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Magnetic Ionic Liquids as Solvents for RNA Extraction and Preservation

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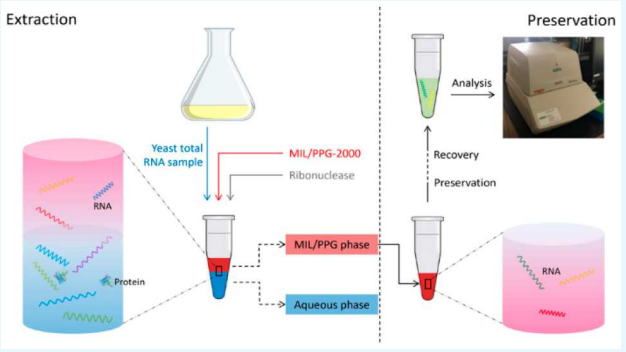
ABSTRACT: Ribonucleic acid (RNA) is particularly sensitive to enzymatic degradation by endonucleases prior to sample analysis. In-field preservation has been a challenge for RNA sample preparation. Very recently, hydrophobic magnetic ionic liquids (MIL) have shown significant promise in the area of RNA extraction. In this study, MILs were synthesized and employed as solvents for the extraction and preservation of RNA in aqueous solution. RNA samples obtained from yeast cells were extracted and preserved by the trihexyl(tetradecyl) phosphonium tris(hexafluoroacetylaceto)-cobaltate(II) ($[P_{66614}^{+}][Co(hfacac)_3^{-}]$) and trihexyl(tetradecyl) phosphonium tris(phenyltrifluoroacetylaceto)cobaltate(II) ($[P_{66614}^{+}][Co(Phtfacac)_3^{-}]$) MIL with a dispersion of the supporting media, polypropylene glycol, at room temperature for up to a 7 and 15 day period, respectively. High-quality RNA treated with ribonuclease A (RNase A) was recovered from the tetra(1-octylimidazole)cobaltate(II) di(L-glutamate) ($[Co(OIM)_4^{2+}][Glu^{-}]_2$) and tetra(1-octylimidazole)cobaltate(II) di(L-aspartate) ($[Co(OIM)_4^{2+}][Asp^{-}]_2$) MILs after a 24 h period at room temperature. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and agarose gel electrophoresis were used to determine the effect of RNA preservation. Furthermore, the preservation mechanism was investigated by exploring the partitioning of RNase A into the MIL using high-performance liquid chromatography.



INTRODUCTION

Ribonucleic acid (RNA) plays a prominent role in regulating gene expression and encoding proteins that are essential for the growth and survival of every living organism.[1−3] Because of its high biological relevance and significant role in gene expression, RNA has attracted notable research interest. However, messenger RNA (mRNA)[4] and small interfering RNA (siRNA)[5] are prone to degradation in a variety of ways including denaturation,[6] oxidization,[7] and nuclease cleavage.[8] For instance, mRNA is particularly prone to rapid degradation by ubiquitous ribonucleases (RNases).[4] In certain conditions, biological samples collected in the field may contain numerous compounds, such as RNase, which can degrade RNA instantly. These samples can only be handled by simple in-field treatments and require preservation before in-lab analysis.[9] Therefore, the isolation of RNA from contaminating RNases and subsequent preservation during sample preparation are critical steps in order to maximize the yield of pure RNA. Moreover, the isolated RNA must be sufficiently pure for analysis with biomolecular techniques such as the reverse transcription polymerase chain reaction (RT-PCR), quantitative RT-PCR (qRT-PCR), and Northern Blot analysis.[4,10−12]

A number of techniques have been developed that preserve RNA over time and protect it from endonuclease degradation *in vitro*. One of the most commonly used methods is diethylpyrocarbonate (DEPC) pretreatment, which can deactivate RNases by forming amide bonds between amino and carboxylic groups.[13,14] However, DEPC is unstable in aqueous solution and can easily react with carbon dioxide or ethanol,[15] which limits its use in certain applications. Other widely accepted methods include the paraffin-embedded tissue process and the formalin-fixed paraffin-embedded tissues (FFPE).[2,16,17] FFPE is especially preferred in tissue sample preparation for downstream analysis involving the polymerase chain reaction (PCR). However, formalin can cross-react with proteins in the sample matrix,[18] leading to the inhibition of reverse transcription for mRNA.[19] In addition, other methods such as lyophilization,[20] formamide protection,[21] and numerous RNase inhibitor treatments[22−24] have been applied for RNA preservation. Unfortunately, drawbacks to these preservation techniques include the requirements of specialized equipment, multiple tedious steps, or a high amount of energy.

