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# Integrated Extraction-Preservation Strategies for RNA Using Biobased Ionic Liquids

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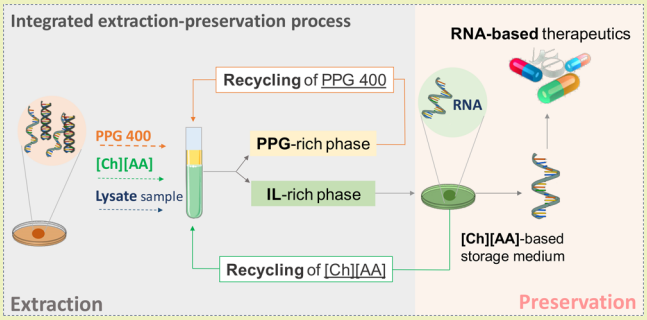
Supporting Information

**ABSTRACT:** The ubiquitous instability of RNA along with issues associated with its purity degree have been preventing its widespread use as low-cost biotherapeutics. On the basis of the well-known capacity of amino acids to specifically interact with RNA when used as chromatographic ligands, a set of amino-acid-based ionic liquids (AA-ILs) was herein investigated, both to act as preservation media and as phase-forming agents of aqueous biphasic systems (ABS). This set of strategies was combined with the goal of developing integrated extraction-preservation platforms. AA-ILs comprising the cholinium cation and anions derived from L-lysine ([Ch][Lys]), L-arginine ([Ch][Arg]), L-glutamic acid ([Ch][Glu]), and DL-aspartic acid ([Ch][Asp]) were studied. It is shown that the stability of RNA is preserved in aqueous solutions of the studied AA-ILs, even in the presence of ribonucleases (RNases). Furthermore, almost all the investigated AA-ILs display no cytotoxicity onto two distinct human cell lines. After identifying the most promising ILs, ABS formed by AA-ILs and polypropylene glycol with a molecular weight of 400 g mol$^{-1}$ (PPG 400) were investigated as extraction and purification platforms for RNA. Both with pure RNA and bacterial lysate samples, RNA is successfully extracted to the IL-rich phase without compromising its integrity and stability. On the basis of these results, the integrated extraction-preservation process for RNA is finally demonstrated. RNA is initially extracted from the bacterial lysate sample using ABS, after which the IL-rich phase can be used as the preservation medium of RNA up to its use. RNA can be then recovered from the IL-rich phase by ethanol precipitation, and the ABS phase-forming components recovered and reused. Although improvements in the purity level of RNA are still required, the approach here reported represents a step forward in the development of sustainable processes to overcome the critical demand of high-quality/high-purity RNA to be used as biotherapeutics.




**KEYWORDS:** *Ribonucleic acid, Amino-acid-based ionic liquids, Aqueous biphasic systems, Integrated extraction-preservation process*



## INTRODUCTION

Ribonucleic acid (RNA) is an essential biopolymer with various biological functions, such as in coding, decoding, regulation, and expression of genes.[1] Accordingly, nucleic acids are powerful biological tools in several fields,[2] ranging from fundamental to applied research. For instance, RNA is a target of study in (1) the quantification of transcripts related to protein levels resulting from the expression of target genes, which are essential to develop drugs that regulate gene expression;[3] (2) structural studies using biophysical methods, to further understand RNA features and functions;[4] and (3) the development of RNA-based therapeutics.[5] In all these biological-based applications, the methods and solvents used for extracting and purifying RNA from various types of cells or other biological matrices, and respective preservation, are critical issues.[3]

RNA research studies and RNA-based therapeutics applications require large amounts of high-purity/high-quality RNA.[6] Accordingly, the RNA synthesis by chemical, enzymatic, and, more recently, by recombinant technologies (using prokaryotic hosts such as *Escherichia coli* and *Rhodovulum sulfidophilum*) has become an indispensable tool.[6] Currently, two methods for isolating RNA prevail, namely, the highly used extraction method based on phenol and chloroform[7] and solid-phase extraction (SPE) using silica-based materials.[8,9] In addition to these recovery protocols, other purification steps including chromatography[10−12] and

