

# Interaction of imidazolium-based ionic liquids with supported phospholipid bilayers as model biomembranes

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## Abstract

The cytotoxicity of Ionic Liquids (ILs) has been raising attention in the context of the biological and environmental impact of their vast field of applications. It is ascertained that the cell membrane is the main target of ILs when they interact with microorganisms, cells and bacteria; nevertheless, studies on the micro- and nanoscale aimed at better understanding the fundamental mechanisms of toxicity of ILs are lacking. In this work, we used atomic force microscopy (AFM) to investigate the impact of room-temperature ILs on the mechanical, morphological and electrostatic properties of solid-supported DOPC phospholipid bilayers, taken as models of biomembranes. In particular, we have characterized the concentration-dependent and time-dependent evolution of the morphological, structural and mechanical properties of DOPC lipid membranes in the presence of imidazolium-based ILs with different alkyl chain length and hydrophilic/hydrophobic character.

The majority of ILs investigated were found to possess the ability of restructuring the lipid bilayer, through the formation of new ILs/lipid complexes, showing distinctive morphological features (increase of area and roughness). The nanomechanical analysis of the lipid membrane exposed to ILs revealed a progressive, concentration-dependent perturbation of the structural ordering and rigidity of the membrane, evidenced by a decrease in the breakthrough force, Young's modulus and area stretching modulus. AFM detected a modification of the electrostatic double-layer at the membrane surface, in terms of a reduction of the original negative surface charge density, suggesting a progressive stratification of cations on the exposed leaflet of the lipid membrane. Our findings may be helpful in designing novel ILs with tailored interaction with biological membranes.

## Introduction

Due to their tunable physico-chemical properties, room-temperature Ionic Liquids (briefly ILs) can be effectively used as solvents in several applications.<sup>1-3</sup> ILs can replace standard aqueous electrolytes in applications aimed at the conversion and storage of energy,<sup>4</sup> such as electrochemical supercapacitors,<sup>5-8</sup> Graetzel solar cells,<sup>9-11</sup> and Li-ion batteries;<sup>12-14</sup> moreover, ILs can be used as lubricants in micro-electro-mechanical devices<sup>15-18</sup> and as synthesis media.<sup>1, 19</sup>

Due to the ILs intrinsic controlled flammability, miscibility and negligible vapor pressure, ILs are often assigned a green label.<sup>20-22</sup> In fact, ILs are not intrinsically green, although in principle they can be designed to be environmentally more sustainable.<sup>23</sup> The increasing spread of ILs technologies and their applications is raising several concerns upon their accidental leakage in the environment (soil or aqueous ecosystem),<sup>24, 25</sup> as well as contact with biological organisms and human beings.<sup>26, 27</sup> It is matter of concern, for example, that the ionic liquid cation [C<sub>8</sub>MIM]<sup>+</sup> was recently detected in urban landfill sites as an exobiotic and major pollutant, causing toxicity in liver model cell line.<sup>28</sup> In this framework, the investigation of ILs toxic character represents a challenge due to their different nature and variety of contacts with different environments and biological organisms. In-depth and rigorous investigations are very important to determine the mechanism of interaction of ILs with living organisms in order to control and design environmentally safe ILs or establish a safety protocol for existing ionic liquids.

Investigations focused on biological interaction of ionic liquids have highlighted a moderate to severe effect of several ILs on living organisms.<sup>29-31</sup> Moreover, an eco-toxicity potential risk has been forecast due to accumulation in the environment because ILs structures are very stable and are not easily biodegradable.<sup>27, 32</sup> In particular, the general toxic action of ILs on living organisms seems to be connected to the functional modification (or even poration/destruction in severe case) of plasma membrane due to the nonspecific lipophilic interaction between the lipid membrane of the cells and the alkyl chain of the cations.

Toxicity indicators (in terms of critical concentrations, such as the half maximal effective concentration EC50) were found to be in linear correlation with lipophilicity parameters (i.e. water/octanol partition coefficient), highlighting the primary interaction of ILs on lipid-rich membrane.<sup>33, 34</sup> In a previous study from our group,<sup>35</sup> the detection limits (LOD) of electrochemical techniques for imidazolium ILs, (along with octanol/water partitioning coefficients) were quantitatively correlated with toxicity data, highlighting the role of the biomembrane in the toxicity mechanisms. The general observation of toxic/pharmacological effects are explained based on the assumption that ILs based compounds adsorb on to and/or intercalate into biomembranes,<sup>36, 37</sup> resulting in the structural perturbation of the biomembrane, its poration, even disruption in severe cases.<sup>35</sup> A variety of experimental and simulated conditions were investigated,<sup>27, 32, 38, 39</sup> confirming a direct link between the IL cation lipophilicity and toxicity, in particular related to the adsorption/intercalation of cations into biomembranes resulting in membrane perturbation/disruption and, eventually, cellular uptake.<sup>32-34, 36, 40</sup> Cations with long

hydrocarbon chains showed an increased lipophilic and therefore toxic character in comparison with shorter chain analogues.<sup>41</sup> Bhattacharya et al.<sup>42</sup> reported that the in-plane elasticity of supported monolayers decreases upon addition of the IL suggesting a relaxation of the rigid structure of lipids in monolayer. Benedetto et al.<sup>43</sup> performed molecular dynamics simulations suggesting the microscopic mechanism of the IL–biomembrane interaction, consisting in the cations ingress into phospholipid bilayer. The uptake of ILs is enhanced by increasing temperature and fluidity of lipid phase.<sup>44</sup>

Studies about the toxic character of ILs pave the way for their exploitation in biological field attracting noteworthy interest in pharmaceuticals.<sup>39</sup> The state of art research for pharmaceutical applications is investigating promising paths in using ILs: 1) to enhance the performance of active pharmaceutical ingredients (APIs), providing novel drug-delivery options and serving as a solution for polymorphic molecules solvation;<sup>45, 46</sup> 2) as APIs themselves, modifying cations or anion chemical structure, in order to exploit their biological related properties.

Due to the importance of the interaction of ILs with the cell membrane in the determination of the IL cytotoxicity, reconstituted biomembranes (such as solid-supported phospholipid bilayers) exposed to ILs can be used as model interfaces, to be studied by means of different surface techniques, for example atomic force microscopy (AFM),<sup>36, 47, 48</sup> neutron scattering,<sup>49</sup> X-ray,<sup>36, 42, 50</sup> quartz crystal balance,<sup>51</sup> nanoplasmonics,<sup>52</sup> electrochemical impedance measurements,<sup>35</sup> as well as by molecular dynamic simulations.<sup>43, 53-55</sup> Given the observed structural modifications induced by ILs on biomembranes, AFM is a promising technique to study the cytotoxicity mechanisms of ILs, since it allows to perform a combined topographic and mechanical investigation of lipid membranes with nanoscale spatial resolution.<sup>56-60</sup> Indeed, AFM has been recently used to investigate the morphological and mechanical modifications caused by the interaction of model biomembranes with ILs;<sup>48</sup> aiming at targeting a more complex biological interface, we have recently performed a similar study on living cells exposed to ILs.<sup>61</sup>

In this study, we used AFM to carry out a systematic study of DOPC phospholipid bilayers interacting with imidazolium-based ILs at different concentrations. Combining topographic imaging to the mapping of several interfacial properties, such as Young's modulus of elasticity, adhesion force, surface charge density, we were able to characterize the concentration dependent morphological, structural and mechanical evolution of the lipid membrane in the presence of ILs with different alkyl chain length and hydrophilic/hydrophobic character.

## Experimental section

### Ionic Liquids and imaging solutions

Ionic liquids used in experiments, with their specific nomenclature and abbreviation, are: 1-butyl -3-methyl imidazolium chloride ( $[C_4MIM][Cl]$ ), 1-butyl- 3-methyl imidazolium tetrafluoroborate ( $[C_4MIM][BF_4]$ ), 1-butyl-3-methyl imidazolium bis(trifluoro methyl sulfonyl)imide ( $[C_4MIM][NTf_2]$ ), 1-methyl-3-octyl imidazolium chloride ( $[C_8MIM][Cl]$ ), and 1-dodecyl-3-methyl imidazolium bis(trifluoro methyl sulfonyl)imide ( $[C_{12}MIM][NTf_2]$ ). All the ionic liquids were purchased from Sigma Aldrich, each with purity grade greater than 98.0%. In order to be stored in a clean way and to reduce water contamination, all the used ILs have been maintained in a desiccator under mild vacuum condition (around  $10^{-2}$  mbar).

Ionic Liquid	Density (g/cm <sup>3</sup> )	Melting point (°C)	CMC (mM)	Character	K <sub>ow</sub>
$[C_4MIM][Cl]$	1,086	70	>1000 <sup>62</sup>	Hydrophilic	0.004 <sup>63</sup>
$[C_4MIM][BF_4]$	1,21	-75	>1000 <sup>64</sup>	Hydrophilic	0.003 <sup>63</sup>
$[C_4MIM][NTf_2]$	1,43	-4	12 <sup>64</sup>	Hydrophobic	0.37 <sup>63</sup>
$[C_8MIM][Cl]$	1,01	12	200 <sup>62</sup>	Hydrophilic	0.25 <sup>63</sup>
$[C_{12}MIM][NTf_2]$	1,25	21.9	0.06 <sup>65</sup>	Hydrophobic	150 <sup>a</sup>

Table 1. Physicochemical parameters of ionic liquids used in this work. CMC is the critical micellar concentration; K<sub>ow</sub> is the octanol/water partition coefficient. <sup>a</sup>Extrapolated from C<sub>n</sub>MIM series data curve.

Aqueous electrolytic solutions containing ILs at different concentrations were prepared prior to AFM experiments on phospholipid bilayers. For different ILs, Table 1 reports some of the physicochemical properties relevant in this work, in particular density, melting point, the critical micelle concentration (CMC), the main character(hydrophobic/hydrophilic) and the octanol/water partition coefficient (K<sub>ow</sub>) as quantification of hydrophilic character. In principle, the concentration of ILs were chosen based on their hydrophobicity as well as on their CMC. Imaging buffer was prepared with a solution of pH 7.4 phosphate buffered saline (PBS) or HEPES-buffered saline (HBS). In particular, considering hydrophobic ionic liquids, starting solutions at high concentration were prepared in methanol, while final operative concentrations were prepared maintaining methanol content below 5%.

### Phospholipid bilayer deposition

The phospholipid molecules (dioleoyl phosphatidylcholine (DOPC) from Avanti polar lipids) were initially dissolved in a chloroform solution at a concentration of 10 mg/ml. The dried lipids (0.1 mg) are rehydrated using 1 ml of high ionic strength buffer solution 450 mM KCl, 25 mM HEPES (4-(2-hydroxy ethyl)-1-piperazine ethane sulfonic acid). A high ionic strength is necessary to shield effectively the electrostatic repulsive interaction between the negatively charged mica surface and phospholipids heads.<sup>66-68</sup> After rehydration, and just before sample preparation for the AFM investigation, lipids are vortexed and sonicated for 10 minutes. The effect of sonication

is to break the bigger multi-layered vesicles, favoring the formation of smaller unilamellar vesicles (better results were obtained using an ultrasonic tip rather than an ultrasonic bath). After sonication, the solution containing the lipids is deposited on a freshly cleaved mica surface in the amount of 40  $\mu\text{l}$  with 15 minutes incubation time. The deposition protocol proceeds with the elimination of deposition buffer (HEPES + KCl), obtained with a gentle flux of nitrogen. Finally, the sample was rehydrated to perform AFM measurement in fluid environment using the imaging buffer with dissolved ILs. The overall procedure was tested several times in order to ensure repeatability.

