Highlighted Article

New room temperature ionic liquids with interesting ecotoxicological and antimicrobial properties

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A B S T R A C T

A new set of room temperature ionic liquids (RTIL), tetrabutylammonium (TBA) salts: formate, acetate, propionate, butyrate, benzoate, nitrobenzoate, cinnamate, salicylate, sulfanilate, linoleate, and oleate, were prepared by neutralization of tetrabutylammonium hydroxide (TBA OH) and the corresponding acid. The compounds showed interesting chemical and biological properties. They are soluble in water and organic solvents producing conducting solutions and are effective against certain Gram-negative as well as Gram-positive bacteria. Notably, they affected some proteins such as bovine serum albumin (BSA) and catalase (CAT) as inferred by following the fluorescence emission spectra.

1. Introduction

Ionic liquids (ILs) are salts that result from the combination of organic cations and various anions. They usually exist as liquids below 100 °C, or even at room temperature (Singh et al., 2008; Liu et al., 2005). The physicochemical properties of ILs depend on the nature and size of both of their cation and anion constituents (Liu et al., 2005). The two most common room temperature ionic liquids (RTILs) contain dialkylimidazolium ion or N-alkylpyridinium ion. The relatively large size of these organic cations compared to simple inorganic cations accounts for the low melting points observed for these organic cations when paired with a variety of anions such as BF$_4^-$, PF$_6^-$, CF$_3$SO$_3^-$(or other complex anions (Luo et al., 2006). The main advantage of ILs is to reduce or eliminate the hazards associated with organic liquids, so they have received considerable interest in various fields of chemistry such as green solvents and separation (Bonhôte et al., 1996; Charles, 2001; Dai et al., 1999; Earle and Seddon, 2000; Gorman-Lewis and Fein, 2004; Nguyen et al., 2007; Pandey, 2006; Piao et al., 2004; Rogers and Seddon, 2003; Schrecker et al., 2008; Shul et al., 2006; Wei and Ivaska, 2003; Zhang et al., 2005). Moreover, enhanced reaction rates obtained in ILs allow the reduction of solvent volumes in the appropriate technological processes, thus reducing costs, risks and possible wastes (Łuczaka et al., 2008).

Some studies (e.g., Gorman-Lewis and Fein, 2004) have demonstrated that ILs can successfully substitute for traditional organic solvents in a wide variety of industrial applications based on Diels-Alder, Heck and Friedel-Crafts reactions as well as others (Earle and Seddon, 2000) where continued development and use of ILs require a better understanding of their effects on the environment. In addition, they are used as acidic catalysts in many reactions (Wasserscheid and Reim, 2000), for their tunable acidity, and as excellent electrolytes because of their large electrochemical window and high ionic conductivity (Zhang et al., 2005). Furthermore, ILs can be used in electrochemical sensors (Wei and Ivaska, 2008) such as plasticizers in ion-selective electrodes. RTILs can be made hydrophobic while retaining ionicity (Bonhôte et al., 1996); this dual property forms the basis for their use as unique separation media for the solvent extraction of ionic species. However, little attention has been paid to possible uses of these salts in pharmaceutical formulation and processing, perhaps because of lack of information on their toxicity. RTILs are capable of dissolving high concentrations of a wide range of organic and inorganic molecules, making them efficient reaction media for complex organic reactions. Their good solvent properties and low vapor pressure make them useful in liquid/liquid extraction processes as alternative to organic solvents (Wilkes, 2004).
RTILs have unique physical, chemical and biological properties and the evolution of the scientific focus on these compounds developed from their physical through their chemical and now biological property sets (Wasserscheid and Keim, 2000). The toxicity, a biological property, has been one of the most highly debatable topics in this field (Shul et al., 2006). Biologically active ions have been used to make new ILs; however, the primary impetus for these materials have been the use of ions of known low toxicity to obtain the IL physical property set (Hough et al., 2007). One of the most exciting recent developments is the use of enzymes and other types of biotransformations in ILs. Antimicrobial quaternary ammonium cations have been recently shown to retain their biological activity (Hough and Rogers, 2007). It was found that IL showed antimicrobial activities against cocci, rods and fungi where a relationship was revealed between the structure of the cation and the antimicrobial activities. Furthermore, in certain limited cases RTILs may adversely affect certain protein functions (Carter et al., 2004). Recently, ionic liquid-based microdrop LPME technique has been successfully demonstrated for the extraction of polycyclic aromatic hydrocarbons, alkylphenols and chloroarainanes (Basheer et al., 2008; Liu et al., 2004).

