Green Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Plasma membrane permeabilisation by ionic liquids: a matter of charge

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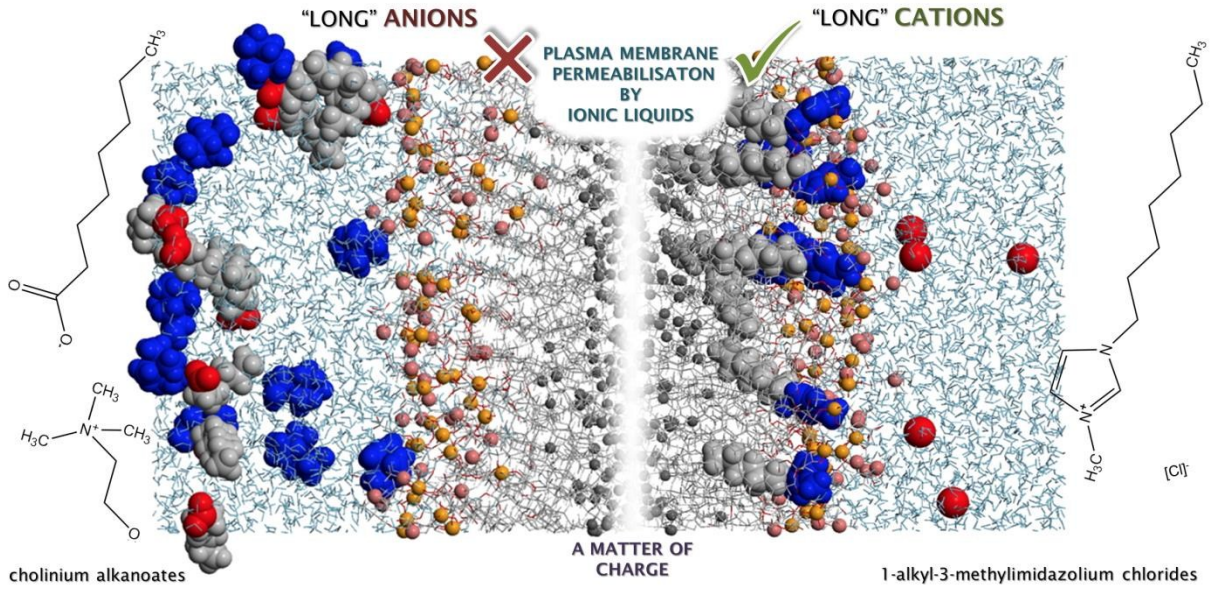
Keywords: filamentous fungi; gene expression; ionic liquids toxicity; plasma membrane permeabilisation, molecular dynamics simulation

Graphical Abstract



The membrane negatively charged surface hinders permeabilisation by ionic liquids long lipophilic anions – a new rule of thumb for the design of greener solvents.

Abstract

Understanding the mechanisms of toxicity of ionic liquids at a molecular level is crucial for their conscious design as to promote higher acceptability as green solvents in a wide range of applications. In this systematic study, we investigated the effects of three families of ionic liquids on the plasma membrane of the filamentous fungus *Aspergillus nidulans*. Using fluorescence microscopy and gene expression analysis, we were able to demonstrate that the widely studied 1-alkyl-3-methylimidazolium chlorides with long alkyl substituents cause membrane permeabilisation. In opposition, cholinium alkanoates biocompatibility was reinforced here, even though their toxicity also increases with the elongation of the anion. Further investigating the effects of charge on membrane permeabilisation, we observed that a series of alkyl-(2-hydroxyethyl)-dimethylammonium bromides led to permeabilisation of the fungal plasma membrane by increasing the length of one alkyl substituent in the cholinium cation. We hypothesise that the chemical nature of the plasma membrane, which presents a heterogeneous charge distribution along its surface, is pivotal for the membrane permeabilising effects of ionic liquids. This study may inspire a new trend in the ionic liquid field: to give preference for the development of compounds carrying functionalised anions as greener alternatives to those carrying functionalized cations.

Introduction

Ionic liquids are generally defined as salts that are liquid below 100 °C,[1] and their potential has been widely explored over the last decades. Millions of possible formulations are estimated, and several hundred ionic liquids are already well known and characterised.[1, 2] Extensive data on their chemistry and physical properties are available, and numerous applications have been proposed.[3] Their enormous potential is often derived from their structural diversity and tuneable properties. Despite their excellent solvation capacity, negligible vapour pressure, and bulk non-flammability,[4] which rendered them the classification of "green" solvents, these organic salts comprise a very heterogeneous group of fluids that are not intrinsically green. Reviews on their environmental impact and biodegradability highlight the toxic nature and recalcitrance of many of these compounds.[5-7]

Imidazolium-based ionic liquids were the first to find applications on an industrial scale and are probably the most investigated group of ionic liquids.[3] Naturally, the first systematic studies on the eco-toxicity of ionic liquids included those containing imidazolium-based cations,[6] particularly focussing on their antimicrobial properties against relevant microbial strains.[8] The effect of the cations lipophilicity appeared to be dominant, since elongation of one alkyl chain considerably increased the toxicity of 1-alkyl-3-methylimidazolium chlorides and bromides, amongst others.[9] It was postulated that those carrying long alkyl chains would interact and disrupt biological membranes, as correlated with the increased 1-octanol/water partition coefficients.[10] Another group of interest amongst ionic liquids are the quaternary ammonium salts. Their properties also depend on the chain length and functional groups of the cation and the anion.[11] These salts are well known and widely used in numerous applications *e.g.* as disinfectants, surfactants, antistatic agents and catalysts.[12] The most interesting group comprises those containing the cholinium cation, mainly due to its benign nature.[13-19] Cholinium chloride is a natural compound, part of the vitamin B complex, and its combination with biocompatible anions constituted a significant advance in the

conscious design of ionic liquids.[18-25] These cholinium-based ionic liquids were proven to present low toxicity in several studies, including the toxicological assessment of a series of cholinium alkanoates against filamentous fungi from the genus *Penicillium*[25] and human cell lines.[26] These cholinium alkanoates were also shown to be highly biodegradable and some presented an outstanding solvent ability towards *e.g.* suberin[27] and stearic acid.[28, 29] As an excellent example of their applications, cholinium hexanoate was successfully employed in the extraction of the plant polyester suberin from cork, partially preserving the native structure of the polymer, which allowed the formation of films with antibacterial properties.[27, 30-32]

Notwithstanding the plethora of published eco-toxicological studies on ionic liquids, the majority relies solely on measurements of their inhibitory and lethal concentrations.[6] A true understanding of their effects still remains largely overlooked. This requires in-depth, molecular analyses of their mechanisms of toxicity, as already considered in a few elegant studies. As an example, the molecular mechanisms underlying the resistance to 1-ethyl-3-methylimidazolium chloride were investigated in the soil bacterium *Enterobacter lignolyticus*.[33] We have also analysed the impact of this ionic liquid, as well as that of cholinium chloride, on the proteome of the model fungi *Aspergillus nidulans* and *Neurospora crassa*.[34] The toxic nature of the imidazolium-based ionic liquid was emphasized by the increase in drug transporter proteins, anti-ROS defence and induction of autolysis. Despite its benign nature, excessive up-take of cholinium may result in cyanide production, a possible explanation for the observed growth inhibition. Our group also demonstrated, through targeted gene expression analyses, that the mechanisms of toxicity of a series of alkyltributylphosphonium chlorides towards *A. nidulans* include plasma membrane permeabilisation and cell wall damage.[35]

In the present study we aim at investigating how structural changes either in the cation or the anion of ionic liquids, particularly the elongation of an alkyl chain, impact the effects of these compounds on biological membranes. Our study organism, the filamentous fungus *Aspergillus*

nidulans, has its genome completely sequenced, which allows the selection of specific target genes for expression analysis. Through fluorescence microscopic, quantitative real-time PCR (*q*RT-PCR) analyses of genes involved in plasma membrane biosynthesis, and molecular dynamics simulations, we elucidated here the effects of systematic elongations of an alkyl chain in either the cation or the anion. We have focussed on series of 1-alkyl-3-methylimidazolium chlorides, cholinium alkanoates and alkyl-(2-hydroxyethyl)-dimethylammonium bromides (Figure 1). Overall, we demonstrate that the charged nature of biological membranes rules the interaction of ionic liquids organic ions with the cell surface. Membrane permeabilisation is ultimately defined by the structure of these ions and is strongly hampered in long lipophilic anions. The evidences reported here constitute rules of thumb for the conscious design of biocompatible ionic liquids.

Experimental

Chemicals

All compounds used in the preparation of minimal media and sample processing were purchased from Sigma-Aldrich, with the exception of NaCl (Panreac, 99.5%). The solvents used in the chromatographic analyses were of the highest purity grade and purchased from Fisher Chemical (Fisher Scientific).

Fungal strain

Aspergillus nidulans strain FGSC A4 was cultivated on dichloran-glycerol (DG18) agar (Oxoid) for five to six days at 27 °C. Conidia (asexual spores) were harvested with a solution of 0.85% w/v NaCl and filtered through glass wool. Conidia were used immediately or stored at -80 °C after being resuspended in a cryoprotective solution containing 0.85% w/v NaCl and 10% v/v glycerol.

Ionic liquids

The 1-alkyl-3-methylimidazolium chlorides ([$C_n$mim]Cl, n = 2, 4, 6, 8 or 10) were purchased from Iolitec, Ionic Liquids Technologies. The cholinium alkanoates (anions = ethanoate, [eth]$^-$;

butanoate, [but]$^-$; hexanoate, [hex]$^-$; octanoate, [oct]$^-$; or decanoate, [dec]$^-$) were synthesised and characterized as previously described.[25]

The synthesis of the alkyl-(2-hydroxyethyl)-dimethylammonium bromides ([$N_{1\,1\,n\,2OH}$], n = 2, 4, 6, 8 and 12) has been previously described by us.[36] The ammonium salts were synthesised by alkylation of 2-(dimethylamino)ethanol with the correspondent brominated derivative (details in ESI). Both salts [$N_{1\,1\,2\,2OH}$]Br and [$N_{1\,1\,4\,2OH}$]Br, were collected as a white solid in 96% yield. The ionic liquids [$N_{1\,1\,6\,2OH}$]Br, [$N_{1\,1\,10\,2OH}$]Br and [$N_{1\,1\,12\,2OH}$]Br, were obtained as a white solid in 95%. For [$N_{1\,1\,8\,2OH}$]Br, a white salt was obtained in 93%. Spectral data for ^{1}H and ^{13}C NMR were identical to previously reported ones.[36-41] For all tested ionic liquids, the 1-octanol/water distribution coefficients (logD) of isolated cations (for 1-alkyl-3-methylimidazolium chlorides and alkyl-(2-hydroxyethyl)-dimethylammonium bromides) or anions (for cholinium alkanoates) were calculated using Chemicalize.org software (http://www. chemicalize.org) by ChemAxon.

Toxicity tests

The toxicity of ionic liquids to *A. nidulans* was evaluated by determining their minimal inhibitory (MIC) and fungicidal concentrations (MFC), distinguishing between growth inhibition and death, respectively. The minimal medium containing glucose (1.0 g l^{-1}) and $K_2[HPO_4]$ (1.0 g l^{-1}) was dissolved in distilled water, sterilised in an autoclave (20 min; 121 °C), and supplemented with the mixture of essential salts, previously sterilised by filtration: $NaNO_3$ (3.0 g l^{-1}), $ZnSO_4{\cdot}7H_2O$, (0.01 g l^{-1}), $CuSO_4{\cdot}5H_2$ O(0.005 g l^{-1}), $MgSO_4{\cdot}7H_2O$ (0.5 g l^{-1}), $FeSO_4{\cdot}7H_2O$ (0.01 g l^{-1}) and KCl (0.5 g l^{-1}). Ionic liquids were added to the culture media at final concentrations ranging from 100 μM to 4 M. Each liquid medium (1 ml) was inoculated with 10^5 conidia and divided into four wells (0.25 ml each) of a 96-well plate. Cultures were incubated at 27 °C, for seven days. Fungal growth (or lack thereof) was evaluated at the end of incubation, gauging by eye the formation of mycelium (turbidity). The lowest concentration that inhibited growth was taken as the MIC. In all samples where no active growth was detected were used as inocula (1 μl) and spread onto malt

extract agar medium (Oxoid). The plates were incubated at 27 °C, for seven days. The lowest concentration which resulted in unviable conidia was taken as the MFC. MIC and MFC values should not be interpreted as absolute ones, but as an indication of the inhibitory and the fungicidal upper concentration limits.

