

# Antimicrobial effects of short chained imidazolium-based ionic liquids—Influence of anion chaotropicity

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## ARTICLE INFO

### Article history:

Received 8 January 2014

Received in revised form

27 August 2014

Accepted 28 August 2014

Available online 22 October 2014

### Keywords:

Ionic liquids

Hofmeister effects

Antimicrobials

Toxicity

*Listeria monocytogenes*

*Escherichia coli*

## ABSTRACT

Ionic liquids (ILs), a new solvent class composed solely of ions, have already found their way into numerous chemical and biochemical applications. Due to their unique properties and wide application range, research utilizing this new technology for biotechnological applications is steadily increasing. However, progress is hampered by lack of toxicological data, especially concerning IL anions and their general underlying toxicity mechanisms. The present study investigated for the first time the influence of the chaotropicity of the anion for nine imidazole based ILs on their antimicrobial behavior. The results indicate that for ILs with small cations ( $[C_n\text{mim}]^+$  with  $n=2$  and 4), the chaotropicity of the anion is a major factor regarding antimicrobial behavior, while for  $[C_6\text{mim}]^+$  based ILs a surfactant-like behavior was identified that explains their high toxicity. It could also be shown that with increasing anion chaotropicity the surfactant-like behavior of the cation is strengthened. Identification of chaotropicity as an underlying mode of antimicrobial action of ILs presents a new point of adjustment for future design with regard to their toxicological behavior.

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## 1. Introduction

Over the past decade a new solvent class with unique physicochemical properties, called ionic liquids (ILs), has produced enormous interest from both researchers and industry. Much of the interest in ILs originated from their possible application as environmentally favorable ‘green’ alternatives to organic solvents in chemical processes (Sheldon et al., 2002) and the possibility to combine various anions and cations to create new ILs with unique properties. Due to their initial application as organic solvent replacements, the main focus lay on IL properties, such as negligible vapour pressure, chemical and thermal stability, nonflammability, high ionic conductivity, wide electrochemical potential window and solvation ability. Since their initial success in chemical applications, ILs have also received attention as co-solvents for water for biochemical applications, such as enzymatic assays, protein refolding, crystallization and many others (Fujita et al., 2005; Liu et al., 2009; Sheldon et al., 2002; Yang, 2009).

Since it became apparent that IL usage will not be limited to academic research, but that they will also be implemented in industrial processes, the ecotoxicology and environmental fate of

ILs have come into focus. ILs were considered as ‘green chemistry’ mainly due to their negligible vapor pressure and the resulting reduced air pollution compared to volatile organic compounds (VOCs). Although therefore ILs are unlikely air contaminants or inhalation toxins, they can be potential soil and water pollutants in the case of an industrial spillage event or via wastewater. There have been some studies so far that studied the environmental fate of ILs in terms of their persistence in air, water, and soil; (bio) degradation; migration in groundwater; bioaccumulation in aquatic or terrestrial organisms and the (eco)toxicity (Bernot et al., 2005; Pham et al., 2010; Pretti et al., 2006; Stolte et al., 2007; Ventura et al., 2012; Zhao et al., 2007; Bubalo et al., 2014; Das and Roy, 2013). In this context, to date, a strong relationship between the length of the alkyl-side chain as well as ring planarity of the cation and the respective IL toxicity has been identified, at least for the imidazolium based ILs (Docherty and Kulpa, 2005; Pham et al., 2010; Ranke et al., 2004, 2007; Ventura et al., 2012; Viboud et al., 2012; Wood et al., 2011).

In contrast to the level of understanding about the impact of different structural elements of the cation, the contribution of the anion to the overall toxicity of ILs is still not clear. So far there seems to be a marginal influence of the anion on the toxicity of ILs, despite some disagreement in the literature (Arning et al., 2008; Stolte et al., 2006; Ventura et al., 2012; Wang et al., 2011). The most toxic ‘common’ anions reported so far are bis

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(trifluoromethylsulfonyl)imide and octylsulfate, while tetrafluoroborate and hexafluorophosphate were considered less toxic. The only systematic explanation of anion induced toxicity found a relationship between the lipophilicity or the number of fluorine atoms and their toxicity towards prokaryotic cells (Cho et al., 2008; Stolte et al., 2006; Wang et al., 2011). To the best of our knowledge, so far Hofmeister effects have not yet been considered as a systematic approach for studying the anion induced toxicity towards microorganisms, although Constantinescu et al. (2007) found a clear connection between inhibition of enzyme activity with increasing chaotropicity of IL anions. This was surprising, as Hofmeister effects are well known to be connected to the toxicity of different ions towards microorganisms (Kunz et al., 2004). In general, Hofmeister effects or sequences refer to the relative effectiveness of anions or cations, on a wide range of phenomena in aqueous solution such as solubility of salts, to electrolyte activities, surface tensions to ion exchange resins, micellar cmcs, cloud points of non-ionic surfactants, ion binding to micelles, proteins and membranes, transport across membranes and colloid stability (Kunz et al., 2004). In previous studies, our group identified the chaotropicity of the anion as a major factor regarding the protein-destabilizing properties of imidazolium based ILs with short side chain lengths (< 8) (Mester et al., 2010, 2012).

This work addresses the toxicity of such imidazolium based ILs towards two model microorganisms with a focus on the chaotropicity of the anion. The final goal is to improve understanding of the active role of the anion on toxicity towards microorganisms.