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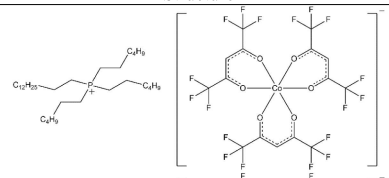
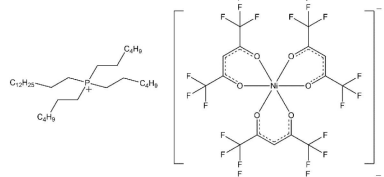
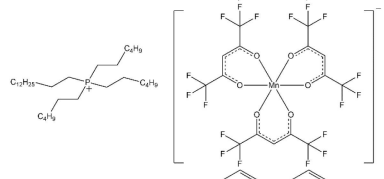
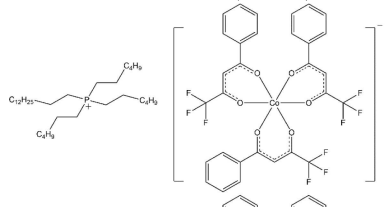
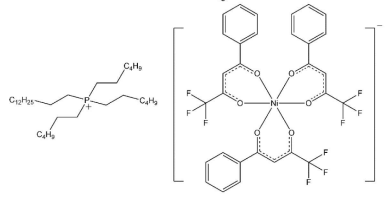
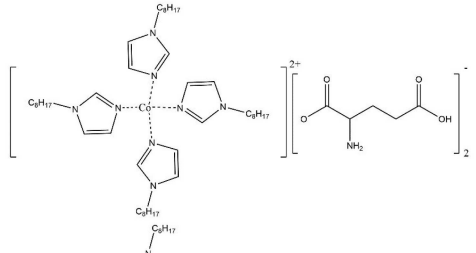
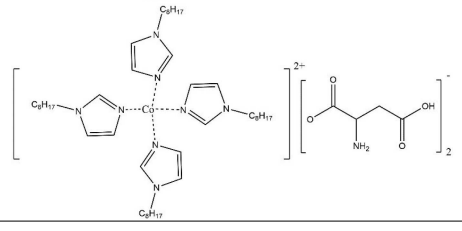
Table 1. Chemical Formulas and Structures of MILs 1−7 That Were Investigated in This Study

MIL	Chemical Formula	Structure
1	$[P_{66614}^{+}][Co(hfacac)_3^{-}]$	
2	$[P_{66614}^{+}][Ni(hfacac)_3^{-}]$	
3	$[P_{66614}^{+}][Mn(hfacac)_3^{-}]$	
4	$[P_{66614}^{+}][Co(Phtfacac)_3^{-}]$	
5	$[P_{66614}^{+}][Ni(Phtfacac)_3^{-}]$	
6	$[Co(OIM)_4^{2+}][Glu^{-}]_2$	
7	$[Co(OIM)_4^{2+}][Asp^{-}]_2$	

Because of the inherent limitations of current methods, it is important to explore the development of methods that effectively combine sample preparation and RNA preservation to minimize the risk of nuclease contamination and maximize the amount of recovered RNA for downstream analysis.

Recently, ionic liquid (IL)-based materials have been shown to exhibit encouraging compatibility in nucleic acid analysis.[25,26] ILs are organic molten salts that possess melting points at or below 100 °C. Because of their tunable cation and anion structures[27,28] and ability to interact with a variety of biomolecules,[29,30] ILs have demonstrated high potential as nucleic acid preservation and extraction solvents.[31−33] For instance, imidazolium[31] and choline-based[25,34] ILs have been previously reported in RNA preservation applications. They have been demonstrated to preserve RNA by either isolating the target nucleic acid from the sample matrix or by maintaining the stability of RNA within the IL.[35] Magnetic ionic liquids (MILs) are a subclass of ILs that incorporate paramagnetic centers in their chemical structures. Because of their ability to be manipulated by an external magnetic field and affinity for biological molecules such as DNA and RNA, MILs have drawn considerable research interest for nucleic acid extraction[36] and for applications requiring automatic operation.[37] In a previously published study,[38] several MILs with different chemical structures were demonstrated to simultaneously extract DNA from aqueous solutions while

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protecting DNA from deoxyribonuclease I (DNase I) degradation.

In order to stabilize RNA in a hydrophobic microenvironment and prevent degradation, several MILs were designed and synthesized in this study based on previously reported ILs.[35,38] The MILs were investigated for their ability to serve as RNA extraction and preservation media. The trihexyl(tetradecyl) phosphonium tris(hexafluoroacetylaceto)-cobaltate(II) ($[P_{66614}^+][Co(hfacac)_3^-]$) and trihexyl(tetradecyl) phosphonium tris(phenyltrifluoroacetylaceto)-cobaltate(II) ($[P_{66614}^+][Co(Phtfacac)_3^-]$) MILs were dispersed in polypropylene glycol (PPG), average Mn ≈ 2000 (PPG-2000). The MIL/PPG-2000 system was investigated for the capability of extracting and preserving yeast total RNA from aqueous solution which could subsequently be analyzed via the qRT-PCR. In addition, another two MILs, namely, tetra(1-octylimidazole)cobaltate(II) di(L-glutamate) ($[Co(OIM)_4^{2+}][Glu^-]_2$) and tetra(1-octylimidazole)cobaltate-(II) di(L-aspartate) ($[Co(OIM)_4^{2+}][Asp^-]_2$) were capable of protecting yeast total RNA from RNase A degradation. Reversed-phase ion-pair liquid chromatography was used to investigate the RNase A extraction efficiency of the MILs to elucidate the preservation mechanism. Anion-exchange high-performance liquid chromatography (HPLC) and agarose gel electrophoresis were used to quantitatively evaluate the recovery efficiency of yeast total RNA. qRT-PCR was used to evaluate the structural integrity of mRNA from the preserved yeast total RNA.

RESULTS AND DISCUSSION

Partitioning of RNA to MILs. MILs **1**−**3** (Table 1) were initially chosen for RNA extraction and preservation as they have been previously used for DNA extraction.[36,39] Two other MILs (**4** and **5**) incorporating aromatic moieties were also investigated. After a 60 min single-droplet extraction (SDE), the yeast total RNA extracted by the MILs was recovered by a liquid−liquid extraction (LLE) method using ethyl acetate and Tris−HCl/ethylenediaminetetraacetic acid (EDTA) buffer prior to analysis. Different LLE buffer compositions were tested and optimized to maximize the recovery of RNA. Tris−HCl concentrations of 40, 80, 160, and 320 mM, EDTA concentrations of 1, 2, and 3 mM, and pH 7 and 8 were investigated for the RNA back-extraction. Consequently, a LLE buffer consisting of 160 mM Tris−HCl, 2 mM EDTA, and pH 8 was chosen as it afforded the highest RNA recovery. An external calibration curve for yeast total RNA was established and used to calculate the RNA concentration in aqueous solution (Figure S1). The extraction efficiency (E_e) of MILs was determined by comparing the total RNA concentration before (C_{std}) and after (C_{ext}) extraction using eq 1.

