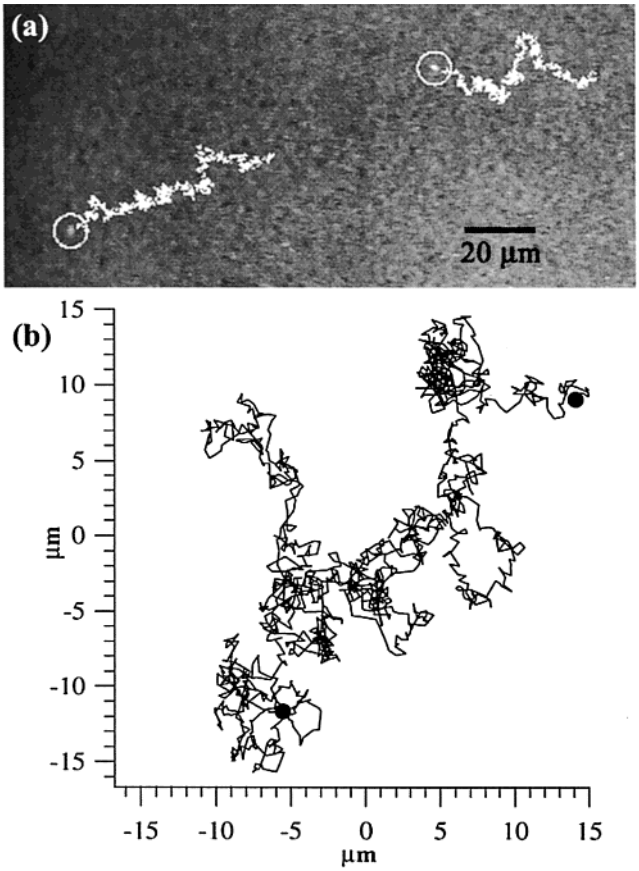

Figure 1. (a) Darkfield picture with two gold particles visible in a DMPC monolayer at 15 mN/m. The white circles pinpoint the actual particles. Superimposed are the tracks of the particles for a time interval of 50 s. (b) Relative motion of particles from Figure 1a, calculated from eq 2. This is a short segment (50 s, 1500 steps) of a full (150 s, 4500 step) track. The beginning and end are marked with filled circles (●).

The scaling of the mean square displacement (MSD) of a diffusing molecule with a time interval Δt distinguishes between normal and anomalous diffusive transport. If the motion is Brownian, leading to normal diffusion, $\mathrm{MSD}(\Delta t) = 4D\Delta t$ for the two-dimensional case, where D is the diffusion coefficient. Alternatively, anomalous diffusion is characterized by $\mathrm{MSD}(\Delta t) \propto \Delta t^{\alpha}$, $\alpha \neq 1$ (α is the scaling coefficient).

As it is impossible to remove all collective drift from the monolayer experimentally (we have reduced drift speed to below 1 μm/s), we use the relative motion of two particles to calculate the mean square displacement due to the diffusive motion alone (see Figure 1b). For one particle with position $\mathbf{a}$, $\mathrm{MSD} = \langle \mathbf{a}^2 \rangle$. Looking at the relative motion of two particles with positions $\mathbf{a}$ and $\mathbf{b}$, the MSD of the relative position (MSD_{rel}) can be expressed by[21]

(16) Galla, H. J.; Hartmann W.; Theilen U.; Sackmann E. *J. Membr. Biol.* **1979**, *48* (3), 215.
(17) Saffman, P. G.; Delbruck, M. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72* (8), 3111.
(18) Egger, M.; Heyn, S. P.; Gaub, H. E. *Biophys. J.* **1990** *57*, 669.
(19) Faulk, W. P.; Taylor, G. M. *Immunochemistry* **1971**, *8*, 1081.
(20) Crocker, J. C.; Grier, D. G. *J. Colloid Interface Sci.* **1996**, *179*, 298.
(21) Gross, D. J.; Webb, W. W. In *Spectroscopic Membrane Probes*; Loew, L. M., Ed.; CRC Press: Boca Raton, FL, 1988; p 19.

