Antibacterial Activity of Short-Chained 1-Alkyl-3-methylimidazolium Bis(trifluoromethylsulfonyl) Imide Ionic Liquids

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(Received 15 April 2014, Accepted 2 September 2014)

The antibacterial activity of a series of ionic liquids containing short-chained 1-alkyl-3-methylimidazolium cations ([C\textsubscript{n}mim\textsuperscript{+}]; n = 2, 4, 6 and 8) and bis (trifluoromethylsulfonyl) imide anion ([Tf\textsubscript{2}N]) against \textit{E. coli} and \textit{B. subtilis} was measured, for the first time. All ILs used in this work were synthesized and analyzed by Fourier transform infrared (FT-IR) spectroscopy, NMR, and Karl-Fischer titration. Antimicrobial activity was determined by the tube dilution method. The ILs investigated showed antibacterial activity against \textit{E. coli} and \textit{B. subtilis}. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were in the ranges of 0.041-6.39 mM and 0.67-12.78 mM, respectively. The antibacterial activity for ionic liquids with longer alkyl chain (n \geq 4) increased with increasing alkyl chain length. The highest inhibition against \textit{E. coli} was found for [C\textsubscript{4}mim][Tf\textsubscript{2}N] (MIC = 0.041 mM and MBC = 0.67 mM). The mechanism of action of these ionic liquids was bacteriostatic.

**Keywords:** Ionic liquid, Antibacterial activity, Imidazolium salts, Bis (trifluoromethylsulfonyl) imide

**INTRODUCTION**

Ionic liquids (ILs), a remarkable class of coulombic fluids, as potential environmentally friendly solvents have garnered widespread attention from the academic and industrial research communities due to their unusual and useful properties [1]. ILs are salts with low melting point that result from the combination of organic cations such as ammonium, imidazolium, pyridinium, pyrrolidinium, quaternary phosphonium and various, usually inorganic anions, such as TfsN (bis (trifluoromethanesulfonyl) imide), BF\textsubscript{4} -, PF\textsubscript{6} -, Br -, etc. Ionic liquids have a diverse array of applications, ranging from synthetic and separation/extraction chemistry to a number of applications in biological processes [2-5]. One of the most common anions in ILs is bis(trifluoromethanesulfonyl) imide anion, TfsN, which apart from its effectively reduced melting point [6], has also reduced density and ionic interactions [8], and enhanced ionic conductivity. These effects have tentatively been attributed to characteristics such as the low symmetry and the bulky nature of TfsN, as well as its flexibility and extensive charge delocalization [6]. The physicochemical and biological properties of ILs depend on the species of cation, anion, and the length of the alkyl groups on the heterocyclic rings [3,9,10]. For example, low symmetry of the cation is believed to be responsible for the low melting points of ILs while the nature of the anion is considered to be responsible for many of the physical properties of ILs such as their miscibility with conventional solvents [11].

ILs have generally known to be bioactive substances and have been widely used because of their broad spectrum of biocide activity [12]. Numerous studies have demonstrated

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the toxicity of various classes of ionic liquids towards both prokaryotic and eukaryotic organisms [13-17]. The antimicrobial activity of imidazolium, pyridinium, and quaternary ammonium ionic liquids was evaluated against both environmental and clinically important microorganisms [18-23]. The most extensively studied type of ILs is the 1-alkyl-3-methylimidazolium range of salts, mainly because of their air and moisture stability. DemberelNyamba et al. described the antimicrobial effects of a series of 1-alkyl-3-methylimidazolium halides [Cn mim]X (where [Cn mim] = 1-alkyl-3-methylimidazolium; Cn = C1nH2n+1; n = 4, 6, 8, 10, 12, 14, 16, 18; and X = Cl, Br) against seven strains of bacteria and fungi [18]. Ranke et al. reported that ILs containing 1-propyl-3-methylimidazolium to 1-decyl-3-methylimidazolium cations and Cl, BF4, and PF6 anions were active against vibrio fischeri, IPC-81 cells, and C6 glioma cells [24]. Pernak’s group evaluated the antibacterial activity of a series of pyridinium, imidazolium, and quaternary ammonium ionic liquids against various bacteria including rods, cocci, and fungi [25,19-23].