### **AFM Imaging**

AFM imaging experiments were performed in PeakForce Tapping mode in liquid environment using both Multimode and Bioscope Catalyst (Bruker) AFMs mounting ScanAsyst-Fluid probes (Bruker, nominal elastic constant  $k = 0.7 \text{ N/m}$ ). For each morphological image, scan parameters were optimized in order to minimize the interaction force between probe and sample ( $F < 0.1 \text{ nN}$ ), still maintaining the highest quality possible. Topographic maps were typically acquired with a sampling resolution of 512 X 256 points, ramping frequency of 1 kHz and scan rate of 1 Hz, leading to a total acquisition time for each map of approximately 4 min. All AFM raw data were analyzed using custom Matlab routines. AFM images were flattened by subtracting up to 3<sup>rd</sup> order polynomials line-by-line, upon suitable masking of protruding features and defects, using the exposed substrate as reference, whenever possible, or alternatively the uniform flat bilayer surface.

During data analysis, every image can be converted in quantitative histogram helping to perform quantification of thickness ( $h$ ), bilayer coverage ( $A$ ) and rms roughness ( $R_q$ ). The relative increase in the surface area of the lipid bilayer upon interaction with the IL was calculated from the height histograms as  $(A-A_0)/A_0$ , where  $A$  and  $A_0$  area the peak values with and without IL.

### **AFM nanomechanical and Force Spectroscopy measurements**

AFM nanomechanical and force spectroscopy experiments were performed using the same Bioscope Catalyst or Multimode Nanoscope (Bruker) instruments equipped with soft silicon nitride MLCT-E probes (Bruker, nominal elastic constant  $k = 0.1 \text{ N/m}$ , nominal radius  $R = 20 \text{ nm}$ ) or other probes with similar or lower stiffness, allowing to cover the  $10\text{-}10^4 \text{ pN}$  force interaction range. The spring constant of each cantilever was calibrated by means of the thermal noise method.<sup>69 70</sup>

#### *Force curves*

Nanomechanical and force spectroscopy analysis is based on the acquisition of raw force curves (FCs), consisting in the acquisition of the raw cantilever deflection signal from the photodetector (in volts) as a function of the z-piezo approaching (retracting) distance (in nanometers), while the AFM tip is ramped vertically on the sample.<sup>71</sup> The tip reverses the motion when a setpoint deflection is reached, typically corresponding to a maximum

force large enough to penetrate the lipid bilayer. Force vs distance curves in physical units of nN and nm, respectively, like those shown in Figure 1, are obtained by rescaling the raw FCs as explained in detail elsewhere.<sup>71, 72</sup> Briefly, the tip-sample distance (or indentation) is obtained by adding the cantilever deflection (in nm) to the z-piezo displacement; the force exerted by the tip (in nN) is retrieved by multiplying the raw photodetector deflection signal by the deflection sensitivity  $S$  (in nm/V) and by the cantilever spring constant  $k$  (in N/m). The deflection sensitivity  $S$  is measured as the inverse of the slope of the contact part of the FC after the probe breaks through the bilayer, reaching the substrate; the spring constant  $k$  is calibrated using the thermal noise method based on the equipartition theorem.<sup>70, 73</sup>

### *Force mapping*

By collecting several FCs distributed regularly across the sample surface (the dataset is also known as Force Volume, FV) it is possible to perform a spatially resolved force spectroscopic or nanomechanical analysis. From each FC, the local height of the sample, relative to a common point along the  $z$  axis, is calculated;<sup>72, 74</sup> it is therefore possible to reconstruct the topographic map of the sample under investigation, which is in one-to-one correspondence with the maps of other physical observables extracted from the FCs. Each FV, recorded in macroscopically separated locations, typically spans an area of  $10 \mu\text{m}^2$  and consists of  $64 \times 64$  or  $10 \times 10$  FCs, for high-resolution and fast mapping purposes, respectively. Two distinct nanomechanical experiments are then performed. First, we recorded fast maps on freshly prepared bilayers, each time changing the IL concentration. This approach retrieves no information on the evolution dynamics of the system, but allows a precise control of both the interaction time and the IL concentration. Secondly, in order to study the evolution of ILs interaction compared with control, high-resolution datasets were recorded: the scan area was fixed on a region of interest and datasets were acquired while increasing IL concentration. In these conditions, the effective interaction time is longer than the nominal one, because further increments of ILs are relative to a system that had already been exposed to lower concentrations; however, the interaction dynamics can be captured, thus complementing results from high-resolution scans and allowing for a more complete description of the ILs-bilayer interaction.

### *Force curve analysis on phospholipid bilayers*

A typical FC acquired on a phospholipid bilayer is shown in Figure 1a. Considering the vertical approach on phospholipid bilayer, the FC presents specific and recognizable features.<sup>75-77</sup> Upon contacting the surface at the contact point, the tip exerts a compressive stress on it, which first induces an elastic deformation of the bilayer, and then the rupture and penetration of the bilayer when a force threshold is reached (*breakthrough event*, corresponding to a breakthrough force  $F_{bt}$ ). The nonlinear force vs distance dependence in the compressive part of the FC accounts for the elastic resistance of the bilayer ( $\delta_{\text{max}}$  is the maximum compression, or indentation). Upon contact with the underlying hard substrate, the FC becomes vertical (tip-sample distance does not change). The distance between breakthrough event and substrate is defined as the breakthrough distance  $d_{bt}$ . Since we deal with

a large number of FCs, we developed a statistical automatic procedure to identify the relevant points and quantities in the FCs (in particular contact point and breakthrough event), which is based on the study of the histogram of distances in the FC (Figure 1b). The histogram typically contains distinct peaks corresponding to the distances at which the FC is steeper, which are the loading region on the bilayer from the contact point up to the breakthrough, and the contact region on the substrate. The onset of the peak corresponding to the loading of the bilayer can be identified with the contact point. We fit a Gaussian curve to the histogram to characterize the center of the distribution and its width  $w$ ; the contact point is identified as the point that is found at a distance  $2.5*w$  before the center. We tested the validity of this empirical procedure on subsets of FCs and fixed it for all FCs; moreover, the same procedure is used for both the control system (bilayer without IL) and the system under study (bilayer with IL). In Table SI1 we present the error propagation on mechanical parameters when a  $\pm 0.5$  nm uncertainty is artificially introduced in the contact point determination following the described procedure. We found out that the thickness maps reconstructed from the contact points are comparable within the error to the topographic maps acquired in standard imaging modes, including PeakForce Tapping.

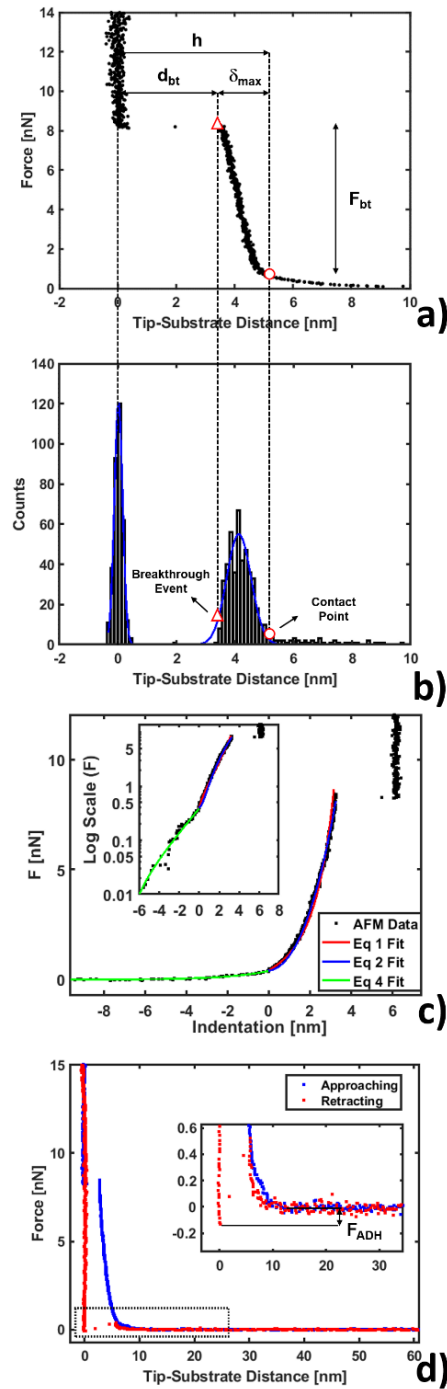


Figure 1. AFM force curve analysis for indentation on phospholipid bilayer. a) Example of force vs. tip-substrate distance curve performed on DOPC bilayer supported on mica surface, showing contact point (o) and breakthrough event ( $\Delta$ ). b) Correspondent histogram on distance axis showing the Gaussian fit to individuate substrate and layer. c) Example of fit using Eq. 1, Eq. 2 and Eq. 4 (red, blue and green lines) with log scale inset to magnify the non-contact region. d) approaching and retracting curves overlapped with a magnification showing the adhesion well and closure of phospholipid bilayer.



## Mechanical analysis

After contact point determination, the distance axis of the FC is shifted (like in Figure 1c) so that the zero of the distance axis corresponds to the upper surface of the lipid bilayer. With this representation, the positive distance axis is defined as the indentation  $\delta$ . Here the force-indentation relations can be employed to analyze the elastic properties of the lipid bilayers. Firstly, we obtain the Young's modulus by fitting the Hertz model for a parabolic indenter to the force vs indentation curves, with the finite-thickness correction valid for not bonded thin elastic layers supported on solid substrate (Eq. 1):<sup>59, 72, 78, 79</sup>

$$\left\{ \begin{array}{l} F = \frac{4}{3} \frac{E\sqrt{R}}{(1-\nu^2)} \delta^{3/2} [1 + 0.884\chi + 0.781\chi^2 + 0.386\chi^3 + 0.0048\chi^4] \\ \chi = \frac{\sqrt{R\delta}}{h} \end{array} \right. \quad \text{Eq.1}$$

, where  $F$  is the applied force,  $\delta$  the indentation (not exceeding half the bilayer thickness  $h$ ),  $\nu$  the Poisson's ratio (set equal to 0.5),  $E$  the Young's modulus of the lipid bilayer, and  $R$  the radius of the spherical probe (set to its nominal value,  $R = 20\text{nm}$ ). The adimensional parameter  $\chi = \sqrt{R\delta}/h$  represents the ratio of the contact radius to the bilayer thickness and is the driving parameter of the finite thickness correction. For the calculation of  $\chi$ , as well as for the fit with Eq. 2, we have used the value of thickness  $h$  measured as described above. The Hertz equation is reasonably accurate also for spherical indenters as long as the indentation is small compared to the radius. Given the truly nanoscale structuring of the lipid bilayer, the relatively large deformations and the limits of validity of the continuous mechanics models we employ, we must consider the obtained elastic parameters as effective ones.

Additionally, we also fitted to the data the theoretical model described by Connel et al. for the indentation of a lipid bilayer, which should intrinsically take into account the finite thickness of the lipid structure and its supported nature:<sup>60</sup>

$$F = \pi\kappa_A R \left( \frac{\delta}{h-\delta} \right)^2 \quad \text{Eq.2}$$

where  $\kappa_A$  represents the area stretching (or compressibility) modulus of the lipid bilayer. Young's and area stretching moduli are connected by Eq. 3:<sup>58, 59</sup>

$$\kappa_A = \frac{Eh}{(1-\nu^2)} \quad \text{Eq.3}$$

We show in Figure 1c the results of fitting the loading part of a force vs indentation curve using Eq. 1 and Eq. 2 (red and blue line respectively). In particular, the fit follows experimental data until indentation reaches about half of the lipid bilayer thickness, at which point the elastic energy of the deformed bilayer overcomes the

energetic cost of forming a hole and the tip penetrates the full bilayer in a breakthrough event. The results of the fit from Figure 1c are shown in Table SII, specifically the first line with precise determination of contact point. We notice that the area compressibility modulus from Eq. 2 and from Eq. 3 are very similar ( $198 \pm 12$  and  $190 \pm 9$  mN/m, respectively), therefore we decided to base our analysis on the results of the Hertzian fit (much simpler to implement) and to employ the Young's modulus as the main descriptor of elasticity in lipid bilayers.

