Ionic liquids as solvents for in situ dispersive liquid–liquid microextraction of DNA

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1. Introduction

Deoxyribonucleic acid (DNA) is an important biomolecule containing the genetic information necessary for the viability of virtually every organism. It is widely investigated within biological and life sciences fields, including genetic engineering [1], DNA profiling [2], and DNA nanotechnology [3]. In chemically complex biological samples that contain proteins, polysaccharides and a variety of metabolites, extracting nucleic acids from this matrix can be challenging and can significantly influence experimental results [4]. Additionally, many experiments are performed on very small samples of DNA. When extracting DNA for challenging downstream experiments, purification and preconcentration of DNA is vital, particularly for trace genetic analysis and amplification using polymerase chain reaction.

A variety of methods have been developed for the extraction of DNA from different biological matrices. Traditionally, the phenol/chloroform method was applied for the isolation of DNA from DNA–protein complexes [5]. This method is based on the fact that proteins can generally be denatured and dissolved into an organic solvent (phenol–chloroform–isopropanol (25:24:1, v/v/v)), while DNA remains in aqueous solution. Eventually, DNA is precipitated by adding ethanol to the aqueous solution. This method has been successfully adopted to isolate DNA from a wide variety of samples including whole blood, platelets, lymph nodes, and bone marrow [6]. Different surfactants, such as cetyltrimethylammonium bromide (CTAB) or sodium dodecyl sulfate (SDS), have been employed in this extraction method [6]. However, this approach has several disadvantages. Firstly, organic solvents are used during the extraction procedures and are often not environmentally benign. Also, the entire extraction process is time consuming (generally requiring 3–4 h), tedious, and requires multiple steps [6]. Several washing and centrifugation steps are often needed for the extraction process thereby increasing the risk of sample contamination or damage [7]. Control over temperature as well as special buffer solutions is often required during the extraction process [5,6].

Currently, commercial DNA extraction kits are available which minimize the use of organic solvent, decrease the risk of sample contamination, and accelerate the extraction process. However, the price of these kits is high and the number of extractions that can be performed is limited. Additionally, the recovery, sensitivity, and purity of DNA extracted using different commercial kits can be highly variable. Some extraction kits require specialized equipment [8,9].

Recently, ionic liquids (ILs) have emerged as novel solvent systems employed in DNA separations [10], ion conductive DNA films [11], and DNA biosensors [12]. ILs are a class of non-molecular solvents with low melting points (<100 °C) produced...
from the combination of various organic cations and organic or inorganic anions. Common IL cations include imidazolium, pyrrolidinium, and phosphonium whereas anions include halides, tetrafluoroborate (BF$_4^-$), hexafluorophosphate (PF$_6^-$), and bis[(trifluoromethyl)sulfonyl]imide (NTf$_2^-$). The different combination of cations and anions produces ILs which possess unique physicochemical properties including nearly negligible vapor pressure at room temperature, wide ranges of viscosity, high chemical and thermal stabilities, and the ability to solvate a wide variety of molecules. In addition, some classes of ILs exhibit lower toxicity than some organic solvents. A previous study by Wang and co-workers employed the 1-butyl-3-methylimidazolium hexafluorophosphate (BMIM-PF$_6$) IL for the direct extraction of DNA from aqueous solution using liquid–liquid extraction (LLE) [13]. It was stated that electrostatic interactions between the cation of the IL and the phosphate groups within DNA played a major role in the extraction. While the study showed the utility of DNA extraction with ILs, more can be done. For example, the authors studied only one IL as the extraction solvent. In addition, relatively large IL volumes in the range of 500–700 μL were employed for each extraction and the extraction was only suitable for DNA samples at low concentrations (lower than 0.01 mg mL$^{-1}$). When samples containing a higher concentration of DNA (0.1 mg mL$^{-1}$) were examined, the extraction efficiency of DNA decreased to below 70% when 700 μL of the IL was used.

