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

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ABSTRACT

Six ionic liquids (ILs) were applied for the first time as solvents in the extraction and preconcentration of deoxyribonucleic acid (DNA) using an *in situ* dispersive liquid–liquid microextraction (DLLME) approach. The effect of different IL substituents and functional group on the extraction efficiency of DNA was investigated. The highest extraction efficiencies of DNA were obtained using 1-(1,2-dihydroxypropyl)-3-hexadecylimidazolium bromide (C_{16}POHIM-Br) and *N*,*N*-didecyl-*N*-methyl-D-glucaminium bromide [$(C_{10})_2$NMDG-Br]. Extraction efficiencies higher than 97% were obtained using small amounts of IL (0.50 mg) for each extraction. The extraction of DNA from a sample matrix containing metal ions and protein revealed that the metal ions did not interfere with the extraction of DNA and that the co-extraction of protein can be mitigated by performing the extraction under moderately acidic conditions. Data from ^{31}P NMR spectroscopy suggest that a combination of electrostatic and π–π interactions dominate IL–DNA complexation and that the extraction is concentration dependant.

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

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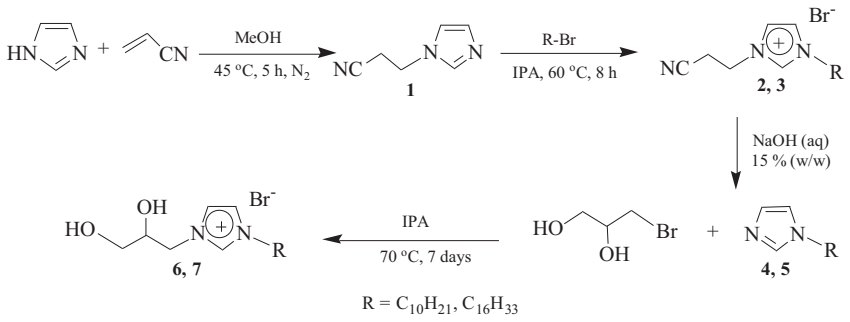

Fig. 1. Synthesis of 1-(1,2-dihydroxypropyl)-3-decylimidazolium bromide (C_{10}POHIM-Br) and 1-(1,2-dihydroxypropyl)-3-hexadecylimidazolium bromide (C_{16}POHIM-Br) ILs.

from the combination of various organic cations and organic or inorganic anions. Common IL cations include imidazolium, pyridinium, 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 (TILDLME) [16], and ultrasound-assisted ionic liquid dispersive liquid-phase microextraction (UILDLME) [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), 1-(1,2-dihydroxypropyl)-3-hexadecylimidazolium bromide (C_{16}POHIM-Br) and *N*,*N*-didecyl-*N*-methyl-D-glucaminium 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_2O) 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 = 1.3×10^6, 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-cyanoethylimidazole **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 C_{10}POHIM-Br **6** or C_{16}POHIM-Br **7** in high purity. These two ILs were characterized by ^{1}H 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-20A liquid chromatograph (Shimadzu, Japan) with two LC-20AT pumps, a SPD-20 UV/VIS detector, and a DGU-20A_3 degasser. All separations were carried out using an anion exchange column (TSKgel DEAE-NPR, 35 mm × 4.6 mm i.d., 2.5 μm particle size) with a guard column (TSKgel DEAE-NPR, 5 mm × 4.6 mm i.d., 5 μm particle size) from Tosoh Bioscience (Bellefonte, 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.