Plasma membrane permeabilisation assay

Ionic liquids with the testing concentrations of 0.01, 0.1, 1, 10 and 100 mM (also 1 and 2 M for some cases) were used to assess plasma membrane permeabilisation. In order to facilitate the comparison of ionic liquids effects, despite some exceptions, the selected concentrations ranged below and above the previously obtained MFC values. A suspension of 10^6 freshly-harvested conidia *per* ml of ionic liquid solution was incubated for one hour at 27 °C, under agitation (90 rpm). The conidia suspension was centrifuged (15 000 rpm) and washed three times with a saline solution to remove the ionic liquid. Conidia were then incubated with propidium iodide (PI) (λ_{ex} = 538 nm, λ_{em} = 617 nm, red), at the final concentration of 20 μM, for 15 min, at 27 °C in the dark and under agitation. Residual dye was removed by centrifugation and washing three times, and conidia were resuspended in 100 μl of saline solution with 10% v/v glycerol. Slides were mounted with 10 μl of the obtained suspension, in three technical replicates. Conidia were observed with an Axio Imager.M1 fluorescence microscope (Zeiss) using a 15 AlexaFluor 546 filter set (for PI-stained conidia) and differential interference contrast (DIC, for total number of conidia). The objective was an EC Plan-Neofluar with 40× magnification and images were captured with an ORCA-ER digital camera (Hamamatsu). Three fields of view from each slide were chosen randomly. Counting of conidia was carried out manually in JMicroVision v1.27. The percentage of membrane-damaged cells was obtained as (number of PI-stained conidia / total number of conidia) × 100. The experiment was repeated three times.

Biodegradability assessment

Biodegradability of ionic liquids was assessed by the determination of their concentration in the culture media before and after incubation with *A. nidulans*. The initial concentrations of ionic liquids used in these assays were approximately one half of the previously determined MIC values, except for [C_2mim]Cl, [C_4mim]Cl, [$N_{1\,1\,1\,2OH}$][eth], [$N_{1\,1\,1\,2OH}$][but], [$N_{1\,1\,2\,2OH}$]Br and [$N_{1\,1\,4\,2OH}$]Br, for which the maximum concentration tested was 100 mM. Fungal cultures were inoculated in 96-well plates as described above and incubated at 27 °C for one, two, five and seven days. The cultivation media were recovered and concentrations of the ionic liquids cations and anions (alkanoates only) were determined by liquid chromatography. The chromatographic conditions used for each ionic liquid family are described in Table S1.

Experimental conditions for gene expression analysis

A suspension of 10^6 freshly-harvested conidia *per* ml of medium was incubated in 25 ml of minimal medium for 24 hours at 27 °C, without agitation. The minimal culture medium containing glucose (10.0 g l^{-1}) and K_2HPO_4 (1.0 g l^{-1}) was dissolved in distilled water, sterilised in an autoclave (10 min; 115 °C), and supplemented with the mixture of essential salts (as described above). After 24 hours of growth, ionic liquids ([$C_n$mim]Cl, where n = 6, 8 or 10; [$N_{1\,1\,1\,2OH}$][hex]; [$N_{1\,1\,1\,2OH}$][oct]; [$N_{1\,1\,1\,2OH}$][dec]; or [$N_{1\,1\,n\,2OH}$]Br, where n = 6, 8, 10 or 12) were added to the culture media to obtain a final concentration corresponding to 80% of the MIC of each compound and incubated for one, two or four hours. A control without ionic liquids was also included.

Total RNA extraction and cDNA synthesis

Mycelia from controls or exposed to ionic liquids were recovered by filtration (0.45 µm membrane filters, Millipore) and immediately frozen in liquid nitrogen. Approximately 50 mg of mycelia was grounded with poly(vinylpolypirrolidone) (0.4 mg *per* mg of mycelia) with a mortar and pestle. The final powder was used in the extraction and purification of total RNA using the RNeasy Plant Mini Kit (QIAGEN), according to the manufacturer's protocol. Genomic DNA digestion was done with the RNase-Free DNase Set (QIAGEN). Quality, integrity and quantity of the total RNA were

analysed in a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and by running 2 µg of RNA into 1% agarose gels in TBE (Tris-boric acid-EDTA) buffer. The complementary DNA (cDNA) was synthesised from 100 ng of the total RNA using an iScript cDNA Synthesis Kit (Bio-Rad) in an Applied Biosystems 2720 Thermal Cycler. The reaction protocol consisted of 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C.

Quantitative real-time PCR analysis

For gene expression analysis, oligonucleotide pairs for specific *A. nidulans* genes (Table S2), were designed using the GeneFisher2 web tool (http://bibiserv.techfak.uni-bielefeld.de/genefisher2), and produced by Thermo Fisher Scientific.[35] The *q*RT-PCR analysis was performed in a CFX96 Thermal Cycler (Bio-Rad), using the SsoFast EvaGreen Supermix (Bio-Rad), 250 nM of each oligonucleotide and the cDNA template equivalent to 1 ng of total RNA, at a final volume of 5 µl *per* well, in three technical replicates. The PCR conditions were: enzyme activation at 95 °C for 30 s; 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 59 °C for 30 s; and melting curve obtained from 65 °C to 95 °C, consisting of 0.5 °C increments for 5 s. Data analyses were performed using the CFX Manager software (Bio-Rad). The expression of each gene was taken as the relative expression compared to the time-zero (before incubation with the tested compounds). The expression of all target genes was normalised by the expression of γ-actin (internal control). Three biological replicates were performed. Statistical analysis of the *q*RT-PCR data was performed in the GraphPad Prism v6.0 software. Treatments with ionic liquids were compared with the control for every respective hour of exposure by multiple Student's *t*-test. Differences in gene expression with a *p*-value below 0.05 were considered statistically significant.

Molecular dynamics simulation

Molecular dynamics simulations of lipid bilayers with aqueous solutions of two distinct ionic liquids, namely $[C_8mim]Cl$ and $[N_{1\,1\,1\,2OH}][oct]$, were carried out using the DLPOLY package.[42] Water and all ionic liquids were modelled using, respectively, SPC model[43] and a previously

described all atom force field (CL&P)[44-46], which is based on the OPLS-AA framework[47] but was to a large extent developed specifically to encompass entire ionic liquid families. Similarly, the bilayer (composed of 76% palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 24% palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine with potassium as counter ion) was modelled using OPLS-AA. For each simulation run, starting initial configurations composed of 4600 water molecules, 16 ion pairs (either in the aqueous phase or embedded in the lipid bilayer) and 100 phospholipids was constructed with Packmol.[48] The boxes were equilibrated under isothermal-isobaric ensemble conditions for 1 ns at 298K and 1 atm using the Nosé-Hoover thermostat and isotropic barostat with time constants of 0.5 and 2 ps, respectively. Several (at least four) consecutive simulation runs of 1 ns were used to produce equilibrated systems at the studied temperature. Electrostatic interactions were treated using the Ewald summation method considering six reciprocal-space vectors, and repulsive−dispersive interactions were explicitly calculated below a cut-off distance of 1.6 nm (long-range corrections were applied assuming the system has an uniform density beyond that cut-off radius). Details concerning this type of simulation can be found elsewhere.[44-46]

Results and Discussion

In this study, a systematic investigation of the toxicity of a series of imidazolium- and cholinium-based ionic liquids (Fig. 1) against *A. nidulans* was performed. We focussed on understanding their effects on the fungal cell envelope, more specifically on the plasma membrane.

Imidazolium-based ionic liquids are recalcitrant and more toxic than cholinium alkanoates

The MIC values of [$C_n$mim]Cl (n = 2, 4, 6, 8 or 10) against *A. nidulans* were distributed over a broad range, from 0.2 mM to 1.39 M (Fig. 2A, Table S3). Their MFC values were nearly identical to the MIC, except for [C_2mim]Cl, which was not lethal even at the highest tested concentration, *i.e.* 4 M. The toxic effect of [$C_n$mim]Cl was defined solely by the cation structure, increasing almost exponentially with the elongation of the alkyl chain (Fig. 2A). The MIC values of cholinium

alkanoates (anion = $[eth]^-$, $[but]^-$, $[hex]^-$, $[oct]^-$ or $[dec]^-$) ranged from 4.5 mM to 1.45 M, whereas their MFC values ranged from 5 mM to 1 M, with the exception of $[N_{1\,1\,1\,2OH}][eth]$ that was above 4 M (Fig. 2B). Their inhibitory and fungicidal concentrations were at least one order of magnitude higher, for longer anions, than $[C_n mim]Cl$. The toxic effect of cholinium alkanoates was defined by the anion structure, increasing exponentially with the elongation of the anion in a similar trend to that observed for $[C_n mim]^+$.

Despite its limitations, the trend observed for both families of ionic liquids could be explained by the 1-octanol/water partition (logP) or distribution (logD) coefficients. The calculated logD values for $[C_n mim]Cl$ (n = 2, 4, 6, 8 or 10) cations ranged linearly from -2.47 to 2.11, suggesting an increasing hydrophobicity for $[C_n mim]^+$ with longer alkyl chains ($n > 4$). For cholinium alkanoates, the logD values of the isolated anions were predicted to increase linearly from -0.265 to 1.52. In both cases, this suggests a scale of lipophilicity for these cations and anions, and has already been taken as a possible explanation for the toxic effects of these ionic liquids.[6, 25]

The biodegradation of $[C_n mim]Cl$ (n = 2, 4, 6, 8 or 10) and cholinium alkanoates (anion = $[eth]^-$, $[but]^-$, $[hex]^-$, $[oct]^-$ or $[dec]^-$) was assessed by liquid chromatography after one, two, five and seven days of growth of *A. nidulans* in media supplemented with *ca.* half the previously determined MIC values. While the $[C_n mim]^+$ cation was recalcitrant to degradation under the conditions tested, keeping its levels constant until the seventh day of incubation, cholinium alkanoates were readily degraded by the fungus (Table 1). The alkanoates were consumed at a greatest rate than the $[N_{1\,1\,1\,2OH}]^+$ cation. For example, after five days *A. nidulans* is able to deplete almost the totality of the anion of $[N_{1\,1\,1\,2OH}][hex]$, while only 9% of the cation was consumed. Moreover, longer anions were more efficiently degraded than the shorter ones. These observations are consistent with that previously reported for filamentous fungi of the genus *Penicillium*.[25] The ability of *A. nidulans* to degrade cholinium alkanoates further reinforces their biodegradable nature.

Imidazolium-based ionic liquids permeabilise the fungal plasma membrane

The toxicity trend observed for both [$C_n$mim]Cl and cholinium alkanoates, correlated with increased lipophilicity,[49] has already been observed for other imidazolium, ammonium and phosphonium derivatives.[50-52] It is believed that through interaction with the biological membranes these ionic liquids can permeabilise the lipid bilayer and cause loss of its integrity, leading to leakage of intracellular material and, ultimately, cell death.

Using a microscopic assessment,[52] we investigated the ability of ionic liquids to permeabilise the plasma membrane of *A. nidulans*. Conidia were exposed to [$C_n$mim]Cl (n = 2, 4, 6, 8 or 10) or cholinium alkanoates (anion = $[eth]^-$, $[but]^-$, $[hex]^-$, $[oct]^-$ or $[dec]^-$) for one hour and stained with propidium iodide (PI). PI can only enter membrane-damaged cells, where it binds to nucleic acids (detected as red fluorescence). For each testing condition the percentage of cells with membrane damage was determined. The saline solution control (0.85% wt. NaCl) showed approximately 6% of membrane-damaged conidia, considered a basal level of injured cells or a consequence of the harvesting procedure. The number of membrane-damaged conidia after exposure to [$C_n$mim]Cl concentrations below MFC values, *i.e.* leading to cell death, were reasonably similar to the control (Fig. 3A, Table S4). Slightly higher concentrations, still below the MFC, resulted only in a minor increase in the number of membrane-damaged cells. However, at concentrations above the MFC values, it was clear that [$C_n$mim]Cl with longer alkyl chains ($n \geq 6$) induced a great increase in the number of membrane-damaged conidia. For example, 100 mM of [C_8mim]Cl or 10 mM of [C_{10}mim]Cl damaged as much as 90% of the conidia. Cholinium alkanoates were unable to cause membrane damage, even at concentrations leading to cell death (Fig. 3A, Table S4). At the highest tested concentration (100 mM), [$N_{1\,1\,1\,2OH}$][dec] damaged only 26% of the conidia, whereas 10 times lower concentration of [C_{10}mim]Cl injured as much as 95%. Despite presenting similar trends regarding their toxic effects, [$C_n$mim]Cl with longer alkyl substituents ($n \geq 6$) induce membrane permeabilisation, while cholinium alkanoates are virtually unable to disrupt the lipid bilayer.