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denaturing polyacrylamide gel electrophoresis[13] are applied to remove process-associated impurities.[14]

In RNA bioprocessing, besides high purity, other parameters are essential, namely, the biopolymer integrity and biological activity. Both features are particularly relevant for their use as biotherapeutics, which should fulfill the requirements of regulatory authorities.[10−12] RNA is a highly labile polymer with inherent chemical instability and can suffer rapid degradation by nucleases.[1] Therefore, RNA is normally preserved in high-purity water or sterile phosphate buffered saline (PBS) aqueous solutions under refrigeration at temperatures below 4 °C or at −80 °C for short- and long-term applications, respectively.[1]

On the basis of the described drawbacks, the development of effective and sustainable recovery processes and the identification of new preservation media are of paramount relevance, where ionic liquids (ILs) can play a role in both approaches. Some published works in the past few years described the strong affinity between ILs and different biomolecules, showing that ILs are solvents with a high potential for biomolecules preservation and extraction,[15] including nucleic acids.[16] However, in most of these studies, no extractions from real complex samples have been performed, and DNA is usually the target. This preference is mainly due to the conditions required to work with RNA, as a consequence of its poor stability (short half-life) when compared with DNA and ubiquitous presence of ribonucleases (RNases).[1]

Among the few reports comprising ILs and RNA, Mazid et al.[17] proved that aqueous solutions of cholinium dihydrogen phosphate ([Ch][DHP]) are able to prolong the shelf life of siRNA up to three months in the presence of RNase A. Mamajanov et al.[18] evaluated the stability of a duplex RNA, which adopts a A-form helix in several aqueous buffers. The authors showed that the RNA melting temperature in aqueous solutions of cholinium chloride is lower than in other salt aqueous solutions.[18] Fister et al.[19] successfully used imidazolium-based ILs to disintegrate virus particles and isolate RNA. In summary, these works dealing with ILs and RNA are focused on two strategies: (i) to use ILs as alternative solvents to maintain or increase the stability and integrity of RNA; or (ii) to use ILs as alternative media to isolation and recover the target nucleic acid. The exploration of integrated systems, that is, by using the same solvent in both the recovery and purification steps and as preservation media, was not attempted hitherto.

ILs can be used to form liquid−liquid systems to be used in separation processes, in which aqueous biphasic systems (ABS) are a promising alternative when dealing with biologically active products.[20] Albeit ABS have been described as biocompatible separation systems because of their high water content, most of the studied IL-based ABS are formed by imidazolium-based ILs,[20] which may be deleterious for biomacromolecules. This fact prompted the research on ILs based on natural feedstocks; for instance,[21] biocompatible ILs formed by the cholinium cation and anions derived from essential amino acids were reported in 2005 by Ohno and coworkers.[22] After this pioneering work, an increasing number of reports focusing on their properties[23−25] have emerged. However, cholinium amino-acid-based ILs (AA-ILs) have been scarcely applied in the development of separation processes. AA-ILs have been combined with inorganic salts ($K_3PO_4$ and $K_2CO_3$)[26] and polypropylene glycol[27] to form IL-based ABS, where a comprehensive characterization of the respective phase diagrams was performed. In two of these works, the extraction capacity of ABS for two model proteins (bovine serum albumin and trypsin[27]) and for phenylalanine enantiomers was evaluated.[28] Still, the performance of these systems was not appraised in the separation of target compounds from real matrices.