The numerous studies showed that the antimicrobial activity of ILs depends on the alkyl chain length of the cation [18-23,25,26]. The antibacterial activity of butyl, hexyl and octylimidazolium and pyridinium bromide ILs on the growth of Bacillus subtilis (B. subtilis), Escherichia coli (E. coli), Pseudomonas fluorescens (P. fluorescens), Staphylococcus aureus (S. aureus), and Saccharomyces cerevisiae (S. cerevisiae) was investigated by Docherty and Kupla. They found that the antibacterial activity improved by increasing the alkyl group chain length as well as increasing the number of alkyl groups substituted on the cation ring [27]. Some studies showed that the anion of the ILs containing imidazolium cation does not have any significant effect on their antimicrobial activity [19,21,27,28]. This is not true for phosphonium ILs [25], as Cieniecka-Ros1konkiewicz et al. showed that both cation structure and the type of anion have an influence on the biological activity of alkyltriethylphosphonium ILs. They showed that replacement of the halide anion with more complex anions causes the antimicrobial properties to decrease [25]. However, numerous studies in the literature showed that 1-alkyl-3-methylimidazolium ILs have antibacterial activity. Most of studied anion counterparts were halides, tetrafluoroborate, and hexafluorophosphate. Few studies reported the antibacterial activity of short-chained 1-alkyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide ionic liquids ([Cn mim][Tf2N]). Lee et al. investigated the antibacterial activity of [C6 mim][Tf2N] and [C6 mim][Tf2N] only against E. coli [29]. The dependence of the antimicrobial activity of imidazolium ILs on chemical structure-chain length and anion type of 30 ILs was investigated by Łuczak et al. [14]. They showed that short-chained imidazolium ILs [C2 mim] and [C4 mim] with chloride anion have weak antimicrobial activity. Exchanging the chloride anion for BF4 or OctOSO3 resulted in an increase of antibacterial activities of ILs containing short-chained cations against E. coli and S. cerevisiae. Increasing the hydrocarbon chain length of [C2 mim] to give [C8 mim] resulted in a 15- and 130-fold decrease in the MIC for ILs with OctOSO3 and Cl anions, respectively. They showed that the antifungal activities of ILs possessing the cation [Cn mim] and different anions decrease in the order of Tf2N > OctOSO3 > BF4 > pTs > MeOSO4 > Cl > TFMS [14].

The optimum biological activity of ILs is affected by the combination of several physicochemical parameters including hydrophobicity, critical micelle concentration, adsorption, aqueous solubility, and transport in the test medium [30]. The solubility of imidazolium-based ILs in water is dependent on both the cation alkyl chain length and the anion identity [31]. Freire et al. [31] observed that an increase in the alkyl chain length on the imidazolium ring causes a decrease in the solubility of imidazolium-based ILs with water. They also found that the hydrophobicity of the anions decreases in order of BF4 > C(CN)3 > PF6 > Tf2N [31]. Despite the lowest solubility of [Cn mim][Tf2N] in water [31], this species showed the highest antifungal activity against Candida albicans compared to some other types of anions [14]. There seems to be an improved biological activity for Tf2N anion with short chain alkyl imidazolium cations.

Gram negative E. coli and Gram positive B. subtilis are common pathogens in human diseases [32-34]. These are yet safe, cultured fairly easily, inexpensively in a laboratory setting, and widely used to bacterial experiment. They also show their unique cell envelope structure of Gram negative and Gram positive bacteria. Therefore, we selected E. coli.
and *B. subtilis* for the antibacterial study. Here, we investigate the antimicrobial activity for four short chain alkyl imidazolium ILs: 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide ([C$_2$ mim][Tf$_2$ N]), 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide ([C$_3$ mim][Tf$_2$ N]), 1-hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide ([C$_6$ mim][Tf$_2$ N]), and 1-octyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide ([C$_8$ mim][Tf$_2$ N]) against *E. coli* and *B. subtilis*. To the best of our knowledge, this is the first report on the antimicrobial activity of short-chained imidazolium ILs (n = 2, 4, 6, and 8) with a Tf$_2$ N$^-$ anion against *B. subtilis*. Furthermore, no study has investigated the antibacterial activity of 1-ethyl- and 1-octyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide.