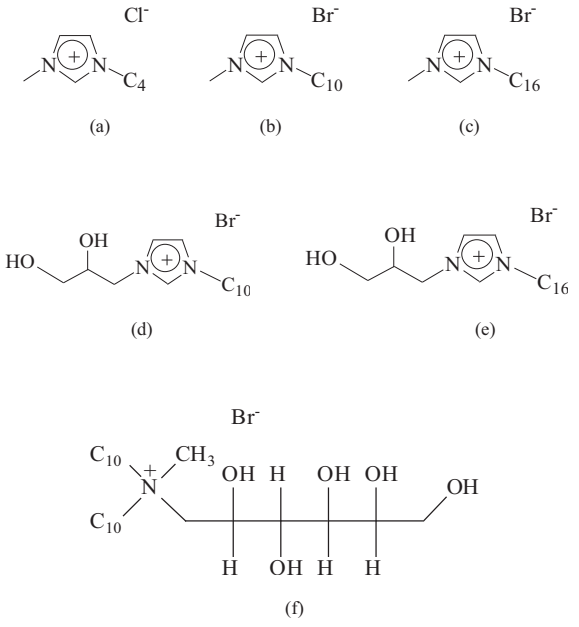

Fig. 2. Structures of studied ILs used for the *in situ* DLLME of DNA: (a) 1-butyl-3-methylimidazolium chloride (BMIM-Cl), (b) 1-decyl-3-methylimidazolium bromide (C_{10}MIM-Br), (c) 1-hexadecyl-3-methylimidazolium bromide (C_{16}MIM-Br), (d) 1-(1,2-dihydroxypropyl)-3-decylimidazolium bromide (C_{10}POHIM-Br), (e) 1-(1,2-dihydroxypropyl)-3-hexadecylimidazolium bromide (C_{16}POHIM-Br), and (f) *N*,*N*-didecyl-*N*-methyl-D-glucaminium bromide [$(C_{10})_2$NMDG-Br].

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). ^{31}P 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} C_{16}POHIM-Br IL were individually prepared in deionized water. The samples for ^{31}P NMR analysis were prepared by adding different volumes of these stock solutions into deuterated dimethylsulfoxide (d_6-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 C_{16}POHIM-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 $LiNTf_2$ 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 $LiNTf_2$ 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}), $CaCl_2{\cdot}2H_2O$ (5.07 mg mL^{-1}), $MgCl_2{\cdot}6H_2O$ (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

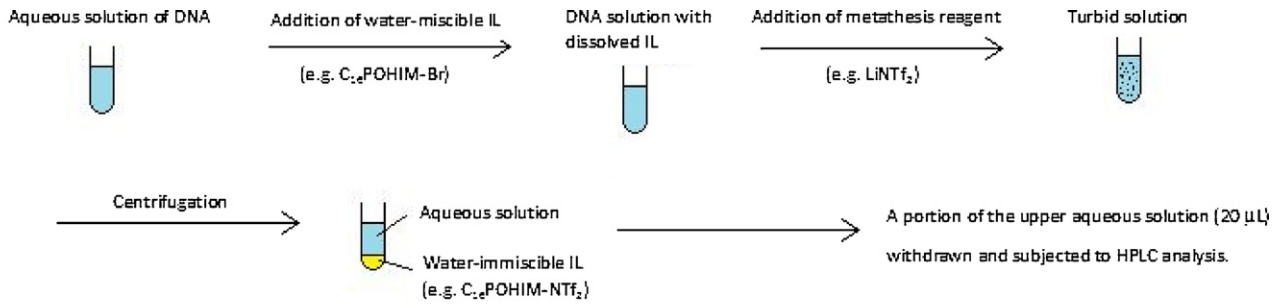

Fig. 3. Schematic diagram of the IL-based *in situ* DLLME method used in this study.

determination of residual DNA. The extraction performance was evaluated using Eq. (1):

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

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 alky 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-NTf_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-NTf_2 to 53.6% for C_{16}MIM-NTf_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}POHIM-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_{10}POHIM-NTf_2 IL (52.4%) is higher than that of C_{10}MIM-NTf_2 IL (46.3%), while the extraction efficiency for the C_{16}POHIM-NTf_2 IL (95.2%) is significantly higher than that of the C_{16}MIM-NTf_2 IL (53.6%). Another IL, *N*,*N*-didecyl-*N*-methyl-D-glucaminium bromide [$(C_{10})_2$NMDG-Br] IL, which possessed five hydroxyl groups within the carbohydrate moiety and two long alky 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.

Table 1
Effect of IL substituent alkyl chain length on DNA extraction efficiency.

