Correlated diffusion in lipid bilayers

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Lipid membranes are complex quasi-two-dimensional fluids, whose importance in biology and unique physical/materials properties have made them a major target for biophysical research. Recent single-molecule tracking experiments in membranes have caused some controversy, calling the venerable Saffman–Delbrück model into question and suggesting that, perhaps, current understanding of membrane hydrodynamics is imperfect. However, single-molecule tracking is not well suited to resolving the details of hydrodynamic flows; observations involving correlations between multiple molecules are superior for this purpose. Here dual-color molecular tracking with submillisecond time resolution and submicron spatial resolution is employed to reveal correlations in the Brownian motion of pairs of fluorescently labeled lipids in membranes. These correlations extend hundreds of nanometers in freely floating bilayers (black lipid membranes) but are severely suppressed in supported lipid bilayers. The measurements are consistent with hydrodynamic predictions based on an extended Saffman–Delbrück theory that explicitly accounts for the two-leaflet bilayer structure of lipid membranes.

Significance

Dynamic processes on membrane surfaces are essential for biological function. Traditionally, quantitative measurements of lipid/protein motion have been interpreted in the framework of membrane hydrodynamics. However, some recent single-molecule tracking studies have proven difficult to interpret via hydrodynamic arguments. Does this suggest a failure of hydrodynamic theory or simply highlight the dangers in attempting to extend hydrodynamic arguments down to molecular scales? Intermolecular correlations are superior to single-molecule observations for studying hydrodynamics due to the longer length scales involved. The current work reports dynamic pair correlations of lipids in model membranes. Submicron distance-dependent correlations are well resolved, and complementary numerical calculations indicate that hydrodynamic theory can predict membrane dynamics over distances of tens of nanometers and longer.

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failures as stemming from the ill-advised application of hydrodynamic theory down to molecular scales (22–24, 26), a far more satisfactory resolution would be achieved by directly testing the performance of SD-like hydrodynamic models via measurements that are less sensitive to molecular-level details.

Two-particle microrheology measures hydrodynamic interactions via the correlated thermal motions of particle pairs (27). Unlike self-diffusion, hydrodynamic interactions are quite insensitive to the size and shape of probe particles, so long as one confines measurements to probe separations larger than the probe size (27–29). Furthermore, measurement of probe correlations over a wide range of separations provides a far more detailed test of hydrodynamic predictions than is possible via comparison to a single (or few) self-diffusion coefficient. For these reasons, it would be highly desirable to perform two-particle microrheology (or similar) measurements in lipid bilayer systems as a stringent test of SD and related hydrodynamic theories. One recent study has investigated the correlated motion of membrane proteins in neuronal cell membranes (30). However, this study involved intact living cells where all manner of complications are expected to influence protein motion. Although protein–protein correlations were observed, it proved impossible to explain them via hydrodynamic theory.

Correlated Brownian motions in model lipid membranes are expected to decay slowly (logarithmically and algebraically) but with weak amplitudes that necessitate measurements on the μm scale or shorter. Further, the rapid motion of particles in such membranes averages their positions during the measurement and complicates analysis of correlations. This work introduces the methodology necessary to overcome these difficulties. In particular, SPT with submillisecond time resolution is developed and combined with a theoretical analysis that explicitly accounts for the finite experimental acquisition times. This allows the measurement of correlated Brownian motion of lipid pairs as a function of distance in model lipid bilayers. In particular, two differently labeled lipids are tracked within freely floating black lipid membranes (BLMs) as well as within supported lipid bilayers (SLBs; Fig. 1). Using two colors allows for the study of lipid pairs with arbitrarily small interparticle distances, limited only by localization precision rather than optical diffraction. The experimental measurements are compared to an SD-like hydrodynamic model (18, 31) that accounts for the finite experimental acquisition times, limited only by localization precision rather than optical diffraction. The angular brackets indicate an averaging over the random Brownian motion of the particles. The experimental measurements are compared to an SD-like hydrodynamic model (18, 31) that accounts for the finite experimental acquisition times, limited only by localization precision rather than optical diffraction. The angular brackets indicate an averaging over the random Brownian motion of the particles. The experimental measurements are compared to an SD-like hydrodynamic model (18, 31) that accounts for the finite experimental acquisition times, limited only by localization precision rather than optical diffraction. The angular brackets indicate an averaging over the random Brownian motion of the particles.

