Diffusion in single supported lipid bilayers studied by quasi-elastic neutron scattering†

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It seems to be increasingly accepted that the diversity and composition of lipids play an important role in the function of biological membranes. A prime example of this is the case of lipid rafts; regions enriched with certain types of lipids which are speculated to be relevant to the proper functioning of membrane embedded proteins. Although the dynamics of membrane systems have been studied for decades, the microscopic dynamics of lipid molecules, even in simple model systems, is still an active topic of debate. Neutron scattering has proven to be an important tool for accessing the relevant nanometre length scale and nano to picosecond time scales, thus providing complimentary information to macroscopic techniques. Despite their potential relevance for the development of functionalized surfaces and biosensors, the study of single supported membranes using neutron scattering poses the challenge of obtaining relevant dynamic information from a sample with minimal material. Using state of the art neutron instrumentation we were, for the first time, able to model lipid diffusion in single supported lipid bilayers. We find that the diffusion coefficient for the single bilayer system is comparable to the multi-lamellar lipid system. More importantly, the molecular mechanism for lipid motion in the single bilayer was found to be a continuous diffusion, rather than the flow-like ballistic motion reported in the stacked membrane system. We observed an enhanced diffusion at the nearest neighbour distance of the lipid molecules. The enhancement and change of character of the diffusion can most likely be attributed to the effect the supporting substrate has on the lipid organization.

Although phospholipid membranes have been studied for decades[1] there is still no consistent picture of lipid dynamics, even in simple model systems. While techniques such as fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) are capable of measuring the diffusion of lipid molecules on micrometre length scales, quasi-elastic neutron scattering (QENS) has proven to be an important tool to study nanoscale dynamics and diffusion on a nanometre length scale. In their seminal 1989 paper, Pfeiffer *et al.*[2] studied the local dynamics of lipid molecules in stacked fluid lipid membranes using incoherent neutron scattering techniques. Here a detailed model of lipid dynamics, including rotations, librations and diffusion, was proposed. More recently, this topic was revisited by Busch *et al.*[3] using state of the art neutron scattering techniques. Their primary finding was that the molecular mechanism for long range lipid diffusion in stacked membranes is a flow-like motion rather than the traditional diffusive motion. Ballistic motion was speculated to be a more efficient search strategy as compared to a Brownian motion.[4,5]

Despite their potential relevance to bioengineering applications, such as biosensors and surface functionalization[6,7] dynamical neutron scattering experiments in single supported bilayers have been limited in the past, as the minimal amount of sample material in a single bilayer results in a low scattering signal. Recent developments in neutron scattering instruments, and increasingly powerful neutron sources, now make it possible to observe dynamics in single bilayers. How the interaction between membrane and substrate affects lipid organization and dynamics, as compared to multi-lamellar or free-standing membranes, is a fundamental question.

We have studied the nanosecond lipid diffusion in a single phospholipid bilayer supported on a silicon wafer. From quasi-elastic neutron scattering, acquired using a backscattering spectrometer, the mechanism of the lipid diffusive motion could be modeled. While flow-like motion was reported in stacked bilayer systems,[3] we find evidence for a continuous diffusion with an enhanced diffusion at the nearest neighbor distance.

Single-supported bilayers of the model system 1,2-dimyristoyl-sn-*glycero*-3-phoshatidylcholine (DMPC) were prepared by vesicle fusion and hydrated by heavy water (D_2O).[8] 100 double-side polished, 2″, 300 μm thick, Si(100)-wafers were cleaned by immersion in an $H_2O_2^+$ sulfuric acid mixture (volume fraction of 70%

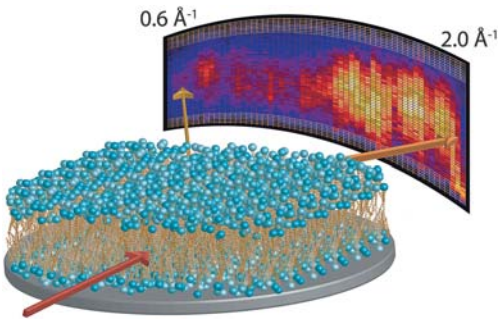

Fig. 1 A sketch of the experimental setup. Each solid supported single bilayer is aligned such that the plane of the membrane is in the scattering plane. In-plane q between 0.6 and 2.0 Å^{-1} are then measured simultaneously.

