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PAPER IN FOREFRONT

Extraction of DNA with magnetic ionic liquids using in situ dispersive liquid–liquid microextraction

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Abstract

A new class of magnetic ionic liquids (MILs) with metal-containing cations was applied in in situ dispersive liquid–liquid microextraction (DLLME) for the extraction of long and short double-stranded DNA. For developing the method, MILs comprised of N-substituted imidazole ligands (with butyl-, benzyl-, or octyl-groups as substituents) coordinated to different metal centers (Ni^{2+}, Mn^{2+}, or Co^{2+}) as cations, and chloride anions were investigated. These water-soluble MILs were reacted with the bis[(trifluoromethyl)sulfonyl]imide anion during the extraction to generate a water-immiscible MIL capable of preconcentrating DNA. The feasibility of combining the extraction methodology with anion-exchange high-performance liquid chromatography with diode array detection (HPLC-DAD) or fluorescence spectroscopy was studied. The method with the Ni^{2+}- and Co^{2+}-based MILs was easily combined with fluorescence spectroscopy and provided a faster and more sensitive method than HPLC-DAD for the determination of DNA. In addition, the method was compared to conventional DLLME using analogous water-immiscible MILs. The developed in situ MIL-DLLME method required only 3 min for DNA extraction and yielded 1.1–1.5 times higher extraction efficiency (EFs) than the conventional MIL-DLLME method. The in situ MIL-DLLME method was also compared to the trihexyl(tetradecyl)phosphonium tris(hexafluorocetylaceto)nickelate(II) MIL, which has been used in previous DNA extraction studies. EFs of 42–99% were obtained using the new generation of MILs, whereas EFs of only 20–38% were achieved with the phosphonium MIL. This new class of MILs is simple and inexpensive to prepare. In addition, the MILs present operational advantages such as easier manipulation in comparison to hydrophobic MILs, which can have high viscosities. These MILs are a promising new class of DNA extraction solvents that can be manipulated using an external magnetic field.

Keywords DNA · Magnetic ionic liquids · In situ dispersive liquid–liquid microextraction · High-performance liquid chromatography · Fluorescence spectroscopy

Introduction

DNA is often regarded as the central database of the cell, controlling cell growth, maintenance, and replication [1]. DNA analysis is routinely used in many fields including forensics [2], anthropology [3], clinical diagnostics [4], genetics [5], and pharmaceuticals [6]. Isolating genomic DNA from cells is often the first step in numerous molecular biology procedures used in these fields such as quantitative polymerase chain reaction (qPCR), gene expression, and gene therapy [7, 8]. These techniques require high quality DNA and their success is often affected by DNA purity and integrity [9]. Obtaining high yields and pure DNA from complex biological matrices presents a significant sample preparation challenge in nucleic acid analysis.

Current methodologies for nucleic acid purification involve solution-based or column-based protocols [7]. Many commercially available kits use solid or semisolid sorbent phases such as anion-exchange spin columns, silica-based membranes, and magnetic particles [7]. However, these kits often are very expensive and have limited reusability. Conventional liquid–liquid extraction (LLE) approaches to purify nucleic acids use phenol and chloroform [7]. Although high quality nucleic acid

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can be obtained, some organic solvents such as phenol and chloroform can be toxic and environmentally unfriendly. In order to reduce the use of harmful organic solvents and provide selectivity in the extraction, ionic liquids (ILs) and their magnetic analogs, magnetic ionic liquids (MILs), have grown in popularity as extraction solvents over the past decade [10–18].

ILs are molten salts with melting points below 100 °C and are comprised entirely of ions [19]. ILs have negligible vapor pressure at room temperature and relatively high thermal stability, as well as variable viscosity [13]. MILs are a subclass of ILs which contain a paramagnetic metal in the cation or anion, allowing the compound to possess magnetic properties [10]. The cationic and anionic moieties of ILs and MILs can often be tuned for specific applications, including for nucleic acid extractions [11, 20, 21].

Previous studies have used ILs or IL-modified materials [22] in different extraction and microextraction techniques for nucleic acids, including single-drop microextraction (SDME) [21, 23], dispersive liquid–liquid microextraction (DLLME) [20, 21, 23–27], aqueous biphasic systems (ABS) [28, 29], and solid-phase microextraction (SPME) [30–34]. SPME often requires long extraction and desorption times; therefore, IL-based liquid-phase extraction techniques such as DLLME are generally preferred [35]. Conventional DLLME involves the rapid injection of a mixture of extraction and dispersive solvents into an aqueous sample, resulting in the enrichment of analytes from the sample matrix [36]. In this technique, a hydrophobic IL is typically used as the extraction solvent. However, the use of MILs containing paramagnetic anions has also been described [37, 38]. In this case, magnetic separation of the MIL can be performed after DLLME, which simplifies the overall procedure. In a variation of the technique called in situ DLLME, a hydrophilic IL is mixed with a metathesis reagent, promoting an anion-exchange reaction that generates a hydrophobic IL. This reaction creates numerous finely dispersed hydrophobic IL microdroplets capable of interaction with analytes. The anion-exchange process also increases the surface area of the IL extraction solvent, generally leading to higher extraction efficiencies [17, 39, 40]. The use of MILs for in situ DLLME was recently possible due to the design of MILs that possess a paramagnetic component in the cation of their structure [41–43]. Since the paramagnetic component is within the cation of the MIL, it is not exchanged during the metathesis reaction allowing magnetic separation to be performed.