In an attempt to examine a larger scope of IL extraction solvents as well as investigate the feasibility of employing a microextraction method that consumes a smaller amount of IL, this study describes an in situ dispersive liquid–liquid microextraction (DLLME) method for the extraction and preconcentration of DNA. IL-based in situ DLLME, first developed by our group in 2009 [14], employs a hydrophilic IL dissolved in an aqueous solution that promotes interactions between analytes and the IL. A metathesis reagent, such as lithium bis[(trifluoromethyl)sulfonyl]imide (LiNTf$_2$), is added to the solution to perform an in situ metathesis reaction producing a water-immiscible IL. Typically, a turbid solution of fine IL microdroplets is formed during the ensuing metathesis reaction that facilitates preconcentration. This approach differs from other IL-based DLLME methods including ionic liquid dispersive liquid–liquid microextraction (IL-DLLME) [15], temperature-controlled ionic liquid dispersive liquid phase microextraction (TILDLLME) [16], and ultrasound-assisted ionic liquid dispersive liquid-phase microextraction (ULIDLME) [17]. These methods utilize organic solvent, heat or ultrasound to disperse the IL phase, respectively. In comparison with the IL-based LLE method, the increased surface area of the IL extraction solvent in the in situ DLLME method often results in higher analyte preconcentration and often eliminates the need of organic dispersive solvents. In addition, the in situ DLLME method often decreases the overall extraction time and requires smaller volumes of the extraction solvent.

In this study, six ILs, namely, 1-butyl-3-methylimidazolium chloride (BMIM-Cl), 1-decyl-3-methylimidazolium bromide (C$_{10}$MIM-Br), 1-hexadecyl-3-methylimidazolium bromide (C$_{16}$MIM-Br), 1-(1,2-dihydroxypropyl)-3-decylimidazolium bromide (C$_{10}$POHIM-Br) and N,N-didecyln-methyl-phosphonium bromide ([C$_{10}$]$_2$NMDG-Br) were applied as extraction solvents in the extraction of DNA. This constitutes the first study to employ DLLME in the extraction of nucleic acids using ILs comprised of various substituents appended to the cation. Using this approach, the extraction of DNA from a complex sample matrix containing metal ions and proteins was studied.

### 2. Experimental

#### 2.1. Reagents

- Imidazole, 1-methylimidazole, 1-chlorobutane, 1-bromodecane, 1-bromohexadecane, acrylonitrile, 3-bromo-1,2-propanediol, benzene and phosphoric acid solution (NMR reference standard, 85% in D$_2$O) were obtained by Sigma–Aldrich (St. Louis, MO, USA). Deuterated dimethylsulfoxide (d$_6$-DMSO) was obtained by Cambridge Isotope (Andover, MA, USA). Sodium chloride, sodium hydroxide, potassium chloride, calcium chloride dihydrate, magnesium chloride hexahydrate, and tris[(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) were supplied by Fisher Scientific (Fair Lawn, NJ, USA). Lithium bis[(trifluoromethyl)sulfonyl]imide (LiNTf$_2$) was purchased from SynQuest Labs, Inc. (Alachua, FL, USA). Albumin, from chicken egg white, and DNA sodium salt from salmon testes (molecular weight = $1.3 \times 10^6$, approximately 2000 bp) were supplied by Sigma–Aldrich. SYBR® Safe DNA gel stain was purchased from Invitrogen (Carlsbad, CA, USA). Stock solutions of albumin and DNA were prepared individually by dissolving 500 μg of each in 1 mL of 20 mM Tris–HCl buffer and the pH adjusted to 8.0 using NaOH. The solutions were stored at –38°C. A working solution of SYBR® Safe DNA gel stain was prepared by dissolving 1.0 μL of stock solution in 10 mL of 20 mM Tris–HCl buffer and the pH adjusted to 8.0 using NaOH. All solutions were prepared with deionized water (18.2 MΩ cm) obtained from a Milli-Q water purification system (Bedford, MA, USA).