The properties of ILs can be tailored by modification of their structure was the motive that prompted exploration for new ones by preparation, characterization and description of their properties so that they can be properly utilized in various physical, chemical, biological and medical applications. Towards this end, a new set of tetrabutylammonium (TBA) salts, such as formate, acetate, propionate, butyrate, linoleate and oleate were synthesized and found effective on Gram-negative bacteria like Escherichia coli, Klebsiella sp., Pseudomonas aeruginosa and the Gram-positive Staphylococcus sp. but no effect was observed on the yeast, Candida albicans. In addition, they were effective on the protein bovine serum albumin (BSA) and the catalase enzyme (CAT). The influence of these RTILs on the intramolecular forces that are participating in the folding and stabilizing of the secondary and the tertiary structures of the previously mentioned proteins (BSA and CAT) were investigated.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals used in this study were purchased from a variety of sources and used without further purification. Tetrabutylammonium hydroxide (TBA OH) as a 0.05 M solution of the base in toluene-methanol, propionic acid and linoleic acid were purchased from BDH. Salicylic acid was purchased from Hi Media Laboratories. Formic acid, benzoic acid, butyric acid, nitrobenzoic acid, sulfanilic acid and cinnamic acid were purchased from Reidel de Haen. CAT and BSA were purchased from Sigma. Methylene chloride, acetic acid and other common chemicals were commercially available. Both CAT and BSA were stored frozen at (−20 °C) in phosphate buffer solution, pH 7.0. Concentrations of CAT and BSA were determined spectrophotometrically using molar extinction coefficients of 2.9 × 10^4 M^−1 cm^−1 (at 280 nm) for CAT enzyme (Pernak et al., 2003) and 4.4 × 10^4 M^−1 cm^−1 (at 280 nm) for BSA (Zhang et al., 2008). Conductivity was measured by a PHYWE (C.E.) conductivity meter. Fluorescence spectra were obtained using Perkin Elmer luminescence (series no. 70412) spectrometer equipped with a water-jacketed cuvette holder that was maintained at constant temperature by means of a circulatory water bath. Tests of these compounds for biological activity were made on certain Gram-positive, Gram-negative bacteria and yeast at the Medical Technology Department at the Islamic University of Gaza, as explained below.

2.2. Preparation of RTIL

A solution of 0.1 M of the intended acid was added dropwise to a 200 mL solution of TBA OH until the reaction mixture was acidic. The product, tetrabutylammonium salt, was extracted four times with 20 mL portions of water. The collected aqeous solution was extracted four times with 20-mL portions of methylene chloride and the combined extracts were dried with anhydrous sodium sulfate. The dried extracts were allowed to slowly evaporate at room temperature, leaving a colorless liquid which was dried further by phosphorous pentoxide under reduced pressure.

2.3. Biological activity studies

TBA formate was screened for its antibacterial and antifungal activities. The antimicrobial activities were tested by the agar diffusion plate method (Dos Santos et al., 2000). The bacteria used for the assay were Staphylococcus sp., Klebsiella sp., P aeruginosa, E. coli and the yeast C. albicans. All the microorganisms were obtained from the Microbiology Laboratories of Al-Shifa Hospital, Gaza. The tests were made in the following manner: fresh cultures of the microorganisms were plated on the surface of Muller–Hinton agar plates to yield confluent growth, and sterile filter paper disks (8 mm in diameter) were placed on the surface of the inoculated plates. Thirty microlitre aliquots of different dilutions (1:2, 1:4 and 1:8 in sterile distilled water) of TBA formate (from 0.1 g/ml stock solution) were then applied on top of the disks, and the plates were incubated at 37 °C for 24 h. The diameter of the zone of growth inhibition around the disks was measured to the nearest millimeter. Gentamicin (10 μg) disks were used as a positive control against bacteria.