This study constitutes the first report of in situ DLLME using MILs for the extraction of DNA. The optimal extraction efficiency of different DNA sizes was investigated using ten types of MILs. The extraction procedure is combined with high-performance liquid chromatography with diode array detection (HPLC-DAD) and fluorescence emission spectroscopy. The superior extraction performance of the developed in situ MIL-DLLME method is confirmed by its comparison with conventional DLLME using both MILs with the paramagnetic component in the cation and a previous generation of MIL, with the paramagnetic component in the anion. MILs developed in situ demonstrated superior extraction efficiency and are easier to work with in comparison to hydrophobic MILs, which can have high viscosities and are difficult to pipette [10, 44–46].

Experimental

Chemicals, reagents, and materials

Different sized fragments of double-stranded DNA (dsDNA) (~ 20 kbp salmon testes DNA, stDNA; ~ 250–500 bp stDNA; and 20 bp DNA) were employed in this study. stDNA (approximately 20 kbp) was acquired from Sigma-Aldrich (St. Louis, MO, USA). To generate shorter duplex DNA fragments of approximately 250 to 500 bp, stDNA was sheared for 60 cycles (1 cycle—30 s on and 30 s off) through sonication in an ice bath. Agarose from LabExpress (Ann Arbor, MI, USA) at 1% w/v concentration was employed for electrophoretic separation to confirm the size of the sheared stDNA fragments. SYBR Safe DNA gel stain was purchased from Invitrogen (Waltham, MA, USA). A l-kb Plus DNA Ladder was purchased from Gold Biotechnology (St. Louis, MO, USA). A synthetic oligonucleotide (sequence: 5′-AGG GCG TGA ATG TAA GCG TG-3′ annealed to its complementary strand) was purchased from Integrated DNA Technologies (Coralville, IA, USA). Ethylenediaminetetraacetic acid (EDTA, ACS reagent, 99.4–100.06%) was purchased from Sigma-Aldrich. Tris(hydroxymethyl)aminomethane (Tris base) and the corresponding hydrochloride salt (Tris-HCl) were purchased from Research Products International (Mount Prospect, IL, USA). SYBR Green I (10,000×) was purchased from Life Technologies (Eugene, OR, USA). Sodium chloride (100.1%), sodium hydroxide (99.4%), and *N,N*-dimethylformamide (99.9%) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure water (18.2 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

For the synthesis of MILs, the reagents cobalt(II) chloride (97%), acetonitrile (99.9%), and 1-butylimidazole (98%) were purchased from Sigma-Aldrich. Nickel(II) chloride (98%), 1-benzylimidazole (99%), 1,1,1,5,5,5-hexafluoroacetylacetone (99%), and ammonium hydroxide (28–30% solution in water) were purchased from Acros Organics (Morris Plains, NJ, USA). Manganese(II) chloride tetrahydrate (98.0–101.0%) was purchased from Alfa Aesar (Ward Hill, MA, USA). Trihexyl(tetradecyl)phosphonium chloride (97.7%) was purchased from Strem Chemicals (Newburyport, MA, USA). Lithium bis[(trifluoromethyl)sulfonyl]imide ($[Li^+][NTf_2^-]$) was purchased from SynQuest Laboratories (Alachua, FL,

USA). Anhydrous diethyl ether (99.0%) was purchased from Avantor Performance Materials Inc. (Center Valley, PA, USA). Ethyl alcohol was purchased from Decon Laboratories, Inc. (King of Prussia, PA, USA).

The chemical structures of the ten different MILs examined in this study are shown in Fig. 1. Nine of the MILs have a general chemical structure based on a cation comprised of four N-substituted imidazole ligands (RIm, with R = B for butyl-, Bn for benzyl-, and O for octyl-) coordinated to different metal centers (M = Ni^{2+}, Mn^{2+}, or Co^{2+}) and chloride or bis[(trifluoromethyl)sulfonyl]imide ($[NTf_2^-]$) anions. The water-soluble MILs ($[Ni(BIm)_4{}^{2+}]2[Cl^-]$, $[Ni(BnIm)_4{}^{2+}]2[Cl^-]$, $[Mn(BIm)_4{}^{2+}]2[Cl^-]$, and $[Co(BIm)_4{}^{2+}]2[Cl^-]$) were used for in situ DLLME. The corresponding hydrophobic form of these MILs was generated by a metathesis reaction with $[Li^+][NTf_2^-]$ and used in conventional DLLME. Stock solutions of the MILs in chloride anion form were prepared in ultrapure water at a concentration of 20 mg mL^{-1}, except for the $[Ni(BIm)_4{}^{2+}]2[Cl^-]$ MIL, which had a concentration of 25 mg mL^{-1}. An aqueous solution of $[Li^+][NTf_2^-]$ containing a concentration of 600 mg mL^{-1} was used for in situ DLLME. To compare to previous MILs used in DNA extractions, the trihexyl(tetradecyl)phosphonium tris(hexafluoroacetylaceto)nickelate(II) ($[P_{66614}{}^+][Ni(II)(hfacac)_3^-]$) MIL was used, which is hydrophobic and is composed of Ni(II) coordinated to three hexafluoroacetylacetonate ($[hfacac^-]$) ligands in the anion.

Instrumentation

An Agilent Technologies 1260 Infinity high-performance liquid chromatograph (Santa Clara, USA) consisting of a quaternary pump, column thermostat, manual injector, and diode array detector (DAD) was used for the indirect determination of DNA. All chromatographic separations were performed using an anion-exchange column (TSKgel DEAE-NPR, 35 mm × 4.6 mm i.d., 2.5 μm) equipped with a guard column (TSKgel DEAE-NPR, 5 mm × 4.6 mm i.d., 5 μm) from Tosoh Bioscience (King of Prussia, PA, USA). Mobile phase comprised of (A) 20 mM Tris-HCl (pH 8) and (B) 1 M NaCl/20 mM Tris-HCl (pH 8) at a flow rate of 0.5 mL min^{-1} was employed for the separations. Gradient elution was performed by increasing from 20 to 100% B over 20 min, and detection at 260 nm. The column was maintained at 40 °C.