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concentrated H_2SO_4, 30%H_2O_2 at 90 °C for 30 min). This strongly oxidizing combination removes all organic contaminants on the surface, but does not disturb the native silicon oxide layer. The wafers were then rinsed and stored under distilled water before use. 200 ml of a 5×10^{-3} mol L^{-1} Hepes, 5×10^{-3} mol L^{-1} $MgCl_2$, and 100×10^{-3} mol L^{-1} KCl buffer solution was prepared and heated to 55 °C. DMPC was added to the buffer solution up to a concentration of 1.5 mg ml^{-1}. The milky solution, which initially contained giant multi-lamellar vesicles, was sonicated (50% duty cycle, power level 4) for 15 h until the solution became transparent and small uni-lamellar vesicles formed. The hydrophilic Si wafers were then completely immersed in the solution for 1 h. The lipid solution was kept at 55 °C during the whole process to keep the bilayers in their fluid phase. After 1 h the wafers were thoroughly rinsed with ~2 L of distilled water to remove excess vesicles from the surface. Through this procedure, small bilayer patches initially develop on the substrate, which eventually undergo a transition into a large uniform single bilayer after approximately 20–25 min.[8] The substrates were then annealed for 72 h at 55 °C in an oven in air before mounting in an aluminium sample can, and rehydrated.

Because the sample was hydrated with D_2O, the incoherent neutron scattering experiment is sensitive to local dynamics of the (protonated) lipid molecules. About 75% of the scattering comes from hydrogen atoms attached to the lipid acyl chains in the hydrophobic membrane core, and about 25% from hydrogen atoms located in the head group region. To increase the scattering signal, 100 wafers (resulting in 200 single bilayers) were mounted horizontally into the spectrometer, such that the neutron beam was always in the plane of the membrane ($q_{||}$). In contrast to previous studies, this type of sample orientation probes $q_{||}$ values between 0.6 and 2.0 $Å^{-1}$ simultaneously, corresponding to lateral length scales of 3 Å to 10 Å. The experimental setup is shown in Fig. 1. The sample was sealed in an aluminium sample can and placed inside a closed-cycle refrigerator (CCR) during the experiment. A sufficient amount of D_2O was added before closing to ensure full hydration of the bilayers (26 water molecules per lipid molecule), approximately 100 μL.

Elastic incoherent neutron scattering experiments were carried out using the high flux neutron backscattering spectrometer HFBS at the NIST Center for Neutron Research (NCNR), Gaithersburg, in its standard setup with Si (111) monochromator and analyzer crystals corresponding to an incident and analyzed neutron energy of 2.08 meV ($\lambda = 6.27$ Å). At the ~0.8 μeV resolution used, only hydrogen movements with characteristic times slower than 1 ns are monitored. The hydrogen atoms reflect the movements of larger groups to which they are attached, such as lipid head groups and carbon tails. Freezing and melting of molecular degrees of freedom can easily be identified, as they lead to jumps or kinks in the recorded intensity. Because the elastic intensity is related to the molecular mean-square thermal fluctuations $\langle \delta u^2 \rangle$, $S_{inc}^{el}(Q) \propto \exp\left(-\left\langle \frac{\delta u^2(T)}{6} \right\rangle Q^2 \right)$, each point on an elastic scan provides a value for the mean-square displacement (MSD) at a particular temperature.[9] The MSD was calculated and is shown in Fig. 2 over a temperature range of 240–355 K. Data were taken while heating the sample at a rate of 0.08 K min^{-1}. The inset in Fig. 2 depicts a sketch of a hydrated single supported bilayer as suggested by Nováková *et al.* from X-ray reflectivity. Each lipid molecule was found to be associated with 26 water molecules. Eight water molecules per lipid were found to be located within the head group region, while 18 water molecules per lipid form a hydration layer on the surface of the bilayer. There was also evidence of the presence of water molecules between the substrate and the lower leaflet.[10]

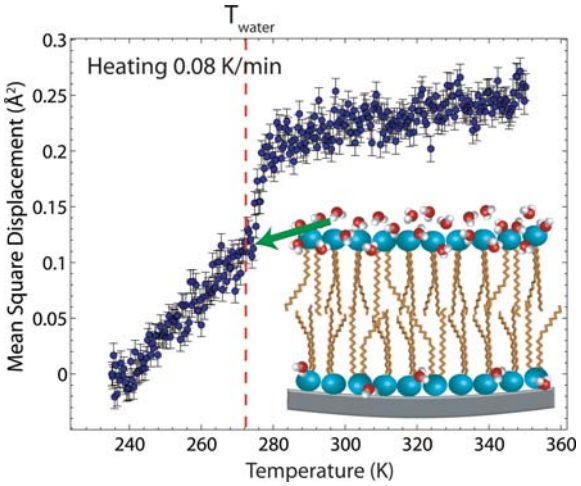

Fig. 2 The MSD of the lipid molecules over a temperature range of 240 to 355 K. The error bars shown are the result of statistical uncertainty. Melting of the hydration water was observed at approximately 273 K. No additional phase transition was observed at higher temperatures.