As result of the high affinity between amino acids and RNA,[29] amino-acid-based affinity chromatography is highly used to purify this biopolymer.[10−12] In addition, amino acids may have an important role in RNA loop recognition through a base-flipping process.[29] This well-known preferential affinity, together with the fact that in general amino-acid-based ILs are nontoxic, biodegradable, and biocompatible,[30] motivated us to investigate the use of aqueous solutions of amino-acid-based ILs as alternative solvents to stabilize and protect RNA from nucleases coupled to their use in the formation of ABS, envisioning the development of integrated preservation-extraction strategies. In affinity chromatography, hydrophilic amino acids (e.g., arginine) are usually used as ligands to improve the RNA biorecognition.[10−12] Accordingly, we selected four amino acids with different polarity (arginine, lysine, aspartic acid and glutamic acid) from the group of aliphatic amino acids to be used as IL anions. AA-ILs composed of the cholinium cation combined with the anions L-lysinate, L-argininate, L-glutamate, and DL-aspartate were synthesized and characterized by us. Their protective effect (aqueous solutions containing 20 wt % of IL) toward RNA, even in the presence of RNases, and respective cytotoxicity onto two human cell lines were evaluated. Zeta potential measurements were additionally carried out to perceive the mechanisms behind the RNA stabilization. The best identified AA-ILs were then investigated as phase-forming components of ABS combined with polypropylene glycol with a molecular weight of 400 g $mol^{-1}$ (PPG 400) with the ultimate goal of extracting RNA from complex samples. Finally, the recovery of the RNA from the IL-rich phase and reuse of the ABS phase-forming agents was addressed, demonstrating that amino-acid-based ILs can be used in the development of integrated processes comprising both the extraction and preservation steps.

## EXPERIMENTAL SECTION

**Materials.** The following ILs were synthesized: (2-hydroxyethyl) trimethylammonium (cholinium) L-lysinate ([Ch][Lys]), cholinium L-argininate ([Ch][Arg]), cholinium L-glutamate ([Ch][Glu]), and cholinium DL-aspartate ([Ch][Asp]). The chemical structures of the synthesized ILs are depicted in the Supporting Information, Figure S1. AA-ILs were synthesized by the neutralization of cholinium hydroxide in methanol solution with amino acids (1:1.05 mol ratio) according to published protocols.[22] Further details on the starting materials purity, source and synthesis procedure are given in the Supporting Information. The purity and structure of the ILs synthesized were confirmed by $^1$H NMR spectroscopy and elemental analysis (cf. the Supporting Information, Figures S2−S5). The ILs synthesis was performed with the following amino acids: L-lysine monohydrated (Lys), purity >98 wt %, from Acros (Belgium); L-arginine (Arg), purity >98 wt %, from Merck (U.S.A.); L-glutamic acid (Glu), purity >99 wt %, from Riedel de Haen (U.S.A.); and DL-aspartic Acid (Asp), purity >99 wt %, from Fluka (UK). Cholinium hydroxide, from Sigma-Aldrich (U.S.A.), at 45 wt % in methanol, was used.

The ABS studied herein were determined using polypropylene glycol with an average molecular weight of 400 g $mol^{-1}$ (PPG 400), supplied by Aldrich, and used as received. All materials used in the experiments were RNase-free. Ultrapure reagent-grade water (Mili-Q



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system, Milipore/Waters) was treated with 0.05% diethyl pyrocarbonate (DEPC, Sigma-Aldrich (U.S.A.)). All reagents used for RNA extraction and fluorescein isothiocyanate isomer 1 (FITC) were acquired from Sigma-Aldrich. Acetonitrile from Sigma-Aldrich (U.S.A.), 99 wt %, and methanol from VWR (U.S.A.), HPLC grade, were used.