It is worth to notice that a thin structured water layer is likely to be present between the hard substrate and the supported phospholipid bilayer. Because of the influence on the results of the mechanical analysis, we included an estimated thickness of the water layer ( $\approx 0.5$  nm) in the effective thickness of the whole bilayer.<sup>80</sup> The model employed with our data was previously confirmed with finite element simulations.<sup>78</sup>

### *Electrostatic analysis and adhesion force*

We analyzed the double-layer (DL) electrostatic interaction force due to the presence of a net surface charge density (typically negative) on both tip and lipid bilayer surfaces.<sup>81</sup> In our measurements, we noticed that before the mechanical contact with the lipid surface, the DL force is small ( $F_{DL} < 0.5$  nN), but not negligible.<sup>82</sup> This force can be appreciated only at very small tip-sample separation, because the high ionic strength of the imaging buffer determines a screening Debye length smaller than 1 nm. The DL interaction should be taken into account in the mechanical analysis, because it could affect the determination of tip-bilayer contact point; moreover, the characterization of the net surface charge density of the lipid bilayer upon interaction with the IL can provide important information on the toxicity mechanisms, since it may be related to IL-related charge stratification at the lipid-water interface, and determine osmotic stresses due to charge gradients.<sup>61, 83</sup> In our case, we used the linearized version of the DL force:<sup>81, 84</sup>

$$F = \frac{4\pi R \lambda_D}{\varepsilon \varepsilon_0} \sigma_P \sigma_S e^{-z/\lambda_D} \quad \text{Eq. 4}$$

, where  $z$  is the tip-surface separation (the negative part of  $x$  axis in Figure 1c),  $\varepsilon$  is the dielectric constant of the liquid medium,  $\varepsilon_0$  the vacuum permittivity,  $\lambda_D$  the Debye's length,  $\sigma_P$  and  $\sigma_S$  the surface charge densities of tip and lipid bilayer, respectively. Considering PBS or HBS at pH 7.4, the  $\text{Si}_3\text{N}_4$  AFM tip is (weakly) negatively charged, assuming a surface charge density  $\sigma_P = -9 \times 10^{-3}$  C/m<sup>2</sup>.<sup>85</sup> Inset of Figure 1c presents an example of the data and fit of Eq. 4 (green line) with force in semi-logarithmic scale, which highlights the linearity of data and fit before the contact point, supporting the exponential character of the interaction, as predicted by Eq. 4, as well as highlighting the difference between electrostatic and mechanical contributions.

Finally, we show a complete force curve cycle in Figure 1d, visualizing approaching and retracting branches. In the retracting phase, the probe moves away from the sample, and the hole formed in the phospholipid bilayer closes behind the retracting tip thanks to the high lateral mobility of the phospholipids. In the retracting FC, the adhesion force  $F_{ADH}$  is defined as the force required by the probe to detach from substrate and reach the non-interacting ‘zero’ force. The typical adhesion well is visible in the zoom of the inset of Figure 1d. Additional details about adhesion analysis can be found in Butt et al.<sup>71</sup>

## Statistics

For a single force mapping dataset, the distribution of the measured values of each quantity (such as  $h$ ,  $F_{bt}$ ,  $E$  etc..) is represented as a histogram, with the corresponding Gaussian fit. The center and the width  $\sigma$  of the Gaussian curve represent the mean value and the standard deviation, respectively, of the single measurement. The uncertainty associated with a single measurement is calculated by summing in quadrature the standard deviation of the mean  $\sigma/\sqrt{N}$ , with  $N$  being the number of FCs in the map, and an estimated overall uncertainty in the probe calibration procedure ( $\approx 10\%$ ). Eventually, the best estimator of the true value for each condition (represented by a group of force maps) was obtained as the mean of the single measurement values, and the corresponding error was calculated as the standard deviation of the mean, adding in quadrature the calibration uncertainty. In case of further operations, such as normalization with respect to the control, error propagation was applied to estimate the final error.

## Results and discussion

### Morphological variations of lipid bilayers interacting with ILs

#### Short-tailed and hydrophilic ILs

We evidenced a general progressive growth of new structures upon addition of ILs, an effect apparently in contrast with the idea of ILs strongly interacting with model membranes creating holes and depletion regions.<sup>86</sup> The growth phenomenon is evident from imaging in Figure 2a, where hydrophilic  $[C_4MIM][BF_4]$  was added at increasing concentrations of 1mM (Figure 2a(ii)), 10 mM (Figure 2a(iii)) and 100 mM (Figure 2a(iv)) every 4 minutes. The height histograms of the 100mM and control samples are shown in Figure 2a(v). As shown in Figure 2a(vi), the bilayer coverage  $A$  shows a relative 48% increase after interaction with 100 mM of  $[C_4MIM][BF_4]$ , which seems to interact more easily with lipids in the amorphous phase (white drops in Figure 2a(i)) rather than with ordered bilayers. Unfortunately, a quantification of thickness from the AFM images through the peak-to-peak distance in the height histogram (Figure 2a(v)) is not reliable at high concentrations of ILs, because the substrate is covered by IL or highly mobile DOPC/ILs aggregates, and the measured thickness can be severely underestimated.

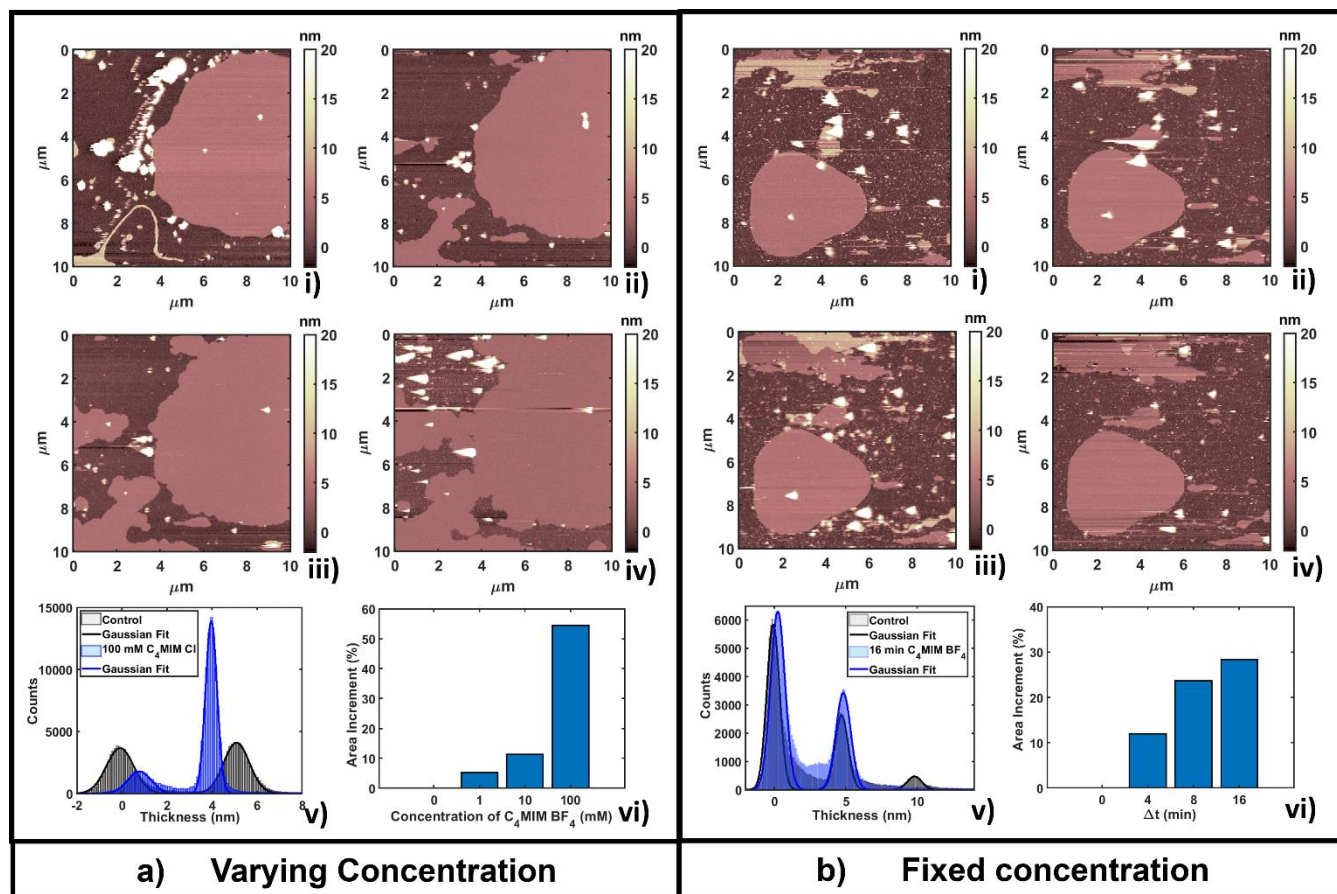


Figure 2. Evolution of a DOPC bilayer as a result of a) adding increasing concentrations of  $[C_4MIM][BF_4]$  at fixed time of measurements  $T = 4$  min after interaction and b) fixing concentration at 10 mM and observing the time evolution. In panel a), image i) represents the control, ii) 1 mM, iii) 10 mM and iv) 100 mM concentration. v) Quantitative height histogram relative to first and last frame; vi) area increment (%) of single bilayer referenced on control. In panel b), image i) represents the control, ii) 4 min, iii) 8 min and iv) 16 min of interaction with 10 mM  $[C_4MIM][BF_4]$ . v) Quantitative height histogram relative to first and last frame; vi) area increment (%) of single bilayer referenced on control.

The time-dependent behavior of  $[C_4MIM][BF_4]$  at fixed concentration of 10 mM is shown in Figure 2b; frames were captured every 4-8 minutes as shown in Figure 2b(i-iv). From statistical analysis in Figure 2b(vi) we observed an increase of coverage for the single DOPC bilayer up to +28% after 16 minutes (see also Figure 2b(iv)), while the thickness of the lipid structures is conserved (Figure 2b(v)). We observed a restructuring of the DOPC material with higher mobility (amorphous and double bilayers) increasing the extent of single bilayer coverage. A similar increase of phospholipid bilayer lateral extension was observed by Schaffran et al.<sup>87</sup> showing the reorganization of liposomes in solution after adding ILs: short-tailed ILs promoted the reorganization of unilamellar liposomes in extended bilayer sheets. The authors explained this fact as the short-tailed ILs act as “edge-actants”, stabilizing and bridging open membrane structures in water. The system in our study is supported

by a solid surface; we argue that ILs with short lateral tails promote the two-dimensional growth of IL-lipid hybrid bilayers from the edges of the pristine DOPC bilayers. Such a hybrid IL-lipid nanostructures were also observed by Jing et al.,<sup>36</sup> who studied the influence of ILs at different concentrations on  $\alpha$ -PC (L- $\alpha$ -phosphatidylcholine), reporting swelling and formation of  $\alpha$ -PC/ILs complexes for concentrations lower than CMC, and bilayer disruption for concentration higher than CMC. Although carried out in different experimental conditions, a recent AFM study by Rotella et al.<sup>48</sup> confirmed how a ‘short tail’ IL such as [C<sub>4</sub>MIM][Cl] enhanced the recovery of uniform lipid bilayer by filling holes and irregularities of original layers.