**EXPERIMENTAL**

**Materials**

All ILs used in this work were synthesized according to the literature [35]. They were analyzed by Fourier transform infrared (FT-IR) spectroscopy, NMR, Karl-Fischer titration for water content, and ion chromatography for chloride content. In all cases, the water mass fraction was found to be less than $8 \times 10^{-4}$ and chloride mass fraction was less than $5 \times 10^{-6}$. The formula, molecular weights, and melting points are summarized in Table 1. All other chemicals were of analytical grade and used as received without further purification. Standard strains of *E. coli* DH 5α (Cinnagen, Iran) and *B. subtilis* PTCC 1365 (persian type culture collection, Iran) were kindly supplied by Dr. Mansour Mashreghi, Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad.

**Characterization Techniques**

The FT-IR spectra of the ILs were recorded at room temperature with a KBr pellet on a Shimadzu 4300 Spectrometer ranging from 450-3600 cm$^{-1}$. The equipment used for NMR spectra is Bruker 300 MHz NMR spectrometer.

**Bacterial Culture and Measurement of Antibacterial Activity**

Antimicrobial activity was determined by the tube dilution method. Two-fold dilutions of each imidazolium salt were prepared in LB (Luria Broth) over the range 0.00024-1% v/v. *E. coli* and *B. subtilis* cultures were prepared by inoculating 5 ml of LB with a colony of bacteria from a stock plate and incubating the inoculum for 18-24 h at 37 ºC on a reciprocal shaker (120 rpm). Overnight broth cultures were serially diluted with LB media and used for each experiment. Suspensions of bacteria at the concentration of $1.2 \times 10^{7}$ colony-forming unit per milliliter (CFU ml$^{-1}$) were added to each dilution mentioned above in 1:1 ratio. The growth of the microorganisms was determined visually after incubation for 24 h at 37 ºC. The lowest concentration at which there was no visible growth (turbidity) was taken as the MIC. After determination of MICs, 25 µl of each tube contents (2 ml) was smeared on LB agar medium and incubated for 48 h at 37 ºC. The lowest concentration of the imidazolium salts supporting no colony formation was defined as the MBC. Positive and negative controls were included for each assay.

**Table 1. Formula, Molecular Weights, and Melting Points of the Studied ILs**

<table>
<thead>
<tr>
<th>ILs</th>
<th>Formula</th>
<th>MW (g mol$^{-1}$)</th>
<th>$T_m$ (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C$_2$mim][Tf$_2$N]</td>
<td>C$<em>8$H$</em>{11}$N$_3$F$_6$S$_2$O$_4$</td>
<td>391.3</td>
<td>288</td>
</tr>
<tr>
<td>[C$_3$mim][Tf$_2$N]</td>
<td>C$<em>{10}$H$</em>{15}$N$_3$F$_6$S$_2$O$_4$</td>
<td>419.36</td>
<td>271</td>
</tr>
<tr>
<td>[C$_6$mim][Tf$_2$N]</td>
<td>C$<em>{12}$H$</em>{19}$N$_3$F$_6$S$_2$O$_4$</td>
<td>447.42</td>
<td>267</td>
</tr>
<tr>
<td>[C$_8$mim][Tf$_2$N]</td>
<td>C$<em>{14}$H$</em>{23}$N$_3$F$_6$S$_2$O$_4$</td>
<td>475.48</td>
<td>N/A</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

A series of short-chained 1-alkyl-3-methylimidazolium bis (trifluoromethylsulfonyl) imide salts ([Cₙmim][Tf₂N]; n = 2, 4, 6 and 8) were prepared. Figure 1 shows the FT-IR spectra of the ILs. The strong peaks around 2851 and 2916 cm⁻¹ were attributed to the alkyl chain of the two characteristic bands of the imidazolium ring and peak around 3100 cm⁻¹ corresponds to the symmetric and asymmetric stretching of the C-H bond in the positions four and five of the imidazolium ring [36]. The bands appeared in 700-900 cm⁻¹ can be mainly ascribed to contributions from ring bending modes of the imidazolium cation. The spectral range of 1000-1400 cm⁻¹ shows the most intense IR bands and is dominated by vibrations of the Tf₂N⁻ anion [37]. The migrations of chemical shifts in the ¹H-NMR spectrum is a good indicator for the electron densities at the H and C atoms in the imidazolium-cation. Figure 2 shows the ¹H-NMR spectra for [Cₙmim][Tf₂N] (n = 2, 4, 6 and 8). The ¹H-NMR spectra show that all nuclei of the imidazolium core up to the second position in the side chain are shifted upfield with values between 0.1 and 0.17 ppm. The upfield shifts in ¹H-NMR spectra indicate higher electron density and weaker interionic interaction through H-bonding [37].