In situ metathesis reaction	Ionic liquid	Extraction efficiency (E) % (n = 3)
BMIM-Cl + $LiNTf_2$	BMIM-NTf_2	8.6 ± 4.5
C_{10}MIM-Br + $LiNTf_2$	C_{10}MIM-NTf_2	46.3 ± 4.2
C_{16}MIM-Br + $LiNTf_2$	C_{16}MIM-NTf_2	53.6 ± 3.7

Conditions: DNA concentration: 0.006 mg mL^{-1}; IL: 0.1 g; $LiNTf_2$/IL (n/n): 1/1; injection volume: 20 μL; extraction time: 30 min; centrifugation time: 10 min; total volume of solution: 400 μL.

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):

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

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 2
Effect of IL hydroxyl groups on DNA extraction efficiency.

In situ metathesis reaction	Ionic liquid	Extraction efficiency (E) % (n = 3)
C_{10}MIM-Br + $LiNTf_2$ [a]	C_{10}MIM-NTf_2	46.3 ± 4.2
C_{10}POHIM-Br + $LiNTf_2$ [a]	C_{10}POHIM-NTf_2	52.4 ± 6.4
C_{16}MIM-Br + $LiNTf_2$ [a]	C_{16}MIM-NTf_2	53.6 ± 3.7
C_{16}POHIM-Br + $LiNTf_2$ [a]	C_{16}POHIM-NTf_2	95.2 ± 0.4
$(C_{10})_2$NMDG-Br + $LiNTf_2$ [b]	$(C_{10})_2$NMDG-NTf_2	92.4 ± 0.4

[a] Conditions: DNA concentration: 0.006 mg mL^{-1}; IL: 0.1 g; $LiNTf_2$/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.

Table 3
Effect of remaining IL in the aqueous phase after extraction.

Ionic liquid	Concentration of the IL after metathesis reaction C_{aq} (mM) (n = 3)	Concentration of the IL in standard solution C_0 (mM) (n = 3)	Percentage of IL remaining S_{IL} % (n = 3)
C_{10}MIM-Br	236.7 ± 5.6	840.2 ± 9.1	28.5 ± 9.1
C_{10}POHIM-Br	393.8 ± 10.0	708.0 ± 7.6	55.6 ± 1.6
C_{16}POHIM-Br	95.6 ± 2.2	286.6 ± 2.2	33.3 ± 7.7

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_{10}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_{16}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_{16}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.

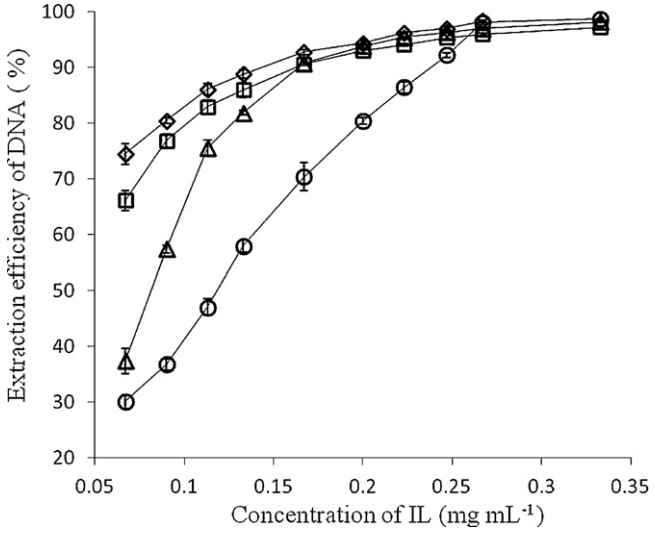

Fig. 4. Effect of IL concentration on the extraction of DNA: (◊) 0.005 mg mL^{-1} DNA, (□) 0.015 mg mL^{-1} DNA, (△) 0.045 mg mL^{-1} DNA, (○) 0.1 mg mL^{-1} DNA. $LiNTf_2$/C_{16}POHIM-Br (n/n): 1/1; sample volume: 10 mL; injection volume: 20 μL; extraction time: 30 min; centrifugation time: 10 min; total volume of solution: 1.5 mL.