### Short-tailed and hydrophobic ILs

For [C<sub>4</sub>MIM][NTf<sub>2</sub>], we initially did not observe two-dimensional growth of the lipid bilayers; instead, we observed the deposition of segregated nano-droplets on top of the bilayers (Figure 3a), especially at 100 and 1000  $\mu$ M, Figure 3a(iii-iv). The size of these droplets increases as the concentration of IL present in solution increases. The morphology of droplets shows directionality along with the scan direction, as expected for a tip interacting with a viscous liquid such as [C<sub>4</sub>MIM][NTf<sub>2</sub>]. The decrease in the bilayer coverage reported in Figure 3a(vi) is apparent: it is due to the IL segregated on top of the DOPC bilayer, which causes a decrease in the area below the peak in the height histogram in Figure 3a(v). Segregation of hydrophobic ILs such as [C<sub>4</sub>MIM][NTf<sub>2</sub>] in solution, forming aggregates on top of lipid membranes, was observed also in computational studies.<sup>53-55</sup>

Figure 3b shows the time evolution of DOPC at fixed concentration (1 mM) of [C<sub>4</sub>MIM][NTf<sub>2</sub>]. It is interesting to observe that longer interaction time causes a transition from segregated liquid (Figure 3b(ii)) to almost complete bilayer covering the substrate (Figure 3b(iv)). A red line is used as a guide to the eye to define the profile of original DOPC bilayer. Generally, we noticed the formation of new layered structures similar to the lipid bilayer but characterized by increased roughness and local irregularities. Because of the good reliability of the flattening procedure on the uniform bilayer surface (Figure 3b(iv)), the roughness analysis is also reliable, and shows an increase, from Rq = 0.30 nm on the pristine bilayer, to Rq = 0.38 nm on newly formed layer. Since holes and drops were excluded from analysis, the roughening process is probably related to the formation of DOPC/ILs hybrid complexes, where ionic liquids act as bridging material between amorphous and regular bilayer structures. Computational analysis was able to observe segregation because of its short time scale;<sup>55</sup> here, by AFM, we could capture the system evolution (lateral growth and roughening of the IL-exposed bilayer) across a much longer time scale. We cannot exclude that these processes are facilitated by the mechanical action of the scanning tip, which may help redistributing and restructuring loosely bound IL-lipid material.

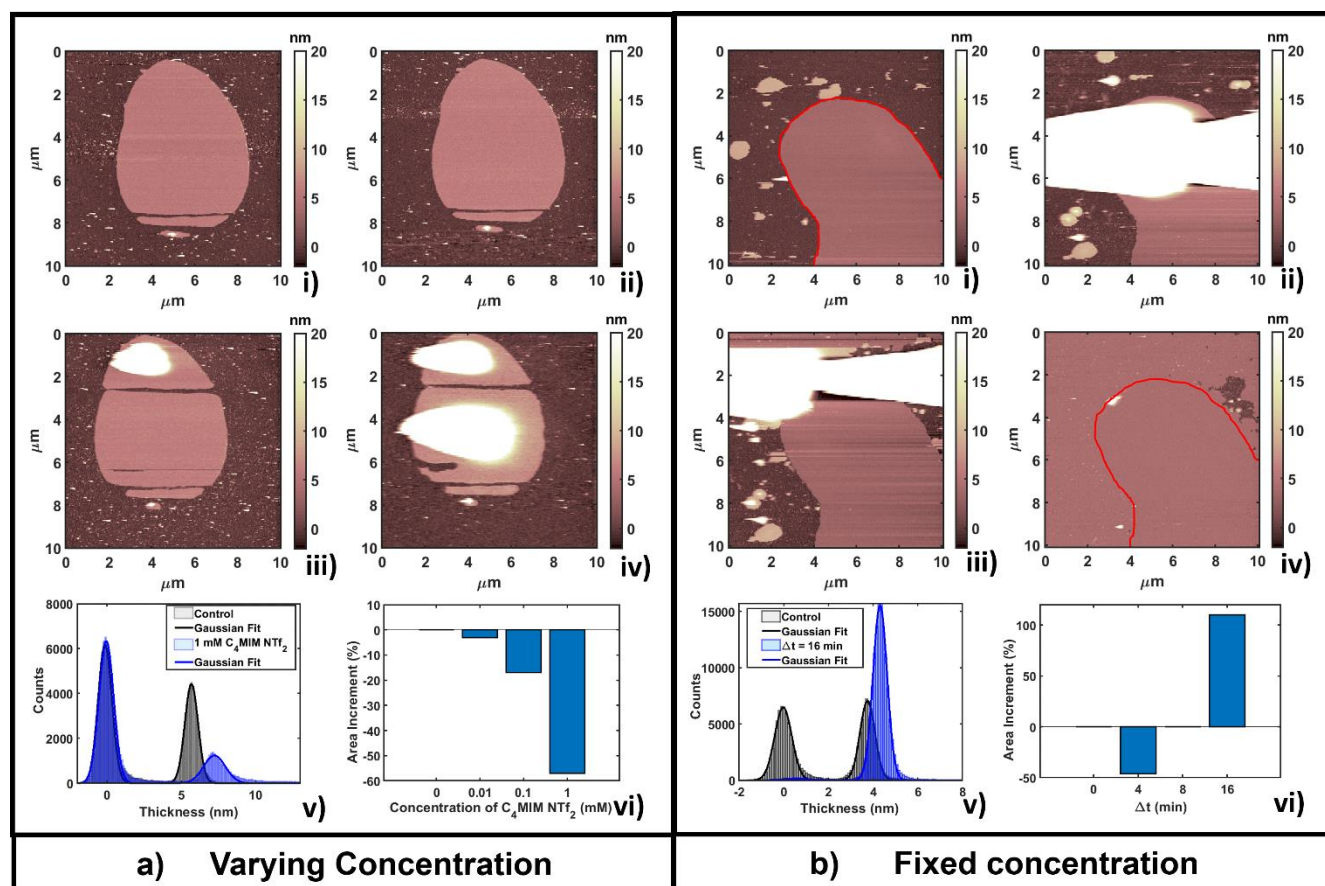


Figure 3. Evolution of a DOPC bilayer as a result of a) adding increasing concentrations of  $[C_4MIM][NTf_2]$  at fixed time of measurements  $T = 4$  min after interaction and b) fixing concentration at 1 mM and observing the time evolution. In panel a), image i) represents the control, ii) 0.01 mM, iii) 0.1 mM and iv) 1 mM concentration. v) Quantitative height histogram relative to first and last frame; vi) area increment (%) of single bilayer referenced on control. In panel b), image i) represents the control, ii) 4 min, iii) 8 min and iv) 16 min of interaction with 1 mM  $[C_4MIM][NTf_2]$ . For better visualization a red line is defining the original bilayer on control and  $\Delta t = 16$  min. v) Quantitative height histogram relative to first and last frame; vi) area increment (%) of single bilayer referenced on control.

### Long-tailed and hydrophobic ILs

We also tested more lipophilic and hydrophobic  $[C_{12}MIM][NTf_2]$  at concentration compatible with the CMC and the lipophilic character of the substance (see Table 1), from 0.1  $\mu M$  up to 10  $\mu M$ . Results are presented in Figure 4a, showing concentration dependent interaction visualized after 4 minutes intervals (Figure 4a(i-iv)). Two main phenomena can be observed: first, the formation and growth of double lipid bilayers with increased roughness ( $R_Q = 3.1$  nm, attributed to  $[C_{12}MIM][NTf_2]/DOPC$ ); second, the deposition of segregated IL droplets on top of flat layers, especially at increasing IL concentrations near the CMC threshold. The reliable height histogram in Figure 4a(v) shows that while the first bilayer is mostly unaffected, a rougher double bilayer develops and grows from the original bilayer edge, until it covers the visible substrate. It seems that the growth of double bilayer is

favored for hydrophobic ILs with respect to hydrophilic ILs, where a single bilayer was observed (Figure 2a). For this reason, the area evolution analysis (Figure 4a(vi)) was focused on the second bilayer, showing that in the case of long-tailed hydrophobic  $[C_{12}MIM][NTf_2]$ , the increase of surface area of the double bilayer is 5 times faster than for the single bilayer of  $[C_4MIM][BF_4]$ .

This behavior can be explained by considering that the high lipophilic character of  $[C_{12}MIM][NTf_2]$  favors the formation of extended (and thicker) composite layers, while the hydrophobic nature favors the segregation of the unreacted fraction (the smallest, brightest spots in Figure 4a(ii-iv)). Formation of multi-layer structures for ILs with longer alkyl chains in the cation was confirmed in other studies.<sup>87</sup> The formation of  $[C_{12}MIM][NTf_2]/DOPC$  complexes was observed by electrochemical methods and simulations.<sup>35, 88</sup> A DOPC monolayer on mercury electrodes underwent irreversible structural reorganization upon interaction with  $[C_{12}MIM][NTf_2]$ , indicating a strong integration of ILs in the regular lipid monolayer structure. The electrochemical fingerprint of IL/DOPC complexes was a convolution of single part signatures (pure DOPC and pure  $[C_{12}MIM][NTf_2]$ ).

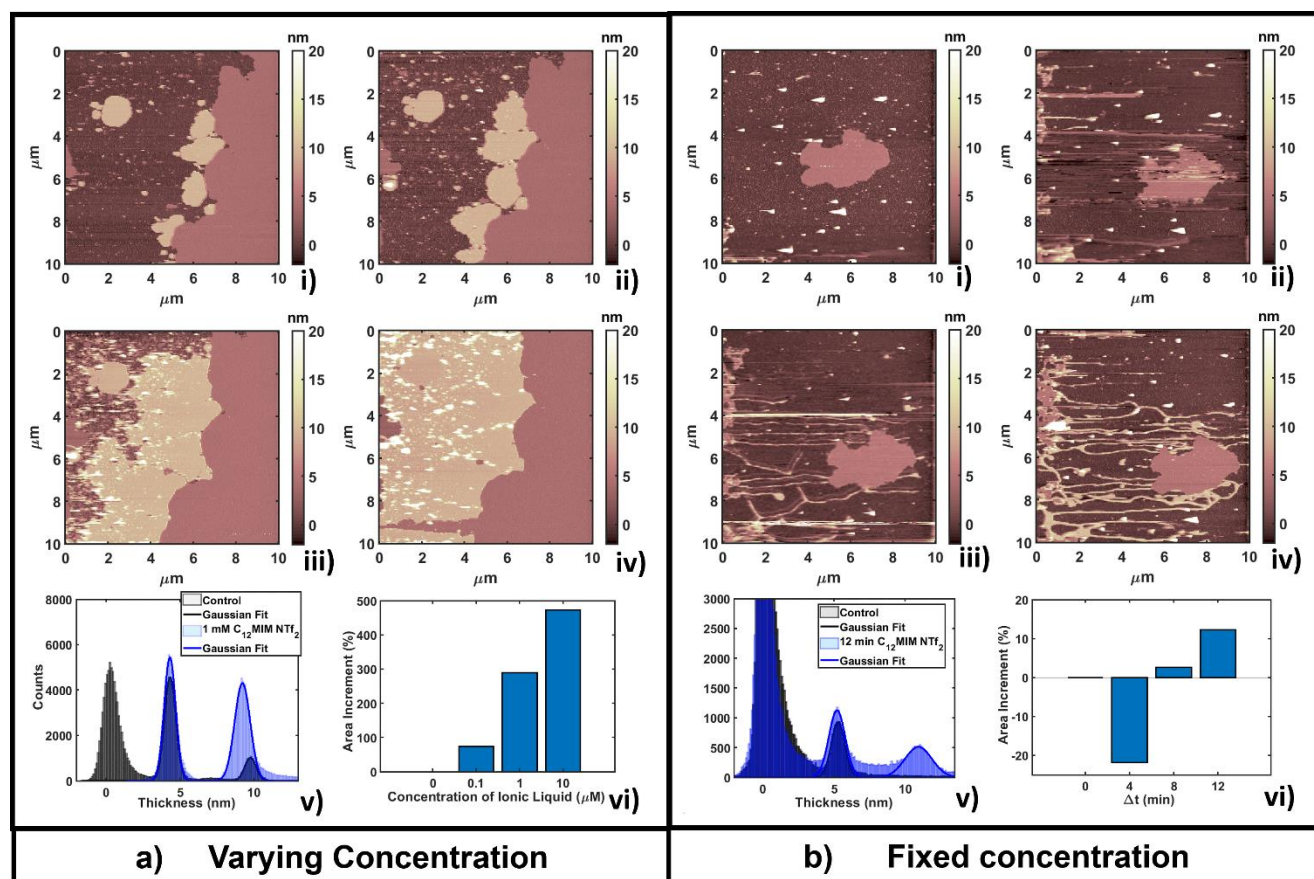


Figure 4. Evolution of a DOPC bilayer as a result of a) adding increasing concentrations of  $[C_{12}MIM][NTf_2]$  at fixed time of measurements  $t = 4$  min after interaction and b) fixing concentration at 0.1  $\mu M$  and observing the time evolution. In panel a), image i) represents the control, ii) 0.1  $\mu M$ , iii) 1  $\mu M$  and iv) 10  $\mu M$  concentration. v) Quantitative height histogram relative to first and last frame; vi) area increment (%) of double bilayer referenced on control. In panel b), image i) represents the control, ii) 4 min, iii) 8 min and iv) 12 min of

interaction with 0.1  $\mu\text{M}$   $[\text{C}_{12}\text{MIM}][\text{NTf}_2]$ . v) Quantitative height histogram relative to first and last frame; vi) area increment (%) of single bilayer referenced on control.