Fluorescence emission spectra were acquired using a Synergy H1 Multi-Mode microplate reader (Winooski, VT, USA) and 384-well plate, black polystyrene, flat bottom microplates (Corning, Corning, NY, USA). Fluorescence emission measurements were obtained at an excitation wavelength of 480 nm. The emission intensity was scanned from 510 to 650 nm with 1 nm resolution. Measurements were acquired in top-read mode.

A Shimadzu AA-7000 atomic absorption spectrophotometer (AAS) equipped with an ASC-7000 auto sampler (Kyoto, Japan) was used for AA measurements. Nickel and

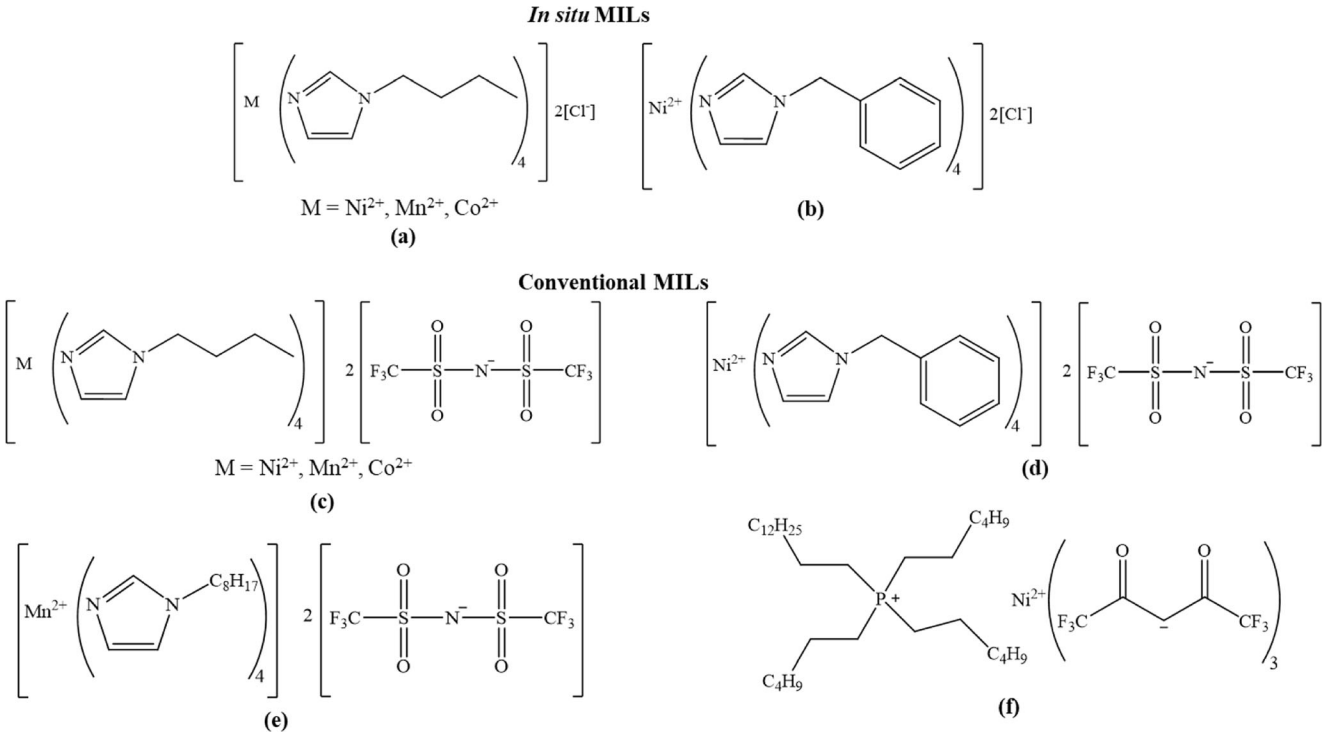

Fig. 1 Chemical structures of the MILs examined in this study: **a** $[Ni(BIm)_4{}^{2+}]2[Cl^-]$, $[Mn(BIm)_4{}^{2+}]2[Cl^-]$, and $[Co(BIm)_4{}^{2+}]2[Cl^-]$); **b** $[Ni(BnIm)_4{}^{2+}]2[Cl^-]$; **c** $[Ni(BIm)_4{}^{2+}]2[NTf_2^-]$, $[Mn(BIm)_4{}^{2+}]2[NTf_2^-]$, and $[Co(BIm)_4{}^{2+}]2[NTf_2^-]$; **d** $[Ni(BnIm)_4{}^{2+}]2[NTf_2^-]$; **e** $[Mn(OIm)_4{}^{2+}]2[NTf_2^-]$; and **f** $[P_{66614}{}^+][Ni(II)(hfacac)_3^-]$

manganese Atomax hollow cathode lamps (PerkinElmer, MedTech Park, Singapore) were used for the determination of nickel and manganese content, respectively. Likewise, a cobalt hollow cathode lamp (Hamamatsu Photonics K.K., Beijing, China) was used for the detection of cobalt.

Procedures

Synthesis of magnetic ionic liquids

The $[P_{66614}^{+}][Ni(II)(hfacac)_3^{-}]$ MIL was synthesized and purified following a previously reported procedure [47]. The MILs used for in situ and conventional DLLME were synthesized according to a recently reported procedure [41]. The $[Ni(BnIm)_4^{2+}]2[Cl^{-}]$ MIL was synthesized following the same procedure, except 3.16 mmol of $NiCl_2$ was added to 12.6 mmol of 1-benzylimidazole in a round bottom flask with 10 mL of water and refluxed at 80 °C for 12 h. The solvent was removed under reduced pressure at 40 °C, and the MIL product was washed with diethyl ether and dried in a vacuum oven for 24 h at 40 °C.

