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# Lipid Membrane Flexibility in Protic Ionic Liquids

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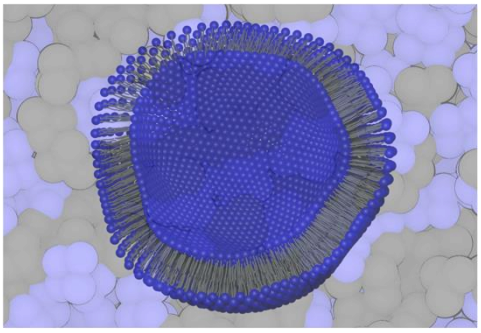
**ABSTRACT:** Here we determine by neutron spin echo spectrometry (NSE) how the flexibility of egg lecithin vesicles depends on solvent composition in two protic ionic liquids (PILs) and their aqueous mixtures. In combination with small-angle neutron scattering (SANS), dynamic light scattering (DLS), and fluorescent probe microscopy we show that the bending modulus is up to an order of magnitude lower than in water, but with no change in bilayer thickness or non-polar chain composition. This effect is attributed to the dynamic association and exchange of the IL cation between the membrane and bulk liquid, which has the same origin as the underlying amphiphilic nanostructure of the IL solvent itself. This provides a new mechanism by which to tune and control lipid membrane behavior.

**TOC GRAPHICS:**



**KEYWORDS:** Lipid Bilayer, Ionic Liquids, Inelastic Neutron Scattering

Ionic liquids (ILs) are a novel class of solvents whose use is rapidly expanding into a wide range of applications. Interest in them primarily arises from their high tunability as solvents enabled by mixing-and-matching the structures of their constituent cations and/or anions.[1] Protic ILs (PILs) are a sub-class that is straightforward to synthesise and economical to produce on industrial scales.[2] PILs are particularly effective solvents for amphiphilic self-assembly of surfactants and lipids into a variety of nanostructures, including unilamellar vesicles,[3-4] and have also shown superior performance for storage of complex biomolecules.[5] Addition of water is commonly employed as a tool to fine-tune solvent properties such as viscosity,[6] especially in biological systems.[5, 7] In particular, alkylammonium PIL-water mixtures have been employed to study protein folding at high protein concentrations and low temperatures, not accessible to conventional solvents.[7-9] When combined with the ease with which PILs and their aqueous mixtures can be supercooled and vitrified, these liquids show great potential as cryopreservatives for biological tissues. Their high biocompatibility, together with their ability to preserve structure and function in biomolecular assemblies shows promise for their use in biotechnology,[10] and even speaks to the possibility of life arising under extreme conditions or in non-aqueous environments.

The diverse functionalities of biological membranes require them to be highly dynamic across a wide range of time- and length-scales. Fast, localized motions ($10^{-9}$ s and $10^{-9}$ m) of individual lipid molecules are essential for rapid membrane reorganization, which influences inter-protein interactions and chemical signalling.[11-12] Meanwhile, the ability to bend and fuse over longer times and on larger scales ($10^{-3}$ s and $10^{-5}$ m) enables bulk deformations crucial for cell growth and division.[13] In between these molecular and macroscopic dynamics, mesoscopic fluctuations ($10^{-7}$ s and $10^{-8}$ m) occur via the collective motions of small clusters of lipid molecules, which can lead to local changes in membrane shape that are closely related to folding and mobility of membrane proteins.[14-15] This is also the class of motions that regulates channel

and vesicle formation,[13, 16] thus governs the transport of nutrients and wastes. Changes in these mesoscopic undulations are also linked to various cell conditions and diseases, including activation of cells during phagocytosis,[17] malaria and other parasitic infections,[18-19] and some studies have suggested cancerous cells are softer for blebbing and migration.[20]

Recent neutron and x-ray studies have shown that many PILs are themselves nanoscopically heterogeneous,[21-22] driven by a solvophobic effect to form distinct polar and apolar domains.[23-24] This renders ILs fundamentally different to molecular solvents, with their nanostructure tuneable through the structure of constituent ions. For example, the amphiphilic nanostructure in ethylammonium formate (EAF) is diminished by replacing the cation with ethanolammonium (EtAF),[21] simply due to the terminal hydroxyl moiety. Mixing with water and other molecular additives also modifies PIL nanostructure.[6, 25] PILs thus provide an avenue towards a more complete picture of bilayer membrane dynamics and functionalities, as well as yielding a novel mechanism by which to control their elastic and viscous properties.