$$\mathrm{MSD}_{\mathrm{rel}}(\Delta t) = \langle(\mathbf{a} - \mathbf{b})^2\rangle = \frac{1}{N-n}\sum_{j=1}^{N-n}\{[\mathbf{a}(j\delta t + n\delta t) - \mathbf{b}(j\delta t + n\delta t)] - [\mathbf{a}(j\delta t) - \mathbf{b}(j\delta t)]\}^2 \quad (1)$$

where $\mathbf{a}(t)$ and $\mathbf{b}(t)$ are the positions of the particles at time t, with δt the time step between two successive pictures taken of the labeled molecules, n is the number of steps such that $n\delta t = \Delta t$, and N is the total number of steps. In our case, $\delta t = 0.033$ s, and N is up to 15 000 steps. In addition, we can decompose $\mathbf{a}$ and $\mathbf{b}$, $\mathbf{a} = \mathbf{a}_{\mathrm{coll}} + \mathbf{a}_{\mathrm{diff}}$, $\mathbf{b} = \mathbf{b}_{\mathrm{coll}} + \mathbf{b}_{\mathrm{diff}}$, where $\mathbf{a}_{\mathrm{coll}}$ and $\mathbf{b}_{\mathrm{coll}}$ are the collective motions (i.e., the drift and vibrations) of the monolayer as a whole and $\mathbf{a}_{\mathrm{diff}}$ and $\mathbf{b}_{\mathrm{diff}}$ are the diffusive motions of $\mathbf{a}$ and $\mathbf{b}$, respectively. Thus

$$\mathrm{MSD}_{\mathrm{rel}} = \langle(\mathbf{a}_{\mathrm{coll}} - \mathbf{b}_{\mathrm{coll}} + \mathbf{a}_{\mathrm{diff}} - \mathbf{b}_{\mathrm{diff}})^2\rangle = \langle(\mathbf{a}_{\mathrm{diff}} - \mathbf{b}_{\mathrm{diff}})^2\rangle = \mathrm{MSD}_a + \mathrm{MSD}_b \quad (2)$$

if the collective motions of the particles are homogeneous throughout the monolayer, which drift-velocity measurements verify in our case, and if there is no correlation of the intrinsic motion of the particles.

To distinguish between anomalous and normal diffusion, we determine the scaling coefficient α of the MSD with Δt by fitting the data linearly to $\log(\mathrm{MSD}) \propto \alpha \log(\Delta t)$. In the case of normal diffusion, we determine the diffusion coefficient D from a linear fit of $\mathrm{MSD}(\Delta t) = 4D\Delta t$.

A special consideration in determining the scaling coefficient is the level of camera noise. The noise level affects the accuracy of the determination of the lipid's position—higher noise levels lead to less accurate position determination. In the case of high noise levels, the error in position is comparable to the step size of the particle, which leads to a distinct overestimation of $\mathrm{MSD}(n\delta t)$ for small n ($n\delta t = \Delta t$). As n becomes large, the calculated MSD approaches the actual MSD from above, leading to apparently slower growth of the MSD with Δt. Thus, high noise levels can lead to a perceived α of 0.4 or smaller. To avoid artifacts caused by camera noise, we only analyzed pictures with a noise level of less than 30% of particle intensity.

Results

We study diffusion on fluid phase DMPC monolayers (i.e., in a surface pressure regime of 5−35 mN/m)[22] to determine to what extent ensemble methods measuring diffusive transport agree with analysis of time tracks of single molecules, as expected from statistical mechanics. In the fluid phase, diffusion coefficients have been measured by ensemble methods,[23] and the monolayer has a simple uniform structure so that normal diffusion is expected. Figure 2a shows a logarithmic $\mathrm{MSD}(\Delta t)$ plot for DMPC, obtained from SPT measurements of the relative motion of two gold-tagged lipids, which is approximately linear over 3 orders of magnitude in time, with a slope of 1.1 ± 0.1.