#### 2.2. Synthesis of ionic liquids

Six different ILs were examined as extraction solvents in this study for in situ DLLME of DNA. BMIM-Cl, C$_{10}$MIM-Br, C$_{16}$MIM-Br, and ([C$_{10}$]$_2$NMDG-Br were synthesized according to previous studies [14,18,19]. The synthesis of two novel ILs, namely C$_{10}$POHIM-Br and C$_{16}$POHIM-Br, is shown in Fig. 1. Imidazole (0.10 mol) and acrylonitrile (0.13 mol) were mixed and stirred in methanol (10 mL).
at 45 °C for 5 h under nitrogen to obtain 1-cyanoethylimidazolium 1. Methanol and residual acrylonitrile were removed under reduced pressure for 3 h at 65 °C. 1-Bromodecane or 1-bromohexadecane (0.11 mol) was mixed with compound 1 and isopropanol (20 mL). This reaction mixture was refluxed at 60 °C for 8 h to obtain 1-cyanoethyl-3-decylimidazolium bromide 2 or 1-cyanoethyl-3-hexadecylimidazolium bromide 3, respectively. After reflux, the residue was dissolved in chloroform and a 15% (w/w) NaOH aqueous solution was added. After stirring for 5 h, the aqueous layer was removed. The chloroform layer was washed using five aliquots (10 mL) of deionized water until a neutral pH was achieved. The product was dried under reduced pressure for 24 h at 70 °C resulting in 1-decylimidazole 4 or 1-hexadecylimidazole 5. Compound 4 or 5 (0.038 mol) was then dissolved in isopropanol (35 mL) and a 10 mL isopropanol solution containing 3-bromo-1,2-propanediol (0.038 mol) was added slowly to the reaction mixture over a span of 1.5 h at 70 °C. This solution was then refluxed for 7 days at 70 °C. Isopropanol was then removed under reduced pressure at 60 °C for 3 h. The crude product was dissolved in water (150 mL) and washed seven times with 100 mL of ethyl acetate. After purification, water was removed under reduced pressure for 24 h at 70 °C and the product subsequently dried in a vacuum oven for 3 days to afford C10POHIM-Br 6 or C16POHIM-Br 7 in high purity. These two ILs were characterized by 1H NMR and ESI-MS, as shown in Fig. S1 of the supplemental information. The structures of all six ILs examined in this study are shown in Fig. 2.

### 2.3. Instrumentation

High-performance liquid chromatographic analysis was performed using a LC-20AT liquid chromatograph (Shimadzu, Japan) with two LC-20A pumps, a SPD-20 UV/VIS detector, and a DPU-20A degasser. All separations were carried out using an anion exchange column (TSKgel DEAE-NPR, 35 mm × 4.6 mm i.d., 25 µm particle size) with a guard column (TSKgel DEAE-NPR, 5 mm × 4.6 mm i.d., 5 µm particle size) from Tosoh Bioscience (Bellevonte, PA, USA). All separations were performed using two mobile phases (A) 20 mM Tris–HCl (pH = 8) and (B) 1.0 M NaCl/20 mM Tris–HCl (pH = 8). In the analysis of DNA, the separation gradient started with 50:50 of mobile phases A and B, and then was gradually increased to 100% B over 10 min. For the separation of DNA and albumin, the gradient began with 100% A and was gradually increased to 100% B in 20 min. The flow rate was set at 1.0 mL min⁻¹. For DNA and albumin, UV detection was accomplished at 260 nm and 280 nm, respectively.

Extractions were performed using either 0.6 or 2.0 mL polypropylene microcentrifuge tubes. All samples were shaken using a mixer from Barnstead/Thermolyne (Dubuque, IA, USA). Centrifugation was performed in a model 228 centrifuge from Fisher Scientific at a rate of 3400 rpm (1380 × g). Absorbance spectra for the ILs were recorded at 208 nm on a Hewlett Packard 8452A Diode Array Spectrophotometer (Santa Clara, CA, USA) with a quartz cuvette (d = 1 cm). 31P NMR spectra of DNA and the DNA–IL complex were obtained at room temperature (293 K) on a Varian VXRS 400 MHz NMR spectrometer at a resonance frequency of 161.9 MHz. Stock solutions of 2.0 mg mL⁻¹ DNA and 8.5 mg mL⁻¹ C16POHIM-Br IL were individually prepared in deionized water. The samples for 31P NMR analysis were prepared by adding different volumes of these stock solutions into deuterated dimethylsulfoxide (d6–DMSO). The chemical shifts were recorded relative to 85% phosphoric acid, which was used as the external standard.

### 2.4. Extraction procedure

#### 2.4.1. In situ DLLME

The IL-based in situ DLLME method used in this study is depicted in Fig. 3. Briefly, 0.5 mg of C10POHIM-Br IL was added to an aqueous DNA solution in a 2.0 mL microcentrifuge tube. The IL was completely dissolved in the aqueous solution after gentle shaking. An aqueous LiNTf2 solution (1.0 g mL⁻¹) was then added to the microcentrifuge tube resulting in the formation of a turbid solution. The molar ratio of IL to LiNTf2 was 1:1. After shaking for 5 min, the turbid solution was centrifuged for 10 min at a rate of 3400 rpm. A portion of the upper aqueous solution (20 µL) was withdrawn into a syringe and subjected to HPLC analysis. The syringe was rinsed with deionized water multiple times to remove any residual ionic liquid.