Results

Preliminaries. It is well known that hydrodynamic interactions between particles in a fluid lead to correlations in the Brownian motion of these particles (9). For two particles in the creeping flow hydrodynamic regime appropriate to this work (32), both these correlations and the single-particle thermal fluctuations associated with self-diffusion are conveniently summarized by the two-body diffusion matrix, $D(r_{12})$. In the membrane geometry relevant to this work, the distinct elements of the diffusion matrix are ($\ell = 1, 2$ indexes the two particles, and $\alpha = +, -$ indexes the two leaflets of the bilayer)

$$D_{\ell}^0 = \frac{1}{\Delta \tau} \left\langle (\Delta x_{\ell})^2 \right\rangle = \frac{1}{\Delta \tau} \left\langle (\Delta y_{\ell})^2 \right\rangle,$$  \hspace{1cm} [1a]

$$D_{\ell}^r (r_{12}) = \frac{1}{\Delta \tau} \left\langle (\Delta x_{\ell})(\Delta y_{\ell}) \right\rangle,$$  \hspace{1cm} [1b]

$$D_{\ell}^t (r_{12}) = \frac{1}{\Delta \tau} \left\langle (\Delta x_{\ell})(\Delta y_{\ell}) \right\rangle,$$  \hspace{1cm} [1c]

where $\Delta x_{\ell}$ and $\Delta y_{\ell}$ are longitudinal (in the direction of the particle separation vector, $r_{12}$) and transverse (perpendicular to

the particle separation vector) probe displacements, respectively, over a short displacement time $\Delta \tau$ [short enough to insure that the associated displacements do not change $r_{12}$ enough to significantly alter the elements of $D(r_{12})$]. The angular brackets indicate an averaging over the random Brownian behavior associated with two particles initially separated by $r_{12}$ but otherwise at thermal equilibrium. $D_0^\ell$ is the usual self-diffusion constant for lipid $\ell$ when it is located in leaflet $\alpha$. [Although $D_0^\ell$ could depend in principle upon $r_{12}$, this dependence is very weak except for separations comparable to particle size (31) and will not be considered in this work.] $D^r_\ell (r_{12})$ and $D^t_\ell (r_{12})$ quantify correlations in the diffusive motion between particles 1 and 2. These correlations derive from hydrodynamic flows in the membrane and surrounding fluids; correlation is strongest for particles that are proximal to one another and falls off as the particles separate. One might also consider $D^r_\ell \big|_{r_{12}} (r_{12})$, which reports on correlations between lipids in opposing leaflets; however, such measurements are more challenging from the experimental perspective and are not considered in this work.

It is stressed from the outset that technical limitations preclude the direct experimental determination of $D^r_{\ell} (r_{12})$ from Eqs. 1b and 1c. Experimentally realizable time scales are not sufficiently short to hold $r_{12}$ nearly constant. (This issue is irrelevant to the measurement of self-diffusion coefficients since $D_0^\ell$ is independent of $r_{12}$.) So, while Eqs. 1b and 1c motivate the measurements described in Measuring and Analyzing Lipid Motion, a quantitative analysis of these measurements requires more elaborate theoretical
considerations than might be naively expected. A description of the required quantitative analysis follows in Hydrodynamics and Lipid Diffusion in Two-Leaflet Membranes.

**Measuring and Analyzing Lipid Motion.** Two-color SPT experiments of fluorescently labeled lipid probes embedded in SLBs and BLMs formed from egg L-α-phosphatidylcholine (EPC) were performed, using a home-built total internal reflection microscope (Methods and SI Appendix, Methods). Simultaneous diffusive trajectories of red and orange labeled lipid probes (Atto647N- and Atto550-labeled DMPE, henceforth “lipid 1” and “lipid 2,” respectively) were obtained by localizing single molecules in individual camera frames and linking them in consecutive frames (33). It is essential to note that lipid molecules embedded in a membrane move a considerable distance during the experimental frame time of \( t_f = 7.76 \) ms. For example, the BLM lipids studied here have self-diffusion coefficients on the order of \( D \approx 10 \) \( \mu \)m\(^2\)/s (see below), leading to an rms displacement of \( \langle \Delta r^2 \rangle^{1/2} = \sqrt{4D t_f} \approx 500 \) nm in each frame. Attempting to infer \( \mu \)m-scale hydrodynamic correlations based upon frame-averaged lipid positions that are inherently uncertain to within half a \( \mu \)m is a dubious proposition at best. To partially mitigate this problem, camera-synchronized excitation pulses were used to obtain effective frame times much smaller than \( t_f \) (34). The method is illustrated in Fig. 2A and B, showing pairs of excitation pulses grouped around every second dead time period between camera frames (\( \Delta t_{off} = 290 \) \( \mu \)s). In the SLB experiments, the excitation pulses have a length of \( \Delta t_{on} = 350 \) \( \mu \)s. This leads to alternating displacement time intervals \( \Delta t_{short} \approx 640 \) \( \mu \)s and \( \Delta t_{long} = 14.9 \) ms. A high laser intensity of \( \sim 5 \times 10^3 \) W/cm\(^2\) was used in order to obtain high-enough signals during the short illumination times. Yet, the long dark time between pairs of pulses allowed sufficient probes to diffuse into the imaging area without being photobleached. BLM experiments were performed using an epi-illumination mode, so that for the same nominal laser intensity the actual excitation intensity was lower by a factor of \( \sim 2 \) to 3. Therefore, longer pulses \( \Delta t_{on} = 1,210 \) \( \mu \)s were used such that \( \Delta t_{short} = 1.5 \) ms.

