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Synergistic effects of polymyxin and ionic liquids on lipid vesicle membrane stability and aggregation

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ABSTRACT

Ionic liquids (ILs) have been investigated for potential antibacterial and antibiotic applications due to their ability to destabilize and permeabilize the lipid bilayers in cell membranes. Bacterial assays have shown that combining ILs with antibiotics can provide a synergistic enhancement of their antibacterial activities. We have characterized the mechanism by which the conventional ILs 1-butyl-3-methylimidazolium chloride (BMICl) and 1-butyl-3-methylimidazolium tetrafluoroborate ($BMIBF_4$) enhance the lipid membrane permeabilization of the well-known antibiotic polymyxin B (PMB). We studied the sizes and membrane permeabilities of multilamellar and unilamellar lipid bilayer vesicles in the presence of ILs alone in aqueous solution, PMB alone, and ILs combined together with PMB. Light scattering-based experiments show that vesicle sizes dramatically increase when ILs are combined with PMB, which suggests that the materials combine to synergistically enhance lipid membrane disruption leading to vesicle aggregation. Lipid bilayer leakage experiments using tris (2,2′-bipyridyl) ruthenium (II) ($Ru(bpy)_3^{2+}$) trapped in lipid vesicles, in which the trapped $Ru(bpy)_3^{2+}$ fluorescence lifetime increases when it leaks out of the vesicle, show that combining $BMIBF_4$ and PMB together permeabilize the membrane significantly more than with PMB or the IL alone. This demonstrates that ILs can assist in antibiotic permeabilization of lipid bilayers which could explain the increased antibiotic activities in the presence of ILs in solution.

1. Introduction

Ionic liquids (ILs) are useful solvents and additives in many chemical and biochemical applications because of their unique benefits including widely tunable properties achieved via a cation-anion mix-and-match approach. It has become common to explore biomedical applications of ILs in aqueous environments, and ILs have been proposed for enzyme inhibition and modulation [1–4], drug delivery [5–8], and antibiotic enhancement [9–13]. Combined with proteins in aqueous solution, ILs can exhibit properties similar to electrolytic salts explained by the Hofmeister series [14–18] or behave in a surfactant-like manner [5,19–22] and partition between aqueous and organic phases [23–27].

Several recent studies have focused on interactions between ILs and lipid vesicles. Gradual addition of low-concentration ILs to 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) vesicles in water increases the bilayer stability without modifying the layer thickness due to an IL screening effect of the polar head groups [28]. This is similar to the effects of salts on DPPC vesicles in water. Hydrophobic ILs insert into lipid bilayers [8,9,13,25–31], which has been credited as a cause of IL cytotoxicity and opened the possibility of using ILs as antibacterial compounds [10,32–35]. ILs inserting into vesicles can alter the membrane curvature leading to a “buckling effect” and micelle nucleation formed by expulsion of lipids from bilayers [25]. Longer alkyl chains on IL cations can more easily embed in the bilayer, increasing their cytotoxicity [26]. IL cationic insertion into the bilayer restructures the membrane [25,29,36] and appears to be energetically favorable, irreversible [25,29], and independent of the lipid type [29].

The membrane insertion and structural destabilization effects of ILs could be beneficial if combined with known membrane-disrupting antibiotic compounds. Antibacterial drug studies on cells have shown that adding ILs enhances the antibiotic activities [8,10,33]. ILs have also been incorporated with enzyme-hydrolyzing groups, alkyl chains, and stable anions with reported antibacterial agents effects [37]. Additionally, a tetracycline-based IL has been shown to insert into liposome membranes with important drug-delivery and antibiotic implications [13]. Molecular dynamics simulations of ILs combined with a gramicidin A dimer on a lipid bilayer show that in the presence of ILs attractive electrostatic interactions between lipid head groups significantly increase the lateral diffusivities of gramicidin A peptides

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and lipids [11,12,38]. Thus, combining ILs with antibiotics has the potential to significantly enhance lipid bilayer membrane destabilization, destructuralization, and overall permeability with applications in antibacterial pharmaceutical techniques.