#### 2.4.2. Extraction of DNA from sample matrix

A sample matrix of higher complexity was prepared by spiking protein (albumin) or metal ions into the aqueous DNA sample solution. An appropriate amount of the protein stock solution, 15–135 µL, was added to the aqueous DNA solution. The concentration of protein ranged from 0.048 to 0.15 mg mL⁻¹ while the concentration of DNA was kept at 0.015 mg mL⁻¹. Four different metal ions were introduced to the aqueous DNA solution by preparing a matrix containing NaCl (20.4 mg mL⁻¹), KCl (10.3 mg mL⁻¹), CaCl2·2H2O (5.07 mg mL⁻¹), MgCl2·6H2O (5.19 mg mL⁻¹) and DNA (0.015 mg mL⁻¹).

### 3. Results and discussion

#### 3.1. Evaluation of IL extraction performance

The amount of DNA extracted into the water-immiscible IL phase was determined indirectly by analyzing the amount of DNA remaining in the aqueous phase after extraction. A portion of the aqueous solution (20 µL) was subjected to HPLC separation for the
determination of residual DNA. The extraction performance was evaluated using Eq. (1):

$$E = \frac{1 - P_{aq}}{P_{st}} \times 100$$  \hspace{1cm} (1)

where $E$ is the extraction efficiency of DNA, $P_{aq}$ is the peak area of DNA in the aqueous solution after extraction obtained from the 20 µL aliquot and the $P_{st}$ is the peak area of DNA in the standard solution (20 µL injection) without addition of ILs or the metathesis reagent.

Until now, the BMIM–PF$_6$ IL has been the most thoroughly investigated IL for the extraction of DNA [13]. In a previous study, the Gibbs free energy of binding for ILs to DNA was investigated and revealed that hydrophobic interactions between the alkyl chain of ILs and DNA can enhance DNA/IL binding [20] and suggest these interactions might be extended to enhance preconcentration in an IL-based extraction approach. To further explore this hypothesis, a broader class of ILs was used in this study. Three imidazolium–based ILs, namely BMIM–Cl, C$_{10}$MIM–Br, and C$_{16}$MIM–Br were applied using in situ DLLME method. These three ILs possess similar structures but differ in the length of the alkyl chain substituents (from butyl to hexadecyl). It should be noted that the ILs are initially added in their halide form (e.g. BMIM–Cl) and are subsequently transformed to a water immiscible IL (e.g. BMIM–NT$_2$) after addition of the metathesis reagent. As shown in Table 1, with an increase in the chain length of the alkyl substituent appended to the IL cation, the extraction efficiency of DNA ($E$) increased from 8.6% for BMIM–NT$_2$ to 53.6% for C$_{16}$MIM–NT$_2$. The longer alkyl chain substituents were observed to significantly increase the total amount of DNA extracted.

Recent studies have shown that hydrogen bonding interactions from between ILs and DNA when using ILs as a solvent for preserving DNA [21,22]. Therefore, the effect of hydrogen bonding interactions between IL and DNA should be considered when designing IL solvents for DNA extraction. Two specifically designed ILs, namely 1-(1,2-dihydroxypropyl)-3-decylimidazolium bromide (C$_{10}$H$_{24}$OPOHIM–Br) and 1-(1,2-dihydroxypropyl)-3-hexadecylimidazolium bromide (C$_{16}$POHIM–Br) were investigated. Both of these ILs contain two hydroxyl groups which are capable of hydrogen bonding. The extraction efficiency ($E$) for these ILs was compared with that of ILs containing similar structure but lacking hydroxyl functionality. As shown in Table 2, the extraction efficiency for the C$_{16}$POHIM–NT$_2$ IL (52.4%) is higher than that of C$_{10}$MIM–NT$_2$ IL (46.3%), while the extraction efficiency for the C$_{16}$MIM–NT$_2$ IL (95.2%) is significantly higher than that of the C$_{16}$MIM–NT$_2$ IL (53.6%). Another IL, N,N-didecyl-n-methyl-D-glucaminium bromide [(C$_{12}$)$_2$NMDG–Br] IL, which possessed five hydroxyl groups within the carbohydrate moiety and two long alkyl chains in the IL cation structure, was also investigated. An extraction efficiency ($E$) of 92.4% was achieved using this IL. Due to the high DNA extraction efficiency achieved by the C$_{16}$POHIM–Br IL, it was chosen as the extraction solvent for all subsequent experiments.