Membrane-permeabilising ionic liquids up-regulate genes involved in saturated fatty acids biosynthesis

Biological membranes are composed of a thin film of lipids and proteins.[53] The lipids are arranged as a double layer, and serve as a relatively impermeable barrier for most water-soluble molecules.[54] In eukaryotic organisms this lipid bilayer is composed mainly of three types of lipids: phospholipids, sterols and sphingolipids.[53] Ergosterol, only found in fungi, is the major sterol present in the plasma membrane of these organisms. Membranes enriched in ergosterol are less fluid, and seem to be more resistant to permeabilising compounds.[55] After exposure to [C$_n$mim]Cl (n = 6, 8 or 10), *A. nidulans* maintained constant the expression levels of *HMGR1* (Fig. 4), the gene coding for a putative 3-hydroxy-3-methylglutaryl-Coenzyme A reductase, involved in the rate-limiting step in ergosterol biosynthesis.[56] Nevertheless, ergosterol is not the only component of the plasma membrane responsible its fluidity and no direct correlation between its amounts and sensitivity or resistance to cationic membrane-permeabilising agents (*e.g.* chitosan) could be observed so far.[57] The balance between saturated and unsaturated fatty acids, nonetheless, seems to play a crucial role in this feature. Filamentous fungi can alter the fatty acid composition of their membranes, regulating its fluidity to overcome adverse environments, such as *N. crassa* upon increased temperatures.[58] *Aspergillus nidulans* has two genes involved in the *de novo* synthesis of saturated long-chain fatty acids, *fasA* and *fasB*.[59, 60] The α subunit of the fatty acid synthase, FasA, of the fungus *Pleurotus tuber-regium* increased after treatment with the nonionic surfactant Tween 80.[61] In the present study, all tested [C$_n$mim]Cl induced a significant up-regulation of both *fasA* and *fasB* in *A. nidulans*, which was dependent on the length of the alkyl chain (Fig. 4). Taken as examples, after only one hour [C$_6$mim]Cl and [C$_8$mim]Cl increased *fasA* 3.2- and 3.8-fold, respectively, whereas *fasB* increased 3.5-fold for [C$_6$mim]Cl. After two hours, *fasA* and *fasB* up-regulation reached, respectively, 4.0- and 5.4-fold for [C$_{10}$mim]Cl. Previous to this study, we have

observed that membrane-permeabilising alkyltributylphosphonium ionic liquids also increase the expression of *fasA* in *A. nidulans*, in a manner dependent on the alkyl chain length.[52]

In filamentous fungi, ceramides are the most common type of sphingolipids, being composed of a sphingoid base and a very-long-chain fatty acid.[62] In *A. nidulans*, two enzymes, encoded by *barA* and *lagA*, are responsible for their condensation. No increase in their expression was observed here after exposure to either [$C_n$mim]Cl (Fig. 4), as previously reported by us for alkyltributylphosphonium chlorides.[52] In fact, as observed in that previous study, while *lagA* expression remained unaltered, *barA* expression levels showed, in general, a significant decrease (Fig. 4). This is consistent with the proposed greater involvement of the latter gene in the filamentous growth of the fungus.[62, 63] Upon exposure to near inhibitory concentrations of ionic liquids, vegetative growth most likely ceases and the biosynthetic machinery is redirected towards the response to the toxic effects of the compounds.

As expected the cholinium alkanoates (anion = $[hex]^-$, $[oct]^-$ or $[dec]^-$), which were virtually unable to cause membrane damage (Fig. 3B), did not lead to increased expression of any of the tested genes (Fig. 4). Even after four hours of exposure, the expression levels of *fasA* and *fasB*, remained constant; supporting them as adequate reporter-genes for determining membrane permeabilisation as a possible mechanism of toxicity of ionic liquids.

The charged nature of the plasma membrane rules the permeabilising ability of ionic liquids

The difference in the effects observed here for [$C_n$mim]Cl and cholinium alkanoates is likely determined by chemical interactions between the composing ions and biological membranes. The plasma membrane is charged along its surface due to the different composition of neutral and negatively charged headgroups of its composing phospholipids. It is likely that the presence of negatively charged phospholipids in the plasma membrane facilitates the interaction of $[C_n mim]^+$ with this cellular structure. Membrane permeabilisation by this cation would then be determined by

the elongation of its alkyl chain. The anions of cholinium alkanoates, on the other hand, are most likely kept away from the membrane surface, repelled by electrostatic forces.

To support this hypothesis, molecular dynamics simulations of lipid bilayers with aqueous solutions of $[C_8mim]Cl$ or $[N_{1\,1\,1\,2OH}][oct]$ were carried out using the DLPOLY package.[42] The bilayer was composed of 76% of the neutral palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 24% of the negatively charged palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; conditions that well represent the heterogeneous charge distribution along the membrane surface (Fig. 5). In an initial assessment, 200 mM of either ionic liquid were added to the aqueous phase and the behaviour of each counter ion was observed. The density of specific atoms of each ionic liquid ion clearly revealed that both cations were more likely to be found near the charged bilayer surface than the anions, which stayed in the aqueous phase (Fig. 6).

Additional simulations were initiated with the ionic liquids embedded in the lipid bilayer (Fig. S1, Fig. S2) to evaluate the interaction of the organic ions ($[C_8mim]^+$ or $[oct]^-$) with the hydrophobic regions of the lipid bilayer and their stability in such environment. $[C_8mim]^+$ remained in the lipid bilayer throughout the simulation run (Fig. 7). Moreover, its long alkyl chain interacted with the hydrophobic regions of the phospholipids, whereas its charged moieties stayed associated with the phosphocholine or phosphoserine headgroups. On the contrary, $[oct]^-$ did not remain inserted in the lipid bilayer, instead it seemed to destabilise and disrupt its original conformation (Fig. 7). This is highly suggestive that the interaction of $[oct]^-$ with the phospholipid hydrophobic regions and headgroups is very unlikely to occur.

Our data is consistent with plasma membrane permeabilisation by ionic liquids as a two-step process, as already suggested for other cationic agents, *e.g* chitosan[55] and cationic antimicrobial peptides.[64] Ionic liquid cations, driven by electrostatic forces, would initially cover the membrane surface in a carpet-like manner. In a second step, hydrophobic moieties in the cation (alkyl chain) would probably interact with the inner regions of the lipid bilayer, facilitating its permeabilisation.

This has been also suggested in simulations of imidazolium-based cations with a single-phospholipid bilayer.[65, 66] Altogether, the molecular dynamics simulations performed here strongly support this two-step mechanism and agree with the observed membrane-permeabilising ability of [$C_n$mim]Cl carrying long alkyl chains ($n \geq 6$) and the lack of a similar effect in cholinium alkanoates. These long anions are likely repelled by the negatively charged phospholipid headgroups, as well as by the overall negative charge of the fungal cell wal l.[67]

Elongating one alkyl chain in the cholinium cation ensues membrane-permeabilising ionic liquids

To further reinforce that the lack of a hydrophobic chain in $[N_{1\,1\,1\,2OH}]^+$ prevents its interaction with the inner regions of the lipid bilayer (Fig. 6), we analysed here a series of ionic liquids in which one alkyl chain in this cation was linearly elongated. The obtained alkyl-(2-hydroxyethyl)-dimethylammonium bromides ($[N_{1\,1\,n\,2OH}]Br$, n = 2, 4, 6, 8, 10 or 12) (Fig. 1) reported MIC values for *A. nidulans* ranging from 0.2 mM to 2 M (Fig. 2C, Table S3). For $[N_{1\,1\,n\,2OH}]Br$, when $n \geq 6$, the MFC values were identical to MIC values, but for shorter alkyl chains they could not be determined. The toxic effects of $[N_{1\,1\,n\,2OH}]Br$ were defined by the cation structure, increasing with the elongation of the alkyl chain (Fig. 2C). Again, this is consistent with the linear increase in logD values predicted for the $[N_{1\,1\,n\,2OH}]^+$, ranging from -3.85 to 0.75. Comparatively, $[N_{1\,1\,n\,2OH}]Br$ with $n \leq 6$ were less toxic than cholinium alkanoates with anions of similar length ($[eth]^-$, $[but]^-$ or $[hex]^-$), whereas for $n \geq 8$ the two series of salts reported similar toxicities. They also showed similar biodegradability, since $[N_{1\,1\,n\,2OH}]^+$ reported degradation levels ranging from 10% to 17% (Table 1).

Testing $[N_{1\,1\,n\,2OH}]Br$ at concentrations below the MFC value the number of membrane-damaged conidia were similar to that of the control (Fig. 3C, Table S4). However, at concentrations above the MFC, $[N_{1\,1\,n\,2OH}]Br$ with long alkyl substituents ($n \geq 8$) led to great percentages of membrane-damaged conidia, reaching more than 90% for 100 mM of ionic liquid. Their

membrane-permeabilising ability was further reinforced by the up-regulation of *fasA* and *fasB* (Fig. 4). This effect was also dependent on the alkyl chain length. Taken as an example, *fasB* increase 2.2- and 2.6-fold, after two hours, for [$N_{1\,1\,6\,2OH}$]Br and [$N_{1\,1\,8\,2OH}$]Br, respectively, and 3.1- and 3.4-fold, after four hours, for [$N_{1\,1\,10\,2OH}$]Br and [$N_{1\,1\,12\,2OH}$]Br, respectively. As expected, neither *HMGR1, barA* or *lagA*, were significantly up-regulated even after four hours of incubation (Fig. 4). As hypothesised, elongating one alkyl chain in the cholinium cation resulted in membrane-permeabilising ionic liquids, notwithstanding their biodegradability and lower toxicity when compared with [$C_n$mim]Cl carrying alkyl chains of similar length.

Conclusions

It is largely accepted that ionic liquids carrying long alkyl chains would interact and disrupt biological membranes. This has been for long supported by the 1-octanol/water partition or distribution coefficients, correlating elongation of ionic liquids alkyl chains with increased lipophilicity. Despite their wide use to explain toxicity trends of ionic liquids, our data rejects the use of these coefficients as a valid indicator of membrane permeabilisation. We revealed here that, although both [$C_n$mim]Cl and cholinium alkanoates present similar trends of toxicity and of increase in 1-octanol/water distribution coefficients, their effects on biological membranes are exceptionally different. [$C_n$mim]Cl with long alkyl substituents ($n \geq 6$) are able to permeabilise the fungal plasma membrane, leading to cell death. This has been directly inferred by microscopy as well as indirectly by the up-regulation of the saturated fatty acid biosynthesis genes, namely *fasA* and *fasB*. On the contrary, cholinium alkanoates were observed here to be devoid of membrane-permeabilising ability, possibly because the lipophilic moiety is negatively charged. Molecular dynamics simulations of the interaction of ionic liquids organic ions with the negatively charged phospholipids of the plasma membrane strongly validate this hypothesis. Overall, our findings reinforce that plasma membrane permeabilisation by ionic liquids takes two steps. Electrostatic

forces facilitate the interaction of the organic cations with the membrane surface, crucial to promote the interaction of their long alkyl chains with the hydrophobic regions of the lipid bilayer, enabling its permeabilisation. Like so, anions carrying long alkyl moieties are most likely repelled by the negatively charged phospholipid headgroups, as well as by the by the overall negative charge of the fungal cell wall mannoproteins. Further supporting this hypothesis, we experimentally demonstrated here that the elongation of one alkyl chain in the cholinium cation ($[N_{1\,1\,n\,2OH}]Br$, n = 2, 4, 6, 8, 10 or 12), resulted in membrane-permeabilising ionic liquids.