Although the structure of PILs has been extensively characterised,[1] little is known about how their underlying nanostructure affects the dynamics of embedded amphiphile aggregates. Egg lecithin and other phospholipids can readily be assembled into unilamellar vesicles in EAF, EtAF,[3] and their mixtures with water, making them ideal model systems in which to examine the effect of solvent structure on lipid bilayer dynamics. This study explores how the dynamics of lipid bilayers can be tuned via solvent composition and nanostructure. Here we report the first study of the nanoscale dynamics of model cell membranes in PILs and their aqueous mixtures. We employed neutron spin echo (NSE) spectroscopy on the IN15 beamline at the Institut Laue-Langevin, which allows access to very long relaxation times needed for high viscosity solvents, and low-q for observing dynamics at the appropriate mesoscopic length scale.[26] Details of the experimental procedures are provided in Supplementary Information.

Figure 1 shows small-angle neutron scattering (SANS) and dynamic light scattering (DLS) measurements on 1% w/v egg lecithin in pure, perdeuterated EAF, EtAF, $D_2O$, and some representative PIL/$D_2O$ mixtures. Fitting DLS autocorrelation functions to a stretched-exponential model confirm the presence of large, unilamellar vesicles with low polydispersity, and their hydrodynamic radii are 90 ± 2, 74 ± 2 and 127 ± 3 nm in $D_2O$, EAF and EtAF, respectively (using experimental viscosities of hydrogenous PILs, complete data sets in Table S1). SANS curves follow the expected $q^{-2}$ scaling for bilayers in the low-q region down to the detector limit of $q < 0.002$ Å$^{-1}$. Weak oscillations seen for the samples in $D_2O$, EAF, and their mixtures are not simply explained, but may be attributed to a population of smaller sized vesicles of rather well-defined mean size. The lack of a pronounced structure factor in SANS curves is expected since lipid concentration is low, this also confirms the absence of significant interactions between vesicles.

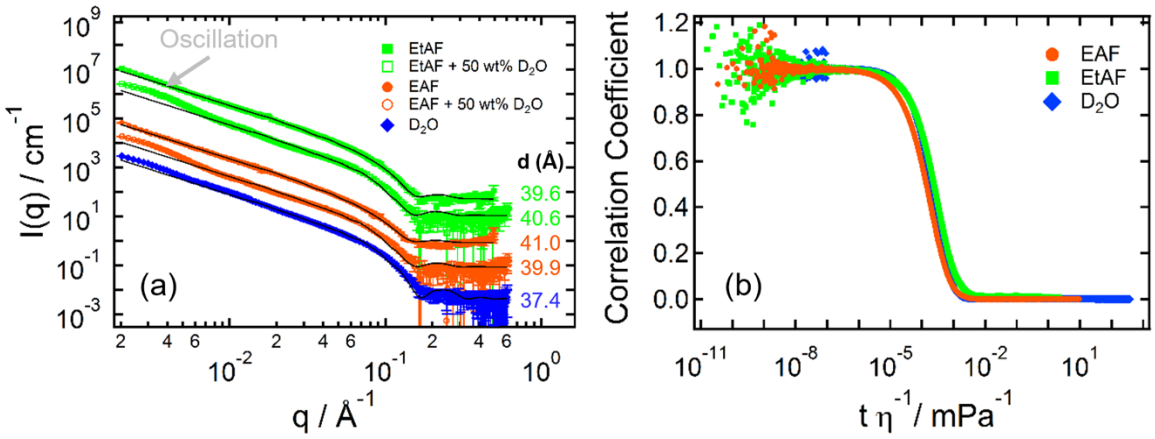



**Figure 1**. SANS curves (a) and DLS correlation functions normalised by solvent viscosity (b) of 1% w/v egg lecithin vesicles in $D_2O$ (blue), ethylammonium formate (EAF, red) and ethanolammonium formate (EtAF, green). SANS curves shown by filled markers were collected on the D11 beamline; hollow markers were collected on the QUOKKA beamline approximately 3 months after the D11/NSE measurements were completed. SANS curves are offset for clarity, with solid lines showing calculated SANS curves for arbitrarily large unilamellar vesicles using