**Small RNA Biosynthesis and Isolation.** A cell culture of *E. coli* DH5$\alpha$ strain modified with plasmid pBHSR1-RM containing the sequence of human pre-miR-29b was used for the small RNA (sRNA) production, namely, tRNA (tRNA), pre-miR-29b, and 6S RNA. Growth was carried out in a rotary shaker at 37 °C and 250 rpm using shake-flasks of 1 L, containing 0.25 L of Terrific Broth medium (12 g/L Tryptone, 24 g/L Yeast extract, 4 mL/L glycerol, 0.017 M $KH_2PO_4$, and 0.072 M $K_2HPO_4$) and supplemented with 30 $\mu$g/mL of kanamycin. Cell growth was evaluated by measuring the optical density at 600 nm of the cell culture and suspended at the beginning of the logarithmic decline phase ($OD_{600}$ after ca. 8 h), as previously described.[31] The cultivated cells of *E. coli* DH5$\alpha$ were recovered by centrifugation at 4500*g* and 4 °C for 20 min, and the pellets stored at −20 °C. The recovery of sRNA was carried out according to the acid guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski et al.,[32] with some modifications as described. Bacterial pellets (100 mL) were resuspended in 5 mL of denaturing cell lysis solutions (4 M guanidinium thiocyanate; 0.025 M sodium citrate, pH 7.0; 0.5% (w/v) *N*-laurosylsarcosine and 0.1 M $\beta$-mercaptoethanol) to perform the lysis. After they were incubated on ice for 10 min, cellular debris, genomic DNA, and proteins were precipitated by mixing 5 mL of water-saturated phenol and 0.5 mL of 2 M sodium acetate (pH 4.0). The sRNA isolation was achieved by adding 1 mL of chloroform/isoamyl alcohol (49:1, v:v) and by vigorously mixing until two immiscible phases were obtained. The upper aqueous phase, which contains mostly RNA, was recovered and concentrated by the addition of 5 mL of ice-cold isopropanol. Precipitated molecules were recovered by centrifugation at 10 000*g* for 20 min at 4 °C and resuspended in 1.5 mL. sRNA molecules were concentrated again with 1.5 mL of ice-cold isopropanol. After it was subjected to centrifugation for 10 min at 10 000*g* (4 °C), the RNA pellet was washed with 7.5 mL of 75% ethanol and incubated at room temperature for 10 min, followed by a 5 min centrifugation at 10 000*g* (4 °C). The air-dried RNA pellet was solubilized in 1 mL of 0.05% DEPC-treated water. Finally, the 260 and 280 nm absorbance of the samples were measured using a Nanodrop spectrophotometer to assess the RNA quantity, and agarose gel electrophoresis was performed to assess the RNA purity.

**Aqueous Solutions of Ionic Liquids as Stabilization Media of RNA.** RNA was dissolved in aqueous solutions of different AA-ILs, at 20 wt %, and the mixtures incubated at room temperature, (25 ± 1 °C), for 1 h and 15 days. The period of 15 days was selected because it is considered an average time between extraction and use of RNA samples, being in agreement with our previous experience[33] and with the study performed by Mathay et al.[34] The shorter incubation period of 1 h was set to mimic typical incubation periods often performed in different laboratory protocols involving RNAs, while allowing to observe the effect of the IL in the stability of the biomolecule at two different periods. RNA was precipitated by adding 2 volumes of pure alcohols (butanol in the case of [Ch][Arg], and ethanol for the remaining) and further incubated for 2 h at −80 °C, until it was recovered by centrifugation at 15 000*g* for 30 min at 4 °C. Nucleic acids pellets were air-dried for 10 min at room temperature and solubilized in 0.05% DEPC-treated water.

**Agarose Gel Electrophoresis.** The integrity and identification of sRNAs recovered after incubation with ILs were evaluated using agarose gel electrophoresis. Twenty microliters of sRNA corresponding to ca. 48 $\mu$g was analyzed by horizontal electrophoresis using 0.8% of agarose gel (Hoefer, U.S.A.) and carried out at 110 V for 30 min with TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA, pH 8.0). The bands corresponding to sRNA molecules were visualized in the gel using the UVItec FireReader system (UVItec, U.K.) after they were stained with 0.01% GreenSafe Premium (NZYtech, Portugal).