$$\text{Extraction efficiency } (E_e\ \%) = \left(1 - \frac{C_{ext}}{C_{std}}\right) \times 100\% \quad (1)$$

As shown in Figure S2, MIL **4** exhibited the highest E_e (72.79 ± 5.66%) of MILs **1**−**5**, close to double the E_e of MIL **1** (39.34 ± 2.65%) though they have the same metal center and cation in their chemical structures. In addition, MIL **5** exhibited an E_e (33.12 ± 3.64%) higher than that of MIL **2** (21.02 ± 2.68%). The reason for this dramatic increase in the E_e could be due to the aromatic moieties in MILs **4** and **5**, which may interact via $\pi-\pi$ stacking interaction with the exposed bases in RNA.[40] A positive control and a no reverse transcriptase (NRT) control were performed together. As shown in Figure 1, mRNA recovered from MILs **1**−**3**

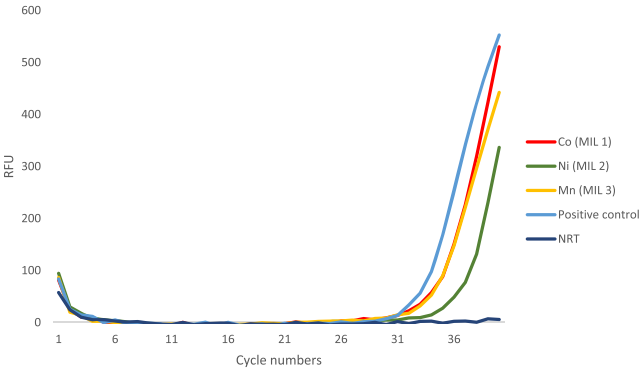

Figure 1. Representative qRT-PCR amplification of cDNA following extraction of 100 ng of RNA with 5 μL of MILs **1**-**3** and LLE recovery. Positive control: recovery of 100 ng of RNA by LLE.

produced complementary DNA (cDNA), indicating intact mRNA. Subsequently, the cycle of quantification (Cq) values generated by qRT-PCR were compared with the positive control (33.03). The Cq value is related to the amount of cDNA, and each decrease of one Cq value represents a 2-fold increase in the mass of nucleic acid. MIL **1** and **3** did not show a significant increase in the Cq value (MIL **1**: 34.00, MIL **2**: 36.31, MIL **3**: 34.13), suggesting only limited RNA loss during the extraction and recovery process. As an example, mRNA recovered from MIL **1** produced approximately 49% less cDNA than the positive control. The nonamplified NRT control ensured that there was no false-positive amplification caused by leftover cDNA sequences in the total yeast RNA. Based on these experiments, MILs **1**, **4**, and **5** were chosen to further examine their preservation ability.

Initially, an identical RNA extraction procedure using MILs **1**, **4**, and **5** was performed with a RNA aqueous solution containing 500 ng yeast total RNA. The biphasic mixtures were stored for another 6 h before recovery. In addition, another 500 ng of yeast total RNA was directly stored for 6 h at −20 °C and used as a positive control. As shown in Figure 2, neither qRT-PCR or agarose gel electrophoresis showed a significant amount of RNA recovered from MIL **5**. However, a portion of mRNA was recovered from MIL **1**, as demonstrated by a Cq value of 38.82. MIL **4** afforded a higher mRNA recovery producing a Cq value of 34.86, approximately 16-times greater than MIL **1**. Agarose gel electrophoresis also indicated that MIL **4** exhibited a superior ability to preserve extracted RNA from degradation compared to the other two MILs. Comparing the results shown in Figures 2 to S2, the MIL with a higher extraction efficiency produced a lower Cq value, except for MIL **5**. Surprisingly, MIL **5** produced the highest Cq value (no amplification in 40 cycles), and no bright cDNA band was observed in the agarose gel, suggesting that the metal center in the MIL structure may play a role in extraction as well as recovery of nucleic acid.[41] In addition, the amount of RNA recovered from MILs **1**, **4**, and **5** was no better than the positive control (Cq = 29.11). The yeast total RNA recovered from MILs **1**, **4**, and **5** was not able to be detected by either HPLC or qRT-PCR after preservation at room temperature for 24 h (Figure S3). While these results are encouraging, additional conditions must be explored to increase the stability and preservation time of RNA in MILs.

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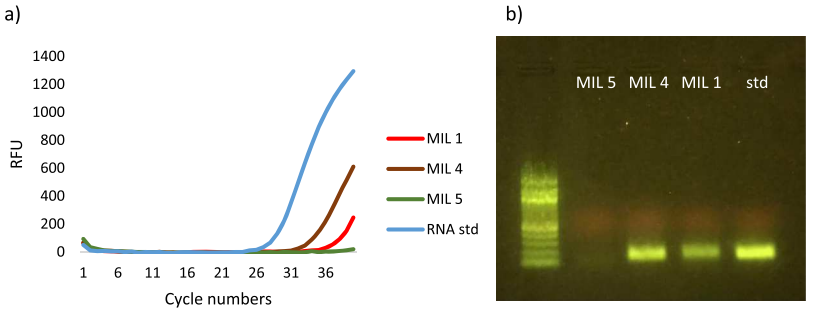

Figure 2. (a) Representative qRT-PCR amplification of cDNA following preservation of 500 ng of RNA for 6 h. (b) Agarose gel electrophoresis of cDNA after qRT-PCR amplification (left lane: 100 bp DNA ladder, New England BioLabs).