Our main motivation in the present study was to push our understanding of ionic liquids toxicity, especially as to provide meaningful data for guiding the conscious design of novel compounds. We propose here genes involved in *de novo* fatty acid synthesis (*fasA* and/or *fasB*) as robust bio-indicators of membrane permeabilisation by ionic-liquids. Notwithstanding, the mechanism behind cholinium alkanoates capacity to inhibit fungal growth (which increases exponentially with the elongation of anion) remains partially unsolved. Overall, our study reinforces the need of in-depth, molecular analyses of the mechanisms of toxicity of ionic liquids, as well as the need to better understand the biology of potential target organisms. We are optimistic that the findings presented here may alter the paradigm of ionic liquid research which has largely favoured the development of ionic liquid families with cations carrying long alkyl chains. Finally, the use of functionalised benign cations carrying long alkyl chains may also constitute an unexpected solution for the development of novel antifungal agents.

Acknowledgements

D. O. H. and K. S. are grateful to *Fundação para a Ciência e a Tecnologia* (FCT) for the fellowships SFRH/BD/66396/2009 and SFRH/BPD/94291/2013, respectively. The work was partially supported by FCT through grants PTDC/QUI-QUI/120982/2010, PTDC/AAC-CLI/119100/2010, PTDC/QEQ-PRS/2824/2012 and Research unit GREEN-it "Bioresources for Sustainability" (UID/Multi/04551/2013). K. S. and J. N. C. L. thank FCT for the support provided through projects FCT-ANR/CTM-NAN/0135/2012 and PTDC/QUI-QUI/117340/2010. The fluorescence microscope used in this study belongs to the Cell Imaging Unit (UIC) at the Gulbenkian Science Institute, Oeiras, Portugal.

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Tables and Figures Captions

Table 1. Biodegradation assessment of 1-alkyl-3-methylimidazolium chlorides ([$C_n$mim]Cl, n = 2, 4, 6 8 or 10), cholinium alkanoates (anion = [eth]$^-$, [but]$^-$, [hex]$^-$, [oct]$^-$ or [dec]$^-$) and alkyl-(2-hydroxyethyl)-dimethylammonium bromides ([$N_{1\,1\,n\,2OH}$]Br, n = 2, 4, 6, 8, 10 or 12) after incubation with *Aspergillus nidulans* for one, two, five and seven days. The amounts of ionic liquids organic ions in the culture media were measured chromatographically.

Fig. 1. Chemical structures of ionic liquids (cations and anions) used in this study, namely 1-alkyl-3-methylimidazolium chlorides ([$C_n$mim]Cl, n = 2, 4, 6 8 or 10), cholinium alkanoates (anion = [eth]$^-$, [but]$^-$, [hex]$^-$, [oct]$^-$ or [dec]$^-$) and alkyl-(2-hydroxyethyl)-dimethylammonium bromides ([$N_{1\,1\,n\,2OH}$]Br, n = 2, 4, 6, 8, 10 or 12)

Fig. 2. Minimal inhibitory and fungicidal concentrations (MIC and MFC, respectively) defined for *Aspergillus nidulans*. A) 1-alkyl-3-methylimidazolium chlorides ([$C_n$mim]Cl, n = 2, 4, 6, 8 or 10). B) Cholinium alkanoates (anion = [eth]$^-$, [but]$^-$, [hex]$^-$, [oct]$^-$ or [dec]$^-$). C) Alkyl-(2-hydroxyethyl)-dimethylammonium bromides ([$N_{1\,1\,n\,2OH}$]Br, n = 2, 4, 6, 8, 10 or 12). Values are represented in mM and are plotted on a logarithmic scale. Open triangles represent the highest value tested for the ionic liquid (not the MFC).

Fig. 3. Percentage of membrane-damaged conidia after one hour of incubation with ionic liquids, obtained as: (number of propidium iodide-stained conidia / total number of conidia) × 100. A) 1-alkyl-3-methylimidazolium chlorides ([$C_n$mim]Cl, n = 2, 4, 6 8 or 10). B) Cholinium alkanoates (anion = [eth]$^-$, [but]$^-$, [hex]$^-$, [oct]$^-$ or [dec]$^-$). C) Alkyl-(2-hydroxyethyl)-dimethylammonium bromides ([$N_{1\,1\,n\,2OH}$]Br, n = 2, 4, 6, 8, 10 or 12).

Fig. 4. Relative expression of genes involved in plasma membrane biosynthesis (*HMGR1*, *fasA fasB*, *barA* and *lagA*,) by *q*RT-PCR. *Aspergillus nidulans* was exposed to 1-alkyl-3-methylimidazolium chlorides ([$C_n$mim]Cl, n = 6, 8 or 10), cholinium alkanoates (anion = [hex]$^-$, [oct]$^-$ or [dec]$^-$) or alkyl-(2-hydroxyethyl)-dimethylammonium bromides ([$N_{1\,1\,n\,2OH}$]Br, n = 6, 8, 10 or 12) for one (white), two (grey) or four hours (black). The y axes represent the fold-change of gene expression relative to the culture at time zero (before exposure). γ-actin gene was used as

internal control. The asterisk marks significant difference (p-value < 0.05) in expression of each treatment when compared to the control for the same period of incubation.

Fig. 5. Simulation snapshot of an equilibrated section of a mixed phospholipids bilayer (76% palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 24% palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine) in water. The counter-ions of the serine-based phospholipids are potassium ions (not-shown). The graphic shows the density profiles along the direction normal to the bilayer (given as discrete probability distribution functions) of water molecules (light blue) and phosphate (orange), ammonium (pink) and methyl (dark grey) groups of the phospholipid species. The bottom line shows space-filled representations of the ionic liquid ions tested in subsequent simulation runs. They are color-coded in order to highlight the charged headgroups of the cations (dark blue) and the anions (red), and the nonpolar alkyl chains of the ions (light grey).

Fig. 6. Simulation snapshots of a section of a mixed phospholipids bilayer equilibrated with 200 mM solutions of $[C_8mim]Cl$ (top) and $[N_{1\ 1\ 1\ 2OH}][oct]$ (bottom). Each system comprises 16 ionic liquid ion pairs. The graphs show the density profiles of the molecules/groups depicted in Fig. 5 plus those of the charged headgroups of the cations (dark blue) and the anions (red), and nonpolar alkyl chains of the ions (light grey).

Fig. 7. Simulation snapshots of a section of a mixed phospholipids bilayer in water equilibrated with 16 ion pairs of $[C_8mim]Cl$ (top) and $[N_{1\ 1\ 1\ 2OH}][oct]$ (bottom) initially incorporated in the bilayer. The graphs show the density profiles of the molecules/groups depicted in Fig. 5 plus those of the charged headgroups of the cations (dark blue) and the anions (red), and nonpolar alkyl chains of the ions (light grey).

Table 1

Ionic liquid	Initial concentration	Ion	Amount in culture (%)			
			1 day	2 days	5 days	7 days
1-alkyl-3-methylimidazolium chlorides						
[C_2mim]Cl	100 mM	+	99.2 ± 1.1	100.1 ± 0.2	97.8 ± 1.3	100.8 ± 0.3
[C_4mim]Cl	100 mM	+	100.1 ± 0.1	99.8 ± 0.2	99.3 ± 0.2	98.9 ± 0.1
[C_6mim]Cl	3.5 mM	+	100.2 ± 0.0	100.1 ± 0.1	99.0 ± 0.5	99.3 ± 0.1
[C_8mim]Cl	0.45 mM	+	104.5 ± 0.1	104.1 ± 0.3	102.6 ± 0.1	102.7 ± 0.2
[C_{10}mim]Cl	0.1 mM	+	96.0 ± 0.6	94.7 ± 0.6	93.05 ± 0.6	92.4 ± 0.0
cholinium alkanoates						
[$N_{1\,1\,1\,2OH}$][eth]	100 mM	+	99.9 ± 0.0	99.3 ± 0.2	87.1 ± 0.7	77.2 ± 0.2
		–	94.1 ± 0.5	63.1 ± 0.6	n.d.	n.d.
[$N_{1\,1\,1\,2OH}$][but]	100 mM	+	100.1 ± 0.1	99.3 ± 0.1	91.8 ± 0.2	77.2 ± 0.1
		–	99.3 ± 0.4	96.5 ± 0.3	69.1 ± 0.3	59.1 ± 0.0
[$N_{1\,1\,1\,2OH}$][hex]	28 mM	+	100.0 ± 0.1	99.4 ± 0.2	98.8 ± 0.1	91.1 ± 1.1
		–	87.6 ± 0.2	57.4 ± 0.3	0.4 ± 0.0	0.4 ± 0.0
[$N_{1\,1\,1\,2OH}$][oct]	9.9 mM	+	98.1 ± 0.8	98.2 ± 0.3	97.3 ± 0.6	80.5 ± 0.8
		–	96.1 ± 0.7	91.4 ± 0.5	33.0 ± 0.0	11.1 ± 0.0
[$N_{1\,1\,1\,2OH}$][dec]	2.2 mM	+	94.2 ± 2.1	91.8 ± 0.8	82.7 ± 2.6	36.2 ± 0.3
		–	97.7 ± 0.2	90.1 ± 0.2	54.2 ± 0.1	23.8 ± 0.1
alkyl-(2-hydroxyethyl)-dimethylammonium bromides						
[$N_{1\,1\,2\,2OH}$]Br	100 mM	+	99.6 ± 0.2	99.2 ± 0.04	97.7 ± 0.1	90.2 ± 0.3
[$N_{1\,1\,4\,2OH}$]Br	100 mM	+	100.1 ± 0.1	99.9 ± 0.2	99.2 ± 0.1	83.9 ± 0.4
[$N_{1\,1\,6\,2OH}$]Br	52.5 mM	+	99.6 ± 0.8	99.7 ± 0.04	95.1 ± 0.8	83.1 ± 0.5

Figure 1.

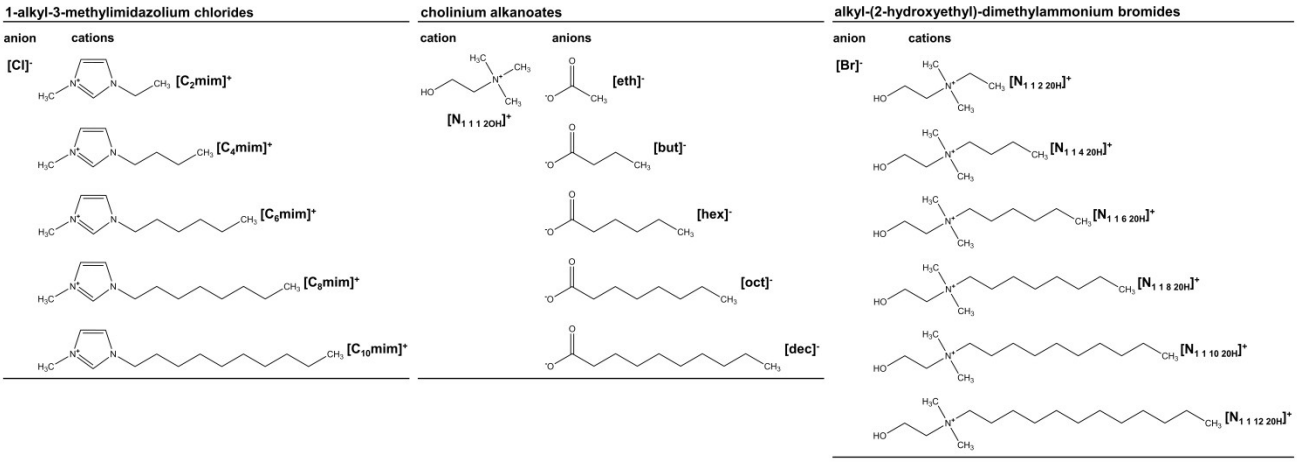


Fig- 2.