### 3.2. Residual IL in aqueous phase

After performing the metathesis reaction, not all of the halide-based IL is transformed to the water immiscible IL due to the solubility of the IL and metathesis reagent in the aqueous solution [23]. Therefore, it must be considered that IL remaining in the aqueous phase after metathesis reaction may interact with DNA thereby playing a role in preventing it from being extracted from the aqueous phase. Stock solutions of C$_{10}$MIM–Br, C$_{10}$POHIM–Br and C$_{16}$POHIM–Br ILs were individually prepared in deionized water. Calibration curves were generated by measuring the absorbance of these ILs at different concentrations using a UV–vis spectrophotometer at 208 nm. The calibration curves were used to determine the concentration of ILs remaining in the upper aqueous phase after extraction. The effect of IL remaining in the aqueous phase after extraction was evaluated using Eq. (2):

$$S_{IL} = \frac{C_{aq}}{C_0} \times 100$$  \hspace{1cm} (2)

where $S_{IL}$ is the percentage of IL remaining in the aqueous phase after extraction, $C_{aq}$ is the concentration of IL in the aqueous phase after extraction and $C_0$ is the original concentration of IL in the

### Table 1

<table>
<thead>
<tr>
<th>In situ metathesis reaction</th>
<th>Ionic liquid</th>
<th>Extraction efficiency ($E$) % ($n = 3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMIM–CI + LiNT$_2$</td>
<td>BMIM–NT$_2$</td>
<td>8.6 ± 4.5</td>
</tr>
<tr>
<td>C$_{10}$MIM–Br + LiNT$_2$</td>
<td>C$_{10}$MIM–NT$_2$</td>
<td>46.3 ± 4.2</td>
</tr>
<tr>
<td>C$_{16}$MIM–Br + LiNT$_2$</td>
<td>C$_{16}$MIM–NT$_2$</td>
<td>53.6 ± 3.7</td>
</tr>
</tbody>
</table>

Conditions: DNA concentration: 0.006 mg mL$^{-1}$; IL: 0.1 g; LiNT$_2$/IL (n/n): 1/1; injection volume: 20 µL; extraction time: 30 min; centrifugation time: 10 min; total volume of solution: 400 µL.

### Table 2

<table>
<thead>
<tr>
<th>In situ metathesis reaction</th>
<th>Ionic liquid</th>
<th>Extraction efficiency ($E$) % ($n = 3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_{10}$MIM–Br + LiNT$_2$</td>
<td>C$_{16}$MIM–NT$_2$</td>
<td>46.3 ± 4.2</td>
</tr>
<tr>
<td>C$_{10}$POHIM–Br + LiNT$_2$</td>
<td>C$_{10}$POHIM–NT$_2$</td>
<td>52.4 ± 6.4</td>
</tr>
<tr>
<td>C$_{16}$MIM–Br + LiNT$_2$</td>
<td>C$_{16}$MIM–NT$_2$</td>
<td>53.6 ± 3.7</td>
</tr>
<tr>
<td>C$_{10}$POHIM–Br + LiNT$_2$</td>
<td>C$_{10}$POHIM–NT$_2$</td>
<td>95.2 ± 0.4</td>
</tr>
<tr>
<td>(C$_{12}$)$_2$NMDG–Br + LiNT$_2$</td>
<td>(C$_{12}$)$_2$NMDG–NT$_2$</td>
<td>92.4 ± 0.4</td>
</tr>
</tbody>
</table>

* Conditions: DNA concentration: 0.006 mg mL$^{-1}$; IL: 0.1 g; LiNT$_2$/IL (n/n): 1/1; injection volume: 20 µL; extraction time: 30 min; centrifugation time: 10 min; total volume of solution: 400 µL.

b DNA concentration: 0.063 mg mL$^{-1}$; total volume of solution: 800 µL; other conditions kept the same.
standard aqueous solution before extraction. Therefore, $S_{il}$ represents the percentage of IL that did not undergo metathesis reaction.

As shown in Table 3, a large amount of the $C_{16}$POHIM-Br IL remained in the aqueous phase and did not participate in the metathesis reaction, which may be one reason why a relatively low extraction efficiency (52.4%) was observed when using this IL. In the case of the $C_{10}$POHIM-Br IL, a significantly smaller amount of it remained in the aqueous solution after extraction, which may be one reason why a higher extraction efficiency (95.6%) was observed for this IL. Therefore, the amount of IL remaining in the aqueous phase after extraction appears to play a role in influencing the extraction efficiency.