In situ dispersive liquid–liquid microextraction

A general schematic of the in situ DLLME process is shown in Fig. 2. All extractions were performed in 4 mL clear glass vials with a screw hole cap containing a polytetrafluoroethylene (PTFE)/silicone septum (Supelco, Bellefonte, PA). An aqueous solution of the MIL in the chloride form (17 to 24 μmol) was added to an aqueous solution of DNA (~ 20 kbp stDNA, ~ 250–500 bp stDNA, or 20 bp DNA). The DNA concentration and the total extraction volume were kept constant at 2 nM and 2 mL, respectively. A volume of 300 μL of dimethylformamide was then added as a dispersive solvent. The vial was homogeneously mixed with a vortex from Fisher Scientific at 2100 rpm for 10 s, followed by the addition of the $[Li^{+}][NTf_2^{-}]$ anion-exchange reagent at a MIL:$[Li^{+}][NTf_2^{-}]$ molar ratio of 1:1.5, 1:2, 1:2.5, or 1:2.8, depending on the experiment. The vial was then mixed by vortex for 3 min to facilitate the metathesis reaction and form a hydrophobic MIL droplet. After droplet formation, the in situ generated hydrophobic MIL settled at the bottom of the vial, and an aliquot of the upper aqueous phase was used for indirect determination of the DNA extraction efficiency (EF) by HPLC-DAD or fluorescence emission spectroscopy. The specific conditions for each MIL are shown in Table S1 of the Electronic Supplementary Material (ESM).

Conventional dispersive liquid–liquid microextraction

The overall process for the conventional DLLME method is shown in Fig. S2 of the ESM. An amount of 16 to 21 μmol of the hydrophobic MIL (in $[NTf_2^{-}]$ form) was dissolved in 300 μL of dimethylformamide as a dispersive solvent. The mixture was then added to a 2-nM aqueous solution of DNA (~ 20 kbp stDNA, ~ 250–500 bp stDNA, or 20 bp DNA) in a 4-mL extraction vial and homogeneously vortexed at 2100 rpm for 5 min to promote dispersion of the hydrophobic MIL throughout the aqueous phase containing the DNA. The MIL was allowed to settle at the bottom of the vial, and an aliquot of the aqueous phase was taken for indirect

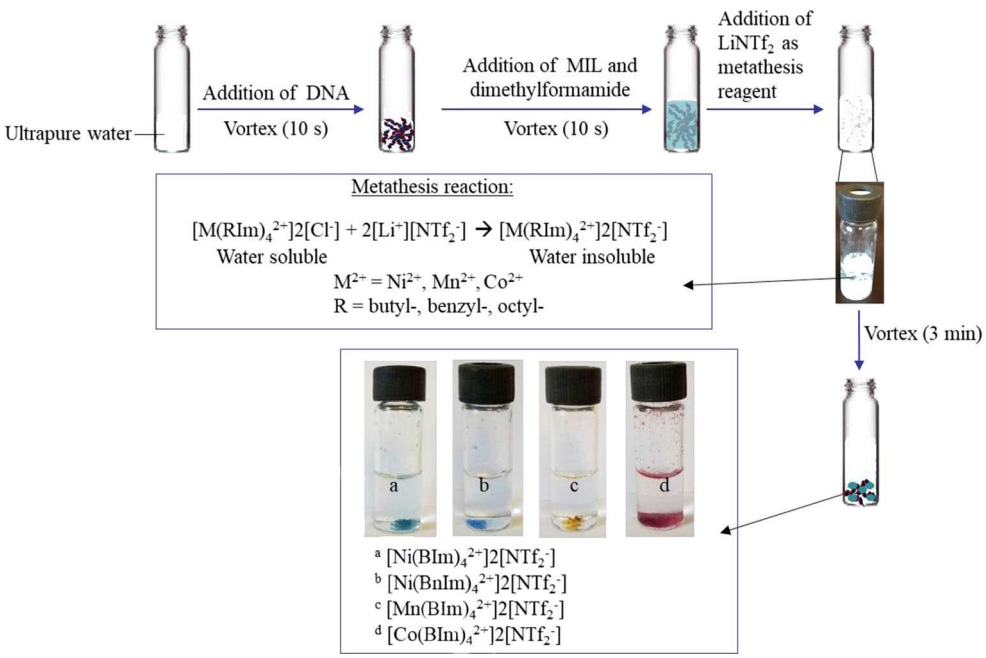

Fig. 2 Schematic describing the in situ DLLME method using MILs for the extraction of DNA

determination of DNA EF by HPLC-DAD or fluorescence emission spectroscopy. The same procedure was followed for the $[P_{66614}^{+}][Ni(II)(hfacac)_3^{-}]$ MIL, except 15 μmol of the MIL was directly added to the aqueous DNA solution without the addition of dimethylformamide, to prevent the MIL from dissolving completely without droplet formation. The amount of each MIL used for extractions is provided in Table S2 of the ESM.

Determination of extracted DNA and free metal remaining in aqueous phase

Indirect determination of the extracted DNA was performed by two different methods: HPLC-DAD and fluorescence spectroscopy. HPLC-DAD separation and detection of the DNA was performed by injecting 20 μL of the aqueous phase after extraction to the system using the conditions detailed in the "Instrumentation" section. Fluorescence emission spectra were obtained by adding 0.2 μL of a 50× SYBR Green I stock solution to a 9.8-μL aliquot of the aqueous phase after extraction. The SYBR Green I dye and the aqueous phase aliquot were mixed for 5 s with a vortex mixer (Barnstead Thermolyne Type 16700, Dubuque, IA, USA) and centrifuged for 3 s (Eppendorf Centrifuge 5424, Hamburg, Germany). The solutions were transferred to the wells in a black microplate and measurements were performed in triplicate at room temperature (~ 23 °C).