**Circular Dichroism (CD) Spectroscopy.** CD experiments were performed in a Jasco J-815 spectropolarimeter (Jasco, U.S.A.), using a Peltier-type temperature control system (model CDF-426S/15). CD spectra were acquired at a constant temperature of 25 °C using a scanning speed of 10 nm/min, with a response time of 1 s over wavelengths ranging from 210 to 320 nm. The recording bandwidth was of 1 nm with a step size of 1 nm using a quartz cell with an optical path length of 1 mm. Three scans were averaged per spectrum to improve the signal to-noise ratio, and the spectra were smoothed by using the noise-reducing option in the operating software of the instrument. CD melting experiments were performed in the temperature range from 10 to 110 °C, with a heating rate of 1 °C/min, by monitoring the ellipticity at 265 nm. Data were converted into fraction folded ($\theta$) as detailed in the literature.[35] Data points where then fitted to a Boltzmann distribution (OriginPro 2015), and the melting temperatures ($T_m$) were determined from the two-state transition model used, using the first derivative method.[36]

**Zeta Potential.** The zeta potential of RNA samples in presence and absence of [Ch][AA]-ILs were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS particle analyzer (Malvern Instruments, U.K.), equipped with a He−Ne laser, at 25 °C. These measurements were performed in disposable capillary cells and computed using the Henry's [F(Ka) 1.5] and Smoluchowsky models. All data were treated with the Zetasizer software v 7.03 and the analyses were held in triplicate with an average of 30 measurements per sample. RNA was incubated with IL (20 wt %) aqueous solutions during 1 h at room temperature (25 ± 1 °C), at a total RNA concentration of 200 $\mu$g/mL. The values given represent the average of three independent samples and respective standard deviation.

**Cell Viability.** The cellular viability of RNA in AA-ILs aqueous solutions was achieved by the MTS assay (shown as percentage of control (%)) using the Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega), according to the manufacturer's instructions. Human fibroblasts and cervical cancer cells (HeLa) were cultured at 37 °C, in a humidified atmosphere containing 5% $CO_2$. Three different passages of each cell line were seeded at a density of $1 \times 10^4$ cells per mL in a 96-well plate; after 24 h, the cell culture medium was replaced by serum-free culture medium. After 12 h, the different formulations of ILs with RNA (10 $\mu$L of a mixture containing 200 $\mu$g/mL RNA and RNA in 20 wt % ILs aqueous solutions) were added to the cells and transfection was carried out for 24 h. The culture medium was exchanged by fresh medium and a mixture of MTS/phenazine metasulfate (PMS) was added to each well, and cells were incubated for 2 h at 37 °C in a humidified atmosphere containing 5% $CO_2$, protected from light. Following incubation, absorbance measurements of the soluble brown formazan produced by MTS were performed in a microplate reader (Bio-Rad) at 490 nm. Cells incubated with absolute ethanol were used as positive control for cytotoxicity, while untreated cells were used as negative controls. The values given correspond to the average percentage values relative to untreated cells of three independent experiments and respective standard deviation.

**Extraction of RNA Using AA-IL-Based ABS and Recyclability of Phase-Forming Components.** Aiming to address the possibility of using AA-IL-based ABS as alternative extraction and purification strategies of RNA, the respective ABS phase diagrams (water + AA-IL + PPG 400), comprising the binodal curve, tie-lines (TLs), and respective tie-line length (TLL), were determined by the cloud point titration method at 25 °C and atmospheric pressure, as described in the literature.[37] Aqueous solutions of each IL in the range from 50 to 70 wt %, and aqueous solutions of PPG 400 from 50 to 80 wt % were gravimetrically prepared and used for the determination of the binodal curves. The dropwise addition of each aqueous solution of IL to the PPG 400 aqueous solution was carried out until the detection of a cloudy (biphasic) solution, followed by the dropwise addition of distilled water until a complete limpid solution (monophasic region) is obtained. This procedure was carried out under constant stirring, and the ternary system compositions were determined by weight quantification of all components ($\pm 10^{-4}$ g) using an analytical balance



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Mettler Toledo Excellence XS205 DualRange. Each ABS phase was identified by conductivity (experimental procedure and data are given in the Supporting Information).