We plot the scaling coefficient α as a function of the time span used for the fit procedure to determine the time range that provides a reliable fit (Figure 2b). For very short times (up to the first decade), there are not enough separate time points to confidently determine α, and at long time scales (the last decade) we have little intrinsic data separated by a time interval $\Delta t = n\delta t$ to average (see eq 1 as $n \rightarrow N$), and thus the MSD values become more scattered. With less than about 1000 steps in a track, these time scales begin to overlap, and it becomes difficult to determine scaling laws.

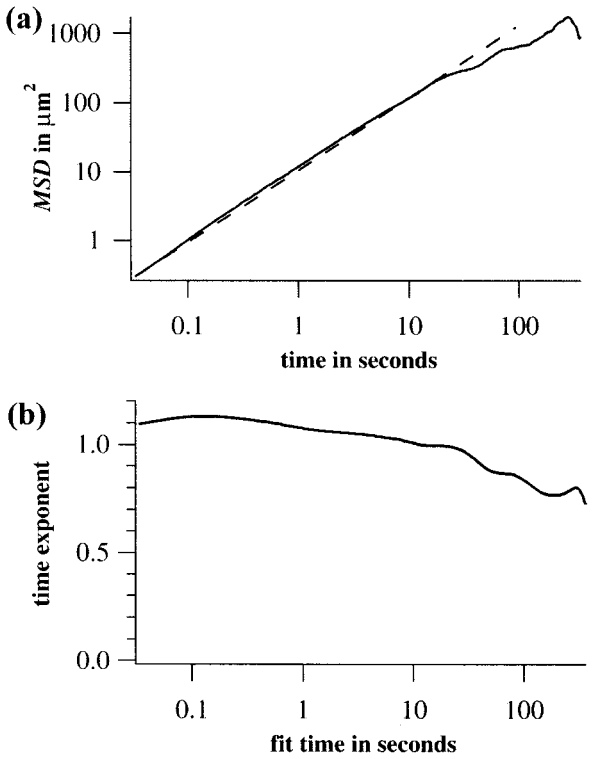

Figure 2. (a) $\mathrm{MSD}(\Delta t)$ for pair of lipids in a DMPC monolayer at 30 mN/m. The superimposed line has a slope of 1.0, corresponding to normal diffusion. The reason for the noise at long times (>20−30 s here) is the lack of independent data for each time point (note, the experimental MSD also deviates upward). (b) The scaling exponent α as a function of fit time for the system in Figure 2a.

DMPC diffusion is Brownian over a range of surface pressures between 5 and 35 mN/m (Figure 3a), with a scaling exponent α of 1.0 ± 0.1. Since the diffusion is normal, the diffusion coefficient can be determined as a function of surface pressure (see Figure 3b). The average value of $(1.1 \pm 0.2) \times 10^{-8}$ cm^2/s is consistent with previously published values, obtained by various ensemble methods such as FRAP.[24,25] Our method is not sensitive enough to resolve the small changes in the diffusion coefficient, within the surface pressure studied, that might be expected from the free-volume model. Indeed, ensemble methods are suited to obtaining the large amounts of data needed to resolve such small changes; we are more interested in fundamental modes of diffusion, for which SPT is better.

Combining Stokes friction with the Einstein relation, the diffusion coefficient of an object that can be approximated by a sphere is $D = k_{\mathrm{B}}T/(6\pi\eta a)$, where $k_{\mathrm{B}}T$ is the temperature, η is the viscosity of the medium, and a is the radius of the object in question. This implies that 30-nm gold particles should have a diffusion constant more than 3 times that of 100-nm gold. Since we do not see differences between the diffusion of lipids tagged with 30- or 100-nm gold beads (Figure 3), we infer that the gold

(22) Mingotaud, A.; Mingotaud C.; Patterson L. K. *Handbook of Monolayers*; Academic Press: San Diego, CA, 1993.
(23) Möhwald, H. In *Phospholipid Handbook*; Cevc, G., Ed.; Marcel Dekker: New York, 1993; p 579.
(24) Alecio, M. R.; Golan, D. E.; Veatch, W. R.; Rando, R. R. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *7*, 5171.
(25) Blume, A. In *Phospholipid Handbook*; Cevc, G., Ed.; Marcel Dekker: New York, 1993; p 455.