![Fig. 2. Measuring and analyzing lipid motion.](image-url)
Full trajectories obtained using illumination pulsing are shown in Fig. 2 C and D for the SLB and BLM cases. Analysis of self-diffusion and correlated diffusion relies only on the lipid vector displacements \( \langle \Delta r \rangle \) observed between adjacent “on” pulses with \( \Delta t_{\text{short}} = 640 \mu s \) for the SLB and \( \Delta t_{\text{short}} = 1.5 \) ms for the BLM cases (indicated by the blue segments in Fig. 2 C and D). For the remainder of the paper, therefore, the notation \( \Delta t_{\text{short}} \equiv \Delta t \) is used for simplicity.

Measurements in BLMs were performed on lipid probes diffusing in one leaflet only, which was achieved by quenching probes in the other leaflet using iodine ions. Distributions of the scalar displacements \( \Delta r = |\Delta r| \) for Atto647N-DMPE (i.e., lipid probe \( \ell = 2 \)) are shown in Fig. 2 E and F for BLMs formed from a \( n \)-hexadecane solution or from an \( n \)-decane solution, designated BLM\(_a \) and BLM\(_b \) respectively. These distributions are unimodal. In contrast, trajectories collected from SLBs demonstrate bimodal lipid dynamics with two populations, fast and slow, as shown in Fig. 2G. The two populations of molecules were distinguished using a two-state hidden Markov model (HMM) analysis (33). As shown previously using iodine quenching (33), the slow and fast diffusive modes are associated with the proximal and distal leaflets of the SLB, respectively.

Fitting the distributions in Fig. 2 (and similar distributions for Atto647N-DMPE) to the appropriate expression (SI Appendix, Single-Particle Tracking) yields the self-diffusion coefficients. For BLM\(_a \), \( D_1 = 13.6 \pm 0.3 \mu m^2/s \) and \( D_2 = 13.0 \pm 0.3 \mu m^2/s \), while for BLM\(_b \), \( D_1 = 20.6 \pm 0.4 \mu m^2/s \) and \( D_2 = 20.5 \pm 0.5 \mu m^2/s \). Due to the symmetry of BLMs, the values are the same in both leaflets. Clearly, the diffusion is slower in BLM\(_a \) than in BLM\(_b \), which is in agreement with the trend of slower diffusion with increasing \( n \)-alkane chain length reported previously (35). The two different probes share the same diffusivities to within experimental uncertainty. By fitting the distributions in Fig. 2 (and similar distributions shown in Fig. 3A) to the appropriate expression, as will be detailed in Hydrodynamics and Lipid Diffusion in Two-Leaflet Membranes.

Hydrodynamics and Lipid Diffusion in Two-Leaflet Membranes. The results presented below involve quantitative comparisons between experimental data and hydrodynamic predictions for the behavior of lipids diffusing in bilayer membranes. The underlying hydrodynamic model (18, 33) used for these comparisons should be viewed as the natural extension of the venerable SD model to membranes with a two-leaflet structure (14, 17) and (in the case of SLBs) an underlying solid support (8, 11–13). A two-leaflet description is required since the lipid probes do not span the entire membrane but reside in a single monolayer. Fig. 3C provides a cartoon illustrating the underlying physics. The membrane is modeled as two
thin fluid sheets (the monolayers), each with surface viscosity \( \eta_m \), which slide against each another with an intermonolayer friction coefficient \( b \). The monolayers are coupled to the surrounding bulk fluids via no-slip boundary conditions. These bulk fluids may have different viscosities above (\( \eta_u \)) and below (\( \eta_l \)) the bilayer. Diffusing lipids are assumed to have a radius \( R \). For SLBs, the separation distance between membrane and the underlying support \( h \) is finite, but it is infinite for the BLMs. In the traditional SD model, the membrane is represented by a single thin sheet (the bilayer) bounded on both sides by infinite fluids that share the same viscosity. The present model reduces to SD in the limit where \( b \to \infty \), \( h \to \infty \) and \( \eta_u = \eta_l \). The infinite friction forces the monolayers to move in lockstep, resulting in a single sheet with a bilayer viscosity \( \eta_b = 2\eta_m \). \( R = 0.5 \text{ nm} \) (36), \( \eta_f = 0.001 \text{ Pa s} \) [bulk water value (37)], and \( h = 1 \text{ nm} \) [for SLB cases (38, 39); \( h = \infty \) for BLM cases] will be assumed throughout this work.