Additionally, solutions of the other TBA salts (0.1 g/ml) were prepared and tested for their biological activity as described above.

2.4. Fluorescence measurements

Spectrofluorimetric titration study of the interactions of TBA salts with BSA and CAT was performed at 25 °C. Fluorescence emission was recorded for the free enzyme and following each addition of 20 μl aliquots of 0.5 M of TBA salts (incubation time was 2.0 min). Samples of the examined proteins of 2.0 μM each (in 3 mL and a 10 mm path length quartz cuvette) were excited at 295 nm, and emission was recorded in the wavelength range 250–600 nm for both BSA and CAT. Excitation and emission bandwidths were 10 nm.

3. Results

Tetrabutyl ammonium hydroxide and the corresponding monoprotic acids react in stoichiometric amounts in 1:1 ratios forming the TBA salts. These products are liquids that are soluble in water and in organic solvents. The observations obtained from solubility tests are presented in Table 1. The compound was considered soluble, designated solubility by (+), if a drop of the compound dissolves in a few drops (1–5) of the solvent. It was considered sparingly soluble, designated by (±), if it dissolves in more than 10 drops of the solvent. It was considered insoluble, designated by (−) if it did not dissolve in 2 mL of the solvent, as was observed in hexane. In addition, solubility of the examined ionic liquids in water were measured using the relation \( A_M = k / C \), where \( C \) is the molar concentration. The molar conductances of these compounds presented in Table 1. These observations indicate that the solutions of these salts in water are electrically conducting.

The results of antimicrobial activity of TBA formate are presented in Table 2. The data indicate that TBA formate has an

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>W</th>
<th>ALC</th>
<th>ACE</th>
<th>EA</th>
<th>THF</th>
<th>CHL</th>
<th>TOL</th>
<th>HEX</th>
<th>( \lambda_M ) (M^−1 cm^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TBA benzoate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>87.5</td>
</tr>
<tr>
<td>2</td>
<td>TBA butyrate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>105.02</td>
</tr>
<tr>
<td>3</td>
<td>TBA acetate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>95.14</td>
</tr>
<tr>
<td>4</td>
<td>TBA propionate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>97.41</td>
</tr>
<tr>
<td>5</td>
<td>TBA nitrobenzoate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>70.23</td>
</tr>
<tr>
<td>6</td>
<td>TBA salicylate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>81.45</td>
</tr>
<tr>
<td>7</td>
<td>TBA sulfanilate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>82.64</td>
</tr>
<tr>
<td>8</td>
<td>TBA cinnamate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>74.36</td>
</tr>
<tr>
<td>9</td>
<td>TBA linoleate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>29.83</td>
</tr>
<tr>
<td>10</td>
<td>TBA oleate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>31.11</td>
</tr>
<tr>
<td>11</td>
<td>TBA formate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>93.96</td>
</tr>
</tbody>
</table>

Abbreviations: W; ethanol; ALC; aceton; ACE; ethyl acetate; EA; tetrahydrofuran; THF; chloroform; CHL; toluene; TOL; hexane; HEX; +; soluble; −; insoluble; ±; sparingly soluble
inhibitory effect on the growth of the three types of the Gram-negative bacteria. TBA formate was most effective against *Escherichia coli* (as determined by the clear “growth inhibition” zones surrounding the disks).

The biological activity of the other room temperature ionic liquids, tetrabutylammonium salts, are presented in Table 3. All these solutions affected Gram-positive bacteria (*Staphylococcus* sp.) to various degrees. Compounds 6–10 affected the Gram-negative bacteria (*Escherichia coli*) as well. In addition, the effect of these ionic liquids on the proteins BSA and CAT was followed spectrofluorimetrically. Fluorescence emission spectra of the studied enzymes (BSA and CAT) with increasing concentrations of TBA formate are shown in Fig. 1(A and B).