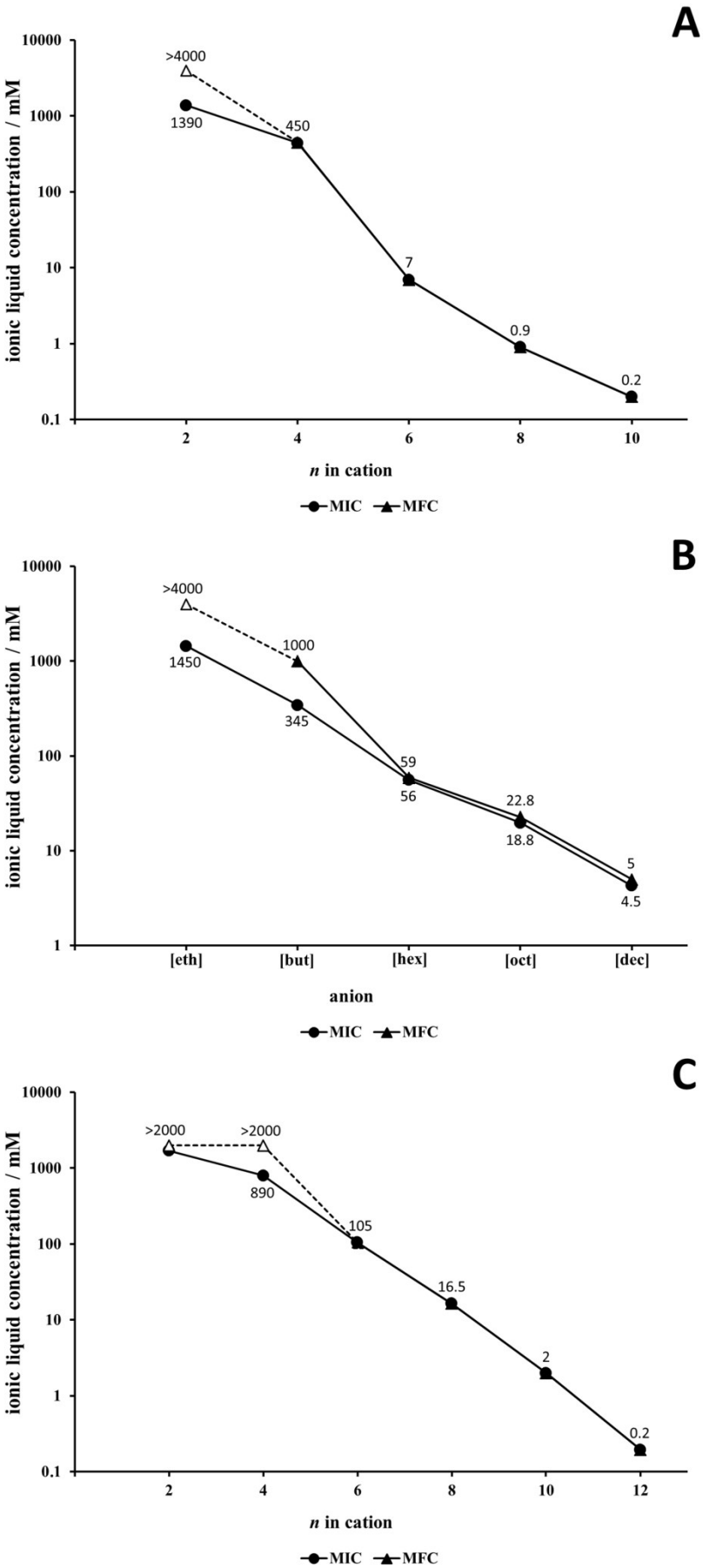


Figure 3.

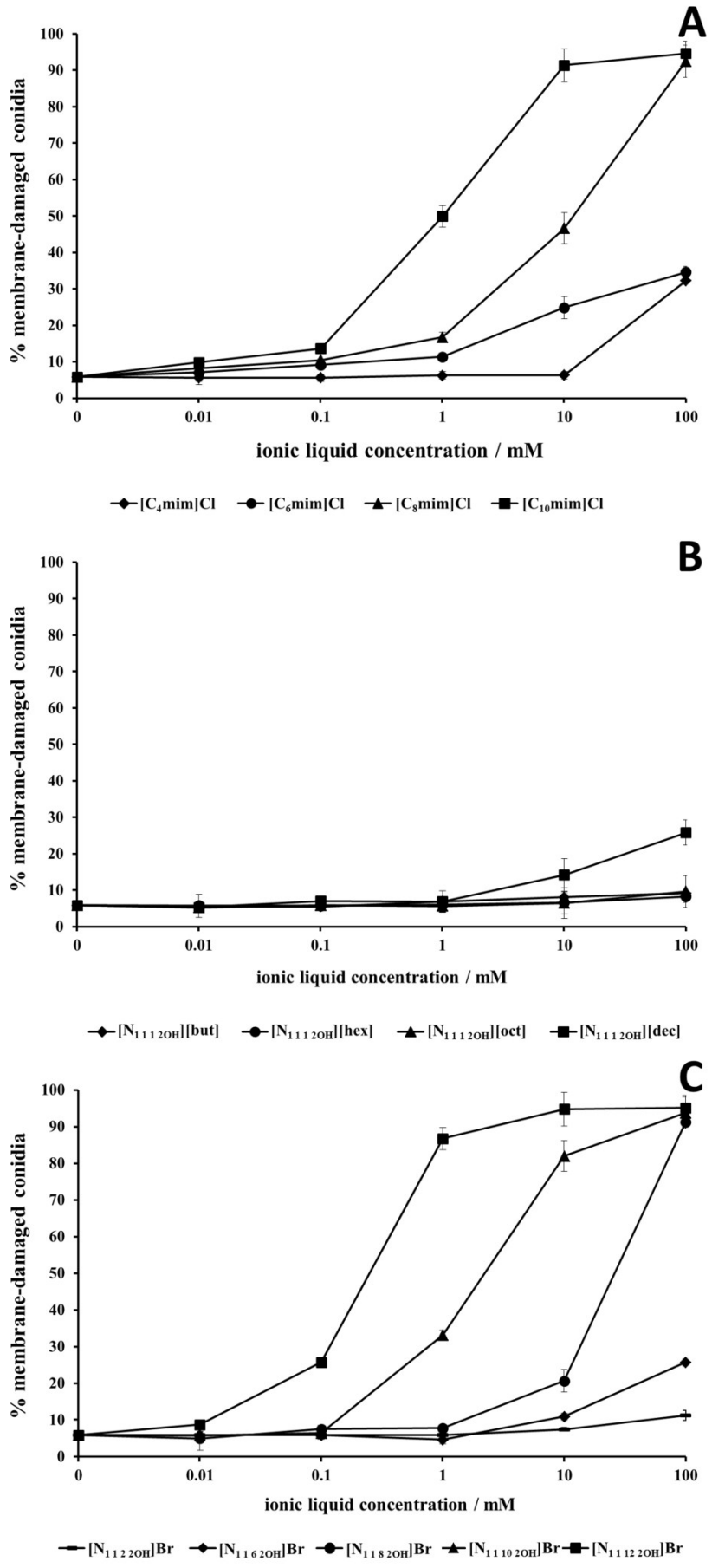


Fig. 4.

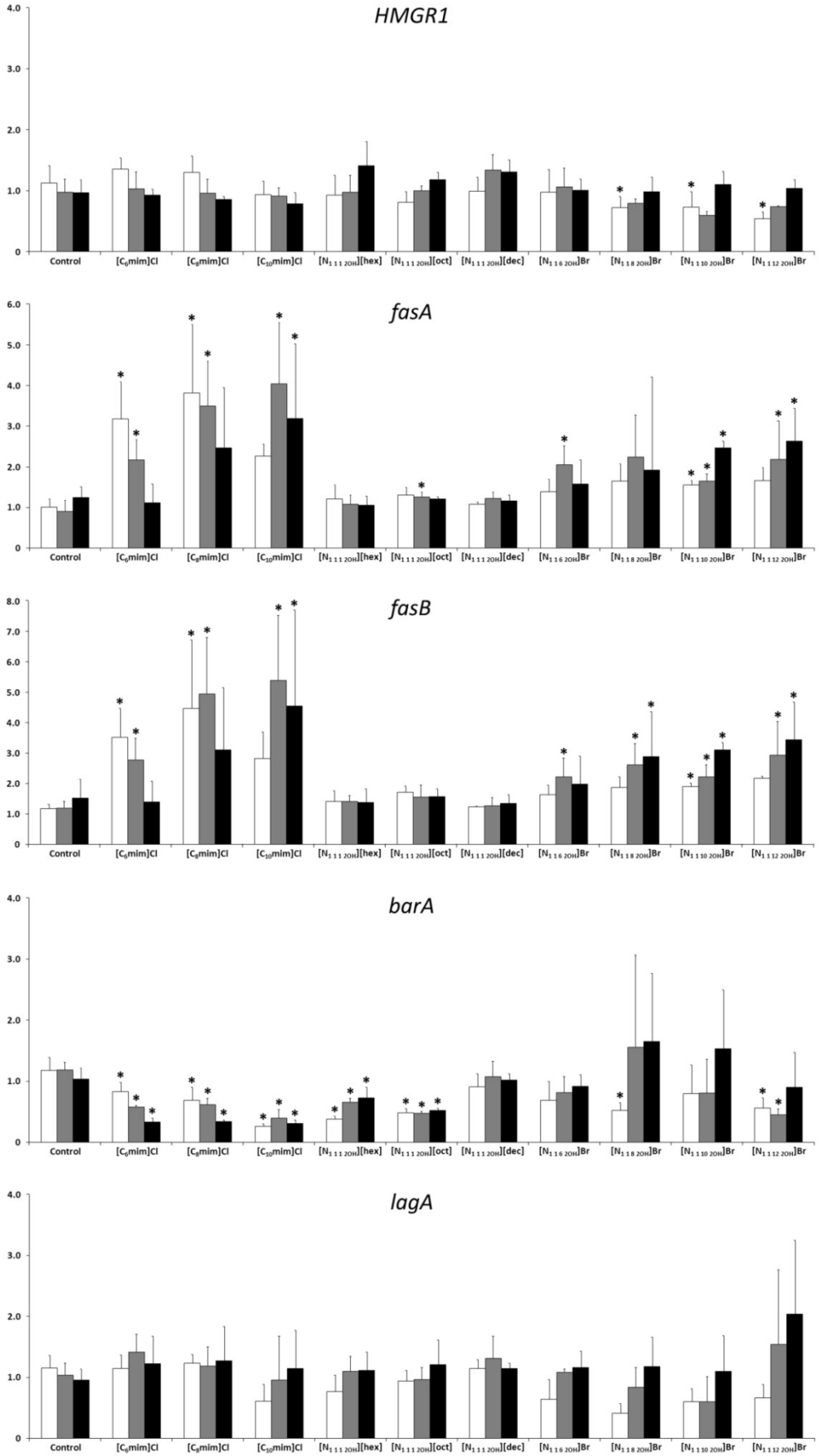


Fig. 5.

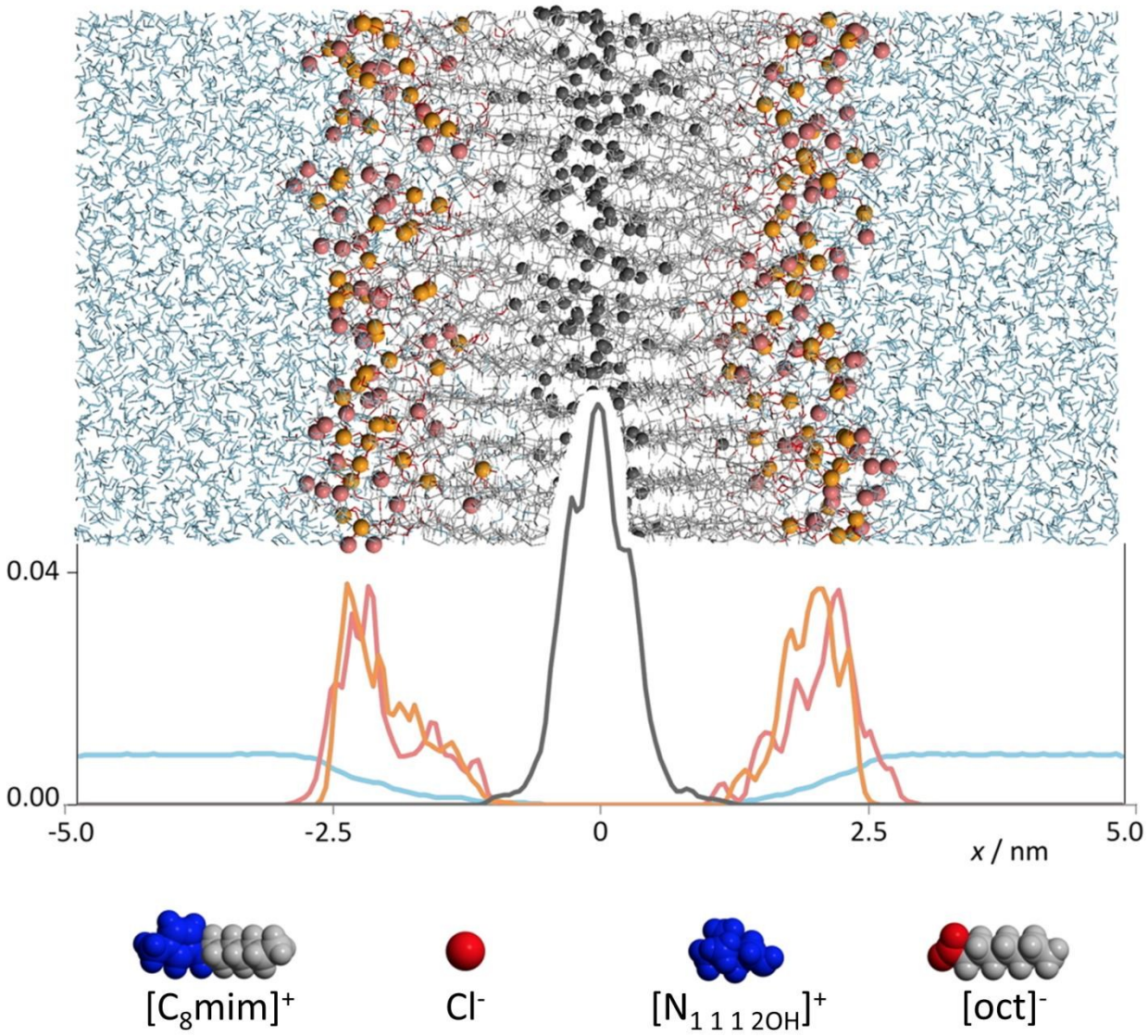


Fig. 6.