All synthesized [Cₙmim][Tf₂N] were screened for antibacterial activity. The calculated average MIC values for E. coli and B. subtilis are shown in Fig. 3 as a relationship between the alkyl chain length of cation and antimicrobial activity. As the figure shows, all the ILs are active against E. coli and B. subtilis and increasing the chain length results in decreasing values of MIC. This indicates that an elongation of the alkyl substituent strongly increases the antimicrobial activity of the ILs. Our results are in a good agreement with observations made by previous studies [13,14,19,21,27,38-40]. Various authors have reported that the aromatic cations (imidazolium and pyridinium) are more toxic than the non-aromatic ILs (pyrrolidinium, piperidinium, phosphonium and ammonium) [41-44]. If the material being tested is bactericidal, the absence of bacterial colonies will be observed upon plating an aliquot of 24-h exposure cultures with no visible turbidity. MBC was recorded as a lowest concentration of an antimicrobial agent killing 99.9% of the bacterial inocula after 48 h incubation at 37 °C. The MBC is usually the same as the MIC, and not more than four times higher than the MIC for bactericidal agents [45,46]. A bacteriostatic agent cannot kill, but inhibits the growth of bacteria. The bacteria will grow when

Fig. 1. The FT-IR spectra of the ILs.

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Fig. 2. $^1$H-NMR spectra of neat (a): [C$_2$ mim][Tf$_2$ N], (b): [C$_4$ mim][Tf$_2$ N], (c): [C$_6$ mim][Tf$_2$ N], (d): [C$_8$ mim][Tf$_2$ N].
Fig. 2. Continued.

it is removed and colonies are observed upon plating. The MBC of bacteriostatic agents are many fold higher than their MIC [47,48]. Bacterial colonies were observed after plating an aliquot of the overnight bacterial cultures containing \([\text{C}_n\text{mim}][\text{Tf}_2\text{N}]\) ILs on LB agar plates. As shown in Table 2, the ratio of MBC to MIC was more than 4 for \([\text{C}_6\text{mim}][\text{Tf}_2\text{N}]\) and \([\text{C}_8\text{mim}][\text{Tf}_2\text{N}]\) ILs. Hence, the mechanism of action is bacteriostatic against both bacterial strains [45,46]. Various mechanisms have been proposed for the antibacterial activity of ILs, including their interaction with cytoplasmic membrane of bacteria and the subsequent loss of permeability properties of the membrane, their interference with respiration and ATP synthesis, membrane leakage in sufficient concentrations, release of the cellular constituents, and even cell death, their aggregation properties like micellation and other association patterns of amphiphilic ILs, and their ability to inhibit acetylcholinesterase [49-51].

According to the baseline toxicity, the hydrophobicity of the cation is responsible for a non-specific disturbance of the structure functioning of biological membranes [52]. Disruption to cell membrane depends on the alkyl chain length of the cation ring and the type of cation present in the IL [13]. Our results showed that the antimicrobial effects of the hexyl- and octyl-substituted ILs depend upon the examined microorganism. The Gram-negative \(E. \text{coli}\) was more susceptible to \([\text{C}_6\text{mim}][\text{Tf}_2\text{N}]\) and \([\text{C}_8\text{mim}][\text{Tf}_2\text{N}]\) compared with the Gram-positive \(B. \text{subtilis}\), similar to

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**Fig. 3.** Relation between alkyl chain length and average log (1/MIC) for \(E. \text{coli}\) (□) and \(B. \text{subtilis}\) (■).