Degradation of RNA under Various Conditions. To further investigate the preservation process of RNA in MILs, MIL **4** was chosen to examine additional conditions as it possessed the highest E_e of the MILs previously tested. Initially, sodium dodecyl sulfate (SDS) was incorporated in the yeast total RNA standard solution based on previous studies and commonly used RNA preservation methods.[8,17] Consequently, SDS increased the stability of yeast total RNA extracted by MIL **4**. RNA could be recovered and detected by qRT-PCR after a 1 day preservation period, as shown in Table 2. However, the RNA still suffered from degradation after a 3-day storage period as no amplification was detected by qRT-PCR. In addition, as RNA can be degraded at a relatively faster rate in aqueous solution than in an anhydrous environment,[16] the presence of water is considered an essential component during RNA degradation. Therefore, removing water from the biphasic mixture after the extraction process by directly pipetting out was also investigated. As shown in Table 2, the amount of recovered RNA from MIL **4** dramatically increased when compared to the previous experiment where water remained in contact with MIL after extraction. In addition, other methods of water removal such as applying vacuum did not significantly affect the amount of RNA recovered. In comparison, Table 2 reveals that no amplification was detected for the negative control where RNA was directly stored in aqueous solution at room temperature for a period of 1 day. A possible explanation of this observation may be that the extracted nucleic acid remains on the surface of the MIL. The contact between RNA and water leads to an increase in the degradation rate. Another possible explanation considered that the degradation of RNA in the aqueous phase may shift the equilibrium in the biphasic system and cause more RNA to be degraded. Although removal of water can increase the preservation time and improve stability, RNA directly

Table 2. Comparison of the RNA Preservation Conditions of Using 0.1% SDS and the Separation of MIL 4 from the Aqueous Phase

0.1% SDS	aqueous phase	preservation time (days)	Cq value
√	√ (remained)	1	36.45
√	√ (remained)	3	no amplification
×	× (separated)	1	27.76
×	× (separated)	3	no amplification
√	× (separated)	1	33.06
×	√ (remained)	1	no amplification

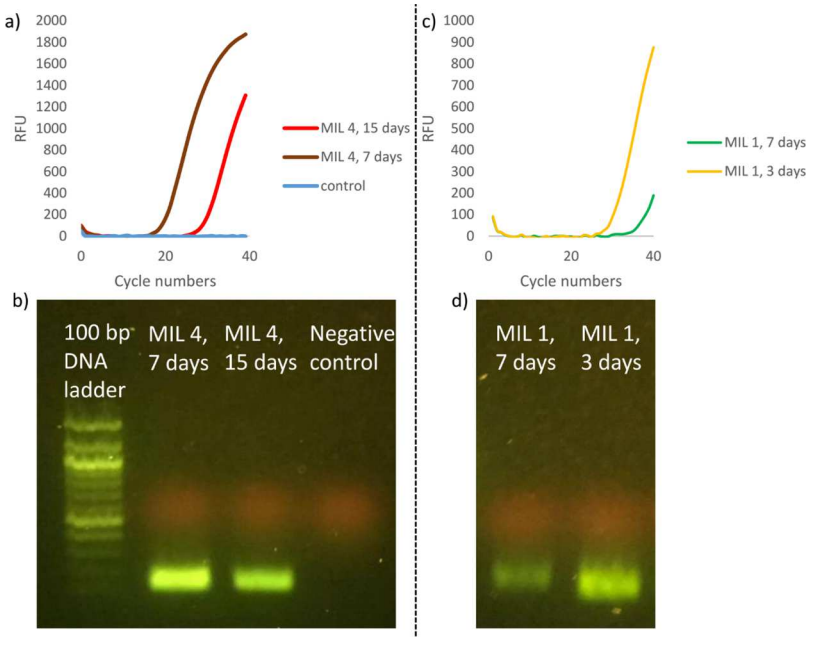

Figure 3. qRT-PCR amplification and agarose gel electrophoresis of cDNA after preserving 5 μg of RNA in MIL **4**/PPG-2000 for 7 and 15 days (a,b), MIL **1**/PPG-2000 for 3 and 7 days (c,d).

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preserved by the MIL cannot be detected by the qRT-PCR after a 3-day preservation period (Table 2).

PPG-2000 Enhances Preservation of RNA in MILs. A number of polymers such as polyethylene glycol (PEG), PPG, and polyvinylpyrrolidone (PVP) have been used to vary the partitioning of various compounds including nucleic acid and proteins between the phases in a two-phase system.[42] In order to increase the preservation time of RNA, PPG-2000 was introduced as a hydrophobic supporting solvent for RNA preservation. More specifically, MIL **4** was dissolved in PPG-2000 in a 1:5 (v/v) ratio before the yeast total RNA extraction process. LLE using ethyl acetate and Tris−HCl/EDTA buffer was no longer effective because of the presence of PPG-2000. Thus, an ethanol precipitation method was used to recover the yeast total RNA from the MIL/PPG-2000 phase. As a result, the amount of RNA recovered from MIL **4**/PPG-2000 increased dramatically. A direct comparison of the amount of yeast total RNA recovered from MIL/PPG-2000 revealed a 4.92 times higher amount of RNA recovered from the MIL, as shown in Figure S4. To study the effect of PPG-2000, an additional experiment was performed using the same procedure with PPG-2000 but without MILs. As shown in Figure S5, the PPG-2000 had a very limited effect on extraction efficiency of yeast total RNA, confirming its role as a supporting medium for MIL-based extraction. Furthermore, the recovery was determined using eq 2 using the HPLC peak area of recovered RNA (A_{re}), RNA standard (A_{std}), the volume of resuspended DEPC-treated water (V_w), and RNA standard (V_{std}). Although the recovery of RNA varied from MIL **1** (0.66% RNA was recovered after 3 days) and MIL **4** (1.00% RNA was recovered after 3 days), the preservation time had a significant increase after applying PPG-2000. As shown in Figure 3, preserved RNA could still be detected by the qRT-PCR and agarose gel electrophoresis after a 15-day preservation period. In contrast, the RNA directly stored in water was completely degraded after 7 days.