### 3.3. Effect of IL concentration on DNA extraction efficiency

The effect of IL concentration on the extraction efficiency of DNA was evaluated by increasing the concentration of the $C_{12}$POHIM-Br IL from 0.07 to 0.33 mg mL$^{-1}$ using samples containing four different concentrations of DNA. As shown in Fig. 4, when an IL concentration of 0.07 mg mL$^{-1}$ was employed, the extraction efficiency ($E$) of DNA decreased dramatically from 74.5% to 30.2% when the DNA concentration was increased from 0.005 to 0.10 mg mL$^{-1}$. These relatively low extraction efficiencies appear to be from the saturation effect due to the small volume of IL phase formed [13,24].

As the concentration of IL was increased, more DNA was extracted into the IL phase resulting in an exponential increase in the extraction efficiency for all DNA samples. When the concentration of IL was increased to 0.27 mg mL$^{-1}$ and above, which was much higher than the highest concentration of DNA (0.10 mg mL$^{-1}$), similar extraction efficiencies were observed for all DNA samples.

![Fig. 4. Effect of IL concentration on the extraction of DNA: (●) 0.005 mg mL$^{-1}$ DNA; (○) 0.015 mg mL$^{-1}$ DNA; (△) 0.045 mg mL$^{-1}$ DNA; (□) 0.1 mg mL$^{-1}$ DNA. LNTf$_2$/C$_{16}$POHIM-Br (n/n): 1/1; sample volume: 10 mL; injection volume: 20 μL; extraction time: 30 min; centrifugation time: 10 min; total volume of solution: 1.5 mL.](image)

Therefore, in order to obtain high extraction efficiency while minimizing the amount of added IL, an IL concentration of 0.33 mg mL$^{-1}$ (a total of 0.50 mg IL) was selected for all subsequent studies. Compared to the IL-based LLE method [13], the amount of IL used for each extraction decreased from 690 or 970 mg to 0.50 mg when using the in situ DLLME method without diminishing the extraction efficiency.

### 3.4. Effect of extraction time

In DLLME, the extraction time is often defined as the time interval from the addition of extraction and dispersive solvents to the time that the centrifugation step is initiated [25]. In this study, the effect of extraction time on DNA extraction efficiency was investigated by shaking the turbid solution after the addition of the metathesis reagent for a range of 1–30 min. As shown in Fig. 5, the extraction efficiency increased from approximately 90–98% as the extraction time was increased. In an effort to compromise extraction efficiency while minimizing the overall extraction time, an extraction time of 5 min was chosen for all subsequent studies. Compared with the extraction time of the IL-based LLE method (10 min) [13] and commercial DNA extraction kits (from 16 to 40 min) [8], the IL-based in situ DLLME method utilizes a shorter extraction step.

![Fig. 5. Effect of extraction time on the DNA extraction efficiency. Concentration of DNA: 0.045 mg mL$^{-1}$; $C_{16}$POHIM-Br: 0.33 mg mL$^{-1}$; LNTf$_2$/C$_{16}$POHIM-Br (n/n): 1/1; injection volume: 20 μL; centrifugation time: 10 min; total volume of solution: 1.5 mL.](image)

### 3.5. Extraction of DNA from a complex sample matrix

Biological DNA samples often contain many other components, such as metal ions and proteins [26]. To be comparable with other DNA extraction methods, it is important to evaluate the effect of these impurity components on the extraction performance using the in situ IL-DLLME method. A complex sample matrix was created by spiking metal ions (NaCl, KCl, CaCl$_2$, 2H$_2$O and MgCl$_2$, 6H$_2$O) or albumin into the aqueous DNA solution prior to extraction.

As shown in Table 4, no significant variation in the extraction efficiency of DNA was observed when the extraction was performed in the presence of the added metal ions. This observation is consistent with results of a previous IL-based DNA extraction study [13]. The effect of added protein on the extraction efficiency of DNA was studied by spiking albumin to the aqueous DNA solution. As shown in Table 5, the extraction efficiency of DNA decreased when the
Table 4

<table>
<thead>
<tr>
<th>Conditions concentration of DNA: 0.015 mg mL⁻¹; C₆₀POHIM-Br: 0.5 mg; LiNTf₂/C₆₀POHIM-Br (n/n): 1/1; injection volume: 20 μL; extraction time: 30 min; centrifugation time: 10 min; total volume of solution: 1.5 ml.</th>
<th>Extraction efficiency (E) % (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neat DNA solution</td>
<td>97.2 ± 0.2</td>
</tr>
<tr>
<td>DNA solution containing metal ions*</td>
<td>98.0 ± 0.1</td>
</tr>
</tbody>
</table>

Conditions concentration of DNA: 0.015 mg mL⁻¹; C₆₀POHIM-Br: 0.33 mg mL⁻¹; LiNTf₂/C₆₀POHIM-Br(n/n): 1/1; injection volume: 20 μL; extraction time: 30 min; centrifugation time: 10 min; total volume of solution: 1.5 ml.