The remaining parameters vary from system to system and will be discussed further in Results. Since \( R = 0.5 \text{ nm} \) is adopted for both lipid probes, \( D_b^f = D_b^s \) is enforced within the modeling, in agreement with the experimental findings of Measuring and Analyzing Lipid Motion.

Unfortunately, the two-leaflet approach does not yield analytical predictions for the dynamics of objects within the membrane [unlike the traditional single-sheet SD model, which does lead to analytical predictions (5, 10)]. Rather, it is necessary to perform numerical calculations to predict elements of the mobility/diffusion matrix (32, 40) and related quantities. The regularized Stokeslets (RS) (41, 42) technique has been used in this study to generate the diffusion matrix \( \mathbf{D}(r_{12}) \); full details are available in refs. 18, 31, 33, and SI Appendix, RS Calculations provides a brief overview. For the present discussion, it suffices to recognize that the RS approach solves boundary value problems associated with particulate suspensions in the creeping flow hydrodynamic regime by treating the entire system (fluids and embedded particles) as fluid-like but with additional geometric constraints that impose rigid-body motion on those portions of the fluid associated with particles. This approach provides potentially exact solutions to the relevant hydrodynamic equations but is limited in practice by discretization issues. The calculations performed in this work were converged to an error of 2% or less.

As mentioned above, Eq. 1a holds true for arbitrary \( \Delta t \), but Eqs. 1b and 1c are restricted to the limit of small \( \Delta t \). Unfortunately, even the short experimental pulses and intervals discussed above are far too long to allow an elementary data analysis based on Eqs. 1b and 1c. The experimental measurements reflect an inherent averaging of \( r_{12} \) during the \( \Delta t \) pulses and considerable changes in \( r_{12} \) during the \( \Delta t \) displacement interval. Finite-time correlations analogous to those in Eqs. 1b and 1c can be defined as

\[
\langle \chi_1 \rangle (A) = \frac{1}{2\Delta t} \int_0^{\Delta t} dt \chi_1(t),
\]

\[
\langle \chi_2 \rangle (A) = \frac{1}{2\Delta t} \int_0^{\Delta t} dt \chi_2(t),
\]

\[
\mathbf{D}(r_{12}) = \frac{1}{\Delta t} \int_0^{\Delta t} \chi_1(t) \chi_2(t^*) dt + \frac{1}{\Delta t} \int_0^{\Delta t} \chi_1(t) \chi_2(t^*) dt + \frac{1}{\Delta t} \int_0^{\Delta t} \chi_1(t) \chi_2(t^*) dt + \frac{1}{\Delta t} \int_0^{\Delta t} \chi_1(t) \chi_2(t^*) dt.
\]

The experimental measure-

Here \( \mathbf{A} \) plays a role analogous to \( r_{12} \), but it is critical to recognize that \( \mathbf{A} \) reflects an averaged lipid separation over the entire first pulse. The constraints in Eqs. 5 and 6 serve to define the \( x \) axis as the direction of longitudinal displacement, as in Fig. A4, with a first-pulse averaged lipid–lipid separation \( \mathbf{A} \). (The centering of lipid one at the origin is arbitrary but convenient.) The \( s_{1b}^*(A) \) notation facilitates comparison with \( s_{1b}^*(1_{1b}^f, r_{12}) \) below. Unlike \( s_{1b}^*(1_{1b}^f, r_{12}) \), \( s_{1b}^*(A) \) is well suited for direct experimental comparison. The \( s_{1b}^*(A) \) curves can be generated by Brownian dynamics simulations using the separation-dependent RS-generated diffusion matrix, \( \mathbf{D}(r_{12}) \), for the evolution. However, this procedure is cumbersome when simulations must be repeatedly run to fit experimental data to hydrodynamic parameters. In practice, Eqs. 3a and 3b were calculated via a linear-response formalism, treating the correlation elements of the diffusion matrix as perturbations to independent diffusion by the two lipids. It was verified that this procedure yields good agreement with full Brownian dynamics simulations (43) for the range of parameters studied in this work; further details are provided in SI Appendix, Brownian Dynamics Simulations and Linear Response Formalism.

Correlated Motion in BLMs. The diffusive trajectories collected for Atto647N-DMPE and Atto550-DMPE in BLMh and BLMd were used to determine experimental correlations according to Eq. 2. As above, fluorescence was quenched in one leaflet, and the analysis applies only to probe pairs occupying the same leaflet.