Flame atomic absorption spectroscopy (FAAS) was performed to determine the amount of metal remaining in the aqueous phase after the in situ DLLME procedure. The method of standard addition was employed by adding a fixed volume of 250 μL of the aqueous phase after in situ DLLME to different $NiCl_2$, $MnCl_2$, or $CoCl_2$ standards with concentrations ranging between 0 and 100 μM.

Results and discussion

Comparison of stDNA extraction efficiency using different methods

In this study, up to ten different MILs were applied in two different extraction methods (in situ DLLME and conventional DLLME). Different MIL:$[Li^{+}][NTf_2^{-}]$ molar ratios were selected for performing in situ DLLME experiments, as explained in the "In situ dispersive liquid–liquid microextraction" section. Different molar ratios were needed, based on the nature of the MIL and in order to obtain a magnetic liquid after the metathesis reaction. HPLC-DAD was initially studied as the separation and indirect detection method. However, this approach was time consuming and required greater amounts of solvents and frequent cleaning of the column to prevent analyte carryover. Pressure issues in the system were observed after subsequent injections. This problem was likely due to interactions with components remaining in the aqueous phase after extraction (such as the MIL, metal, ligands, and unreacted $[Li^{+}][NTf_2^{-}]$). Another possible reason for pressure issues could be the weak anion-exchange groups modified on the surface of the stationary phase. Fluorescence emission spectroscopy was studied as an alternative to HPLC-DAD and was found to be simpler and required less solvent. In both methods, the aforementioned components remaining in the aqueous phase after extraction can possibly affect the signal.

A comparison of the results obtained with these two analytical techniques was carried out for all extractions performed with different MILs. These experiments were performed with stDNA spiked samples. The EF was calculated using Eq. (1).

$$\mathrm{EF} = \left(1 - \frac{P_{\mathrm{aq}}}{P_{\mathrm{std}}}\right) \times 100 \quad (1)$$

where P_{aq} is the peak area of DNA in the aqueous phase after extraction in HPLC-DAD or the mean maximum relative fluorescence units (mean max. RFU) obtained from the emission spectrum in fluorescence measurements. Similarly, P_{std} is the peak area or mean max. RFU obtained from measurement of a 2-nM DNA standard solution, which corresponds to the initial DNA concentration used in extractions. A comparison of the EF values was established by using Student's *t* test at a 95% confidence level (Table S3 of the ESM). In general, no significant EF differences were found for various MILs using the HPLC-DAD or fluorescence emission detection methods (Table S3 and Fig. S3 of the ESM). These results indicate that SYBR Green I underwent a selective interaction with the DNA remaining after extraction, and no other component of the aqueous sample caused an interference in the determination. An exception of this behavior was observed with the $[Mn(BIm)_4^{2+}]2[Cl^{-}]$ MIL for which the performed statistical analysis revealed differences between the detection methods, likely due to the formation of Mn(II,III) oxide precipitates (i.e., MnO, Mn_2O_3, MnO_2, and Mn_2O_7 [48]). In fact, the formation of a precipitate was observed after storing aqueous solutions of $[Mn(BIm)_4^{2+}]2[Cl^{-}]$ longer than 2 h. The $[Mn(BIm)_4^{2+}]2[Cl^{-}]$ MIL precipitate was characterized by Raman spectroscopy and X-ray diffraction (XRD) and compared to those of manganese(II) oxide and manganese(III) oxide. Both the Raman spectrum and the XRD pattern of the $[Mn(BIm)_4^{2+}]2[Cl^{-}]$ MIL precipitate and Mn(II,III) oxides were similar (Fig. S6 of the ESM). $[Mn(BIm)_4^{2+}]2[Cl^{-}]$ was the only MIL in which the formation of precipitate was observed, likely due to the weaker stability of Mn(II)–imidazole complexes compared to the analogous Co(II)– and Ni(II)–imidazole complexes. The reported stability constants (log *K*), found through potentiometric pH titrations ($I = 0.5$ M, $NaNO_3$; 25 °C), were 1.42 ± 0.01, 2.48 ± 0.02, and 3.09 ±

0.01, for the complexes [$MnIm^{2+}$], [$CoIm^{2+}$], and [$NiIm^{2+}$], respectively [49]. With these considerations, HPLC-DAD was chosen to perform indirect detection of DNA with all of the studied Mn(II)-based MILs, including [$Mn(BIm)_4^{2+}$]2[Cl^-], [$Mn(BIm)_4^{2+}$]2[NTf_2^-], and [$Mn(OIm)_4^{2+}$]2[NTf_2^-].

Student's *t* test was not used to compare detection methods for the [$Ni(BnIm)_4^{2+}$]2[Cl^-], [$Co(BIm)_4^{2+}$]2[Cl^-], and [$Co(BIm)_4^{2+}$]2[NTf_2^-] MILs because no stDNA was detected in HPLC-DAD. These results indicated almost quantitative extraction of the DNA, but also that the indirect method was not the most suitable for studying the extraction performance of the DLLME methods. The data obtained with the benzylimidazole-based MILs using HPLC-DAD agreed with those obtained by fluorescence, for which an EF up to 99% was obtained, indicating quantitative extraction of stDNA.

Figure 3 shows the EF obtained for the extraction of three different DNA sizes with all MILs. The extraction method was combined with HPLC-DAD or fluorescence, and the detection method was based on the aforementioned considerations (i.e., experiments using [$Mn(BIm)_4^{2+}$]2[Cl^-], [$Mn(BIm)_4^{2+}$]2[NTf_2^-], and [$Mn(OIm)_4^{2+}$]2[NTf_2^-] MILs were analyzed by HPLC-DAD, and the remaining MILs using fluorescence spectroscopy). Relative standard deviations (RSD) lower than 20% were obtained in all cases except for the Mn(II)-based MILs, where RSD values below 25% were achieved. The obtained EF values were 1.1–1.5 times higher when in situ DLLME was employed compared to conventional DLLME, an increase that is directly related to the metathesis reaction. These results were in agreement with previously reported in situ DLLME methods [39, 40, 42, 43, 50]. Furthermore, in both methods, the use of dimethylformamide as disperser solvent and vortex mixing increased the dispersion of the hydrophobic MIL in the aqueous solution, maximizing the contact area between the aqueous solution and MIL (see Fig. 3).