Conditions: concentration of metal ions: NaCl: 20.4 mg mL⁻¹; KCl: 10.3 mg mL⁻¹; CaCl₂ 2H₂O: 5.07 mg mL⁻¹; MgCl₂ 6H₂O: 5.19 mg mL⁻¹. Other conditions kept the same.

Table 5

<table>
<thead>
<tr>
<th>Concentration of protein* (mg mL⁻¹)</th>
<th>Extraction efficiency of DNA E (%) (n=3)</th>
<th>Extraction efficiency of protein E (%) (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>97.2 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>0.048</td>
<td>92.3 ± 3.2</td>
<td>26.4 ± 0.3</td>
</tr>
<tr>
<td>0.095</td>
<td>85.2 ± 1.2</td>
<td>55.7 ± 3.0</td>
</tr>
<tr>
<td>0.15</td>
<td>84.0 ± 0.9</td>
<td>70.9 ± 1.2</td>
</tr>
</tbody>
</table>

* Conditions: concentration of DNA: 0.015 mg mL⁻¹; C₆₀POHIM-Br: 0.33 mg mL⁻¹; LiNTf₂/C₆₀POHIM-Br(n/n): 1/1; injection volume: 20 μL; extraction time: 30 min; centrifugation time: 10 min; total volume of solution: 1.5 ml.

3.6. Insight into IL/DNA electrostatic interactions using ³¹P NMR

³¹P NMR is a powerful tool in the study of DNA complexes in aqueous solution [31]. Using 85% phosphoric acid (H₃PO₄) and D₂O as a standard, the ³¹P resonance of DNA can be found at a chemical shift between −0.3 and −1.5 ppm since the phosphate groups are in the form of phosphodiester [32,33]. The electrostatic interaction between the IL cation and phosphate groups of DNA have been previously investigated using ³¹P NMR [13,34]. Comparing the ³¹P signal in a pure DNA solution, the ³¹P signal of DNA shifts slightly upfield after the addition of IL due to the IL cation interacting with the DNA phosphate group through substitution of the countercations (Na⁺), thereby leading to a change in the chemical environment of the phosphorous atom [13,34]. However, the effect of IL concentration on the ³¹P chemical shift of DNA has not been investigated in previous studies. In addition, a thorough reproducibility study of DNA–IL complexes should be considered since the difference in chemical shift between pure DNA and the DNA–IL complexes is quite small and can be difficult to identify (from −0.72 to −0.94 ppm [13] and from −12.21 to −12.22 ppm [34]).

In this study, the C₆₀POHIM-Br IL was added to the pure DNA solution to investigate the electrostatic interaction between the IL cation and DNA. Since the pKa of phosphate group of DNA is lower than the pH of the sample solution (pH = 7.5), they should be negatively charged and able to interact with the IL cation. The effect of IL concentration on the chemical shift of DNA was examined by increasing the IL concentration from 0.14 to 0.34 mg mL⁻¹ while keeping the DNA concentration constant at 1.0 mg mL⁻¹. In addition, 1-hexadecylimidazole, benzene, potassium chloride, and SYBR® Safe DNA Gel Stain were studied to evaluation their propensities at prompting a change in the ³¹P chemical shift. 1-Hexadecylimidazole and benzene are aromatic neutral molecules with 1-hexadecylimidazole being a reaction intermediate (compound 5 in Fig. 1) possessing a similar structure to the C₆₀POHIM-Br IL. SYBR® Safe DNA gel stain is a highly sensitive stain for the visualization of DNA in agarose or acrylamide gels that possesses a positive charge and multiple aromatic moieties. As shown in Table 6, the ³¹P signal of the phosphate groups in DNA appeared at a chemical shift of −0.52 ppm in the absence of the C₆₀POHIM-Br IL. The inter-day reproducibility of the chemical shift was determined using three individual sample solutions for each concentration level in different days and yielded relative standard deviation (RSD) values ranging from 1.4% to 3.7%. As the concentration of the IL was increased from 0.14 to 0.43 mg mL⁻¹, the signal shifted upfield from −0.73 to −0.93 ppm indicating a stronger electrostatic interaction with the increase of IL concentration (see Fig. S2 in the supplemental information for all NMR spectra). In the case of 1-hexadecylimidazole and potassium chloride, chemical shifts of −0.53 ppm and −0.55 ppm, respectively, were observed. The chemical shift of benzene was observed at −0.68 ppm, which may be due to the fact that this planar, aromatic molecule can stack between two base pairs of DNA and change the P–O bond angle of the phosphate groups [35]. The chemical shift of SYBR® Safe DNA gel stain was observed at −0.90 ppm, which is similar with that of the C₆₀POHIM-Br IL. The results seem to indicate that the C₆₀POHIM-Br IL is interacting with DNA through a combination of electrostatic and π–π interactions.