Correlation Functions. The correlations \( c_L(r_{12}) \) and \( c_T(r_{12}) \) for BLMh are shown in Fig. 4 A and B, while the correlations \( c_L(r_{12}) \) and \( c_T(r_{12}) \) for BLMd are shown in Fig. 4 C and D, respectively. The longitudinal correlation \( c_L(r_{12}) \) is positive at short distances and then decays with interparticle distance \( r_{12} \) over the \( \mu \text{m} \) scale for both BLMs, whereas the transverse correlation \( c_T(r_{12}) \) demonstrates only a weak positive signal for BLMd and is immeasurably small over the whole range for BLMh. The two free hydrodynamic parameters in the BLM geometry available for fitting are the interleaflet drag, \( b \), and the monolayer viscosity, \( \eta_m \). (Note that \( \eta_f = \eta_m = 0.001 \text{ Pa s} \) for the BLM systems.) These parameters were simultaneously fit to the mean lipid self-diffusion coefficient, \( D = (D_1 + D_2)/2 \) (via RS calculations) and to both correlation curves, \( c_L(r_{12}) \) [via comparison to \( s_{1b}^*(1_{1b}^f, r_{12}) \)]. The fitting procedure is detailed in SI Appendix, Correlated Motion in BLMh, BLMd, and BLMc. The best-fit parameters (and associated 68% confidence intervals) are \( \eta_m = 8 \times 10^{-11} \text{ Pa s m} \) (7.6 to 8.2 \times 10^{-11} \text{ Pa s m}) and \( b = 1.8 \times 10^4 \text{ Pa s/m} \). The best-fit diffusion matrix elements \( D_{12}(r_{12}) \) and \( D_{12}(r_{12}) \) are shown in Fig. 4 A and B, respectively, together with the corresponding time-averaged forms \( s_{1b}^*(r_{12}) \) and \( s_{1b}^*(r_{12}) \). The value obtained for the leaflet viscosity agrees well with a previous measurement of membrane viscosity for a BLM formed from a solution of similar lipids in \( n \)-dodecane (21). For BLMh, the available correlation data provide insufficient information to convincingly guide the fit. (The data quality for BLMh and BLMd are comparable; however, BLMh involves a more challenging parameter regime.) If it is assumed that the leaflet viscosity of BLMh is the same as that of BLMd (i.e., \( \eta_m = 8 \times 10^{-11} \text{ Pa s m} \)), then \( b = 1.8 \times 10^4 \text{ Pa s/m} \) can be inferred solely from the self-diffusion numbers. This higher \( b \) value (consistent with lipid bilayers devoid of cosolvent) strongly couples the two leaflets together and causes the membrane to behave largely as a traditional SD single-sheet membrane with \( L_{SD} = 2\eta_m/2\eta_f \approx 80 \text{ nm} \). Predicted diffusion elements \( D_{12}(r_{12}) \) and \( D_{12}(r_{12}) \) assuming these values for \( \eta_m \) and \( b \) are shown in Fig.
C and D, respectively, together with the corresponding time-averaged forms $s_L(r_{12})$ and $s_T(r_{12})$. Although these curves rely on an assumed value of $\eta_m$, as opposed to fitting both free parameters independently, they appear consistent with the experimental data.

Lack of Correlations in SLBs. In the case of the SLB, probe behaviors in the distal and proximal leaflets differ drastically, as evidenced by the self-diffusion coefficients. The experimental correlations for the SLB in the distal leaflet, $c^+_{L}(r_{12})$ and $c^+_T(r_{12})$, are shown in Fig. 5 A and B, respectively, while the related correlations for the proximal leaflet, $c^-_{L}(r_{12})$ and $c^-_T(r_{12})$, are shown in Fig. 5 C and D. To within the experimental resolution, no detectable correlations are observed, unlike the case of the BLMs.

In the SLB geometry, there are in principle three hydrodynamic parameters available for fitting: $\eta_m$, $b$, and $\eta_f$. However, as in the case of BLMh, the available correlation data are not helpful in determining these parameters. Adopting the value $\eta_m = 8 \times 10^{-11}$ Pa s m, as determined for BLMd, and fitting to the self-diffusion coefficients using the RS method yields $b = 7.3 \times 10^7$ Pa s m and $\eta_f = 7.3$ Pa s. This analysis suggests that

![Fig. 4](image-url) Correlated Brownian motion as a function of distance $r_{12}$ for BLMs and comparison with hydrodynamic theory. The panels show the experimental correlation functions, $c_L$ or $c_T$ (continuous line with error bars), as well as coupling diffusion coefficients calculated using the hydrodynamic theory, $D_L$ or $D_T$ (black dotted line), and the corresponding time-averaged forms, $s_L$ or $s_T$ (colored dotted line). (A and B) BLMd. (C and D) BLMh.