Polymyxin B (PMB) is a strongly cationic polypeptide antibiotic originally derived from *P. polymyxa* that acts by binding to the lipopolysaccharide (LPS) components of the bacterial membrane of Gram-negative bacteria [39,40]. The charged amino acid residues in PMB interact with the lipopolysaccharide carboxyl and phosphate groups to first displace Ca^{2+} and Mg^{2+}, followed by a deeper insertion into the bilayer which disrupts the bacterial outer membrane [39–44]. Subsequent action on the cytoplasmic membrane has also been reported [40]. Because of the membrane-centric mechanism of action, numerous biophysical studies on the interaction of PMB with model bilayers and vesicles have followed [45–48]. We can investigate the potential for ILs to assist and/or enhance antibiotic activities by characterizing the effects of different ILs combined with PMB on model lipid vesicles in solution. By studying the effects of membrane-permeable molecules on simple model lipid vesicles, and focusing on the membrane destabilization and permeabilities, we can elucidate the fundamental mechanisms of IL-enhanced antibiotic activity.

In this work we study the combination of the ILs 1-butyl-3-methylimidazolium chloride (BMICl) and 1-butyl-3-methylimidazolium tetrafluoroborate ($BMIBF_4$) with PMB and the effects of this combination on model lipid vesicles. In controlled environments, vesicle size changes reflect changes in their membrane structure and stability which provides insights to the effects of the permeability of molecules across the bilayer [49–51]. We utilize conventional biophysical chemistry techniques including light scattering to quantify vesicle size changes. Direct measurements of the lipid bilayer permeability changes induced by ILs, PMB, or both were investigated using PMB dye leakage assays. These experiments rely on fluorescent molecules entrapped within the lumen of lipid vesicles which exhibit differential fluorescence properties upon leakage out of the vesicle and are thus probes of membrane transportability and permeabilization [52,53]. The effects of BMICl and PMB together on model vesicles are mostly additive, but the combined effects of $BMIBF_4$ and PMB are synergistic in that the change in vesicle size and membrane permeabilization is much more dramatic when the IL and antibiotic are combined than with either species alone.

2. Experimental

2.1. Scattering-based assay for vesicle size changes

1,2-Dioleoyl-*sn*-glycero-3-Phospho-rac-(1-glycerol) (DOPG) and 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids, Inc. DOPC and DOPG stock solutions were prepared with concentrations of 31.8 and 28.9 mM, respectively, in chloroform. Multilamellar vesicles (MLVs) were prepared by mixing 98% DOPC with 2% DOPG in chloroform and evaporating the solvent under nitrogen flow followed by further drying under vacuum for 1 h. The resultant film was dissolved in 5 mM pH 7 HEPES buffer; the MLVs formed spontaneously after vigorous mixing. The final lipid concentration for all samples was 2 mM.

The ILs 1-butyl-3-methylimidazolium chloride (BMICl), and 1-butyl-3-methylimidazolium tetrafluoroborate ($BMIBF_4$) were purchased from Sigma Aldrich. IL stock solutions were prepared at 3.4 M concentration in HEPES buffer and used immediately after preparation to avoid IL decomposition. Polymyxin B Sulfate (PMB) was purchased from Calbiochem, and a PMB stock solution was prepared at 22.5 mM in HEPES buffer. Solutions were prepared with final concentrations of 2 mM MLVs, ILs, and 1.5 mM PMB in HEPES buffer.

Solution absorbance values at 550 nm were measured in a Molecular Devices Spectramax M5 multimode plate reader. The 5 mM HEPES buffer was used as a blank reference. The absorbance values are proportional to the light scattered by the MLVs. When MLV sizes and/or shapes change, the amount of light scattered changes and hence the absorbance values change. We measured the absorbance at 550 nm of BMICl, $BMIBF_4$, and PMB in HEPES buffer for reference; these absorbance values were negligible and hence any absorbance changes can be attributed primarily to changes in light scattering due to MLV size/shape changes. Absorbance data were corrected for any sample volume changes (which would chance the concentration), but these changes were very small (only ~2–3%). This absorbance-based scattering experiment was run twice with similar results for both experiments. For each experiment at least 4 replicates were measured. Reported values are averages and standard deviations of these replicates.