bilayer thicknesses determined from Kratky-Porod analysis (see text and SI). The weak oscillation near low-q is marked by the grey arrow.

Figure 2 shows the normalised intermediate scattering functions obtained from NSE, $I(q, t)/I(q, 0)$,[27] versus Fourier time, $t$, of 1 wt% egg lecithin in $D_2O$ at nine wave vectors spanning $0.0260 \leq q \leq 0.0807$ Å$^{-1}$, corresponding to lipid domain (plaque) sizes between 7.8 and 24.1 nm. These plaques are all significantly smaller than the size of vesicles, which enables the separation of local bilayer dynamics (i.e. movement of these plaques) from diffusion of vesicles using a global fit to the Zilman-Granek model with a translational diffusion term (Eq. 1):[28-29]

$$\frac{I(q,t)}{I(q,0)} = e^{-Dtq^2} e^{-(t\Gamma q^3)^{\frac{2}{3}}} \qquad \text{Eq. 1}$$

where $D$ is the experimental translational diffusion constant of vesicles obtained with the in-line DLS, leaving a single relaxation rate ($\Gamma$) for lipid bilayer undulations in each solvent environment as the only free parameter to fit.

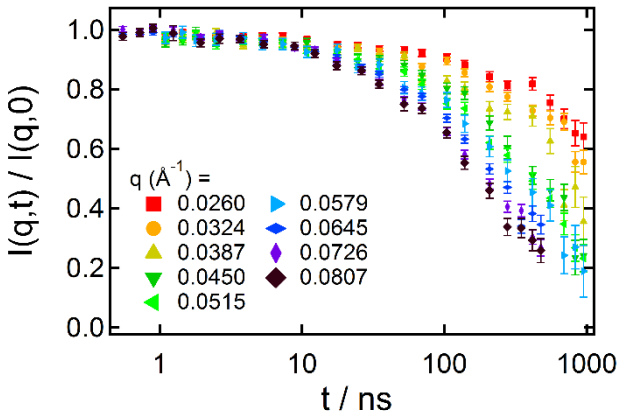



**Figure 2**. Intermediate scattering functions (NSE spectra) of egg lecithin vesicles in $D_2O$.

A lipid membrane undergoing transverse motion displaces solvent, so the relaxation rate depends on solvent viscosity, $\eta$. The bending modulus, $\kappa$, of the lipid bilayer is obtained from Eq. 2:[28-36]

$$\Gamma = 0.0069\gamma_k \left(\frac{k_B T}{\kappa}\right)^{\frac{1}{2}} \frac{k_B T}{\eta} \qquad \text{Eq. 2}$$

where $k_B$ is the Boltzmann constant, and $T$ is temperature. The semi-empirical pre-factor 0.0069 accounts for friction between lipid monolayers. $\gamma_k$ is a weak, monotonic, increasing function of $\kappa/k_BT$ that approaches 1 for rigid membranes.[29]

NSE results shows that the bilayers are markedly more flexible in the presence of these PILs than in water, where the fitted bending modulus of $25.2k_BT$ in $D_2O$ is comparable to values previously reported for pure phosphatidylcholine fluid bilayer membranes (Figure 3).[35-37] In the PILs, the bending modulus is reduced by a factor of 5-10 (Table 1), being lower in EAF than EtAF. Due to increasing solvent viscosity (see Table 1), relaxation rates decrease with increasing IL content to such an extent that we are unable to directly observe significant relaxation in pure EtAF within the accessible time range. Bending constants for both pure ILs were estimated by extrapolating a fit of $\Gamma\eta$ vs water content to 0. This was consistent with best fit to pure EAF results (Figure S1. Full spectra with fits are shown in SI.)