![Fig. 5](image-url) Correlated Brownian motion as a function of distance $r_{12}$ for an SLB and comparison with hydrodynamic theory. Each panel shows experimental correlations, $c^+_L$ or $c^+_T$ (continuous line with error bars), as well as coupling diffusion coefficients calculated using hydrodynamic theory, $D^+_L$ or $D^+_T$ (black dotted line), and the corresponding time-averaged forms, $s^+_L$ or $s^+_T$ (colored dotted line), respectively. (A and B) Distal leaflet. (C and D) Proximal leaflet. While the predicted coupling diffusion coefficients are nonzero in a very narrow window close to $r_{12} = 0$, this is completely washed out by time averaging; the experiments correspondingly display no evidence of correlations.
the interleaflet friction is slightly larger than the BLMh case, and the subphase viscosity is nearly four orders of magnitude larger than the viscosity of bulk water (0.001 Pa s) (37). The coupled diffusion coefficients, \(D^*_r(r_1)\) and \(D^*_s(r_1)\), and corresponding pulse-averaged quantities, \(s^*_r(r_1)\) and \(s^*_s(r_1)\), implied by these parameters are shown in Fig. 5. The theoretical predictions are barely visible on these axes, occurring only in the immediate vicinity of \(r_1 = 0\) for \(D^*_r(r_1)\), and that behavior is washed away upon pulse averaging. It is clear that the experimental uncertainties in \(r_1\) ((\(\Delta r_1\)) \(\approx\) 150 nm in the distal leaflet and \(\Delta r_2\) \(\approx\) 50 nm in the proximal leaflet), coupled with finite acquisition times, preclude measurement of hydrodynamic correlations in the supported geometry.

The qualitatively different behavior of the SLB correlations as compared to the BLMs is attributable to the large subphase viscosity, \(\eta_s\), and small gap between membrane and support, \(h = 1\) nm. In this regime, the substrate imparts a simple Evans–Sackmann-like (11) drag on the proximal leaflet with friction coefficient \(b_{ds} = \frac{\eta_s}{h} = 7.3 \times 10^6\) Pa m/s, and as far as the distal leaflet is concerned, the proximal leaflet is largely locked in place by this strong friction. The interleaflet friction is two orders of magnitude smaller, so sliding motions predominately involve leaflet–leaflet slipping as opposed to leaflet–substrate slipping, and the distal leaflet behaves effectively as an Evans–Sackmann sheet above a support with effective friction coefficient \(b_{ds} = b\). Hydrodynamic interactions in the distal leaflet are thus screened (8, 11, 18, 44) for separations exceeding \(L_{ab} = \frac{b_0 \eta_s^{1/2}}{\sqrt{C_0}} \approx 1\) nm. The range of interactions in the proximal leaflet is even smaller since there is no way to avoid the direct effect of \(b_{ds}\). It is not surprising that hydrodynamic interactions cannot be detected, given the experimental resolution.

It is believed that some dyes interact directly with the glass substrate when incorporated into SLBs, leading to reduced dye diffusivities (45, 46). Thus, it might be tempting to attribute the small \(D^-\) values measured for the proximal leaflet of the SLB to dye molecules dragging on the substrate, as opposed to the elevated viscosity of trapped water suggested above. This mechanism was considered but was found to be inconsistent with the entirety of the data collected for the SLB system. To wit, if one assumes that the viscosity of water in the trapped layer is identically to that of bulk water (0.001 Pa s), it then proves impossible to reproduce the measured \(D^-\) value for self-diffusion in the distal leaflet. (Even in the extreme limit of \(b = \infty\), the predicted \(D^-\) is too high.) Further, both probes used here are quite hydrophobic and naturally associate with membranes out of aqueous solution (47), so they are less likely to interact with the hydrophilic glass substrate than other common single-molecule labels (48). We cannot completely rule out the possibility of a hybrid mechanism, where both direct dye–substrate interactions and elevated \(\eta_s\) contribute to the observed measurements. However, this case introduces an additional unknown to the theoretical model (the direct friction on labels in the proximal leaflet), and it is impossible to uniquely determine the three free parameters (\(\eta_s\), \(b\), and direct probe friction) on the basis of the two available self-diffusion constants. No matter which mechanism is assumed (elevated \(\eta_s\) or a combination of elevated \(\eta_s\) and a direct probe interaction), it is always the case that the predicted correlations are too short-ranged to be measured experimentally, for any set of parameters capable of reproducing both \(D^-\) and \(D^+\).

**Discussion**

Hydrodynamic interactions within lipid membranes can couple the motion of lipids or of proteins embedded in the membrane over distances much larger than the molecular scale. Collective motion in model lipid bilayers has been studied previously with neutron scattering experiments (49, 50) and molecular dynamics simulations (51, 52), but these methods could probe dynamics only over relatively short (nanometer) length scales. The present work avoids this limitation, allowing the study of distance-dependent correlated Brownian motion of lipid probes within membranes on the scale of hundreds of nanometers to microns. \(T_{\text{corr}} = 0\) for both BLMs and SLBs highlights the considerable dynamical differences between these two systems, despite the fact that they were prepared with identical lipid mixtures. While self-diffusion in the distal leaflet in a SLB is reduced by a relatively modest amount (a factor of 3 to 5) relative to the BLMs, self-diffusion in the proximal leaflet is reduced by two orders of magnitude, and the \(\mu\)-scale correlated motions seen in BLMs are completely suppressed (to within experimental resolution) in both SLB leaflets.