A melting transition at about $T_{water} = 273$ K was observed (see Fig. 2), which can most likely be assigned to the melting of the membrane hydration water. In multi-lamellar DMPC systems the transition from the gel into the fluid phase occurs at $T_m = 23.4$ °C = 296.6 K in fully hydrated bilayers and appears as a pronounced step in the MSD.[11,12] No such transition was observed. It was reported that the transition temperature may shift up to 318 K for nanometre sized patches of single supported bilayers.[13,14] The transition from the gel into the fluid phase involves a drastic increase in volume and area of the lipid molecules. For patches, the volume and area difference of the lipid molecules between the two phases can most likely relax over defects in the bilayer coverage.[13] In our samples, the transition was possibly suppressed due to large uniform and defect free single bilayers. While we cannot exclude the possibility that the upper and lower leaflets have different transition temperatures, the technique used should be sensitive enough to detect a transition in the individual leaflets. The diffusion constant that we determined is an order of magnitude faster than diffusion reported in the gel phase of multi-lamellar systems[2] and corresponds well with the diffusion constants reported in fluid multi-lamellar membranes, as discussed below. At 303 K, the single DMPC bilayers can, therefore, tentatively be characterized to be in a fluid state with an enhanced structural order; however, further structural investigations are required to unambiguously determine the structural state of the bilayer.

To measure the slow nanosecond dynamics, μeV energy resolved spectra were measured on the BASIS backscattering spectrometer at the Spallation Neutron Source, at Oak Ridge National Laboratory. The experiments were performed using a 60 Hz chopper setting with the incident wavelength bandwidth centered at of 6.4 Å. The Si (111) crystal analyzers provide an energy resolution of ~3 μeV, FWHM of the elastic peak, and a Q-range of 0.6 to 2.0 $Å^{-1}$. The dynamic range used was −80 to 80 μeV. Typical counting times were ~24 h.

Different diffusion models have been proposed. For a continuous diffusion, the quasi-elastic broadening is described by a Lorentzian

peak shape, with a full width at half maximum (FWHM) that shows a $q_{\|}^2$ dependence,

$$FWHM_L(q_{\|}) = 2\hbar D q_{\|}^2 \quad (1)$$

where D is the diffusion constant.[15]

A flow motion, as suggested in ref. 3,4 is described by a Gaussian component, where the width of the function has a linear $q_{\|}$ dependence,

$$FWHM_G\left(q_{\|}\right) = 2\sqrt{2ln(2)}\hbar v_o q_{\|}. \quad (2)$$

Here v_o refers to the ballistic velocity of the lipid molecules. In an unconstrained motion, v_o would correspond to the thermal velocity, $\sqrt{2k_BT/m}$.

Fig. 3 (a) and (b) depict exemplary spectra at $q_{\|} = 0.9$ and 1.3 Å^{-1}. The total scattering can be described by a narrow central component due to instrumental resolution and a quasi-elastic broadening between ~1–24 μeV. There is also evidence for a smaller broad component, similar to what has been observed by Busch *et al.* This broad component, most likely due to vibrations and librations of the lipid molecules, has a width of ~200 μeV, but is clearly out of the dynamic range of the instrument. The narrow component was assigned to the lateral diffusion of the lipid molecules. The broadening could well be fitted using a Lorentzian function rather than a Gaussian. The data were fit with three components: an asymmetric Gaussian and a background, a narrow (1–24 μeV) Lorentzian function to describe the lipid diffusion, and a broad (~200 μeV) Lorentzian function. The instrumental resolution was determined by fitting data measured in the frozen state of the bilayers, at $T = 240$ K, using the asymmetric Gaussian function to accommodate the slight asymmetry in the resolution function of the BASIS spectrometer†. The parameters were then used to fit the quasi-elastic broadening observed at $T = 303$ K.