The effects of some of the prepared TBA salts on the mentioned enzymes are presented in Fig. 2.

4. Discussion

A new set of room temperature ionic liquids (TBA salts) was prepared and their properties were explored. These ionic liquids are soluble in water and common organic solvents at room temperature. The solutions of these salts are conductive which confirms their ionic nature. These solvents have a wide range of polarity from highly polar alcohols gradually to weakly polar toluene. This solubility behavior in water and organic solvents is similar to that of surfactants considering their structure. These salts can be environmentally benign with respect to organic solvents where the ever-increasing awareness of the detrimental health and environmental effects of some organic solvents has given chemists the impetus to search for “green” technologies. The ionic liquids are already associated with the term “green” and “green chemistry” (Basheer et al., 2008; Duponta et al., 2000; Kosobucki and Buszewski, 2008). Such solubility in a wide variety of solvents provides flexibility in exploration of their future potential uses.

RTILs might be useful as versatile solvents in the design of controlled release drug delivery systems. Not only there is a wide

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**Table 2**

Biological effect of TBA formate on growth of tested microorganisms.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Growth inhibition zone diameter (mm)*</th>
<th>Klebsiella sp.</th>
<th>Pseudomonas aeruginosa</th>
<th>Escherichia coli</th>
<th>Candida albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBA formate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:2</td>
<td>27</td>
<td>30</td>
<td>37</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>1:4</td>
<td>20</td>
<td>23</td>
<td>32</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>1:8</td>
<td>16</td>
<td>17</td>
<td>20</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 µg disk</td>
<td>10</td>
<td>12</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

* Growth inhibition zone diameters are given in millimeters and represent the clear (no growth) zones surrounding the disks containing TBA formate or the positive control, gentamicin.

**Table 3**

Antimicrobial activity of other TBA salts on Gram-negative and Gram-positive bacteria.

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>“Growth inhibition” zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gram-negative bacteria (Escherichia coli)</td>
</tr>
<tr>
<td>1</td>
<td>TBA benzoate</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>TBA butyrate</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>TBA acetate</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>TBA propionate</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>TBA nitrobenzoate</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>TBA salicylate</td>
<td>17</td>
</tr>
<tr>
<td>7</td>
<td>TBA sulfanilate</td>
<td>18</td>
</tr>
<tr>
<td>8</td>
<td>TBA cinnamate</td>
<td>17</td>
</tr>
<tr>
<td>9</td>
<td>TBA linoleate</td>
<td>16</td>
</tr>
<tr>
<td>10</td>
<td>TBA oleate</td>
<td>18</td>
</tr>
</tbody>
</table>

**Fig. 1.** Representative fluorescence emission spectra of BSA (panel A) and CAT (panel B) with TBA formate both enzymes are 2 µM in phosphate buffer solution pH 7.0 at 25°C. The lines in the direction of arrows correspond to successive additions of 0.5 M TBA formate aliquots. Fluorescence intensities are expressed in arbitrary units (a.u.).
them interesting reservoirs for controlled release (Jaitely et al., 2008). Consequently, the mechanism of action of the compounds in the current contribution seems to be comparable and related to the inhibition of specific components (proteins or lipids) present in the cell envelope of bacteria.

In proteins that contain the three aromatic amino acids tryptophan, tyrosine and phenylalanine, fluorescence is usually dominated by contribution of tryptophan residues, because both their absorbance at the wavelength of excitation and their quantum yield of emission are considerably greater than the respective values for tyrosine and phenylalanine (Ruiz et al., 2003). Tryptophan fluorescence is highly sensitive to environment; therefore, these residues are useful structural probes (Zhang et al., 2008).