$$\text{Recovery}\ (\%) = \frac{A_{re} \times V_w}{A_{std} \times V_{std}} \times 100\% \quad (2)$$

RNA Preservation against RNase A. As mentioned previously, nucleic acid preservation in the presence of endonucleases is a significant challenge during sample preparation. In particular, ubiquitous RNases can degrade RNA instantly. To determine if MIL/PPG-2000 could prevent RNA from endonuclease degradation, RNase A was introduced into the system before extraction. As shown in Figure S6, no RNA was recovered from either MIL **1**/PPG-2000 or MIL **4**/PPG-2000 in the presence of RNase A.

In order to reduce the degradation caused by RNase A, optimized conditions were tested. Consequently, no amplification was observed by qRT-PCR after a 1 h incubation of RNase A followed by a 1-h extraction of yeast total RNA though the experimental conditions for RNA preservation up to 15 days period were applied. Furthermore, the addition of 0.1% SDS solution did not improve RNA preservation, as shown in Figure S6.

Inspired by the work of Freire and co-workers[35] which incorporated amino acids in the chemical structure of ILs, MIL **6**, and **7** were synthesized and investigated. The extraction and preservation steps were performed using the experimental conditions previously described with an additional step of spiking RNase A into the MIL/PPG-2000 system. Consequently, a significant amount of RNA was recovered from both MIL **6**/PPG-2000 (22.01% recovery) and MIL **7**/PPG-2000 (12.61% recovery), as shown in Figure 4. Although the

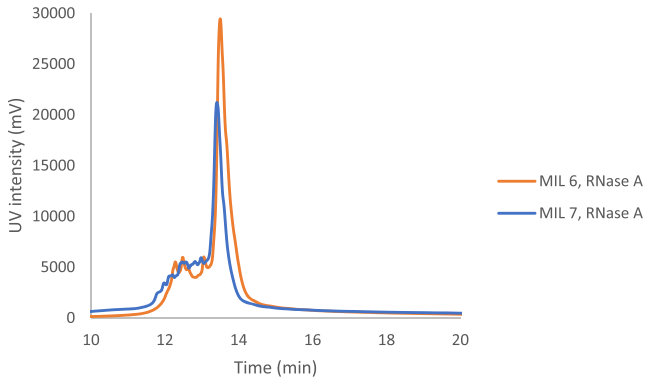

Figure 4. HPLC quantification of 5 μg of RNA recovered from MIL **6**/PPG-2000 spiked with 4 μg RNase A (orange) and MIL **7**/PPG-2000 spiked with 4 μg RNase A (blue).

qRT-PCR results in Figure S7 revealed high Cq values which should represent a low amount of cDNA, these values from the experiments could be due to MIL moieties in the RNA precipitate inhibiting the qPCR amplification rather than insufficient preservation. To test this assumption, agarose gel electrophoresis experiments were performed by directly loading recovered RNA samples. The agarose gel electrophoresis results in Figure 5 demonstrated the preservation of RNA. Bands can be observed even without performing an amplification step, indicating a high quantity of RNA recovery.

Because some ILs may play a role in the preservation of nucleic acids by destabilizing endonucleases,[43] the partitioning of RNase A to MILs was further investigated by reversed-phase ion-pair liquid chromatography. MILs **1**, **4**, **6**, and **7** were tested because of their advanced preservation ability of yeast total RNA. As shown in Figure S8, the amount of RNase A extracted by MILs was not significant at first but slowly increased within 4 h. This suggests that the mechanism of RNA preservation is mainly due to the RNA-MIL interaction. However, the endonuclease destabilization by the MILs may promote the preservation as well if the extraction time is increased. Compared with other MILs, MIL **6** and **7** exhibited a superior ability in protecting RNA from RNase A degradation. Furthermore, the recovered RNA can be detected by agarose gel electrophoresis even after 24 h incubation in the presence of RNase A (Figure 5).

EXPERIMENTAL SECTION

Reagents and Materials. LC−MS grade acetonitrile (≥99.9%), hexane, mixture of isomers (≥98.5%), methanol (≥99.8%), chloroform (≥99.8%), isoamyl alcohol (≥98%), ethyl acetate (≥99.5%), water (DEPC-treated and sterile filtered), trifluoroacetic acid (99%), EDTA (99.4−100.06%), poly(propylene glycol) (PPG, average Mn ∼ 2000), liquified phenol (≥89.0%), cobalt(II) chloride (97%), silver nitrate (≥99.0%), sodium dodecyl sulfate (99%), magnesium chloride (99.0−102.0%), L-glutamic acid, L-aspartic acid, yeast synthetic drop-out medium, Amberlite IRN78 hydroxide form, and acid-wash glass beads were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethyl ether (≥99%) was purchased from Avantor (Center Valley, PA, USA). 1,1,1,5,5,5-Hexafluoroace-