The time dependent investigation of the IL-DOPC bilayer interaction was performed at the lowest fixed concentration (0.1  $\mu\text{M}$ ) of  $[\text{C}_{12}\text{MIM}][\text{NTf}_2]$ . Figure 4b shows that the area and the shape of the lipid bilayer patch do not change dramatically across a time span of 16 min. Area analysis (Figure 4b(vi)) shows that there is an initial area reduction, due to re-equilibration, during the first 8 minutes (Figure 4b(i-ii)), followed by a gradual recovering of the initial value. The appearance (in figure 4b(ii)) and the evolution of filamentous and locally planar structures (such as those in the left border of the scanned area) suggest that loosely bound IL-lipid matter is displaced by the scanning tip and redistributed across the scan area, where it reassembles; the dendritic structures indeed grow preferentially along the tip fast scan direction, suggesting an active role of the scanning tip. Height histogram analysis (Figure 4b(v)) shows that the thickness of the formed structures, either elongated or more extended, is compatible with both a single and a double lipid bilayer (a second peak appears at 10.7 nm, about twice the thickness of the single bilayer). Similar dendritic structures were observed by confocal laser scanner microscopy by Jing et al.,<sup>36</sup> in particular, the coexistence of fibers/tubes with multi-layers aggregates upon interaction with IL (depending on concentration and lipophilicity of cations).

The time lapse analysis supports the hypothesis that the IL is able to interact strongly with the phospholipids, forming a composite material that maintains most of the pristine lipid bilayer properties including the typical bilayer structure. At higher concentration or after a longer interaction time, more extended and less defected layers like those observed in Figure 3b can form, also depending on the hydrophobicity of the IL.

### **Mechanical analysis of DOPC bilayers interacting with ILs**

We analyzed the nanomechanical properties of hydrophilic ILs only, since the observed segregation phenomena suggest that hydrophobic ILs may not be uniformly interacting with the lipid bilayer, therefore making the measurements unreliable. Moreover, we observed already that an AFM tip displacing an IL droplet can be easily and irreversibly covered by an IL layer that would negatively affect all measurements.<sup>89</sup>



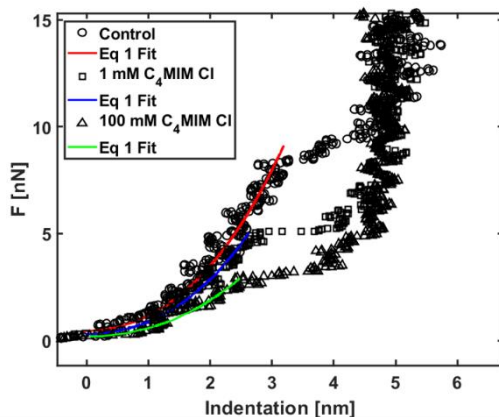


Figure 5. Force curves on phospholipid bilayers interacting with  $[C_4MIM][Cl]$  at different concentrations after equilibration time  $T_{eq} = 20$  min. Typical examples of force vs. Indentation (after contact) with fit of Eq.1.

We used  $[C_4MIM][Cl]$  and  $[C_8MIM][Cl]$  as the most common water-soluble IL with short and long lateral tail to explain mechanical modifications of phospholipid bilayers upon interaction with ILs. The single measurements with their associated errors are presented in Table SI2 (3-5 experiments for  $[C_4MIM][Cl]$  and 1 for  $[C_8MIM][Cl]$ ), where we also highlighted the percentage variation with respect to the control experiment. Examples of approaching force curves collected on a DOPC bilayer before and after interaction with 1 mM and 100 mM of  $[C_4MIM][Cl]$  are shown in Figure 5. The short-scale steps observed before the breakthrough events are artifacts due to either the digitization of the deflection signal or to vibrational noise from the floor, or both, and do not affect the results. Figure 5 shows that the IL weakens the mechanical stability of the DOPC bilayer, i.e., it reduces the breakthrough force, as well as its rigidity: as a matter of fact, the Young's modulus is proportional to the slope of the loading portion of the force curve, and the FCs are less steep after interaction with the IL.

The thickness of phospholipid bilayers can be measured from topographic maps, as explained in the Methods. No significant modification of the height (thickness) of the bilayers was detected, as shown in Figure 6a. In particular, it is interesting that the same thickness was measured by either direct topographic imaging (5.5 nm, shown in previous Figure 2,3,4) or by nanomechanical imaging (4.9-5.9 nm, Figure 6a). Both analyses confirmed that modification of thickness measured by AFM are within the experimental error.

In Figure 6b we show breakthrough force maps and related force histograms for DOPC bilayers on mica before and after interaction for 20 min with 1mM and 100 mM  $[C_4MIM][Cl]$ , respectively. Depending on the probe, average values of breakthrough force can typically vary between 2 and 14 nN. The mean breakthrough force, as extracted through a gaussian fit of the histogram, presents a first -15% variation at 1mM, and a remarkable -62% variation at 100 mM, with respect to the control system (Figure 6b(i)).

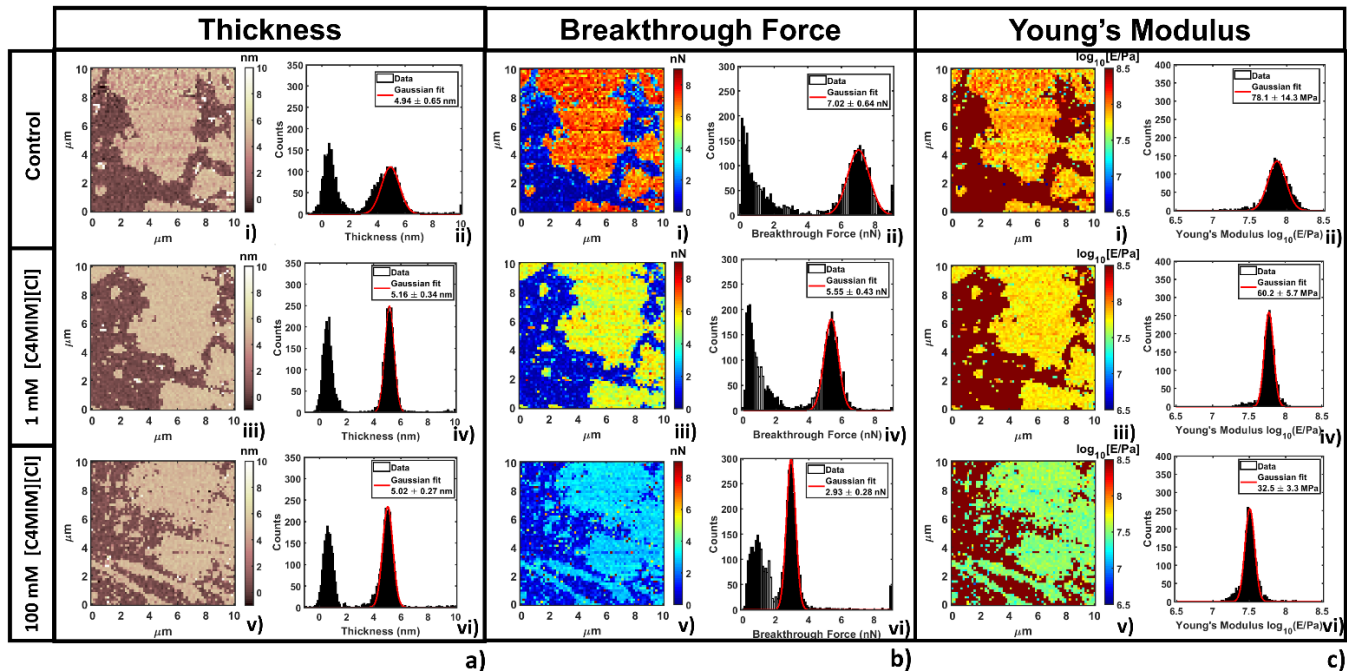


Figure 6. Representative analysis of the evolution of a) thickness, b) breakthrough force and c) Young's modulus for DOPC supported bilayer varying the concentration of  $[C_4MIM][Cl]$ . For each panel, image i) represents the map, image ii) the histogram with Gaussian fit focused on bilayer before interaction. In the same order iii-iv and v-vi images represent the same location after 20 min interaction with 1 mM and 100 mM concentration, respectively.

The maps of Young's modulus and the corresponding histograms for DOPC bilayers on mica before and after interaction for 20 min with 1mM and 100 mM  $[C_4MIM][Cl]$ , respectively, are shown in Figure 6c. Depending on the probe used and different experimental conditions, the Young's modulus of control DOPC is between 20-100 MPa (the Young's modulus of the mica substrate was arbitrarily set at the  $10^9$  Pa in all maps). Similarly to the breakthrough force, the Young's modulus decreases -28% and -49% as the concentration of  $[C_4MIM][Cl]$  increases from 1mM to 100 mM, respectively (see Figure 6c(iv-vi)).

Figure 7 shows the variation of the measured normalized quantities with the concentration of IL, as observed in the course of several different replicated experiments, for the same nominal conditions. The adopted normalization with respect to the measurements performed with the same AFM probe in the absence of IL should remove the dependence on the tip radius, deflection sensitivity and other experimental parameters, which directly influence the measured forces and distances. Given that the same common trends are observed through all the experiments, a certain quantitative variability is observed in the reported concentration-dependent curves for the same ILs, recorded during different experiments performed in the same nominal conditions.