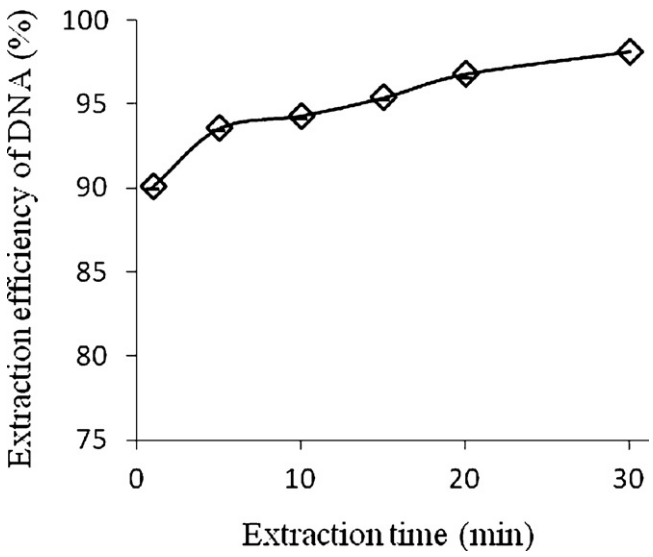

Fig. 5. Effect of extraction time on the DNA extraction efficiency. Concentration of DNA: 0.045 mg mL^{-1}; C_{16}POHIM-Br: 0.33 mg mL^{-1}; $LiNTf_2$/C_{16}POHIM-Br (n/n): 1/1; injection volume: 20 μL; centrifugation time: 10 min; total volume of solution: 1.5 mL.

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.

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, $CaCl_2{\cdot}2H_2O$ and $MgCl_2{\cdot}6H_2O$) 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
Effect of added metal ions on DNA extraction efficiency using C_{16}POHIM-Br +$LiNTf_2$.

	Extraction efficiency (E) % (n = 3)
Neat DNA solution	97.2 ± 0.2
DNA solution containing metal ions[a]	98.0 ± 0.1

Conditions concentration of DNA: 0.015 mg mL^{-1}; C_{16}POHIM-Br: 0.5 mg; $LiNTf_2$/C_{16}POHIM-Br (n/n): 1/1; injection volume: 20 μL; extraction time: 30 min; centrifugation time: 10 min; total volume of solution: 1.5 mL.

[a] Conditions: concentration of metal ions: NaCl: 20.4 mg ml^{-1}; KCl: 10.3 mg ml^{-1}; $CaCl_2 \cdot 2H_2O$: 5.07 mg ml^{-1}; $MgCl_2 \cdot 6H_2O$: 5.19 mg ml^{-1}. Other conditions kept the same.

Table 5
Effect of added protein on DNA extraction efficiency.

Concentration of protein[a] (mg mL^{-1})	Extraction efficiency of DNA E (%) (n = 3)	Extraction efficiency of protein E_P (%) (n = 3)
0	97.2 ± 0.2	–
0.048	92.3 ± 3.2	26.4 ± 0.3
0.095	85.2 ± 1.2	55.7 ± 3.0
0.15	84.0 ± 0.9	70.9 ± 1.2

[a] : Conditions: concentration of DNA: 0.015 mg mL^{-1}; C_{16}POHIM-Br: 0.33 mg mL^{-1}; $LiNTf_2$/C_{16}POHIM-Br(n/n): 1/1; injection volume: 20 μL; extraction time: 30 min; centrifugation time: 10 min; total volume of solution: 1.5 mL.

concentration of albumin within the sample increased. However, an increased extraction efficiency of albumin (E_P) was also observed. This is likely due to the fact that albumin is negatively charged in the sample solution (pH = 7.5) since its isoelectric point (pI = 4.6 [27]) is lower than the pH of the sample solution. Therefore, the negatively charged protein may undergo electrostatic interaction with the IL cation [28], thereby presenting a competitive extraction between DNA and albumin. To explore this hypothesis, the pH of the DNA–protein solution was decreased from 7.5 to 3.6 through the addition of hydrochloric acid while maintaining the concentration of DNA and albumin constant at 0.015 mg mL^{-1} and 0.095 mg mL^{-1}, respectively. In the case of albumin, a dramatic decrease in extraction efficiency was observed, as shown in Fig. 6. The change in the charged state of the protein may have diminished the electrostatic interaction between the protein and the IL cation leading to lower extraction efficiency. On the contrary, no obvious variation in the extraction efficiency of DNA was observed when the pH was decreased. Since the pK_a of the phosphate group (pK_a = 1.2 [29]) within the DNA backbone is lower than the pH range