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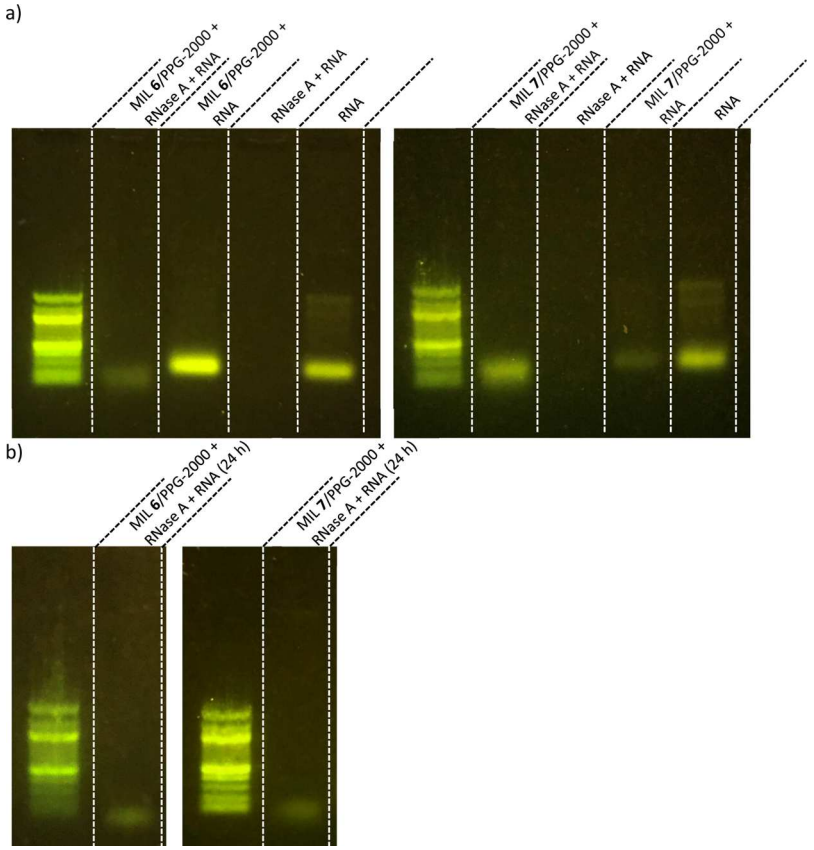

Figure 5. Effect of RNase A on RNA preservation within (a) MIL **6**/PPG-2000 for 2 h and MIL **7**/PPG-2000 for 2 h. (b) MIL **6**/PPG-2000 for 24 h and MIL **7**/PPG-2000 for 24 h. Left lane of each agarose gel: 100 bp DNA ladder (New England BioLabs).

tylacetone (99%), 4,4,4-trifluoro-1-phenyl-1,3-butanedione (99%), glycerol (99+%), and nickel(II) chloride (98%) were purchased from Acros Organics (Morris, NJ, USA). Tris-(hydroxymethyl)aminomethane (ultra pure) and tris-(hydroxymethyl)aminomethane hydrochloride (≥99.0%) were purchased from RPI (Mount Prospect, IL, USA). Sodium hydroxide, glucose (dextrose anhydrous), agarose, sodium chloride, sodium hydroxide, sodium acetate, dimethylsulfoxide (DMSO), acetic acid, and ammonium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The SsoAdvanced Universal SYBR Green Supermix was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Manganese-(II) chloride tetrahydrate (98.0−101.0%) was purchased from Alfa Aesar (Ward Hill, MA, USA). Ethanol (200 proof) was purchased from Decon Labs, Inc. (King of Prussia, PA, USA). Octylimidazole (98%) was purchased from IOLITEC (Tuscaloosa, AL, USA). The Difco yeast nitrogen base w/o amino acid was purchased from Becton Dickinson (Sparks, MD, USA). RQ1 RNase-Free DNase I was purchased from Promega (Madison, WI, USA). The SuperScript III Reverse Transcriptase Kit and SYBR Safe DNA gel stain were purchased from Invitrogen (Carlsbad, CA, USA). RNase A (from bovine pancreas) was purchased from Roche (Mannheim, Germany). All primers were purchased from Integrated DNA Technologies (Coralville, IA, USA).

MIL Synthesis. The chemical structures of all MILs that were examined in this study are shown in Table 1. Among them, the $[P_{66614}^+][Co(hfacac)_3^-]$, $[P_{66614}^+][Ni(hfacac)_3^-]$, $[P_{66614}^+][Mn(hfacac)_3^-]$, $[P_{66614}^+][Co(Phtfacac)_3^-]$, and $[P_{66614}^+][Ni(Phtfacac)_3^-]$ MILs were synthesized according to the previously published procedures.[37,44] The $[Co(OIM)_4^{2+}][Cl^-]_2$ salt was synthesized based on the previously published procedures.[45,46] Each equivalent of the salt was dissolved in methanol, and an anion-exchange reaction was performed in a column filled with 4−6 equiv of the Amberlite IRN78 resin in the hydroxide form. The eluent was reacted with 2.2 equiv of glutamic acid or aspartic acid at room temperature overnight to obtain the $[Co(OIM)_4^{2+}][Glu^-]_2$ and $[Co(OIM)_4^{2+}][Asp^-]_2$ MIL solutions, respectively. The residual neutral amino acid was crystallized in cold acetonitrile and removed by filtration.