Effect of the MIL structure on the extraction of DNA

The DLLME methods described in this work are influenced by the metal center and the ligands that comprise the MIL. The transition metal center within the cation of the MIL can interact with DNA primarily through electrostatic interactions of the negatively charged phosphate backbone of DNA and through metal binding to the nitrogenous bases of DNA [51, 52]. Additionally, MILs and DNA can interact through hydrogen bonding, π-π stacking, and van der Waals interactions [53]. IL/MIL cations can bind to the minor grooves of dsDNA through hydrophobic and polar interactions [54]. According to Fig. 3, the Co(II)-based MILs provided the highest EF values (87% or greater), with almost quantitative extraction of ~ 20 kbp and ~ 250–500 bp DNAs using both in situ and conventional DLLME. The extractions using Mn(II)-based MILs provided lower EF values, likely due to the weaker stability of the MIL (see "Comparison of stDNA extraction efficiency using different methods" section).

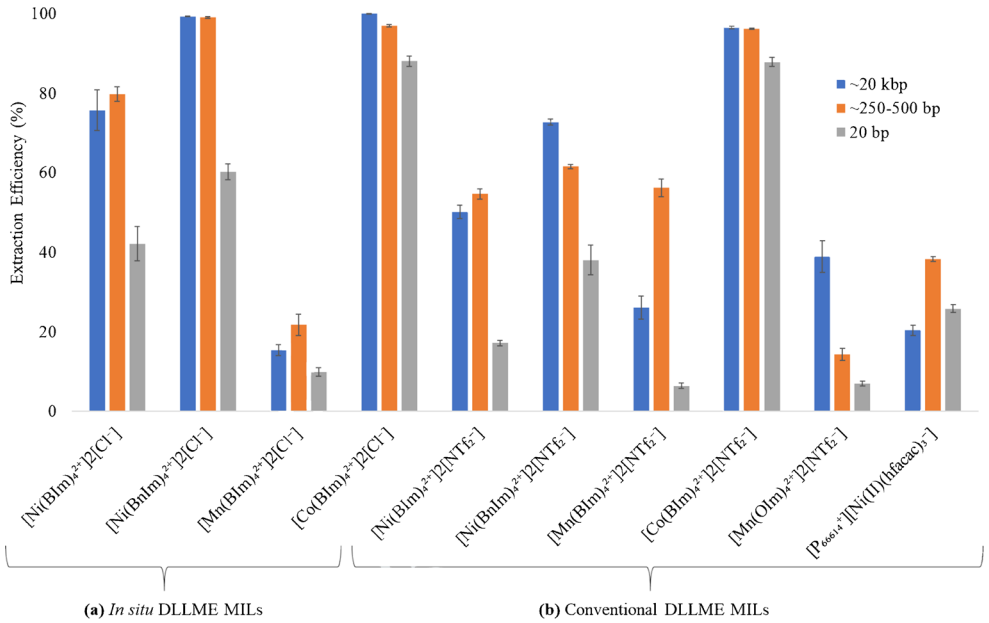

Fig. 3 Extraction efficiencies (% EF) of ~ 20 kbp stDNA (blue), ~ 250–500 bp stDNA (orange), and 20 bp DNA (gray) fragments by each of the MILs using (a) MIL-based in situ DLLME or (b) conventional MIL-DLLME and fluorescence emission spectroscopy detection. Experimental conditions ($n = 3$): 2 nM DNA, 2 mL total extraction volume, 15–24 μmol MIL, 300 μL dimethylformamide dispersive solvent, 3 min vortex at 2100 rpm. Note: For in situ DLLME, a range of 1:1.5 to 1:2.8 M ratio of MIL:[Li^+][NTf_2^-] was used, depending on the MIL. For the [P_{66614}^+][$Ni(II)(hfacac)_3^-$] MIL, no dispersive solvent was used. HPLC-DAD detection was used for the [$Mn(BIm)_4^{2+}$]2[Cl^-] MIL

The substituent groups attached to the imidazole rings within the MIL structure also play a role in the extraction of DNA. Most of the MILs contained BIm as ligand, with the exception of two MILs that contained BnIm (i.e., $[Ni(BnIm)_4^{2+}]2[Cl^-]$ and $[Ni(BnIm)_4^{2+}]2[NTf_2^-]$), and one composed of OIm (i.e., $[Mn(OIm)_4^{2+}]2[NTf_2^-]$). The $[Mn(OIm)_4^{2+}]2[NTf_2^-]$ MIL was only applied for conventional DLLME because its corresponding chloride salt ($[Mn(OIm)_4^{2+}]2[Cl^-]$) was not water soluble; therefore, the metathesis reaction with the DNA spiked sample was not successful. In both extraction modes, higher EF values were obtained with Ni(II)-based MILs containing BnIm as opposed to BIm ligands. In these cases, the BnIm ligands not only provided a more hydrophobic MIL structure but also facilitated π-π stacking of the MIL with DNA. In conventional MIL-DLLME, the $[Mn(OIm)_4^{2+}]2[NTf_2^-]$ MIL provided higher EF than $[Mn(BIm)_4^{2+}]2[NTf_2^-]$ for the extraction of ~ 20 kbp stDNA, providing evidence that imparting more hydrophobicity to the MIL through the addition of longer alkyl chain substituents to the imidazole ligand can enhance the extraction of larger DNA fragments.