Fig. 4 plots the FWHM of the Lorentzian, following deconvolution with the instrumental resolution, as a function of $q_{\|}^2$. The data can well be fit by a straight line, making it possible to determine the diffusion constant using eqn (1), $D = 60 \pm 10 \times 10^{-12}$ m^2 s^{-1}. The data deviate from the straight line around $q_{\|} = 1$ Å^{-1}, giving rise to faster dynamics. Diffusion seems to be enhanced at nearest neighbour distance of the lipid molecules of about 6 Å ($2\pi/1$ Å^{-1}).

When comparing to eqn (1) and (2), the most likely mechanism for diffusion in single supported bilayers seems to be continuous diffusion rather than the flow-like motion reported for multi-lamellar

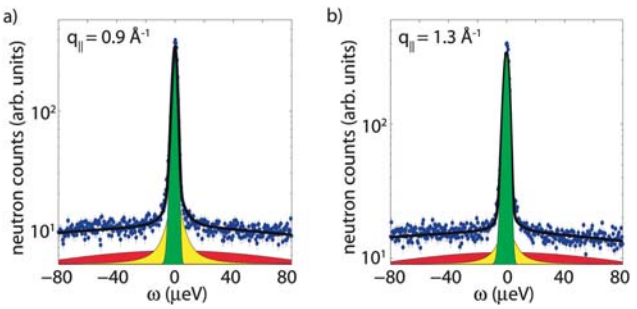

Fig. 3 Spectra for a) $q_{\|} = 0.9$ Å^{-1} and b) 1.3 Å^{-1}. The error bars shown are the result of statistical uncertainty. Data were fitted (black) with the instrumental resolution (green), a Lorentzian quasi-elastic broadening (yellow) and an additional broad Lorentzian (red).

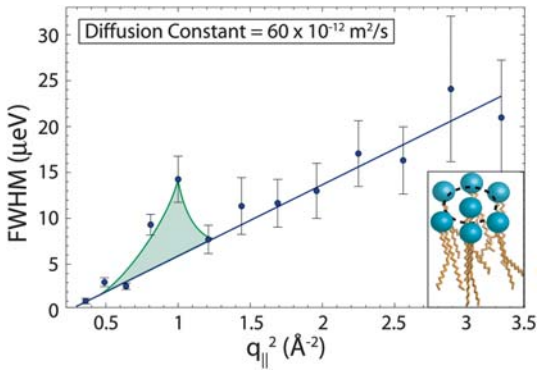

Fig. 4 FWHM of the Lorentzian as a function of $q_{\|}^2$. When excluding data points around $q_{\|} = 1$ Å^{-1}, data can be fit using a linear function. Diffusion seems to be enhanced around $q_{\|} = 1$ Å^{-1}, as indicated by the peak. Error bars correspond to a 10% confidence level in the Lorentzian fit. The inset shows a sketch of the lipid packing that might give rise to an enhanced nearest neighbour diffusion.

membranes stacks. The peak like feature around 1 Å^{-1} in Fig. 4 shows evidence for an enhanced diffusion of the lipid molecules, possibly triggered by voids in the ordered arrangement of lipids. The diffusion constant we found lies well within the range of diffusion constants of fluid phospholipid bilayers reported in the literature (18–180 × 10^{-12} m^2 s^{-1} [2,3,16,17]). However, because we cannot resolve a possible difference in the diffusion constants of the lipids in the upper and lower leaflets of the bilayer, we cannot exclude the possibility that the diffusion in the lower leaflet is suppressed by the presence of the substrate, while the diffusion in the upper leaflet may be enhanced by a highly ordered fluid phase of the lipids, thereby averaging to a diffusion constant which is comparable to multilamellar systems.

This change of character and enhanced nearest neighbour diffusion is most likely to be due to the highly ordered fluid phase of the lipids caused by the confinement of the defect free bilayer on the substrate. Note that the diffusion constants, as determined by incoherent neutron scattering, are usually an order of magnitude above the values obtained by macroscopic techniques in single[18,19] and multi-lamellar systems.[20] This may be due to coherent motion of lipid molecules, which are speculated to move as loosely bound clusters rather than individual molecules.[4,21]

This experiment has also demonstrated the feasibility of performing inelastic neutron scattering experiments on single supported bilayer systems. Complex membranes containing different membrane embedded proteins, as well as cholesterol, can be prepared. By tuning bilayer properties, such as elasticity, the impact of membrane properties on protein function may be elucidated. Inelastic neutron scattering experiments were once limited by the availability of sample material. The sample used for this study contained less than 1 mg of lipids. This opens up new opportunities for dynamical neutron scattering experiments in relevant and highly topical membrane/protein systems.

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