The $[P_{66614}^+][Co(hfacac)_3^-]$, $[P_{66614}^+][Ni(hfacac)_3^-]$, $[P_{66614}^+][Mn(hfacac)_3^-]$, $[P_{66614}^+][Co(Phtfacac)_3^-]$, and $[P_{66614}^+][Ni(Phtfacac)_3^-]$ MILs were synthesized by reacting 10 mmol of ammonium hydroxide with 10 mmol of hexafluoroacetylacetone or 4,4,4-trifluoro1-phenyl-1,3-butanedione. Subsequently, 3.3 mmol of cobalt(II) chloride hexahydrate, nickel(II) chloride hexahydrate, or manganese-(II) chloride tetrahydrate were added and reacted for 24 h at room temperature. The $[NH_4^+][M(hfacac)_3^-]$ and $[NH_4^+]$-$[M(Phtfacac)_3^-]$ salt products were washed with water several times and subsequently reacted with 1 mmol of purified $[P_{66614}^+][Cl^-]$ in methanol for 24 h at room temperature. The MIL products in diethyl ether solution were washed with deionized water and dried at 50 °C overnight under reduced pressure.

Yeast Total RNA Preparation. The total RNA and mRNA samples were both obtained from yeast cells (BY4735). The first generation of yeast cells was transferred into a 100 mL volume of the liquid medium (0.67% yeast nitrogen base,

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0.2% synthetic dropout medium, and 2% glucose) and incubated at 300 rpm for approximately 2 days at 30 °C until OD_{600} > 1.0 (the optical density of the yeast cell suspension measured at 600 nm). After incubation, the yeast cell suspension was transferred into two separate 50 mL centrifuge tubes and centrifuged at 3700 rpm for 5 min at 4 °C. The precipitated cells were washed with DEPC-treated water and centrifuged under the previously described condition. The washed cells were then resuspended in 3 mL (1 volume) RNA extraction buffer which consisted of 50 mM Tris−HCl, 10 mM EDTA, and 0.1 M NaCl, at pH 7.5 with 5% sodium dodecyl sulfate (SDS). One volume of denaturing buffer (phenol (pH 4): chloroform: isoamyl alcohol, v/v/v = 49.5:49.5:1) was added to the resuspended cells along with 1 g of acid-washed glass beads. The mixture was incubated at room temperature for 6 min and vortexed at high speed for 2 min. The suspension was centrifuged, and the supernatant carefully transferred to a new tube. Another volume of denaturing buffer was added and vortexed at high speed for 2 min. After centrifugation, the supernatant was carefully transferred to a new tube. The supernatant was extracted with one volume of denaturing buffer and centrifuged before transferring to another new tube. Subsequently, the chloroform: isoamyl alcohol (v/v = 24:1) buffer was added, and the tubes were vortexed at high speed for 2 min to remove the residual phenol in the supernatant. For each volume of RNA solution, a 0.1 volume of 3 M sodium acetate (pH 5.2) and 3 volumes of iced ethanol stored at −20 °C were added to the aqueous layer. The suspension was incubated at −20 °C for at least 1 h to precipitate the nucleic acid. The precipitate was washed with 70% ethanol after centrifugation and resuspended in DEPC-treated water. The obtained yeast total RNA was further treated with DNase I in 1× Reaction Buffer (New England BioLabs) for 30 min at 37 °C to remove genomic DNA. DNase I was inactivated by an addition of 2 mM EGTA at 65 °C for 10 min subsequently, and the solution was stored at −80 °C. Eventually, the concentration of the yeast total RNA solution was analyzed using a NanoDrop Spectrophotometer (Thermo 2000c).

qRT-PCR Conditions. The reverse transcription reaction was performed using a SuperScript III Reverse Transcriptase Kit (Invitrogen). The reaction mix consisted of 4 μL of 5× First-Strand Buffer, 1 μL of 10 μM reverse primer (5′-TAC CGG CAG ATT CCA AAC CC-3′), 1 μL of 0.1 M DTT, 1 μL of 10 mM dNTP, 1 μL of SuperScript III RT (200 U/μL), RNA sample solution, and Milli-Q water to yield a 20 μL reaction mix. The thermal protocol for all reaction mixes was as follows: 65 °C for 5 min, 4 °C for 5 min, 45 °C for 60 min, 70 °C for 15 min, and hold at 4 °C at the end. After the reverse transcription reaction, 1−2 μL of the solution was subjected to a qPCR reaction mix which consisted of 10 μL of SsoAdvanced Universal SYBR Green Supermix (2×), 2.6 μL of 50 mM $MgCl_2$, 1 μL of DMSO, 0.6 μL of 10 μM forward primer (5′-GAA ATG CAA ACC GCT GCT CA-3′), 0.6 μL of 10 μM reverse primer, and 3.2−4.2 μL of Milli-Q water (20 μL for each reaction mix). The thermal cycling protocol for the qPCR was as follows: an initial denaturation step of 5 min at 95.0 °C followed by 40 cycles of 10 s at 95.0 °C and 30 s at 64.0 °C.

Agarose Gel Electrophoresis Conditions. A 0.8% agarose gel containing 5% SYBR Safe DNA Gel Stain (10,000×) was used for agarose gel electrophoresis. A volume of 20 μL of either the complementary DNA (cDNA) generated by reverse transcription or the RNA was pretreated with 4 μL of 30% glycerol before loading on the gel. Agarose gel electrophoresis was carried out for 30 min at 125 V with 1× TAE buffer.

LLE and Recovery of the RNA Sample. A 5 μL volume of MIL was added directly to the RNA sample solution, and the mixture was incubated without stirring at room temperature for approximately 60 min. The aqueous phase was then separated and prepared for HPLC injection to determine the extraction efficiency of RNA, while the MIL phase was separated and dissolved in 25 μL of ethyl acetate. The RNA in the MIL phase was recovered into the aqueous phase by adding 25 μL of DEPC-treated water or 25 μL of LLE buffer (160 mM Tris−HCl, 2 mM EDTA, pH 8), and the biphasic mixture was vortexed for 1 min. A 12 μL volume of the aqueous phase was subjected to qRT-PCR. The generated and amplified cDNA was further analyzed by agarose gel electrophoresis. A positive control was performed using the same procedure without the MIL present.