![Fig. 6. Effect of added protein under different pH conditions. (A) 0.095 mg mL⁻¹ albumin, (B) 0.015 mg mL⁻¹ DNA, C₆₀POHIM-Br: 0.33 mg mL⁻¹; LiNTf₂/C₆₀POHIM-Br (n/n): 1/1; injection volume: 20 μL; extraction time: 10 min; centrifugation time: 10 min; total volume of solution: 1.5 ml.](image-url)
Table 6
Effect of IL concentration on the 31P chemical shift of DNA.

<table>
<thead>
<tr>
<th>Added compound</th>
<th>Concentration (mg mL⁻¹)</th>
<th>Average chemical shift of DNA peak (ppm) (n = 3)</th>
<th>% RSD (n = 3)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additive</td>
<td>–</td>
<td>−0.52 ± 0.01 (1.0)</td>
<td>2.1</td>
</tr>
<tr>
<td>C16POHMIM-Br</td>
<td>0.14</td>
<td>−0.73 ± 0.01 (1.0)</td>
<td>1.6</td>
</tr>
<tr>
<td>C16POHMIM-Br</td>
<td>0.28</td>
<td>−0.84 ± 0.01 (1.0)</td>
<td>1.4</td>
</tr>
<tr>
<td>C16POHMIM-Br</td>
<td>0.43</td>
<td>−0.93 ± 0.01 (1.0)</td>
<td>1.6</td>
</tr>
<tr>
<td>1-hexadecylimidazole</td>
<td>0.43</td>
<td>−0.53 ± 0.01 (1.0)</td>
<td>2.5</td>
</tr>
<tr>
<td>Benzene</td>
<td>0.43</td>
<td>−0.68 ± 0.02 (1.0)</td>
<td>3.7</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.43</td>
<td>−0.55 ± 0.02 (1.0)</td>
<td>3.3</td>
</tr>
<tr>
<td>SYBR® Safe DNA gel stain</td>
<td>Less than 8.3 × 10⁻⁵³</td>
<td>−0.90 ± 0.02 (1.0)</td>
<td>2.2</td>
</tr>
</tbody>
</table>

¹ 31P NMR spectra (in d₂-DMSO) recorded with a Varian 400 MHz NMR spectrometer at a resonance frequency of 161.90 MHz. The chemical shifts are in ppm relative to 85% phosphoric acid (external standard). Concentration of DNA: 1.0 mg mL⁻¹.
² Experiments were carried out using three individual sample solutions in different days.
³ Exact concentration is proprietary (see Section 2).

4. Conclusions

Six ILs were applied as extraction solvents in the extraction of DNA using in situ DLLME. The effect of IL substituent alkyl chain length and the presence of hydroxyl group substituents were investigated in an effort to increase DNA extraction efficiency.

The optimized method utilized an extraction time of 5 min and an IL concentration of 0.33 mg mL⁻¹. This maximized the rate at which an acceptable extraction performance was achieved. Extraction efficiencies higher than 97% were obtained when using 0.50 mg IL for each extraction. The presence of metal ions in the aqueous sample was not observed to interfere with the extraction of DNA. The pH of the sample can be used as a means to mitigate competitive extraction effects from proteins present in the sample matrix. Electrostatic interaction between the C16POHMIM-Br IL and DNA was monitored using 31P NMR spectroscopy and observed to increase when higher IL concentrations were employed.

The results obtained in this study indicate that the IL-based in situ DLLME method possesses clear advantages over existing DNA extraction protocols due to its speed, low extraction solvent consumption, and high extraction efficiency. Also, this approach not only provides an alternative method for the separation and pre-concentration of trace DNA from complex sample matrices, but may also be applicable to higher concentration levels for isolation and purification. On-going studies in our group are exploring methods capable of separating DNA from the IL after extraction to allow for complete recovery and re-use of the IL solvent.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2012.11.055.

References