Two different BLMs were studied in this work. To understand the differences between them, it is important to review the BLM formation process (53). BLMs are formed over an aperture in a thin hydrophobic sheet from lipids dissolved in a nonpolar organic solvent, often a simple \(n\)-alkane (Fig. 1B). Following formation, the bilayer region remains in chemical equilibrium with a solvent annulus formed in the region of contact with the hydrophobic sheet, with some residual solvent partitioning into the bilayer. The partitioning of the hydrocarbon solvent in the bilayer region of BLMs is known to decrease with increasing chain length of the \(n\)-alkane (54). Indeed, capacitance measurements reveal that bilayers formed from lipids dissolved in \(n\)-decane are 62% thicker than solvent-free bilayers, whereas bilayers formed from lipids dissolved in \(n\)-hexadecane are only 10% thicker than the solvent-free case (55). X-ray diffraction, small-angle neutron scattering, and NMR measurements all suggest that the pronounced thickness increase of BLMs prepared with shorter-chain solvent is associated with the incorporation of solvent molecules into the region between the two leaflets (56–58). On the other hand, alkanes such as \(n\)-hexadecane predominately incorporate within the leaflets and align with the lipid chains, with a relatively low solubility of 1 solvent molecule per 6 to 10 lipid molecules (57).

Based on the differing modes of solvent partitioning, it is to be expected that the opposing leaflets in BLMd should be coupled to each other much more weakly than those in BLMh, which should in turn be coupled more weakly than a solvent-free bilayer. Since the experimental data for BLMh and the SLB cannot unambiguously assign values to both \(b\) and \(\eta_s\), the value \(\eta_s = 8 \times 10^{-11}\) Pa m obtained from BLMh has been assumed for the BLMh and SLB cases. This choice is equivalent to the assumption that variations between BLMh and BLMd are due to changes in the interleaflet coupling, \(b\). This view is affirmed by molecular dynamics simulations, which attribute the dominant effect on BLM properties to the solvent in the bilayer interior (59). A consistent picture emerges from this analysis, with the solvent-free SLB revealing the strongest interleaflet coupling, \(b = 7.3 \times 10^7\) Pa m/s, followed by the nearly solvent-free BLMh with \(b = 1.8 \times 10^8\) Pa m/s and BLMd with \(b = 1.8 \times 10^7\) Pa m/s. Previously reported values for the interleaflet drag in solvent-free SLBs are in the range \(b \approx 1 \times 10^7\) to \(1 \times 10^8\) Pa m/s (60–62), in agreement with this analysis. It is worth noting that \(b = 1.8 \times 10^7\) Pa m/s for BLMd is quite low. One would naively predict \(b \approx n_\eta/H \approx 5 \times 10^8\) Pa m/s for a 2-nm-thick layer \((H = 2\text{ nm})\) of \(n\)-decane \((\eta = 0.0009\) Pa s) (63), coupling the two monolayers together via no-slip boundary conditions on both sides. This discrepancy suggests a pronounced slipping between the \(n\)-decane layer and the two monolayers surrounding it.

In reality, and in contrast to the assumption above, slight differences in viscosity between BLMh and BLMd are probably to be expected. It is known, for example, that bilayer fluidity as measured using fluorescence anisotropy depends moderately on the \(n\)-alkane chain length (64). However, the viscosity of...
BLMh cannot differ drastically from \( \eta_m = 5 \times 10^{-11} \) Pa s m. The correlation data for BLMh may not allow for a full independent determination of \( \eta_m \) and \( b \), but the range of possible viscosity values is only \( \eta_m \approx (6 \to 14) \times 10^{-11} \) Pa s m, allowing for any possible \( b \) value between zero and infinity. These bounds are enforced solely by the self-diffusion measurements. The theoretical coupling diffusion coefficients \( D_{f,T} \) are then almost invariant over a relevant range of the possible viscosities \( \eta_m \) enforced by the self-diffusion measurements. This means, however, that despite the remaining uncertainty in \( \eta_m \), the correct forms of the coupling diffusion coefficients \( D_{f,T} \) could still be obtained (SI Appendix, Fitting of Correlated Motion).