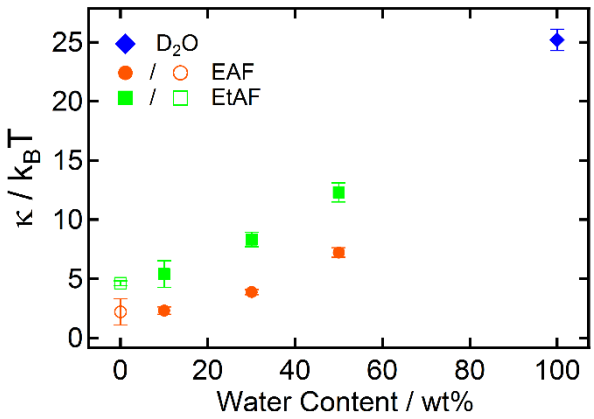



**Figure 3**. Dependence of membrane bending modulus, $\kappa$, on solvent composition of ethylammonium formate (EAF, red circles) and ethanolammonium formate (EtAF, green squares) mixtures with $D_2O$. Extrapolated values for pure ionic liquids are shown as hollow markers (see text).

**Table 1**. Measured vesicle translational diffusion constant ($D$) and undulation relaxation rates ($\Gamma$) of egg lecithin bilayers in $D_2O$ and EAF/EtAF-$D_2O$ mixed solvents. Solvent viscosities ($\eta$) were determined experimentally. Bending moduli ($\kappa$) for the bilayers are obtained from Eq. 2 using $\gamma_k = 1$.

| Solvent | $D$ (x $10^{-14}$ $m^2/s$) | $\Gamma$ (x $10^{-21}$ $m^3/s$) | $\eta$ (mPa·s) | $\kappa(k_BT)$ |
|---|---|---|---|---|
| $D_2O$ | 223 ± 0.88 | 5.16 ± 0.17 | 1.09[38] | 25.2 ± 0.9 |
| EAF + 50 wt% $D_2O$ | 75.1 ± 0.33 | 2.69 ± 0.14 | 3.93 ± 0.07 | 7.2 ± 0.4 |
| EAF + 30 wt% $D_2O$ | 40.9 ± 0.19 | 1.69 ± 0.09 | 8.50 ± 0.06 | 3.9 ± 0.2 |
| EAF + 10 wt% $D_2O$ | 14.9 ± 0.08 | 0.81 ± 0.11 | 23.1 ± 0.13 | 2.3 ± 0.3 |
| EAF | 6.54 ± 0.05 | 0.45 ± 0.11* | 45.6 ± 0.09 | 2.2 ± 1.1* |
| EtAF + 50 wt% $D_2O$ | 61.4 ± 0.27 | 2.25 ± 0.11 | 3.60 ± 0.14 | 12.3 ± 0.8 |
| EtAF + 30 wt% $D_2O$ | 21.9 ± 0.11 | 1.26 ± 0.09 | 7.83 ± 0.06 | 8.3 ± 0.6 |
| EtAF + 10 wt% $D_2O$ | 4.46 ± 0.04 | 0.26 ± 0.05 | 47.1 ± 0.94 | 5.4 ± 1.1 |
| EtAF | 0.87 ± 0.02 | 0.067 ± 0.002* | 197 ± 0.50 | 4.6 ± 0.2* |

*Bending constants for pure ILs from extrapolated $\Gamma\eta$ vs water content to 0.

What is the origin of this increased membrane flexibility? The bending modulus of a bilayer primarily arises from the lateral compressibility of its constituent monolayers, with one expanded while the other is compressed. This in turn depends on interactions between adjacent polar head groups and non-polar alkyl chains, which we consider in turn.

Although the phosphocholine lipid bilayer is neutral, each lipid head group is a zwitterion subject to electrostatic interactions which might render the bending modulus sensitive to ionic strength. Previous work has only reported modest decreases,[39-40] or increases,[41] in rigidity that are negligible compared to the dramatic softening observed here.