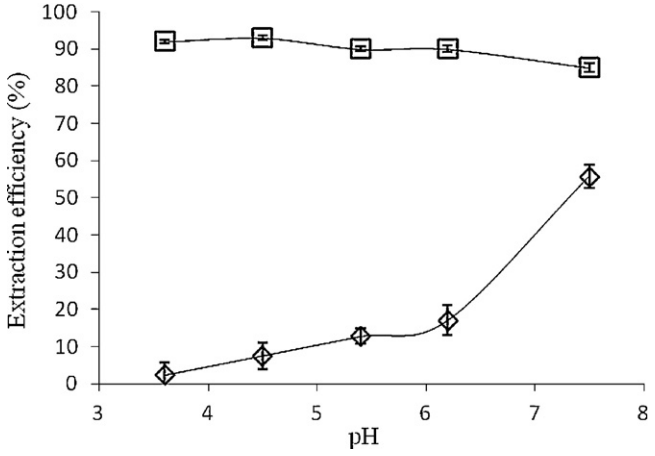

Fig. 6. Effect of added protein under different pH conditions. (◊) 0.095 mg mL^{-1} albumin, (□) 0.015 mg mL^{-1} DNA. C_{16}POHIM-Br: 0.33 mg mL^{-1}; $LiNTf_2$/C_{16}POHIM-Br (n/n): 1/1; injection volume: 20 μL; extraction time: 10 min; centrifugation time: 10 min; total volume of solution: 1.5 mL.

evaluated, the phosphate groups remained negatively charged in this pH range and allows electrostatic interactions between the phosphate group of DNA and the IL cation to persist [30]. In order to minimize the competitive extraction effect from albumin, performing the extraction from aqueous sample at low pH is preferred.

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

^{31}P NMR is a powerful tool in the study of DNA complexes in aqueous solution [31]. Using 85% phosphoric acid (H_3PO_4) and D_2O as a standard, the ^{31}P 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 phosphodiesters [32,33]. The electrostatic interaction between the IL cation and phosphate groups of DNA have been previously investigated using ^{31}P NMR [13,34]. Comparing the ^{31}P signal in a pure DNA solution, the ^{31}P 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 ^{31}P 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 C_{16}POHIM-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 evaluate their propensities at prompting a change in the ^{31}P 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 C_{16}POHIM-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 ^{31}P signal of the phosphate groups in DNA appeared at a chemical shift of −0.52 ppm in the absence of the C_{16}POHIM-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 C_{16}POHIM-Br IL. The results seem to indicate that the C_{16}POHIM-Br IL is interacting with DNA through a combination of electrostatic and π–π interactions.

Table 6
Effect of IL concentration on the ^{31}P chemical shift of DNA.

Added compound	Concentration (mg mL^{-1})[a]	Average chemical shift of DNA peak (ppm) (n = 3)	% RSD (n = 3)[b]
No additive	–	−0.52 ± 0.01	2.1
C_{16}POHIM-Br	0.14	−0.73 ± 0.01	1.6
C_{16}POHIM-Br	0.28	−0.84 ± 0.01	1.4
C_{16}POHIM-Br	0.43	−0.93 ± 0.01	1.6
1-hexadecylimidazole	0.43	−0.53 ± 0.01	2.5
Benzene	0.43	−0.68 ± 0.02	3.7
Potassium chloride	0.43	−0.55 ± 0.02	3.3
SYBR® Safe DNA gel stain	Less than 8.3×10^{-5}[c]	−0.90 ± 0.02	2.2

[a] ^{31}P NMR spectra (in d_6-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}.
[b] Experiments were carried out using three individual sample solutions in different days.
[c] 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 C_{16}POHIM-Br IL and DNA was monitored using ^{31}P 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 preconcentration of trace DNA from complex sample matrixes, 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.

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