Turning to SLBs, several studies indicate differences in melting temperature \( T_m \) between the two leaflets. Atomic-force microscopy measurements have shown the existence of two independent structural phase transitions in SLBs, which were attributed to the independent melting of the two leaflets (65–68). It was correspondingly proposed that the interaction with the support modifies lipid order in the proximal leaflet. A recent neutron reflectometry study, however, revealed only minor leaflet asymmetry in the main melting transition due to the presence of the substrate (69). It is thus unclear whether the viscosities of the two leaflets should differ substantially in SLBs, and the answer may be very system dependent. In the absence of direct experimental guidance to the contrary, the same viscosity was assumed for both leaflets of the SLB. It follows that the substantial measured asymmetry in self-diffusion between the two leaflets requires a subphase viscosity larger by three to four orders of magnitude than that of bulk water (i.e., \( \eta_f = 7.3 \) Pa s). Viscosities larger by two to seven orders of magnitude from bulk water have previously been measured for interfacial water confined between surfaces (70, 71).

A key component of this study has been the deliberate attempt to compare experiment and hydrodynamic theory in a detailed and careful way for membrane systems. This proved to be somewhat more challenging than might be naively expected. The systems studied here (lipid probes in BLM and SLB geometries) break assumptions inherent to the well-known SD model. The required extensions to SD theory to account for two-leaflet dynamics do not yield closed-form analytical solutions. It is thus necessary to invoke numerical schemes that predict elements of the diffusion matrix for possible comparison to experimental measurements: RS calculations served this purpose in the present work. Unfortunately, molecular scale probes diffuse so rapidly that even the submillisecond acquisition times employed here yield data that are not suited for direct comparison to elements of the diffusion matrix. The \( D_{f,T}(r) \) elements vary with probe positions, which are constantly evolving, and it is thus a time-averaged form of these quantities collected experimentally. The corresponding theoretical time averaging can be performed via Brownian dynamics simulations, using the RS-obtained diffusion matrix for the underlying dynamics, but this scheme is prohibitively expensive for the purposes of fitting parameters to the underlying hydrodynamic model. To accelerate this process, linear-response predictions have been derived and implemented numerically to allow for a direct comparison between experimental data and theoretical predictions. Under favorable circumstances, the resulting scheme (two-leaflet hydrodynamic theory → RS calculation of diffusion matrix → linear-response implementation of finite extraction times) fit to experimental data) allows for the extraction of all free parameters in the hydrodynamic model.

This is the case for BLMh. Unfortunately, the BLMh and SLB cases involve parameter regimes that do not allow for unambiguous assignment of all relevant parameters based on the available experimental data. Future experiments might be able to resolve some of the ambiguities reported here, for example, by measuring correlations of particles that span both membrane leaflets.

The comparison between theory and experiment presented here indicates that SD-like hydrodynamic models are consistent with the collective Brownian motions of lipids in lipid membranes. To realize this correspondence, it is essential that the proper generalizations of SD hydrodynamics be employed and that theoretical predictions are crafted to correspond directly with experimental measurements. This is a point we have made previously, in the context of self-diffusion measurements (15, 18, 33). The present study extends this conclusion to correlated diffusion, which is a far more sensitive probe of hydrodynamic predictions than is self-diffusion.

What are the implications of the correlated motions in the membrane plane revealed here for membrane-related biological processes? It is known that hydrodynamic interactions can reduce diffusion-limited reaction rates (72, 73), and the effect is predicted to be especially strong in membranes (74). As observed here, the presence of a support hinders lipid flows relative to free membranes and screens the spatial extent of hydrodynamic interactions, hence potentially also affecting reactions in the membrane plane. Supporting structures such as the actin cytoskeleton are therefore likely to modulate membrane protein interactions by screening hydrodynamic flows. Due to the coupling of the two leaflets observed here, this effect would even translate to peripheral membrane proteins associated with the outer plasma membrane only (75, 76). Flow resistance in biological cells has been attributed primarily to momentum adsorption by immobile cytoskeleton-bound transmembrane proteins acting as direct obstacles for membrane flow (16, 30, 77, 78), while our results indicate that flow resistance may also result from supporting structures that influence the extracellular side of the membrane only indirectly.

Methods
See SI Appendix for a detailed description of the methodology for sample preparation, data collection, and analysis. Briefly, SLBs were prepared by allowing unilamellar vesicles to fuse on the surfaces of flow cells made of microscope cover glasses. The vesicles contained a small fraction of labeled lipids. BLMs were prepared by flowing a lipid n-alkane solution into a cell that contained a polymeric sheet with a single wedge-shaped microaperture of a diameter of \( \sim 100 \) \( \mu \)m. Two-color single-molecule data were collected using a microscope in either total internal reflection mode (SLBs) or epi-illumination mode (BLMs), with pulsed illumination implemented as discussed in Results. Diffusive trajectories were obtained by localizing single molecules in individual camera frames and linking them in consecutive frames. The trajectories were then used to obtain correlated Brownian motion of probe pairs. Finally, the correlation data were analyzed using the RS technique, taking into account the time averaging due to finite camera acquisition times.

Data Availability. All study data are included in the article and/or SI Appendix.

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