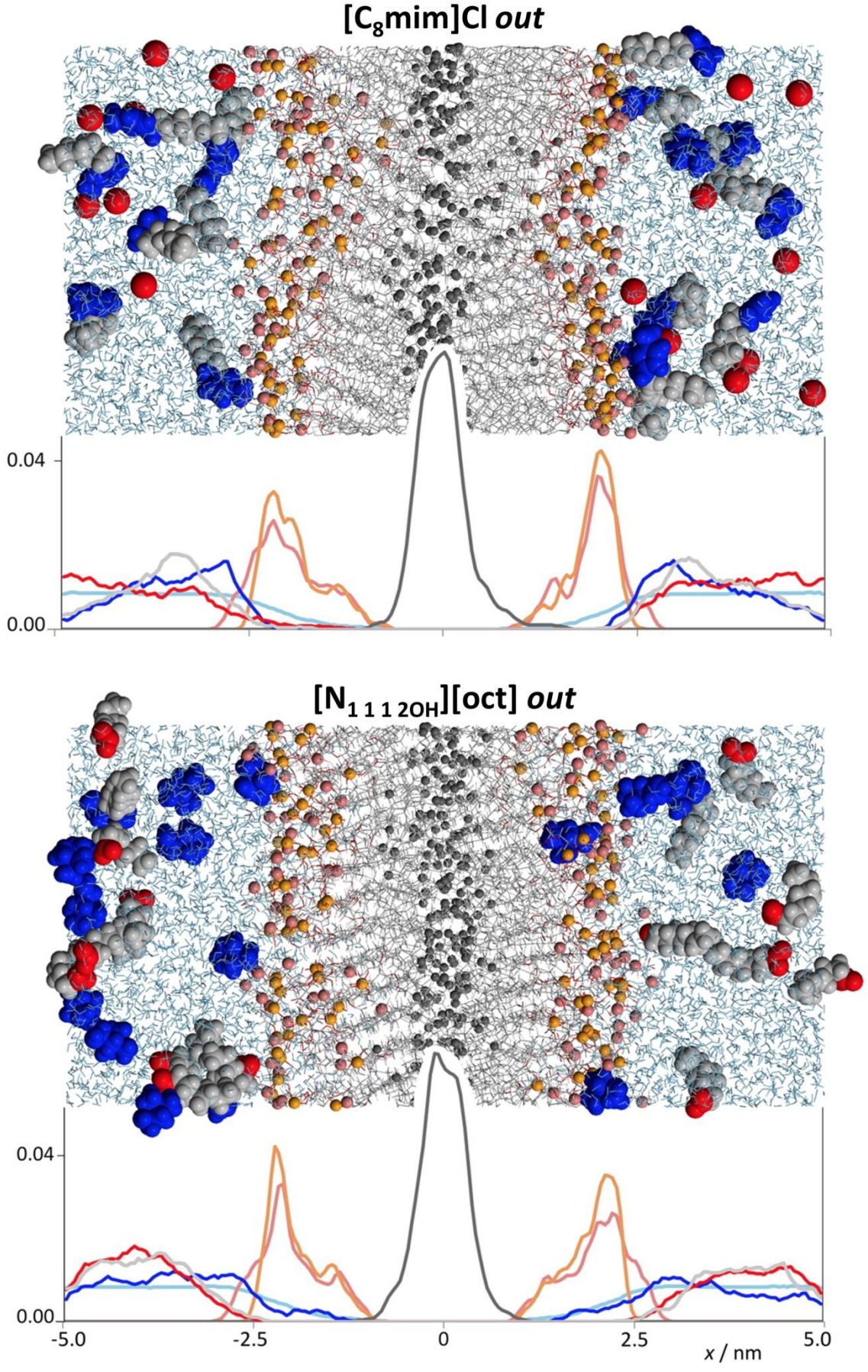
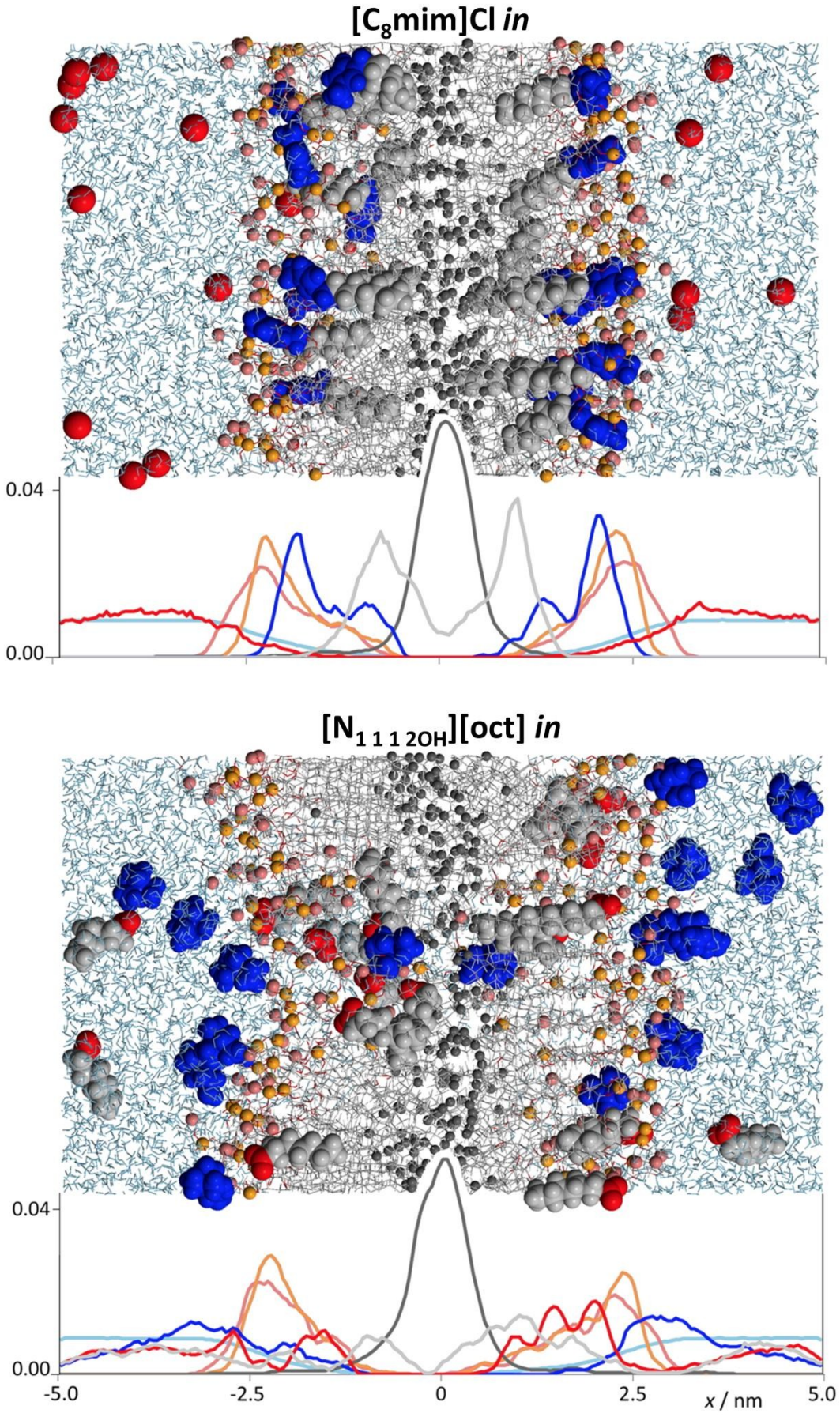


Fig. 7.



Electronic Supplementary Information

Experimental

General remarks

All chemicals, reagents and solvents for the synthesis of the compounds were of analytical grade, purchased from commercial sources, namely Sigma-Aldrich®, Merck and Alfa Aesar and these were used without further purification. The synthesis of the alkyl-(2-hydroxyethyl)-dimethylammonium bromides ([$N_{1\,1\,n\,2OH}$], n = 2, 4, 6 ,8 and 12) has been previously described by us.[1] ^{1}H and ^{13}C NMR spectra were measured on an Ultrashield Bruker Avance II 300 spectrometer and Ultrashield Bruker Avance II 400 spectrometer. Splitting patterns are indicated as *s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *m*, multiplet; *br*, broad peak. The low resolution electrospray ionization mass spectra were recorded using a Micromass® Quattro Micro triple quadruple (Waters®, Ireland) with an electrospray (ESI) ion source. Elemental analysis was performed in a Flash 2000 CHNS-O analyzer (Thermo Scientific, UK). Melting points were determined on a Stuart SMP10 apparatus**.**

General method for the synthesis of alkyl-(2-hydroxyethyl)-dimethylammonium bromides

In a Aldrich ace pressure tube (Z181064) at room temperature, a solution of the correspondent brominated derivative (45 mmol) dissolved in MeCN (5 mL) was added to a solution of 2-(dimethylamino)ethanol (4.1 g, 45 mmol) in MeCN (5 mL) and heated overnight at 60 ºC, unless stated. For the compounds [$N_{1\,1\,2\,2OH}$]Br, [$N_{1\,1\,4\,2OH}$]Br, the salt precipitated during the reaction, while for the compound [$N_{1\,1\,12\,2OH}$]Br, the salt precipitated when the Aldrich ace pressure tube was cooling to room temperature. Diethyl ether was added and the salt was filtered and dried under vacuum. For the compounds [$N_{1\,1\,6\,2OH}$]Br, [$N_{1\,1\,8\,2OH}$]Br, and [$N_{1\,1\,10\,2OH}$]Br, in which the reaction mixture was liquid, the solvent was evaporated and diethyl ether was added to precipitate the product. The ionic liquid was then filtered and dried under vacuum.