In this study the respective minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of each IL was determined by the well established microplate dilution method with Gram-positive *Listeria monocytogenes* and Gram-negative *Escherichia coli*. In addition to the MIC and MBC, the short-term induced (30 min) toxicity was also studied, to compare the results of the toxicity tests with previous studies, which investigated the effect of similar ILs on protein levels.

## 2. Materials and methods

### 2.1. Ionic liquids and bacterial strains

All ILs were provided by Merck (Darmstadt, Germany) and were of synthesis grade. The purity provided by the manufacturer was given with 99.5 percent containing < 800 ppm water and < 100 ppm halides as impurities. A list of all investigated ions is shown in Table 1.

*L. monocytogenes* EGDe (1/2a, internal number 2964) was used as a model organism for Gram-positive bacteria. *E. coli* was used as a model organism for Gram-negative bacteria. The bacteria were maintained at −80 °C using MicroBank™ technology (Pro-Lab Diagnostics, Richmond Hill, Canada) and were part of the collection of bacterial strains at the Institute of Milk Hygiene, Milk

Technology and Food Science, University of Veterinary Medicine, Vienna, Austria. All bacterial strains were grown overnight in tryptone soya broth with 0.6 percent (w/v) yeast extract (TSB-Y; Oxoid, Hampshire, United Kingdom) at respective growth temperatures (37 °C for *L. monocytogenes* and *E. coli*).

### 2.2. Minimal inhibitory (MIC) and minimal bactericidal (MBC) concentration assessments

Minimal inhibitory and bactericidal concentrations of the examined ILs were determined by the serial two-fold dilution microtiter plate method in TSB-Y medium (Morrissey et al., 2009). To ensure a constant cell status at the beginning of each experiment, one ml of the respective overnight culture of either *E. coli* or *L. monocytogenes* was transferred into 9 ml fresh TSB-Y medium and incubated for 3 h at 37 °C to ensure that the cells were in the logarithmic growth-phase. Afterwards each well containing serial diluted ILs was inoculated with  $5 \times 10^5$  CFU/ml of the respective bacterial cells. For MBC determinations the exact initial CFU concentration of each experiment was determined by the plate count method on TSA media. After inoculation with the respective bacteria, the 96-well microtiter plates (Corning B.V. Life Sciences, Amsterdam, Netherlands) were measured at 610 nm wavelength in a TECAN F100 microplate reader (Tecan Austria GmbH., Groedding, Austria) to check for possible interference of the ILs at the given wavelength. The microtiter plates were then incubated for 18 h at 37 °C and afterwards microbial growth was assessed by measuring absorbance at 610 nm. The MIC was defined as the lowest IL concentration where no growth of the respective bacteria could be measured within 18 h. To assess the MBC, the CFU/ml of the four wells containing the lowest concentrations of the respective IL, which showed no bacterial growth (including the MIC), were determined by tenfold dilution series in PBS and the plate count method. The MBC was defined as the lowest concentration leading to 99.9 percent reduction of the initially inoculated CFU. Each experiment was performed in triplicate on different days and the calculated average MIC and MBC values, as well as the standard deviation, are presented. Each experiment included positive and negative controls for the respective bacteria.

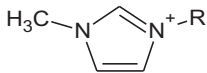
### 2.3. Short-term antimicrobial activity

Short-term exposures (30 min at 37 °C) of different concentrations (0–2 M) of the ILs ([*C<sub>n</sub>*mim][Cl] and [SCN] with *n*=2, 4 and 6; [*C<sub>4</sub>*mim][DCA], [TCA] and [MeOSO<sub>3</sub>]; [*C<sub>4</sub>*pyr][DCA]) in  $1 \times$  phosphate buffered saline with a pH value of 7.4 (PBS) were performed on Gram-negative (*E. coli*) and Gram-positive (*L. monocytogenes*) bacteria. Afterwards, the samples were centrifuged at  $8000 \times g$  for 5 min at room temperature and the supernatant was discarded. The pellet was washed twice in 1 ml PBS with additional centrifugation for 5 min at  $8000 \times g$ . Viable cell quantification from the remaining bacterial pellet was performed using the plate count method (PCM) on nonselective tryptone soya agar plates supplemented with 0.6 percent (w/v) yeast extract (TSA-Y, Oxoid, Hampshire, United Kingdom). All experiments were repeated at least twice.

## 3. Results and discussion

The present work examined the influence and the underlying mechanisms of action of different anions of imidazole-based ILs, with varying cationic side chain lengths, on their antimicrobial activity to Gram-negative *E. coli* and Gram-positive *L. monocytogenes*. For each IL and bacterial species the minimal inhibitory concentration (MIC) as well as the minimal bactericidal

**Table 1**  
Abbreviations and names of all investigated ions.

| Cation                                                                              |                               | Anion                 |                   |
|-------------------------------------------------------------------------------------|-------------------------------|-----------------------|-------------------|
|  |                               | X <sup>−</sup>        |                   |
| Na                                                                                  | Sodium                        | [MeOSO <sub>3</sub> ] | Methylsulfate     |
| [C <sub>2</sub> mim]                                                                | 1-Ethyl-3-methylimidazolium   | [Cl]                  | Chloride          |
| [C <sub>4</sub> mim]                                                                | 1-Butyl-3-methylimidazolium   | [DCA]                 | Dicyanamide       |
| [C <sub>4</sub> pyr]                                                                | 1-Butyl-1-methylpyrrolidinium | [SCN]                 | Thiocyanate       |
| [C <sub>6</sub> mim]                                                                | 1-Hexyl-3-methylimidazolium   | [TCA]                 | Tricyanomethanide |

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