Preservation of RNA within MILs. A 2 μg mass of RNA in aqueous solution or 0.1% SDS solution was extracted by 5 μL of MIL for approximately 60 min. Two different preservation conditions were investigated: (1) the biphasic mixture was directly stored at room temperature or −20 °C and (2) the aqueous phase was carefully removed before the MIL phase was stored at room temperature or −20 °C. Subsequently, the remaining total RNA was recovered by LLE, as previously described, and analyzed by qRT-PCR. Additionally, to increase the preservation time, a 5 μL volume of MIL was dissolved in 25 μL of PPG-2000 before the extraction and preservation of a 5 μg mass of RNA aqueous solution. The aqueous phase was carefully removed subsequently, and the MIL/PPG-2000 phase was stored at room temperature or −20 °C for 7- to 15-day preservation period. Afterward, 150 μL of iced ethanol and 5 μL of 3 M sodium acetate (pH 5.2) was added to precipitate the remaining RNA. The solution was kept at −20 °C for 1 to 2 h and centrifuged at 15,000 rpm for at least 10 min. After carefully removing the supernatant, the RNA was resuspended with DEPC-treated water followed by downstream analysis by HPLC, qRT-PCR, or agarose gel electrophoresis.

Preservation of RNA from RNase A. The RNA preservation experiments were performed by the previously described methods with the addition of RNase A. A 5 μL volume of MIL was initially dissolved in 25 μL of PPG-2000, followed by 4 μL of 1 μg/μL RNase A solution being spiked with MIL/PPG-2000. After an incubation time of 60 min, the aqueous phase was removed, and 5 μg of RNA sample solution was added and incubated for 60 min. The aqueous phase was carefully removed, and total RNA was recovered by ethanol precipitation and resuspended in DEPC-treated water. A negative control was performed using the same procedure without MILs.

Partitioning Behavior of RNA and MILs. The standard RNA solution used in these experiments was a diluted 10 ppm yeast total RNA solution. SDE was performed with 1 μL of MIL and 50 μL of standard solution to determine the partitioning behavior. After 1 h extraction, the residual aqueous solution was analyzed by anion-exchange HPLC using a Shimadzu LC-20AT HPLC chromatograph (Columbia, MD, USA) with a multiwavelength UV−vis detector and separated on a 35 × 4.6 mm i.d. × 2.5 μm TSKgel DEAE-NPR anion exchange column with a 5 × 4.6 mm i.d. × 5 μm TSKgel DEAE-NPR guard column (Tosoh Bioscience, King of Prussia,

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PA). Mobile phase A consisted of 20 mM Tris−HCl (pH 7), and mobile phase B consisted of 1 M NaCl and 20 mM Tris−HCl (pH 7). RNA was detected at 260 nm, and the amount of RNA was determined using an external calibration curve. Gradient elution was performed with the following program: 0% B from 0 to 2 min, increased from 0 to 5% B from 2 to 9 min, increased to 50% B from 9 to 10 min, increased to 100% B from 10 to 15 min, held at 100% B from 15 to 20 min, decreased from 100 to 0% from 20 to 22 min, and held at 0% from 22 to 30 min.

Partitioning Behavior of RNase A and MILs. A 100 μL volume of 1 μg/μL RNase A solution was extracted by 1 μL of MILs using the same SDE method described in the previous experiments. After 1 h extraction, the residual aqueous solution was injected onto a Shimadzu LC-20AT HPLC with a multi-wavelength UV−vis detector and separated by a 50 × 4.6 mm i.d. × 2.7 μm Poroshell 120 EC-C18 reverse phase column (Agilent, Santa Clara, CA, USA). Mobile phase A consisted of 0.1% TFA/H_2O, and mobile phase B consisted of 0.07% TFA/ACN. Gradient elution was performed with the following program: increased from 5% B to 100% B from 0 to 20 min, held at 100% B from 20 to 30 min, and decreased from 100 to 5% B from 30 to 40 min.

CONCLUSIONS

In this study, hydrophobic MILs were prepared and applied as solvents to extract and preserve yeast total RNA from aqueous solution. RNA was able to be preserved for a period of 15 days to facilitate recovery using the $[P_{66614}^+][Co(hfacac)_3^-]$ and $[P_{66614}^+][Co(Phtfacac)_3^-]$ MILs with the aid of an additional dispersion of PPG-2000. Although the recovery was relatively low, the recovered RNA was able to be analyzed by HPLC, qRT-PCR, and agarose gel electrophoresis. In addition, the $[Co(OIM)_4^{2+}][Glu^-]_2$ and $[Co(OIM)_4^{2+}][Asp^-]_2$ MILs demonstrated the capability of extracting and protecting yeast total RNA from RNase A degradation simultaneously, as determined by HPLC and agarose gel electrophoresis. The extraction efficiency of RNase A was found to be lower than RNA, suggesting that the MIL solvent provides an anhydrous microenvironment to prevent RNA from interacting with RNase A. This study offers a new method for RNA preservation and can be highly beneficial for in-field biological sample preparation and storage. Ongoing studies are focused on improving the recovery, in-depth study of preservation mechanism, alleviating qRT-PCR inhibition caused by MIL moieties, and further increasing the preservation time in the presence of RNase A.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01098.

External calibration curve of yeast total RNA. Extraction efficiency of yeast total RNA using different MILs. cDNA amplification curve in different conditions. Chromatograms of RNA recovery in different conditions. Static extraction of RNase A using different MILs (PDF)

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Notes

The authors declare no competing financial interest.

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