2.2. DLS measurements for size quantification of unilamellar vesicles

DOPC/DOPG vesicles were prepared in a manner similar to those in Section 2.1. 98% DOPC and 2% DOPG were mixed in chloroform, and the solvent was removed under nitrogen flow and vacuum. The film was dissolved in 5 mM HEPES buffer pH 7 for a final lipid concentration of 2 mM and vortexed vigorously. Prior to adding ILs or PMB, all solutions were sonicated using a Branson Sonifier 250 with a 1/8 in. tapered Branson microtip for 2 min. Pulses were applied at a duty cycle of 20 and an output of 2. After sonication, the lipids formed relatively monodisperse, small unilamellar vesicles (SUVs). ILs and PMB (prepared in 5 mM HEPES buffer at pH 7) were added to the SUV solutions. Solutions were allowed to equilibrate at room temperature for 30 min before measurements. Particle sizes were measured using a Brookhaven Instruments Corporation dynamic light scattering (DLS) instrument. For each DLS size measurement, 5 DLS scans were averaged together to generate an average particle size. Standard deviations in the particle sizes were all less than 10%.

2.3. $Ru(BiPyr)_3^{2+}$ leakage experiments

Tris (2,2′-bipyridyl) ruthenium (II) ($Ru(bpy)_3^{2+}$) was purchased from Fluka, and a 60 mM stock solution was prepared in HEPES buffer. Lipid vesicles with entrapped $Ru(bpy)_3^{2+}$ were prepared as above with several modifications. The final lipid concentration in the samples for the $Ru(bpy)_3^{2+}$ experiments was 20 mM. After MLV formation, the lipid solution was frozen using liquid nitrogen and thawed in a hot water bath at 55 °C. This freeze-thaw cycle was repeated ten additional times, ending the cycle by thawing the vesicles completely. The vesicles were then extruded twenty-one times through two stacked 0.2 μm polycarbonate filter membranes in a syringe extruder (Avanti Polar Lipids, Inc.).

Vesicles were separated from unincorporated $Ru(bpy)_3^{2+}$ by size exclusion chromatography using G50 Sephadex. The column was allowed to equilibrate in HBS (50 mM HEPES, 50 mM NaCl pH 7) under gravity flow for approximately 1 h before the vesicle solution was loaded onto the column. Fractions were then collected from the column in 1 mL aliquots. Vesicles with $Ru(bpy)_3^{2+}$ entrapped inside exhibited an orange color and were separated from the untrapped $Ru(bpy)_3^{2+}$ (which exhibited a red color).

The two most concentrated fractions of vesicles entrapped with $Ru(bpy)_3^{2+}$ were combined with 0.5 mL of the third most concentrated fraction. The lipid concentration was roughly 1.5 mM based on control dilution experiments. Then, the following samples were made in HEPES buffer with final concentrations of 0.25 mM vesicles in all of them: 0.2, 0.4, 0.6, and 0.8 M $BMIBF_4$, 0.6 M NaCl, 0.6 M BMICl, 1.5 mM PMB with 0.2, 0.4, 0.6, and 0.8 M NaCl, 1.5 mM PMB with 0.2, 0.4, 0.6, and 0.8 M $BMIBF_4$, and 1.5 mM PMB with 0.2, 0.4, 0.6, and 0.8 M BMICl. Samples were incubated at room temperature for 30 min prior to measurement. Fluorescence lifetimes of these samples were measured in a homebuilt laser-induced fluorescence (LIF) spectrometer. This instrument is composed of a Continuum Minilite Nd:YAG laser (Continuum, Inc.) and an Oriel Cornerstone 130 monochromator. The 355 nm output of the laser excited the sample and the fluorescence at