Results obtained using this new generation of MILs were also compared to the $[P_{66614}^+][Ni(II)(hfacac)_3^-]$ MIL, which has been used in previous studies for DNA extractions [23, 25, 55]. In general, higher EF values were obtained for most of the new generation MILs, including those used in both in situ MIL-DLLME and conventional MIL-DLLME. For these materials, the paramagnetic metal is within the cation rather than the anion, and therefore, in situ generation of the hydrophobic MIL was possible without exchanging the paramagnetic metal during the metathesis reaction. Higher EF values were also obtained for the MILs containing $[NTf_2^-]$ anions used in conventional MIL-DLLME compared to the $[P_{66614}^+][Ni(II)(hfacac)_3^-]$ MIL. This may be due to greater electrostatic interaction between the divalent metal in the cation and the negatively charged phosphate groups in the DNA backbone, rather than with the trihexyl(tetradecyl)phosphonium cation of the $[P_{66614}^+][Ni(II)(hfacac)_3^-]$ MIL.

Selectivity of MILs in the extraction of duplex DNA fragments of varying sizes

The selectivity of the MILs in the extraction of different sized fragments of double-stranded DNA was investigated, and the results are shown in Fig. 3. In general, similar EF values for each MIL were observed in the extraction of the ~ 20-kbp stDNA and the ~ 250–500-bp DNA fragments. However, the EF values were partially reduced when the method was applied for the extraction of 20 bp DNA. The larger-sized DNA fragments (~ 20 kbp stDNA and ~ 250–500 bp DNA) provided a more hydrophobic environment, which increased hydrophobic interactions between the DNA and the MIL. Consequently, more DNA was extracted compared to the 20-bp DNA fragments, which are smaller and less hydrophobic. The biggest differences in EF were found with the Ni(II)-based MILs. Higher EF values (between 75 and 99%) were observed for the extraction of ~ 20 kbp stDNA and ~ 250–500 bp DNA fragments with the $[Ni(BIm)_4^{2+}]2[Cl^-]$ and $[Ni(BnIm)_4^{2+}]2[Cl^-]$ MILs, whereas EF values ranging between 42 and 60% were obtained for the 20-bp DNA fragment. The same trend was observed for the $[Ni(BIm)_4^{2+}]2[NTf_2^-]$ and $[Ni(BnIm)_4^{2+}]2[NTf_2^-]$ MILs with EF values between 50 and 73% for the larger DNA fragments and between 20 and 32% for the 20-bp DNA fragment. The $[Mn(BIm)_4^{2+}]2[NTf_2^-]$ MIL extracted the ~ 250–500-bp DNA fragments with the highest EF value of 56%, whereas the EF dropped to 26 and 8% for the ~ 20-kbp stDNA and 20-bp DNA fragments, respectively. If the results of this MIL are compared to those obtained for the $[Mn(OIm)_4^{2+}]2[NTf_2^-]$ MIL, increasing the alkyl chain substituent from butyl to octyl increased the tendency of the MIL to extract DNA fragments of ~ 20 kbp. For the Co(II)-based MILs, more subtle differences were observed in the extraction of different sizes of DNA.

Determination of metal ion concentration in the aqueous phase after extraction

As previously stated, the key aspect of the in situ MIL-DLLME method is the metathesis reaction between the MIL and the metathesis reagent ($[Li^+][NTf_2^-]$). If this reaction is not complete during the extraction procedure, some of the MIL (in the chloride form) can remain unreacted in the aqueous phase. The yield of the metathesis reaction can depend on the extraction conditions, the solubility of the MIL and metathesis reagent in aqueous solution, and the water stability of the MILs. Furthermore, the $[NTf_2^-]$ form of the MIL can also be found in the aqueous phase after extraction, as a result of partial solubility of the MIL in the aqueous solution [56]. In order to verify this hypothesis, AAS using the method of standard addition was applied to determine the amount of metal ion remaining in the aqueous phase after in situ MIL-DLLME. Since the metal is within the cation of the MIL, the amount of metal ion remaining in the aqueous phase after extraction can be directly related to the amount of the remaining MIL in both the $[Cl^-]$ and the $[NTf_2^-]$ forms. The obtained results are shown in Fig. S7 and Table S4 of the ESM. The percentage of the MIL in the aqueous phase after extraction, S_{MIL}, was calculated using Eq. (2).

$$S_{MIL} = \frac{C_{aq}}{C_o} \times 100 \quad (2)$$

where C_{aq} is the concentration of the MIL in the aqueous phase after extraction and C_o is the initial concentration of MIL. The results indicated that 24–59%, depending on the

Table 1 Comparison of the developed method with other reported methods from the literature