The increased flexibility could be due to a reduced membrane thickness, $d$, on which bending modulus depends with an exponent between 2 and 3.[42] Figure 1a) shows the thickness of a

homogenous bilayer determined from SANS curves by Kratky-Porod analysis,[43] in which the scattered intensity for dilute unilamellar vesicles is written as:[44]

$$I(q) = I(0)e^{-q^2 {R_g}^2} q^{-2} \qquad \text{Eq. 3}$$

where $I(0)$ is the zero-angle scattering, and $R_g$ is the radius of gyration of the bilayer thickness. The resulting bilayer thickness, given by $d^2 = 12{R_g}^2$ (Corresponding Kratky-Porod plots shown in Figure S5) is 37.4 Å in $D_2O$, which agrees with published values,[45-46] and is little changed in either pure PIL or PIL/water mixtures (Table 1). This is confirmed by the excellent agreement between experimental data and calculated SANS curves shown as solid lines in Figure 1a (details in SI).

Safran has shown that the energy cost of bilayer bending can be reduced by the expulsion of soluble lipid from the compressed monolayer side into solution, together with the uptake of dissolved lipid on the stretched side. This lowers $\kappa$ by a factor $r = 1 - \frac{\varphi_s^c}{\varphi_s}$,[47] where $\varphi_s$ denotes the concentration of amphiphile and $\varphi_s^c$ its critical concentration for membrane formation, which should be close to the free lipid monomer concentration in solution. Whilst egg lecithin is insoluble in water, we have measured its critical concentration for aggregation or solubility in EAF by surface tension and light scattering to be around 0.02 wt% (Figure S7 and Table S4). However, this is insufficient to account for the observed decrease in bending modulus in EAF (Table 1), which would require a critical aggregation concentration near 0.9 wt%.

The polar-apolar domain nanostructure of ILs and their solutions might lead to partial incomplete segregation of the lipid non-polar chains within the bilayer from the PIL, or, equivalently, finite solubility of PIL within the bilayer acyl chains. We examined bilayer composition using confocal laser microscopy with the apolar solvatochromic dye, Nile Red. Both the emis-

sion spectrum and fluorescence lifetime of the solubilized dye were the same in vesicles prepared in water or a PIL (details in SI), suggesting that the lipid bilayer is fully segregated from all solvents examined.

The derived bending moduli depend on solvent viscosity (Eq. 2), which could be affected by differences in liquid structure or composition (in mixed solvents) near the vesicle surface. Near a macroscopic surface, the isotropic bulk nanostructure of ILs is known to distort into layers, which could lower the viscosity adjacent to the bilayer membrane, thereby increasing the apparent value of $\kappa$ derived from Eq. 2. We dismiss this factor on several grounds. First, it is inconsistent with the observed trends in relaxation rates and bending moduli up to and including the pure ILs, shown in Figure 3. In the limiting case of a pure water layer around the vesicles, relaxation rates would be independent of solvent composition, contrary to our observations. Second, while structural distortion of PILs at interfaces are known, these are only long-ranged at flat interfaces and in strongly-nanostructured liquids.[48-49] Further, estimated amplitudes of the oscillations suggest that this would require an unreasonably thick low-viscosity layer in order to significantly affect hydrodynamic dissipation and alter $\kappa$.[50]

The same solvophobic interactions that drive bulk nanostructure have also been shown to cause both ethylammonium and ethanolammonium cations to preferentially orient at apolar interfaces,[49, 51] and to associate with surfactant aggregates in ILs and their water mixtures, affecting their curvature and equilibrium structure.[6, 52] Larger conventional IL cations have also been shown by contrast variation SANS experiments to become incorporated into DPPC vesicle bilayers.[53] Such association of IL cations between lipid head-groups may change the optimal packing condition away from planar bilayers by increasing the average area per lipid.[54] The theoretically predicted power-law dependence of $\kappa$ on area has an exponent of 7-8,[55] so that a small increase in the area could markedly lower bilayer stiffness. Theoretical studies have also predicted that short-chain additives lower bending energy dramatically by increasing

chain-packing entropy.[56] Such associated IL cations may also soften the membrane by exchanging between bilayer and its own bulk state during deformation in a manner analogous to the effect of a soluble lipid discussed above.