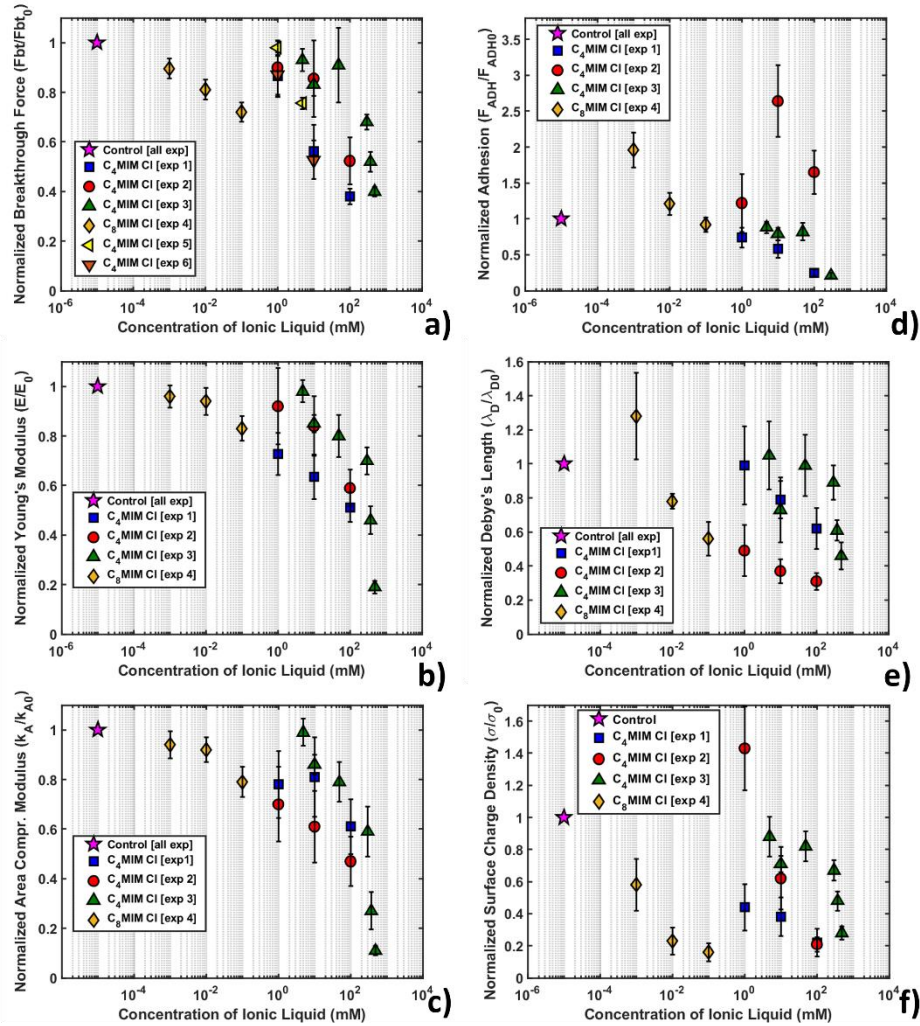


Figure 7. Representation of mechanical and electrostatic normalized quantities after interaction with  $[C_4MIM][Cl]$  and  $[C_8MIM][Cl]$  at different concentrations. a) Breakthrough force ( $F_{bt}$ ), b) Young's modulus ( $E$ ), c) area compressibility modulus ( $\kappa_A$ ), d) adhesion force ( $F_{ADH}$ ), e) Debye's length ( $\lambda_D$ ), f) intensity of surface charge density ( $\sigma_s$ ). For reference, the control data point is showed on the graphs at  $10^{-5}$  mM.

Figure S11 shows normalized thickness, maximum indentation and breakthrough distance versus IL concentration. As the IL concentration increases, we observe an increase of the maximum indentation ( $\delta_{MAX}$ ), while thickness ( $h$ ) and breakthrough distance ( $d_{bt}$ ) are similar to the control values within the experimental error for all concentrations of IL.

All experiments consistently show a remarkable decrease of breakthrough force (Figure 7a), Young's modulus (Figure 7b) and area compressibility modulus (Figure 7c) at progressively increasing concentrations of the IL. The increase of the maximum indentation shown in Figure S11 is compatible with the softening and structural weakening of the lipid bilayer. The interaction of ILs with the lipid membrane is concentration dependent

and inherent to the lipophilic nature of the cation (through the length of the alkyl chain), as demonstrated by the different concentration ranges of [C<sub>4</sub>MIM][Cl] (1-100 mM) and [C<sub>8</sub>MIM][Cl] (1-100 μM). Our results suggest that the hydrocarbon tail of the IL strongly interact with the hydrophobic tails of the phospholipids inside the bilayer, which causes a relaxation of the bilayer structure that makes the layer softer (i.e. reduces the Young's and the area compressibility moduli) and more vulnerable to the tip indentation (i.e. it reduces the breakthrough force).

Despite the decreasing trend of the mechanical quantities is obeyed in all experiments, the rate of decrease is not always the same. As mentioned, normalization with respect to the control without IL should remove any residual dependence on the tip radius and/or on the accuracy of the calibration of both the cantilever spring constant and deflection sensitivity, therefore other factors should explain the observed differences. One such factor could be the different timing of the IL-bilayer interaction in the different experiments; for example, in experiments 1, 4 and 6 (see legends in Figure 7), the combined topographic and mechanical analysis was performed on the same lipid bilayer domains, which have been sequentially exposed to increasing concentrations of ILs, while large datasets (4096 curves) were acquired (with acquisition time of the order of 20 min) (as shown for example in Figure 6). This experimental strategy aimed at studying the kinetics of IL-bilayer interaction on the same location. Clearly, the effective interaction times at a given IL concentration under these experimental conditions were increasingly longer, which may explain why the observed mechanical effects are stronger and faster. Conversely, in experiments 2, 3 and 5, different bilayers have been studied at different IL concentrations, while keeping the interaction time fixed (20 min); smaller datasets (225 curves) were acquired (acquisition time approximately 4 min) in different locations, before and after the interaction with the IL. The effective interaction time under these conditions were both shorter and more precisely controlled, which explains the observed weaker and slower effects.

Nevertheless, we should point out that a supported phospholipid bilayer system is very sensitive to temperature, ionic strength and contamination (for example lipid and IL residues on the AFM tip), especially during the mechanical investigation. Rotella et al.<sup>48</sup> performed indentation experiments on supported POPC bilayers interacting with 0.5M [C<sub>4</sub>MIM][Cl] in deionized milliQ water (instead of physiological buffer like in our experiments), measuring a slight increase of thickness (0.5 nm) and slight increase of all mechanical parameters. To explain this behavior, Dusa et al.<sup>90</sup> using QCM proposed a “carpet” mechanism where ILs first adsorb onto the phospholipid layer and after a certain amount of time, when a time/concentration threshold value is reached, begin intruding into the lipid bilayer. Different working solutions could cause a modification of this threshold value. It is possible that Rotella et al. characterized the bilayer during the initial carpet regime, which could explain the observed trend, which is opposed to the one reported in this work and in others.<sup>42, 53, 91</sup> To support this hypothesis, we report that our research group recently investigated the interaction of a DOPC bilayer with a common lipid dye (Nile red).<sup>78</sup> These hydrophobic molecules are designed to target the lipid layer avoiding structural damage. AFM indentation indeed showed the presence of a stabilizing overlayer of dye on top of exposed leaflet causing an

increase of thickness, breakthrough force and Young's modulus, similarly to Rotella et al.<sup>48</sup> In a recent work, Kumari et al.<sup>91</sup> showed that after 5 hours interaction with DOPC, [C<sub>4</sub>MIM][Cl] can overcome the interaction energy threshold, showing a dramatic decrease of stability and mechanical properties as a consequence of cation tails inserting in the bilayer.

The lowered mechanical stability of the lipid membrane found by AFM nanomechanical measurements is in agreement with previous electrochemical results, where inclusions of IL tails inside the lipid layer contributed to destabilize the lipid structure.<sup>35</sup> A confirmation of the modification of the mechanical properties of the lipid bilayer after interaction with dispersed IL was provided by a simulation works performed by Bingham et.al.<sup>55</sup> and Yoo et al.<sup>53, 54</sup> Bingham et.al.<sup>55</sup> simulated a POPC bilayer in pure water. After addition of [C<sub>4</sub>MIM][Cl] (500 mM) to the solution, the area compressibility modulus decreased by -15%, while in the [C<sub>4</sub>MIM][PF<sub>6</sub>] case, the effect was quantitatively more important, with the variation reaching -45%. Yoo et al.<sup>53, 54</sup> simulated a system similar to our experiments using POPC in buffer solution (NaCl) with [C<sub>4</sub>MIM][Cl]. Variation predicted by simulation show a -40% decrease of area compressibility modulus, in line with the trend of our results. Using pressure-area isotherms, Bhattacharya et al.<sup>42</sup> confirmed penetration of IL molecules in a lipid monolayer and an IL concentration - dependent decrease in the in-plane rigidity, following the trend of our AFM results. Kundu et al.<sup>92</sup> analyzed the effect of ILs on liposomes using dissipative quartz crystal microbalance (QCM-D). Voigt-based viscoelastic modeling and the temperature-dependence of frequency shifts underlined a shear viscosity profile displaying a clear shift downwards of the main phase transition upon the addition of ILs.<sup>51</sup>

### **Adhesive and electrostatic behavior of DOPC bilayers interacting with ILs**

The maps and histograms of adhesion force relative to the control for a DOPC bilayer interacting with 1 mM and 100 mM [C<sub>4</sub>MIM][Cl] are shown in Figure SI2a, while the mean adhesion force versus IL concentration curve is shown in Figure 7d. The adhesion force decreases with increasing IL concentration. To interpret this result, we observe that while, after the breakthrough event, the tip is retracted from the underlying mica substrate, the bilayer closes around it, interacting with the tip walls. We can argue that, as the IL weakens and disrupts the lipid bilayer integrity, the AFM tip encounters less resistance upon extraction. A similar effect was recently visualized studying the interaction with Nile red hydrophobic staining agent on DOPC bilayer, with and without laser illumination.<sup>78</sup> Using Nile red, the adhesion force increases in agreement with the presence of a dye overlayer on top of DOPC molecules, improving their stability (breakthrough force and Young's modulus). Oppositely, when focusing the laser light on the bilayer, the DOPC structure weakens, leading to a decrease of mechanical parameters and adhesion force.

The double layer electrostatic force (Eq. 4) at the water-lipid bilayer interface was studied as a function of the IL concentration. In PBS or HBS at pH 7.4, the Si<sub>3</sub>N<sub>4</sub> AFM tip is (weakly) negatively charged, since the

isoelectric point of silicon nitride is typically between 6 and 7.5 pH units; in order to capture the order of magnitude of the surface charge density of the lipid bilayer, we assumed a surface charge density  $\sigma_P = -9 \times 10^{-3} \text{ C/m}^2$ .<sup>85</sup> Nevertheless, the normalization of the surface charge density with respect to the control sample without IL removes the dependence on both the tip radius and the charge density.

As shown in Figure 1c, a repulsive interaction between the AFM tip and the lipid bilayer is evident in the absence of IL, which suggests a negatively charged DOPC bilayer surface, in agreement with measurements performed in similar conditions.<sup>81</sup> We also observed that the measured Debye length ( $\lambda_D$ ) is between 0.5 and 2 nm for all measurements, as expected for high ionic strength electrolytes. The maps and quantification of Debye length are presented in Figure SI2b, showing a slight decrease (although not significant) while increasing the IL concentration.

As shown in Figure SI2c, the surface charge of DOPC bilayer decreases in absolute value when the IL concentration increases. Considering that cations are firstly interacting with the negatively charged lipid bilayer surface and also through their hydrocarbon tails, our results suggest that the crowding of cations on the bilayer surface is likely, leading to a progressive neutralization of the pristine negative surface charge of the lipid bilayer outer leaflet.

The overall behavior using different probes on  $[\text{C}_4\text{MIM}][\text{Cl}]$  and  $[\text{C}_8\text{MIM}][\text{Cl}]$  was averaged and reported in Table SI3 (with absolute values), while the dependence of the normalized surface charge density on the IL concentration is shown in Figure 7f.