**Table 2.** The MIC and MBC (mM) Values and Ratio of MBC to MIC for ILs

<table>
<thead>
<tr>
<th>Strain</th>
<th>ILs</th>
<th>C(_2\text{mim})</th>
<th>C(_4\text{mim})</th>
<th>C(_6\text{mim})</th>
<th>C(_8\text{mim})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E. \text{coli})</td>
<td>MIC</td>
<td>6.39</td>
<td>5.96</td>
<td>0.17</td>
<td>0.041</td>
</tr>
<tr>
<td></td>
<td>MBC</td>
<td>6.39</td>
<td>5.96</td>
<td>1.40</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>MBC/MIC</td>
<td>1</td>
<td>1</td>
<td>8.23</td>
<td>16.34</td>
</tr>
<tr>
<td>(B. \text{subtilis})</td>
<td>MIC</td>
<td>6.39</td>
<td>5.96</td>
<td>2.79</td>
<td>1.31</td>
</tr>
<tr>
<td></td>
<td>MBC</td>
<td>12.78</td>
<td>11.92</td>
<td>&gt;11.17</td>
<td>&gt;10.52</td>
</tr>
<tr>
<td></td>
<td>MBC/MIC</td>
<td>2</td>
<td>2</td>
<td>&gt;4</td>
<td>&gt;8.03</td>
</tr>
</tbody>
</table>
previous studies by Demberelnyamba et al. [18]. Hence, the microbial inhibition of ionic liquids not only depends on the structure of ILs but also on the structure of the cell membrane. Several studies have demonstrated that the anion contributes to the antibacterial activity of ILs [14,28,43,50,53]. The anions of tetrafluoroborate and hexafluorophosphate were considered less toxic and bis(trifluoromethylsulfonyl) imide and octylsulfate are the most toxic anions [24,27,28,41,43,44,54-56]. Łuczak et al. revealed that both cation structure and the type of anion influence the biological activity of the imidazolium ILs. However, the influence of anion is relatively small. Some differential antibacterial activities were recorded for short-chained cations ([C₄mim] and [C₆mim]) containing different anions by this research group. For example, replacing Cl⁻ with BF₄⁻ was found to result in an increase of antibacterial activity of ILs for these cations. They also observed no difference in antimicrobial activity for ILs with diverse anions and chain lengths of six and more carbons on the imidazolium cation. Lower values of MIC were obtained for [C₄mim][OctOSO₃] (n = 2-8) by Łuczak et al., in which the MIC values were from 22.3 to 2 mM against S. cerevisiae and from 30.1 to 4 mM against E. coli [14]. Here, the calculated average MIC values of studied ILs against E. coli and B. subtilis were in the range of 6.39-0.041 mM and 6.39-1.31 mM, respectively. Gilmore et al. [57] demonstrated that incorporation of tetrachlorocuprate(II) and dibromoargentate(I) anions improved the antimicrobial activity of 1-alkyl-3-methylimidazolium ionic liquids (n = 8-18) compared with the corresponding 1-alkyl-3-methylimidazolium chlorides especially against Gram negative bacteria and fungi. They also reported that [C₄mim][AgBr₂] ILs exhibited higher antimicrobial activity than that of the corresponding [CuCl] salts and the maximum difference between their MIC values was observed for salts with short alkyl chain ([C₄mim]). Some differential antibacterial activities were also obtained by Pernak et al. for imidazolium ionic liquids with an appended alkoxy functional group and anions Cl⁻, BF₄⁻ and PF₆⁻ [21].

By comparing our results with literature data, we suggest that the antimicrobial activity of short-chained [C₄mim][Tf₂N] (n = 2, 4, 6 and 8) ILs may be higher than that of [C₄mim][Cl], [C₆mim][BF₄], and [C₆mim][OctOSO₃] salts. Other than the nature of hydrophobicity, aqueous solubility can affect on the optimum biological activity of ILs and the solubility is the limiting step for the transport in the test medium [30]. Moreover, many bacteria require minute amounts of water to survive, in order to grow and proliferate they need water abundantly. Presumably, a suitable combination of the hydrophobicity and aqueous solubility allow the ILs to have a superior antibiological activity [58]. Therefore, it seems that not only the species of cation and the length of alkyl groups on the heterocyclic rings but also the type of anion are essential factors for designing more effective antimicrobial ILs.

CONCLUSIONS

Four short-chained ([C₄mim][Tf₂N]) (n = 2, 4, 6 and 8) ILs were prepared, characterized, and tested for antimicrobial activity. The imidazolium salts examined in this study exhibit antibacterial activity against E. coli and B. subtilis. The cations with short chains (n = 2 and 4) have low biostatic activities whereas the antimicrobial activity of those with long alkyl chain (n = 6 and 8) show decrease of minimal inhibitory concentrations. The ILs with eight carbon atoms in the alkyl chain of cation shows the highest activity against E. coli.

ACKNOWLEDGEMENTS

The authors express their gratitude to Ferdowsi University of Mashhad for support of this project (grant no. p/2128).

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