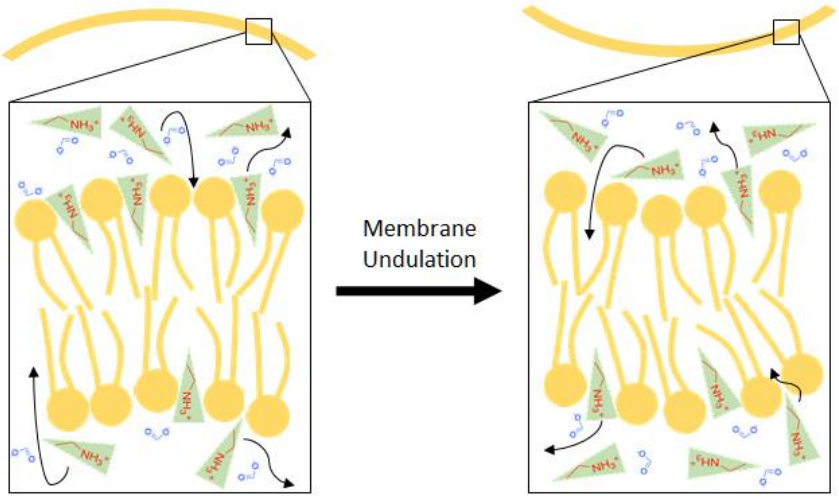



**Figure 4**. Schematic representation of the dynamic association and exchange of ethylammonium cations with the two monolayers of a phospholipid vesicle during an undulation.

Dynamic association of amphiphilic PIL actions, depicted in Figure 4, can thus account for the pronounced reduction in the bending modulus of lecithin bilayers upon exchanging water with the PILs EAF or EtAF. Similar effects have been inferred from AFM studies of supported lipid bilayer membranes upon addition of up to 40% ethanol.[57] This model is also consistent with the observed lowering of the bending moduli even at 50% water content, with the greater effect seen in EAF compared to EtAF, and with the known behaviour of ethylammonium and ethanolammonium cations as self-assembly media in both pure ILs and IL-water mixtures.[52] Scattering length densities obtained from fits to SANS data (Table S3) also suggest that the bilayers incorporate significantly greater amounts of IL than water.

It has previously been noted that chain-melting enthalpies of saturated-chain phosphatidylcholines are lower in EAF than either EtAF or water, suggesting an enhanced stabilization of the liquid state of the bilayer in EAF.[3] These differences are also reflected in the bulk liquid

structure of the ILs themselves; alkylammonium salts are amphiphilically nanostructured, consisting of interpenetrating networks of polar and apolar aggregated domains, but this feature is almost absent in ethanolammonium ILs.[23, 58]

We have shown using NSE spectrometry that lipid bilayer membranes are markedly more flexible in pure PILs and PIL/water mixtures than in water, with their bending moduli reduced by up to a factor of 5 to 10. Bending moduli are lower in systems containing EAF than those with EtAF. This arises from the amphiphilicity of the IL cation, which enables its inclusion in the bilayer as a small, dynamically-exchanging component. This effect has the same origin as the amphiphilic nanostructure within the bulk IL, differing for EAF and EtAF,[59] which we show here impacts not only equilibrium structure but also dynamics of self-assembled solutes. The extremely high flexibility but persistent stability of lipid bilayers in PILs is expected to impact on many dynamic membrane processes, which may make them promising media for diverse biotechnological applications such as the crystallization of membrane proteins,[60] for biosensors,[61] or for topical drug delivery.[62]

**ASSOCIATED CONTENT:**

Supporting_Information.pdf: Details of the materials and methods; details of the raw NSE and DLS data and fitting; the Kratky-Porod analysis to obtain thickness shown in Figure 1a; the SANS raw data and fitting shown in Figure 1a; the supplementary confocal microscopy and surface tension measurements discussed in the text.

**NOTES:**

The authors declare no competing financial interests.

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