Our results show that besides modifying the mechanical stability of lipid bilayers, ILs also modify their surface charge density, an effect that can influence the ionic transport in membranes of living cells and/or produce interfacial crowding of ionic species. For example, Ryu et al.<sup>93</sup> showed that gramicidin channels in phospholipid membranes are influenced by ILs, depending on their concentration and lipophilicity. In particular, the decreased channel conductance upon interaction with ILs was explained by increased cationic positive charge accumulation, an effect that we likely observed in this work. The increase of cations on surface of phospholipids was also observed as an increase of surface potential.<sup>94</sup> Crowding of cations on phospholipid membranes could induce hyperosmotic shock in short interaction time. This effect was visualized on metastatic cancer cells after 20 min,<sup>61</sup> where high concentration (100 mM) of  $[\text{C}_4\text{MIM}][\text{Cl}]$  caused a decreased of cell volume and increase of cell stiffness as consequence of water expulsion, which increased the viscosity of the cytoplasm. On top of this, a cross-talk interaction between mechanical properties and electrostatic effect on charged phospholipid membrane was recently observed.<sup>95</sup> The evolution of surface charge at membrane level is thus important to understand the effect of ILs on natural complex membranes of living cells.

## Conclusions

We have studied the interaction of imidazolium-based ILs with model biological membranes consisting of DOPC phospholipid bilayers supported on mica surfaces by means of atomic force microscopy, carrying out a combined morphological, mechanical and short-range electrostatic characterization of the IL/lipid bilayer interface. Most of ILs interacting with DOPC bilayers showed a visible and measurable effect on their morphological and structural properties. Our findings can be summarized as follows:

- The strength of the IL-DOPC bilayer interaction is related to the length of the alkyl chain of the IL cation. The experimental evidence supports the hypothesis that the specific affinity of the IL alkyl chains for the hydrophobic tails of the phospholipids leads to: a) crowding of the IL cations at the exposed lipid bilayer leaflet, and b) penetration (tail-first) of the cation into the bilayer structure.
- Upon interaction with imidazolium-based ILs, the pristine DOPC bilayer structure is mechanically and structurally weakened, as witnessed by a reduction of the breakthrough force, as well as of the Young's and area compressibility moduli; at higher concentration, the membrane locally reorganizes into a composite phospholipid-IL material and, eventually, is disrupted.

The noticeable decrease of breakthrough force and Young's modulus indicate a lowering in mechanical stability but also that bilayers are more compliant. This finding is important in the IL toxicity scenario, since it suggests that the primary interaction channel of ILs with living cells is localized at the outer cell membrane, where, depending on their concentration and alkyl chain length, the ILs induce a structural reorganization and a softening, and likely a permeabilization of the membrane. On the other hand, the AFM analysis was able to capture a modification of phospholipid bilayer surface charge density coherent with positive cationic crowding on the exposed leaflet.

The results obtained in this work provide information about the activity of widely used and investigated imidazolium based ILs at the biomembrane level, relating their structure to different degrees of interaction. Tuning the interaction of ILs with biomembrane, by modifying the chemical structure of ions, would allow the design of novel ILs that satisfy strict environmental sustainability and low cytotoxicity constraints, but also provide novel membrane targeting molecules for biomedical applications.

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## Conflict of interest

The authors declare no conflict of interest.

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# Interaction of imidazolium-based ionic liquids with supported phospholipid bilayers as model biomembranes

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## SUPPORTING INFORMATION

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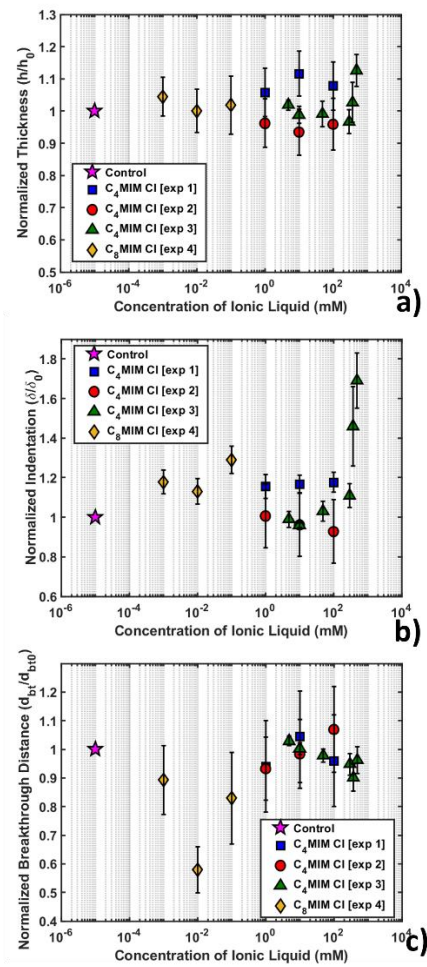


Figure S11. Representation of morphological normalized quantities of DOPC bilayers after interaction with  $[C_4MIM][Cl]$  and  $[C_8MIM][Cl]$  at different concentrations. a) Thickness ( $h$ ), b) maximum indentation ( $d_{MAX}$ ), c) breakthrough distance ( $d_{bt}$ ). For reference, the control data point is showed on the graphs at  $10^{-5}$  mM.

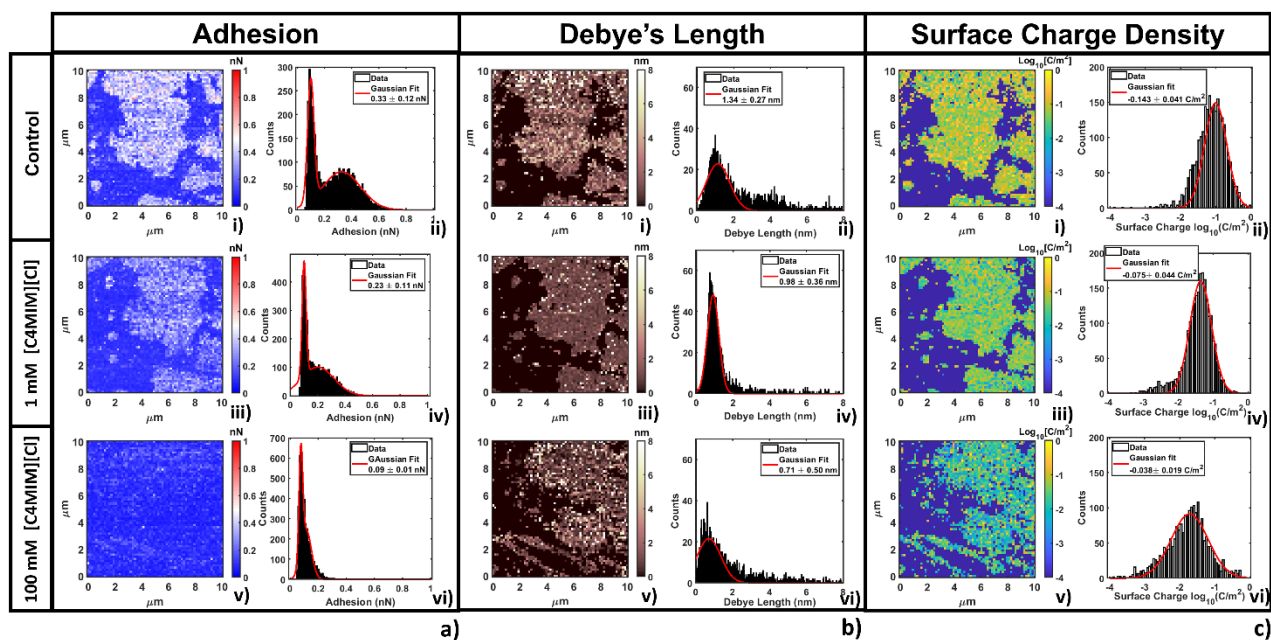


Figure SI2. Representative analysis of the evolution of a) adhesion force  $F_{ADH}$ , b) Debye's length  $l_D$  and c) surface charge density  $s_s$  for DOPC supported bilayer varying the concentration of  $[C_4MIM][Cl]$ . For each panel, image i) represents the map, image ii) the histogram with Gaussian fit focused on bilayer before interaction. In the same order iii-iv and v-vi, images represent the same location after 20 min interaction with 1 mM and 100 mM concentration, respectively.

## Additional Tables

### Impact of the water layer between the mica substrate and the lipid bilayer on the mechanical analysis

Table SI1. Mechanical analysis data after considering an error in the contact point determination ( $\pm 0.5$  nm). The values correspond to the force curve of Figure 1c. Symbol definition: R is tip radius (fixed at  $R = 20$  nm), C is the empirical multiplier of the number of Gaussian peak widths (standard deviations) away from the center (towards larger distances from the bilayer surface) in order to localize the contact point, h is thickness,  $F_b$  is breakthrough force, E is Young's modulus,  $k_A$  is compressibility modulus,  $l_D$  is Debye's length and  $s_s$  is density of surface charges. The equation used to extract mechanical parameter is shown above the respective columns.

			Eq. 1	Eq. 2	Eq. 3	Eq. 4	Eq. 4
C	h (nm)	$F_{bt}$ (nN)	E (MPa)	$k_A$ (mN/m)	$k_A$ (mN/m)	$\lambda_D$ (nm)	$\sigma_s$ (mC/m <sup>2</sup> )
2.5	5.94	8.36	24.0	198	190.1	1.97	-124
2.2	5.46	8.36	28.6	262	208.2	1.39	-532
2.8	6.42	8.36	20.8	148	178.0	1.91	-102

## Mechanical Analysis Data

Each table represents an independent experiment using different probes or testing different conditions. Here,  $h$  represents the thickness of DOPC bilayer,  $F_{bt}$  is the breakthrough force,  $E$  is the corrected Young's modulus. For all the quantities,  $\sigma[ ]$  represents the standard deviation of the mean, while var% is the percentage variation referenced with control (this quantity is averaged on the measurement group).

**Table SI2a.** Experiment 1. Mechanical data of DOPC phospholipid bilayer interacting with  $[C_4MIM][Cl]$  at different concentrations.  $k = 0.14$  N/m, radius  $R = 10$  nm, optical lever sensitivity Defl. Sens. = 22.4 nm/V. Data are shown in Figure 6 main text.

Condition	$h$ (nm)	$\sigma[h]$ (nm)	var%	$F_{bt}$ (nN)	$\sigma[F_{bt}]$ (nN)	var%	$E$ (MPa)	$\sigma[E]$ (MPa)	var%
Control	4.92	0.43		8.07	0.76		41	10.3	
	4.94	0.65		7.02	0.64		39.7	8.1	
1 mM	5.27	0.46		6.60	1.2		28.7	3.2	
	5.16	0.34	5%	5.55	0.43	-14%	30	3.2	-28%
10 mM	5.60	0.23		4.41	0.37		29.5	2.6	
	5.41	0.33	11%	4.06	0.49	-44%	21.9	2.9	-37%
100 mM	5.61	0.43		2.81	0.34		21.3	2.2	
	5.02	0.27	7%	2.93	0.28	-62%	20.1	2.3	-49%

**Table SI2b.** Experiment 2. Mechanical data of DOPC phospholipid bilayer interacting with  $[C_4MIM][Cl]$  at different concentrations.  $k = 0.25$  N/m,  $R = 10$  nm, Defl. Sens = 49.1 nm/V.

Condition	$h$ (nm)	$\sigma[h]$ (nm)	var%	$F_{bt}$ (nN)	$\sigma[F_{bt}]$ (nN)	var%	$E$ (MPa)	$\sigma[E]$ (MPa)	var%
Control	5.96	0.59		9.83	2.2		196.5	14	
	6.39	0.47		12.38	3.12		142	11	
1 mM	5.86	0.34		9.7	1.4		119	33	
	5.06	0.48	-12%	10.3	0.72	-10%	195	21	-8%
10 mM	5.63	0.72		8.31	1.45		120	21	
	4.98	0.64	-14%	10.7	2.8	-15%	167	29	-16%
100 mM	5.4	0.52		6.08	1.5		91.4	17.3	
	5.49	0.73	-12%	5.54	1.41	-48%	111	18	-41%

Table S12c. Experiment 3. Mechanical data of DOPC phospholipid bilayer interacting with [C<sub>4</sub>MIM][Cl] at different concentrations.  $k = 0.72$  N/m,  $R = 10$  nm, Defl. Sens = 13.6 nm/V.