We have explored the effect of TBA formate on two proteins, i.e., BSA and CAT. The most studied model of globular protein is serum albumin which plays a vital role in transporting several small molecules. The BSA molecule is made up of a chain of 580 amino acid residues forming three homologous domains (I, II and III), which are divided into nine loops (L1–L9) by 17 disulfide bonds and two tryptophan residues, at positions 134 and 212 of the chain. The loops in each domain are made up of sequences of large–small–large loops forming triplets. Each domain in turn is the product of two subdomains (IA, IB, etc.) (Rafati et al., 2004). In addition, CAT enzyme which plays a major role in the protection of tissues from toxic effects of hydrogen peroxide and partially reduced oxygen species. It has 11 sulfhydryl groups and 15 tryptophan residues per mole of the enzyme (Coban et al., 2007).

Tryptophan emission spectrum for the folded protein has a maximum emission at 335 nm, which shifts to 350 nm when the protein unfolds. As shown in Fig. 1A and B, the maximum fluorescence emission at 350 nm suggested a higher content of exposed tryptophan residue in each of the two enzymes.

Frequently, in proteins, spectral shifts are observed as a result of several phenomena, such as binding, protein–protein association, denaturation, etc.

Denaturation of enzymes can be monitored by several spectroscopic probes such as CD, UV difference spectroscopy, fluorescence and NMR.

Denaturation of proteins may be made by denaturating agents such as urea, guanidine hydrochloride (GdmCl) and/or ionic surfactants. It is important to note that the mechanism of denaturation of proteins by ionic surfactants would occur at much lower concentration of surfactants than those required for other commonly used denaturants such as urea or guanidine hydrochloride. The denaturation process by ionic surfactants depends primarily on the effect of these compounds on the structure of water encapsulating the protein and weakening of the hydrophobic interactions in its tertiary structure. There is an evidence that the initial interaction between ionic surfactants and proteins is predominantly ionic; as their ions bind to groups of opposing charges on the protein and should facilitate unfolding of the protein, which in turn results in exposure of more binding sites (Rafati et al., 2004).

The influence of RTILs on the studied enzymes can be compared with the action of the ionic denaturant guanidine hydrochloride. These denaturants perturb and weaken the optimized electrostatic interactions involving the charged side chains. Del Vecchio et al. (2002) suggested that the cationic surfactant DTAB binds readily to the more easily unfolded C-terminal part of the BSA molecule (Moosavi-Movahedi et al., 1996).

As shown in Fig. 1 the fluorescence spectra are characterized by emission maxima at 350 nm for both BSA and CAT. It can be

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**Fig. 2.** Effect of the studied ILs on BSA (panel A) and CAT (panel B): TBA formate (○), TBA acetate (●), TBA propionate (□), TBA butyrate (■) and TBA linoleate (▲) at 25°C in 20 mM phosphate buffer solution pH = 7.0.
clearly seen that the fluorescence emission maxima at the specified wavelengths increased as a result of interaction with increasing amounts of TBA formate. This increase may be attributed to the influence that was induced upon addition of TBA formate to the enzyme solution during the spectrofluorimetric titration. Such findings can be correlated to the fact that the fluorescence spectra of proteins with a maximum around 350 nm are characteristic of tryptophan residues well buried in the core of the protein, whereas the fluorescence spectra with a maximum around 350 nm are characteristic of tryptophan residues exposed to the aqueous solvent (Del Vecchio et al., 2002).

Moreover, it is interesting to note that the fluorescence emission spectra at 350 nm were increased continuously on increasing the concentration of the specified TBA salts (Fig. 2) suggesting that more tryptophan residues of the hydrophobic region become exposed or accessible to the aqueous phase due to the interaction with TBA salts (Abdalla et al., 2002).

5. Conclusion

A set of new ionic liquids, tetrabutylammonium salts, were prepared, characterized and tested for biological activity. They are effective against Gram-positive bacteria and/or against Gram-negative bacteria.

Interaction of some of these TBA salts with BSA and CAT was detected by spectrofluorimetric titration method. The change in tryptophan emission spectra as a result of addition of these salts onto both CAT and BSA confirmed the denaturation of both enzymes by the influence of tetrabutylammonium salts.

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References