Sample/DNA size	Extraction method/extraction solvent/sorbent	Analytical technique	Extraction time	EF (%)	Ref.
Aqueous sample/20 bp DNA	In situ MIL-DLLME/$[Co(BIm)_4^{2+}]2[Cl^-]$ and $[Li^+][NTf_2^-]$	FD	3 min	88.05 ± 2.64	This method
Aqueous sample/~250–500 bp stDNA	In situ DLLME/$[Ni(BnIm)_4^{2+}]2[Cl^-]$ and $[Li^+][NTf_2^-]$	FD	3 min	99.04 ± 0.38	This method
Aqueous sample/~20 kbp stDNA	In situ DLLME/$[Co(BIm)_4^{2+}]2[Cl^-]$ and $[Li^+][NTf_2^-]$	FD	3 min	99.97 ± 0.03	This method
Aqueous sample/~20 kbp dsDNA	MIL-DDE[a]/$[P_{6,6,6,14}^+][FeCl_4^-]$	HPLC-UV	30 s	93.8 ± 0.6	[21]
Aqueous sample/single-stranded *KRAS* template DNA	MIL-SDME[b]/$[P_{6,6,6,14}^+][Ni(hfacac)_3^-]$	qPCR	20 min	–	[23]
Meat samples/mitochondrial DNA (mtDNA)	IL-ABS[c]/$[Chol^+][Hex^-]$ (10 w/v%) in sodium phosphate buffer (50 mM, pH = 8.5)	qPCR	15 min	–	[29]
Maize powder/genomic DNA (~10 kbp)	IL-ABS[c]/$[C_2MIm^+][Me_2PO_4^-]$ (10 w/v%) in sodium phosphate buffer (50 mM, pH = 8.5)	qPCR	5 min	–	[28]
Aqueous sample/~20 kbp stDNA	In situ IL-DLLME/$[C_{16}POHIm^+][Br^-]$ and $[Li^+][NTf_2^-]$	HPLC-UV	30 min	95.2 ± 0.4	[20]
Aqueous sample/calf thymus DNA and salmon testes DNA	IL-DLLME/$[BMIm^+][PF_6^-]$	FD	10 min	99.5	[24]

[a] MIL-based dispersive droplet extraction

[b] MIL-based single-drop microextraction

[c] Liquid extraction with an IL–aqueous buffer system

nature of the MIL, remained in the aqueous phase after in situ DLLME (Table S4 of the ESM). Among the different studied MILs, a significant amount of the $[Ni(BIm)_4^{2+}]2[Cl^-]$ MIL was detected in the aqueous phase after extraction (58.8% with respect to the initial amount of spiked MIL). This result is in accordance with the data obtained in Fig. 3 which revealed a relatively low EF for this MIL (75.7% for ~20 kbp stDNA), in comparison to the $[Ni(BnIm)_4^{2+}]2[Cl^-]$ and $[Co(BIm)_4^{2+}]2[Cl^-]$ MILs for which almost quantitative extraction of ~20 kbp stDNA was observed. For the $[Ni(BnIm)_4^{2+}]2[Cl^-]$ and $[Co(BIm)_4^{2+}]2[Cl^-]$ MILs, 46.7 and 38.8% of MIL remained in the aqueous phase after extraction, respectively. Nonetheless, for in situ DLLME with the three aforementioned MILs, the free MIL in the aqueous phase did not significantly affect the fluorescence signal of DNA as the obtained fluorescence data did not statistically differ with respect to the HPLC-DAD data (Table S3 and Fig. S3 of the ESM). Only 24.2% of the $[Mn(BIm)_4^{2+}]2[Cl^-]$ MIL remained in the aqueous phase, indicating that the Mn(II,III) oxide precipitate that formed over time during the preparation of the solutions for fluorescence detection was likely not present in the aqueous phase during the AAS measurement.

Comparison to other reported methods

DNA extraction by the in situ MIL-DLLME method was compared to other IL- and MIL-based extraction methods reported in the literature and is shown in Table 1. All reported extraction methods provided a high EF of DNA with values around 80% or greater. However, the developed extraction method is rapid, especially if the method is compared with those that used ILs [20, 24], which required centrifugation steps to recover the extraction phase. Among the different methods presented in Table 1 using MILs [21, 23], the present method is faster, requiring only 3 min for extraction, with the exception of the method reported by Clark et al. that reported an extraction time of 30 s using MIL-based dispersive droplet extraction (MIL-DDE) with the $[P_{6,6,6,14}^+][FeCl_4^-]$ MIL [21]. In any case, the in situ MIL-DLLME method presented in this work is simple to execute due to the low viscosity of the MIL solution in the chloride form, which is used initially in the extraction. Furthermore, this new generation of MILs is easy to prepare and is water stable (except for Mn(II)-based MILs).

Conclusions

This study constitutes the first report of the in situ formation of hydrophobic MILs for the extraction of DNA. This generation of MILs is easy and inexpensive to prepare making them a more affordable alternative for DNA extraction than commercially available DNA extraction kits. At the same time, these MILs possess three important features: (1) paramagnetic nature, resulting in a hydrophobic MIL droplet that can be retrieved with an external magnetic field; (2) their paramagnetic component is in the cation, which allows for the in situ generation of the hydrophobic MIL during extraction; and (3) low viscosity, which is convenient for its manipulation and transfer.

The in situ MIL-DLLME method can be combined with both HPLC-DAD or fluorescence detection, with the latter method more suitable for faster detection of DNA. Different sized fragments of dsDNA (20 bp, ~250–500 bp, and ~20 kbp DNA) were extracted by in situ MIL-DLLME and conventional MIL-DLLME, where 1.1–1.5 times higher EFs were obtained using in situ MIL-DLLME. Among the different studied MILs, the Co-based MILs ($[Co(BIm)_4^{2+}]2[Cl^-]$ and $[Co(BIm)_4^{2+}]2[NTf_2^-]$) provided the highest EFs (>85%). The Ni-based MILs ($[Ni(BIm)_4^{2+}]2[Cl^-]$, $[Ni(BIm)_4^{2+}]2[NTf_2^-]$, $[Ni(BnIm)_4^{2+}]2[Cl^-]$, and $[Ni(BnIm)_4^{2+}]2[NTf_2^-]$) showed the greatest selectivity in extracting the different sized duplex DNA fragments, with higher EF values obtained for the extraction of ~20 kbp stDNA and ~250–500 bp DNA fragments than the 20-bp DNA fragment.

The in situ MIL-DLLME method showed advantages over existing DNA extraction protocols due to its speed (3 min per extraction) and simplicity. Future studies are focused on the application of the in situ MIL-DLLME method to real biological samples as well as designing a MIL-compatible qPCR buffer in order to facilitate direct analysis of extracted DNA from the MIL droplet.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

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