Condition	h (nm)	$\sigma[h]$ (nm)	var%	F <sub>bt</sub> (nN)	$\sigma[F_{bt}]$ (nN)	var%	E (MPa)	$\sigma[E]$ (MPa)	var%
Control	5.32	0.09		1.54	0.13		66.3	5.75	
	5.43	0.1		1.54	0.11		68.3	4.9	
5 mM	5.5	0.11		1.46	0.13		66	5	
	5.5	0.11		1.41	0.13		67.6	5.2	
	5.44	0.1	2%	1.43	0.08	-7%	65	4	-2%
Control	4.59	0.12		2.52	0.2		53.6	3.2	
	4.61	0.13		2.51	0.21		53.7	3.3	
10 mM	4.59	0.13		2.21	0.22		46.7	3.1	
	4.61	0.15		2.02	0.19		45.8	3.5	
	4.44	0.16	-1%	2.05	0.17	-17%	45.1	3.1	-15%
Control	5.73	0.14		1.66	0.28		75	7.8	
	5.67	0.12		1.59	0.49		80.9	11.8	
	5.97	0.18		1.43	0.43		64.1	9.6	
50 mM	5.74	0.17		1.48	0.32		47.9	9.2	
	5.76	0.19		1.27	0.39		60	10	
	5.73	0.17	-1%	1.74	0.43	-5%	69.8	9.4	-20%
Control	5.32	0.1		2.91	0.15		145.5	5.4	
	5.31	0.09		2.67	0.13		92.1	7.2	
	5.24	0.1		2.89	0.15		63	4.8	
300 mM	4.93	0.15		1.85	0.12		71.3	8.1	
	5.2	0.12		1.86	0.12		85	9	
	5.22	0.17	-4%	2.13	0.16	-32%	56.9	5.4	-30%
Control	5.18	0.28		7.37	0.36		478.8	69	
383 mM	5.07	0.27		4.5	0.45		358	122	
	4.82	0.28		5.17	0.5		221	68	
	6.07	0.45	2%	4.05	0.41	-38%	83	13	-54%
500 mM	6.03	0.18		3.06	0.32		101	16	
	5.73	0.43		2.7	0.26		87	25	
	5.74	0.26	12%	3.17	0.32	-60%	89	27	-81%



Table SI2d. Experiment 4. Mechanical data of DOPC phospholipid bilayer interacting with [C<sub>8</sub>MIM][Cl] at different concentrations.  $k = 1.65 \text{ N/m}$ ,  $R = 10 \text{ nm}$ ,  $Z_{\text{sens}} = 14.6 \text{ nm/V}$ .

Condition	h (nm)	$\sigma[h]$ (nm)	var%	F <sub>bt</sub> (nN)	$\sigma[F_{bt}]$ (nN)	var%	E (MPa)	$\sigma[E]$ (MPa)	var%
Control	4.93	0.44		14.47	1.31		85.6	2.1	
	4.97	0.33		14.6	1.56		88.2	6.0	
	5.27	0.26		14.97	0.62		80.1	5.9	
1 $\mu\text{M}$	5.09	0.18		13.17	1.07		89	4.1	
	5.55	0.47		13.32	0.72		81.3	3.9	
	5.17	0.28	4%	12.92	1.07	-11%	78.6	9.7	-4%
10 $\mu\text{M}$	4.97	0.07		11.95	1.1		79.5	6.2	
	5.19	0.17		11.65	0.92		80	4.4	
	5.2	0.19	0%	12.68	1.03	-19%	77	13	-6%
100 $\mu\text{M}$	5.09	0.28		10.32	1.02		67	4.8	
	5.34	0.18	2%	11.11	0.61	-28%	72.9	6.1	-17%

### Electrostatic Force Analysis Data

Each table represents an independent experiment using different probes or testing different conditions. Here,  $F_{\text{ADH}}$  represents the adhesion force during retraction curve,  $\lambda_{\text{D}}$  is the Debye's length and  $\sigma_s$  is the surface charge density of the bilayer. For all the quantities,  $\sigma[]$  represents the standard deviation of the mean, while var% is the percentage variation referenced with control (this quantity is averaged on the measurement group).

Table SI3a. Experiment 1. Electrostatic data of DOPC phospholipid bilayer interacting with [C<sub>4</sub>MIM][Cl] at different concentrations.  $k = 0.14 \text{ N/m}$ ,  $R = 10 \text{ nm}$ , optical lever sensitivity  $Z_{\text{sens}} = 22.4 \text{ nm/V}$  and probe surface charge density  $\sigma_p = -9 \times 10^{-3} \text{ C/m}^2$ . Data are shown in Figure SI2 in SI.

Condition	F <sub>ADH</sub> (nN)	$\sigma[F_{\text{ADH}}]$ (nN)	var%	$\lambda_{\text{D}}$ (nm)	$\sigma[\lambda_{\text{D}}]$ (nm)	var%	$\sigma_s$ (C/m <sup>2</sup> )	$\sigma[\sigma_s]$ (C/m <sup>2</sup> )	var%
Control	0.283	0.139		0.930	0.540		-0.198	0.070	
	0.348	0.121		1.344	0.273		-0.143	0.041	
1 mM	0.249	0.115		1.271	0.512		-0.076	0.041	
	0.210	0.112	-26%	0.989	0.367	-1%	-0.075	0.044	-56%
10 mM	0.212	0.123		0.910	0.310		-0.069	0.031	
	0.148	0.109	-42%	0.891	0.578	-21%	-0.060	0.029	-62%
100 mM	0.075	0.012		0.719	0.464		-0.036	0.023	
	0.079	0.013	-76%	0.713	0.506	-38%	-0.038	0.019	-78%

Table SI3b. Experiment 2. Electrostatic data of DOPC phospholipid bilayer interacting with [C<sub>4</sub>MIM][Cl] at different concentrations.  $k = 0.25$  N/m, radius  $R = 10$  nm,  $Z_{sens} = 49.1$  nm/V and  $\sigma_P = -9 \times 10^{-3}$  C/m<sup>2</sup>.

Condition	F <sub>ADH</sub> (nN)	$\sigma$ [F <sub>ADH</sub> ] (nN)	var%	$\lambda_D$ (nm)	$\sigma$ [ $\lambda_D$ ] (nm)	var%	$\sigma_s$ (C/m <sup>2</sup> )	$\sigma$ [ $\sigma_s$ ] (C/m <sup>2</sup> )	var%
Control	0.29	0.21		0.48	0.14		-0.127	0.043	
	0.35	0.14		0.46	0.17		-0.133	0.053	
1 mM	0.38	0.13		1.1	0.35		-0.293	0.023	
	0.41	0.17	22%	1.29	0.43	152%	-0.104	0.052	53%
10 mM	1.06	0.67		1.16	0.5		-0.099	0.054	
	0.62	0.17	164%	1.42	0.55	172%	-0.061	0.021	-38%
100 mM	0.41	0.17		1.37	0.47		-0.031	0.008	
	0.64	0.21	65%	1.74	0.36	228%	-0.025	0.006	-79%

Table SI3c. Experiment 3. Electrostatic data of DOPC phospholipid bilayer interacting with [C<sub>4</sub>MIM][Cl] at different concentrations.  $k = 0.72$  N/m,  $R = 10$  nm,  $Z_{sens} = 13.6$  nm/V,  $\sigma_P = -9 \times 10^{-3}$  C/m<sup>2</sup>.

Condition	F <sub>ADH</sub> (nN)	$\sigma$ [F <sub>ADH</sub> ] (nN)	var%	$\lambda_D$ (nm)	$\sigma$ [ $\lambda_D$ ] (nm)	var%	$\sigma_s$ (C/m <sup>2</sup> )	$\sigma$ [ $\sigma_s$ ] (C/m <sup>2</sup> )	var%
Control	0.549	0.065		0.211	0.075		-0.099	0.025	
	0.564	0.062		0.224	0.073		-0.092	0.029	
5mM	0.572	0.075		0.207	0.061		-0.087	0.024	
	0.469	0.067		0.21	0.054		-0.084	0.023	
	0.416	0.070	-12%	0.205	0.066	-5%	-0.084	0.021	-12%
Control	1.063	0.147		0.622	0.084		-0.115	0.006	
	1.096	0.171		0.609	0.076		-0.113	0.017	
10mM	0.969	0.137		0.648	0.064		-0.109	0.009	
	0.788	0.136		1.035	0.168		-0.067	0.009	
	0.82	0.162	-21%	0.973	0.208	+43%	-0.068	0.014	-29%
Control	0.568	0.104		0.714	0.164		-0.087	0.026	
	0.495	0.099		1.15	0.44		-0.043	0.019	
50 mM	0.479	0.108		2.13	0.81		-0.027	0.014	
	0.478	0.14		0.819	0.659		-0.058	0.047	
	0.358	0.096	-18%	0.427	0.132	+20%	-0.076	0.026	-18%
Control	1.398	0.103		0.829	0.132		-0.205	0.018	
	1.057	0.112		0.838	0.156		-0.186	0.034	
	0.783	0.136		0.573	0.078		-0.198	0.024	
300 mM	0.289	0.095		0.882	0.131		-0.157	0.024	
	0.207	0.06		0.649	0.092		-0.111	0.018	
	0.209	0.073	-79%	0.622	0.103	-4%	-0.129	0.022	-33%
Control	1.44	0.42		4.06	0.57		-0.066	0.005	
383mM	1.98	0.3		2.4	0.9		-0.804	0.247	
	2.24	0.33		3.5	2.5		-0.007	0.003	
	1.48	0.35	+32%	3.4	4	-24%	-0.008	0.002	-52%
500 mM	2.31	0.34		1.13	0.79		-0.025	0.007	
	1.79	0.185		1.95	1.05		-0.016	0.004	
	1.79	0.19	+36%	1.92	0.85	-59%	-0.015	0.004	-92%

Table S13d. Experiment 4. Electrostatic data of DOPC phospholipid bilayer interacting with [C<sub>8</sub>MIM][Cl] at different concentrations.  $k = 1.65 \text{ N/m}$ ,  $R = 10 \text{ nm}$ ,  $Z_{\text{sens}} = 14.6 \text{ nm/V}$ ,  $\sigma_p = -9 \times 10^{-3} \text{ C/m}^2$ .

Condition	$F_{\text{ADH}}$ (nN)	$\sigma[F_{\text{ADH}}]$ (nN)	var%	$\lambda_D$ (nm)	$\sigma[\lambda_D]$ (nm)	var%	$\sigma_s$ (C/m <sup>2</sup> )	$\sigma(\sigma_s)$ (C/m <sup>2</sup> )	var%
Control	0.156	0.08		0.95	0.38		-0.831	0.140	
	0.58	0.15		1.48	3.14		-0.091	0.030	
	0.51	0.23		2.59	0.41		-0.145	0.025	
1 $\mu\text{M}$	0.91	0.18		1.69	0.61		-0.364	0.037	
	0.8	0.21		3.32	1.17		-0.128	0.061	
	0.74	0.16	+96%	3.25	1.2	+64%	-0.127	0.039	-42%
10 $\mu\text{M}$	0.78	0.15		1.24	2.23		-0.096	0.017	
	0.71	0.13		3.33	1.22		-0.091	0.033	
	0.78	0.16	-8%	2.29	1.32	-17%	-0.059	0.010	-77%
100 $\mu\text{M}$	0.99	0.14		1.95	1.12		-0.068	0.028	
	0.85	0.16	+21%	3.73	2.45	+18%	-0.045	0.016	-85%