Ethyl-(2-hydroxyethyl)-dimethylammonium bromide ([$N_{1\,1\,2\,2OH}$]Br**).** The reaction mixture was heated for 20 min. The product was collected as a white solid in 96 % yield, m.p. 280-282ºC (Lit. 282 - 284ºC)[2]. ^{1}H NMR (300 MHz, D_2O) δ 4.02-3.97 (m, 2H), 3.47 – 3.39 (m, 4H), 3.08 (s, 6H), 1.35 – 1.28 (m, 3H). ^{13}C NMR (75 MHz, D_2O) δ 64.41, 61.04, 55.39, 50.91, 50.86, 7.73. Anal. Calcd for $C_6H_{16}BrNO$: C, 36.38; H, 8.14; N, 7.07. Found: C, 36.13; H, 8.24; N, 7.24. Spectral data ^{1}H and ^{13}C NMR identical to reported one.[3]

Butyl-(2-hydroxyethyl)-dimethylammonium bromide ([$N_{1\,1\,4\,2OH}$]Br**).** The salt was recovered as a white solid in 96 %, m.p. 110-113 ºC. ^{1}H NMR (300 MHz, D_2O) δ 3.97 (s, 2H), 3.44 – 3.40 (m, 2H), 3.34 - 3.29 (m, 2H), 3.07 (s, 6H), 1.75 – 1.64 (m, 2H), 1.38 – 1.25 (m, 2H), 0.88 (t, J = 7.5 Hz, 3H). ^{13}C NMR (75 MHz, D_2O) δ 65.30, 64.83, 55.39, 51.46, 51.41, 23.98, 19.10, 12.91. Anal. Calcd for $C_8H_{20}BrNO$: C, 42.49; H, 8.91; N, 6.19. Found: C,42.64; H, 9.22; N, 6.43. Spectral data ^{1}H and ^{13}C NMR identical to reported one.[3]

Hexyl-(2-hydroxyethyl)-dimethylammonium bromide ([$N_{1\,1\,6\,2OH}$]Br**).** The reaction mixture was heated during 72 h. The product was obtained as a white gummy in 95 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.79 (s, 2H), 3.44 – 3.41 (m, 2H), 3.33 – 3.27 (m, 2H), 3.08 (s, 6H), 1.48 (s, 2H), 1.05 – 1.01 (m, 6H), 0.59 (t, J = 7.5 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 65.25, 65.86, 55.17, 51.49, 30.60, 25.27, 22.17, 21.79, 13.37. Spectral data ^{1}H and ^{13}C NMR identical to reported one.[3]

Octyl-(2-hydroxyethyl)-dimethylammonium bromide ([$N_{1\ 1\ 8\ 2OH}$]Br**).** A white salt was obtained in 93 %, m.p. 107-110 ºC (Lit. 116 – 118 ºC)[4]. ^{1}H NMR (300 MHz, CDCl3) δ 4.07 (s, 2H), 3.71 - 3.70 (m, 2H), 3.54 – 3.49 (m, 2H), 3.33 (s, 6H), 1.71 (s, 2H), 1.31 - 1.22 (m, 10H), 0.83 (t, *J* = 6.0 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 66.00, 65.56, 55.82, 52.07, 31.61, 29.12, 29.02, 26.24, 22.85, 22.54, 14.05. Spectral data ^{1}H NMR identical to reported one.[4]

Decyl-(2-hydroxyethyl)-dimethylammonium bromide ([$N_{1\ 1\ 10\ 2OH}$]Br**).** The product was obtained as a white solid in 95 %, m.p. 167-168 ºC. ^{1}H NMR (400 MHz, D_2O) δ 3.95 (s, 2H), 3.43 (s, 2H), 3.34 – 3.32 (m, 2H), 3.08 (s, 6H), 1.70 (s, 2H), 1.28 – 1.20 (m, 14H), 0.78 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, D_2O) δ 65.25, 64.95, 55.37, 51.51, 31.69, 29.18, 28.83, 25.95, 22.47, 22.29, 13.77. Anal. Calcd for $C_{14}H_{32}BrNO$: C, 54.19; H, 10.39; N, 4.51. Found: C, 54.19; H, 10.30; N, 4.53. Spectral data ^{1}H identical to reported one.[5]

Dodecyl-(2-hydroxyethyl)-dimethylammonium bromide ([$N_{1\ 1\ 12\ 2OH}$]Br**).** A white solid was recovered in 95 %, m.p. 190-193 ºC (Lit 193 - 196 ºC)[6]. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.04 (s, 2H), 3.69 – 3.66 (m, 2H), 3.52 – 3.46 (m, 2H), 3.30 (s, 6H), 1.68 (s, 2H), 1.28 – 1.18 (m, 18H), 0.80 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 66.01, 65.55, 55.80, 52.06, 31.85, 29.56, 29.46, 29.40, 29.29, 29.21, 26.26, 22.87, 22.64, 14.09. Spectral data ^{1}H NMR identical to reported one.[5]

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4. Calas, M.; Ancelin, M. L.; Cordina, G.; Portefaix, P.; Piquet, G.; Vidal-Sailhan, V.; Vial, H., *J Med Chem* **2000**, *43*, 505-516.
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Table S1. Liquid chromatography conditions for the quantification of ionic liquids organic ions in the culture media. The tested ionic liquids were: 1-alkyl-3-methylimidazolium chlorides ([$C_n$mim]Cl, n = 2, 4, 6 8 or 10), cholinium alkanoates (anion = [eth]$^-$, [but]$^-$, [hex]$^-$, [oct]$^-$ or [dec]$^-$) and alkyl-(2-hydroxyethyl)-dimethylammonium bromides ([$N_{1\,1\,n\,2OH}$]Br, n = 2, 4, 6)[a].

Ion	Detection / nm	Column	Mobile phase[b]	Flow rate / mL min^{-1}
$[C_2mim]^+$	218	Synergi Polar-RP column (150 × 4.6 mm) packed with polar endcapped particles (4 µm, pore size 80 Å) (Phenomenex), set at 26 °C	98% A1 + 2% B1	0.8
$[C_4mim]^+$			94% A1 + 6% B1	1.2
$[C_6mim]^+$			80% A1 + 20% B1	1.2
$[C_8mim]^+$			50% A1 + 50% B1	1.0
$[C_{10}mim]^+$			40% A1 + 60% B1	1.0
$[N_{1\,1\,1\,2OH}]^+$	215	Synergi Polar-RP column (150 × 4.6 mm) packed with polar endcapped particles (4 µm, pore size 80 Å) (Phenomenex), set at 26 °C	98% A2 + 2% B2	1.0
$[N_{1\,1\,2\,2OH}]^+$			95% A2 + 5% B2	
$[N_{1\,1\,4\,2OH}]^+$			70% A2 + 30% B2	
$[N_{1\,1\,6\,2OH}]^+$			45% A2 + 55% B2	
[eth]$^-$	212	Acquity UPLC HSS C18 (2.1 × 150 mm), 1.8 µm particle size column (Waters), set at 25 °C	Linear gradient from 10% to 95% of B3 (90% to 5% of A3) in 5.7min, then to 100% of B3in 1.3 min. Return to initial conditions in 1.5 min, maintaining for 1.5 min	0.4
[but]$^-$				
[hex]$^-$				
[oct]$^-$				
[dec]$^-$				

[a] The $[N_{1\,1\,8\,2OH}]^+$, $[N_{1\,1\,10\,2OH}]^+$ and $[N_{1\,1\,12\,2OH}]^+$ cations could not be quantified since their initial concentrations were below the detection limits of the established method. [b] A1) aqueous solution of 5 mM phosphate buffer, pH 3; B1) acetonitrile; A2) aqueous solution of 0.1% (v/v) heptafluorobutyric acid, pH 6; B2) methanol, A3) aqueous solution of 0.1% (v/v) phosphoric acid; B3) acetonitrile.

Table S2. List of the designed *q*RT-PCR oligonucleotides (forward and reverse) used in this study. Oligonucleotides were designed based on the sequences of *A. nidulans* genes, obtained from the *Aspergillus* Genome Database (http://www.aspergillusgenome.org/). The selected genes are involved in plasma membrane biosynthesis of *Aspergillus nidulans*. The γ-actin (*actA*) gene was selected as housekeeping (internal control).

Name	**Gene (Code)**	**Sequence (5′ to 3′)**
HMGR1f	*HMGR1* (AN3817)	AAGCACTCCATGTCATGGCTA
HMGR1r	*HMGR1* (AN3817)	TAATAGCCTCAGCCACAACTGA
AN9407F	*fasA* (AN9407)	CTTCAAAGCAAGGTCTTCCTGA
AN9407R	*fasA* (AN9407)	CGCCAGGGACTCAATCACA
AN9408F	*fasB*(AN9408)	TTGGAAGACGTGAAGGCTCA
AN9408R	*fasB* (AN9408)	GGTACGTCGATTCCCTTCAGA
barAf	*barA* (AN4332)	GTGGTGCTCAACCTGATGGA
barAr	*barA* (AN4332)	TGGGTACATGATATGGCGTGA
lagAf	*lagA* (AN2464)	TCCCCACAGAGAGCACGAA
lagAr	*lagA* (AN2464)	GATGATGTGGTGGCCCACA
ACTF	*actA* (AN6542)	CTGGGACGACATGGAGAAGAT
ACTR	*actA* (AN6542)	GTAGATGGGGACGACGTGAG

Table S3. Minimal inhibitory and fungicidal concentrations (MIC and MFC, respectively) of 1-alkyl-3-methylimidazolium chlorides ([$C_n$mim]Cl, n = 2, 4, 6, 8 or 10), cholinium alkanoates (anion = [eth]$^-$, [but]$^-$, [hex]$^-$, [oct]$^-$ or [dec]$^-$) and alkyl-(2-hydroxyethyl)-dimethylammonium bromides ([$N_{1\,1\,n\,2OH}$]Br, n = 2, 4, 6, 8, 10 or 12) defined for *Aspergillus nidulans*.

Ionic liquid	**MIC / mM**	**MFC / mM**
1-alkyl-3-methylimidazolium chlorides		
[C_2mim]Cl	1390	>4000*
[C_4mim]Cl	445	450
[C_6mim]Cl	7	7
[C_8mim]Cl	0.9	0.9
[C_{10}mim]Cl	0.2	0.2
cholinium alkanoates		
[$N_{1\,1\,1\,2OH}$][eth]	1450	>4000*
[$N_{1\,1\,1\,2OH}$][but]	345	1000
[$N_{1\,1\,1\,2OH}$][hex]	56	59
[$N_{1\,1\,1\,2OH}$][oct]	19.8	22.8
[$N_{1\,1\,1\,2OH}$][dec]	4.3	5
alkyl-(2-hydroxyethyl)-dimethylammonium bromides		
[$N_{1\,1\,2\,2OH}$]Br	>2000*	>2000*
[$N_{1\,1\,4\,2OH}$]Br	890	>2000*
[$N_{1\,1\,6\,2OH}$]Br	105	105
[$N_{1\,1\,8\,2OH}$]Br	16.5	16.5
[$N_{1\,1\,10\,2OH}$]Br	2	2
[$N_{1\,1\,12\,2OH}$]Br	0.2	0.2

*MIC or MFC could not be determined and is estimated to be above the maximum concentrations tested

Table S4. Percentage of membrane-damaged conidia after one hour of incubation with distinct concentrations of 1-alkyl-3-methylimidazolium chlorides ([$C_n$mim]Cl, n = 2, 4, 6 8 or 10), cholinium alkanoates (anion = [eth]$^-$, [but]$^-$, [hex]$^-$, [oct]$^-$ or [dec]$^-$) or alkyl-(2-hydroxyethyl)-dimethylammonium bromides ([$N_{1\,1\,n\,2OH}$]Br, n = 2, 4, 6, 8, 10 or 12). Values were obtained as: (number of propidium iodide-stained conidia / total number of conidia) × 100. Percentage in the saline solution control was 5.87%. Emboldened values were obtained with concentrations above the minimal fungicidal concentration.