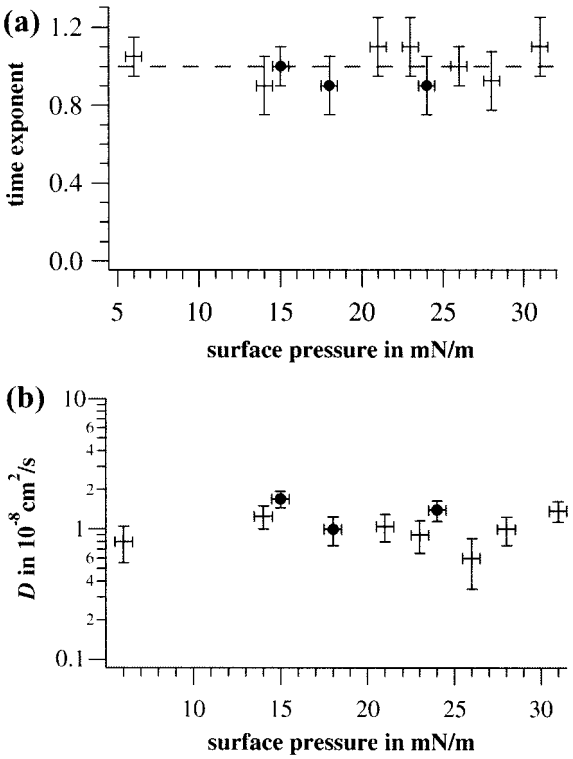

Figure 3. (a) The variation of the time exponent α with surface pressure, with an average value 1.0 ± 0.1. The crosses correspond to lipids tagged with 30-nm gold colloids, the crosses with a filled circle (●) correspond to lipids tagged with 100-nm gold. The scaling error bars are calculated from the standard deviation of the scaling exponent within each run. The surface pressure error is intrinsic to our system. An exponent of 1.0 is normal diffusion and is shown with the dashed line. (b) The variation of the diffusion coefficient with surface pressure. Lipids labeled with 30- and 100-nm gold are plotted as in Figure 3a. For 30-nm gold, $D = (1.1 \pm 0.2) \times 10^{-8}$ cm^2/s; for 100-nm gold, $D = (1.4 \pm 0.7) \times 10^{-8}$ cm^2/s. Errors are determined as above.

particle has a negligible effect on lipid motion. More evidence for this arises from comparing the diffusion constant of gold bound to a lipid (D_L) to that of 30- and 100-nm gold freely diffusing in water (D_{30}, D_{100}, respectively). Our experiments show that $D_{30} \approx 3 \times 10^{-7}$ cm^2/s, $\sim 30 D_L$, and $D_{100} \approx 1 \times 10^{-8}$ cm^2/s, $\sim 10 D_L$. Taking into account the relative size of a gold particle (30, 100 nm) and a lipid (<1 nm), Stokes friction shows that the viscosity of the monolayer is about 1000 times that of the subphase. Thus, at the surface pressure studied, the system is a lipid in a very viscous fluid, dragging a much larger gold particle through an essentially free medium.

Conclusions

We have developed a system that can resolve the motion of individual lipids in a monolayer over wide time intervals and at a variety of packing densities. Our results on DMPC monolayers provide a control for single particle tracking by showing that SPT leads to the same results as the study of diffusive transport by ensemble methods. In addition, our method offers improved statistics and control over SPT on cell membranes, gives more information than ensemble methods since it does not rely on assumptions about diffusive behavior, and can readily distinguish between normal and anomalous diffusive modes. Furthermore, in inhomogeneous lipid monolayers, such as the fluid-crystalline phase, tracks can be correlated with the spatial structures. In such a system, temporal deviations from normal diffusion may be expected. Ultimately, the large amount of data per particle will lead to improved statistics about both the diffusion coefficient and scaling behavior of lipids in monolayer systems.

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