Ionic liquid	**Concentration / mM**						
	0.01	0.1	1	10	100	1000	2000
1-alkyl-3-methylimidazolium chlorides							
[C_2mim]Cl	n.d.	n.d.	n.d.	n.d.	n.d.	7.8 ± 3.6	8.0 ± 3.7
[C_4mim]Cl	n.d.	5.6 ± 0.5	6.3 ± 1.8	6.3 ± 1.1	32.3 ± 2.0	**32.4 ± 2.1**	n.d.
[C_6mim]Cl	7.1 ± 0.7	9.2 ± 1.5	11.4 ± 0.6	**25.0 ± 3.7**	**34.6 ± 1.0**	n.d.	n.d.
[C_8mim]Cl	8.2 ± 2.2	10.5 ± 2.0	**16.8 ± 3.6**	**46.7 ± 3.6**	**92.6 ± 3.8**	n.d.	n.d.
[C_{10}mim]Cl	9.8 ± 1.8	13.6 ± 1.5	**50.0 ± 6.3**	**91.4 ± 4.6**	**94.7 ± 4,1**	n.d.	n.d.
cholinium alkanoates							
[$N_{1\,1\,1\,2OH}$][eth]	n.d.	n.d.	n.d.	n.d.	n.d.	12.4 ± 6.2	27.9 ± 1.9
[$N_{1\,1\,1\,2OH}$][but]	n.d.	5.6 ± 0.2	6.9 ± 1.0	8.1 ± 1.4	9.2 ± 0.9	**31.2 ± 4.1**	n.d.
[$N_{1\,1\,1\,2OH}$][hex]	5.8 ± 0.9	5.7 ± 0.8	6.1 ± 0.3	6.6 ± 1.3	**8.2 ± 1.6**	n.d.	n.d.
[$N_{1\,1\,1\,2OH}$][oct]	5.2 ± 0.9	5.9 ± 1.1	5.7 ± 1.3	6.5 ± 1.1	**9.7 ± 1.9**	n.d.	n.d.
[$N_{1\,1\,1\,2OH}$][dec]	5.3 ± 1.0	7.1 ± 1.0	6.9 ± 2.7	**14.2 ± 1.3**	**25.9 ± 2.7**	n.d.	n.d.
alkyl-(2-hydroxyethyl)-dimethylammonium bromides							
[$N_{1\,1\,2\,2OH}$]Br	n.d.	n.d.	n.d.	n.d.	n.d.	12.8 ± 1.0	n.d.
[$N_{1\,1\,4\,2OH}$]Br	n.d.	5.9 ± 1.0	5.9 ± 0.7	7.5 ± 0.5	11.3 ± 1.4	11.6 ± 2.0	n.d.
[$N_{1\,1\,6\,2OH}$] Br	6.0 ± 2.0	5.9 ± 0.3	4.6 ± 0.5	11.0 ± 0.8	25.8 ± 0.5	n.d.	n.d.
[$N_{1\,1\,8\,2OH}$] Br	5.0 ± 0.8	7.6 ± 1.7	7.8 ± 0.8	20.7 ± 2.4	**91.3 ± 2.6**	n.d.	n.d.
[$N_{1\,1\,10\,2OH}$] Br	5.8 ± 1.7	6.5 ± 2.0	33.2 ± 0.6	**82.0 ± 3.0**	**93.8 ± 1.7**	n.d.	n.d.
[$N_{1\,1\,12\,2OH}$] Br	8.8 ± 1.5	25.8± 0.5	**86.8 ± 5.1**	**94.8 ± 2.1**	**95.2 ± 0.7**	n.d.	n.d.

n.d. = not determined.

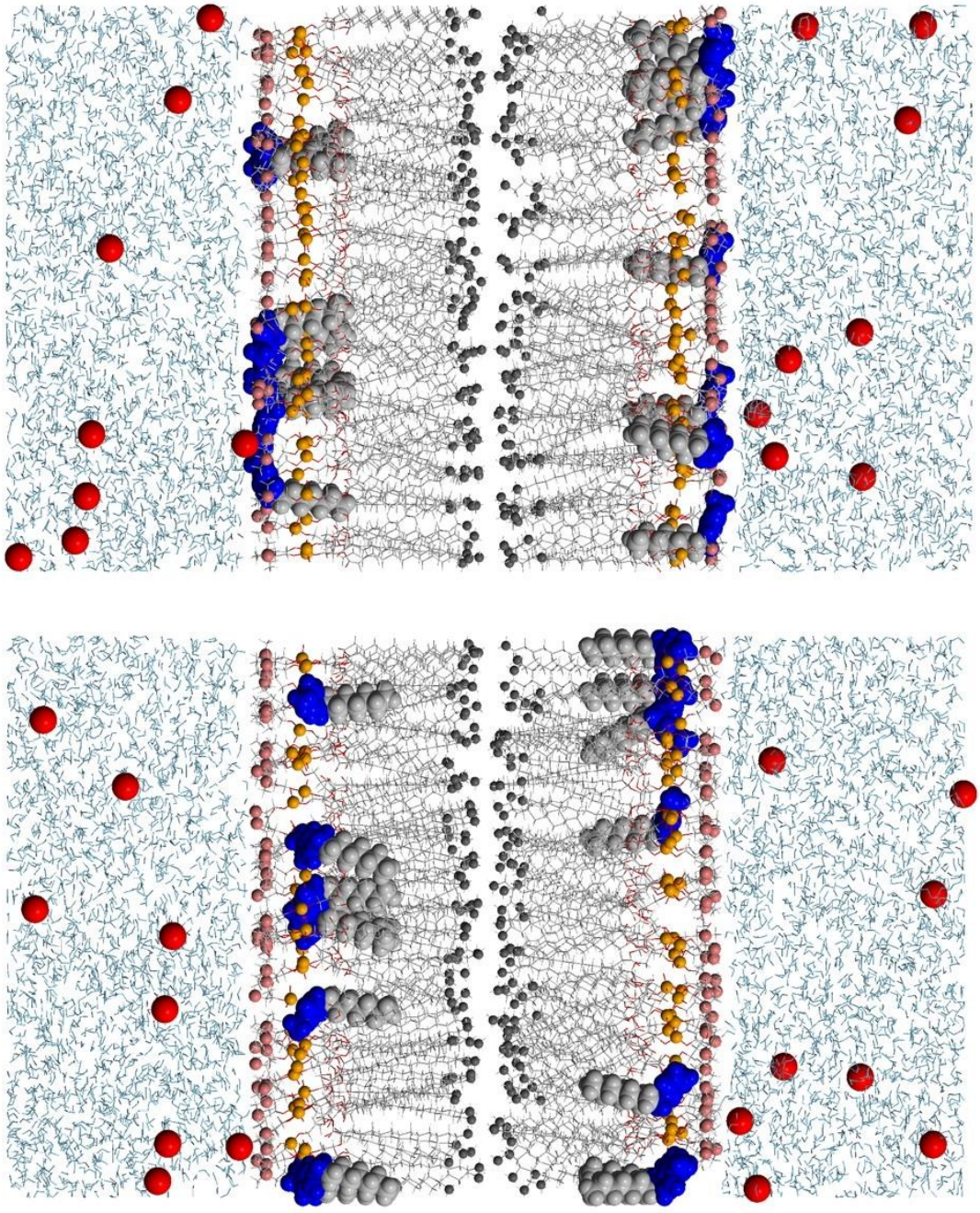


Figure S4. Initial configuration snapshots of a section of a mixed phospholipids bilayer in water with 16 ion pairs (1-octyl-3-methylimidazolium chloride, [C_8mim]Cl) initially incorporated in the bilayer: (top) charged cation headgroups close to the ammonium groups in the phospholipids; (bottom) charged cation headgroups close to the phosphate groups in the phospholipids.

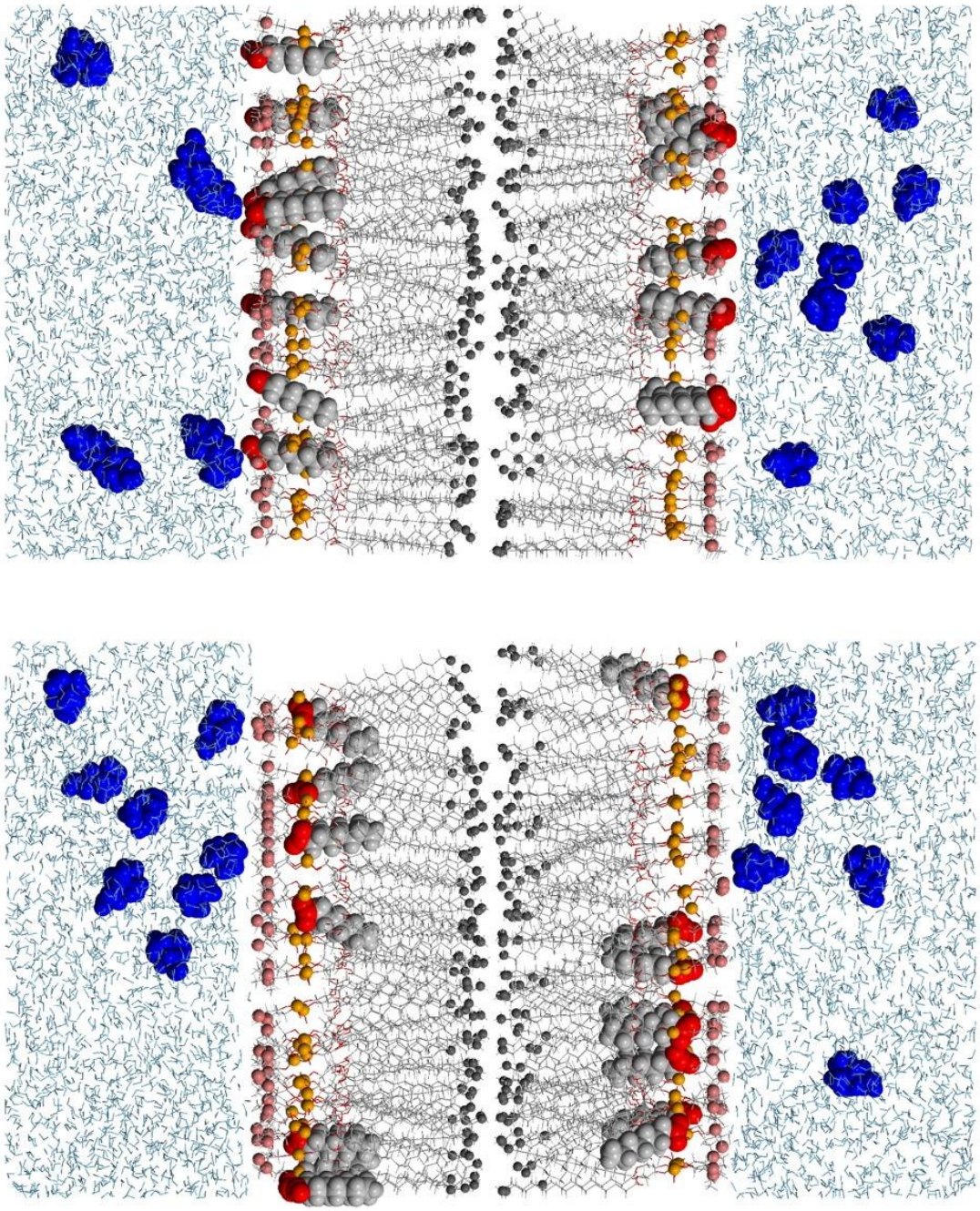
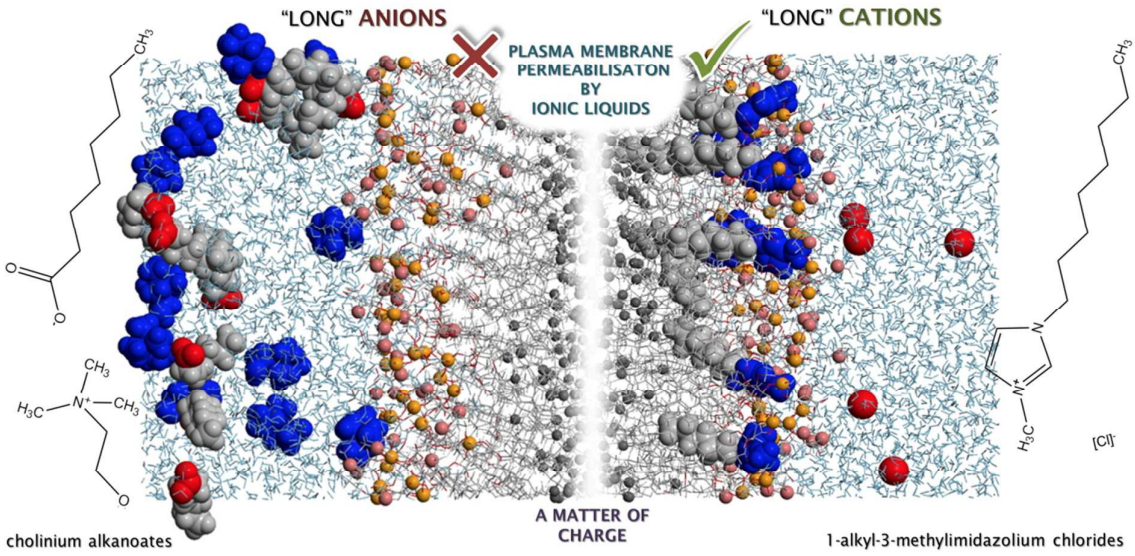


Figure S5. Initial configuration snapshots of a section of a mixed phospholipids bilayer in water with 16 ion pairs (cholinium octanoate, $[N_{1\ 1\ 1\ 2OH}][oct]$) initially incorporated in the bilayer: (top) charged anion headgroups close to the ammonium groups in the phospholipids; (bottom) charged anion headgroups close to the phosphate groups in the phospholipids.

Graphical Abstract



The membrane negatively charged surface hinders permeabilisation by ionic liquids long lipophilic anions – a new rule of thumb for the design of greener solvents.