The ternary mixtures used in the extraction experiments of pure RNA were gravimetrically prepared at the following mixture composition: 20 wt % of PPG 400 + 20 wt % of AA-IL ([Ch][Glu] or [Ch][Asp]) + 60 wt % of DEPC-treated water with 400 $\mu$g/mL RNA. This fixed mixture composition was selected considering the biphasic region of all the studied systems. Furthermore, this mixture point is close to the binodal curves, allowing to have a minimum amount of IL and maximum amount of water in each ABS, while keeping in mind the development of a sustainable method. The TLs for this mixture point in each ABS, which give the composition of each phase, were determined according to the method proposed by Merchuk et al.[38] The compositions of the top and bottom phases and the overall system compositions were determined by the lever-arm rule and mass balance approaches, as described in the literature.[38] Further information is given in the Supporting Information. ABS with a total weight of 1 g were used. All mixtures were carefully stirred and centrifuged for 5 min at 25 °C (5000*g*), followed by the separation of the two phases. RNA was recovered from both phases by alcohol precipitation, as described before. The partitioning of nucleic acids in AA-IL-based ABS was ascertained by agarose gel electrophoresis.

After demonstrating the favorable RNA extraction (with pure RNA samples) to the IL-rich phase, new experiments were carried out envisaging the purification of RNA directly from real matrices, in this work with a bacterial lysate sample comprising RNA, genomic DNA, and some proteins and other impurities. Detailed experimental information on the sample preparation and composition is given in the Supporting Information. The ABS chosen are composed of 20 wt % of PPG 400, 20 wt % of AA-IL, and 60 wt % of lysate. Each mixture composition was weighed, mixed, and centrifuged for 5 min (5000*g*). RNA evaluation was carried out as described before.

With the final goal of developing sustainable separation processes, the recovery and reusability of the ABS phase-forming components was finally ascertained. To this end, the recovery of RNA from the IL-rich phase was first performed by ethanol precipitation, as described above. Then, the volatile solvents present in the IL-rich phase were evaporated under vacuum at room temperature, up to constant weight, leaving only the IL and PPG 400 as the nonvolatile species. The recovered PPG 400 and IL were then reused (two more times) in the ABS formation, and their impact on the integrity of RNA after a new extraction step was assessed by agarose gel electrophoresis. The recovered phase-forming components aqueous solutions were also evaluated in terms of their feasibility for keeping the RNA stability and integrity toward the development of integrated extraction-preservation processes.

## RESULTS AND DISCUSSION

All ILs used, namely, [Ch][Lys], [Ch][Arg], [Ch][Glu], and [Ch][Asp], were synthesized in this work. Their chemical structure and details on their characterization and purity are given in the Supporting Information (cf. Figures S2−S5). These ILs were designed and synthesized on the basis of the specific affinity between amino acids and RNA and their previous use on amino-acid-based affinity chromatography for RNA purification,[10−12] coupled to the fact that amino-acid-based ILs may present low toxicity and low environmental impact.[30]

**Integrity, Stability, and Cytotoxicity of RNA in the Presence of Aqueous Solutions of ILs.** Initial studies on the integrity of RNA at 25 °C in the presence of aqueous solutions of [Ch][Lys], [Ch][Arg], [Ch][Glu], and [Ch][Asp] at 20 wt % were carried out by agarose gel electrophoresis in order to address the potential of the synthesized ILs to act as alternative preservation media. Since we aim to apply ABS as a simultaneous extraction and preservation strategy, the concentration of IL used in the preservation stage should be in accordance with the IL content required to form two-phase systems. Higher concentrations of IL were not investigated since they compromise the sustainability of the process (by increasing both the environmental footprint and cost of the process). The results obtained are depicted in Figure 1, where a well-defined band with a low

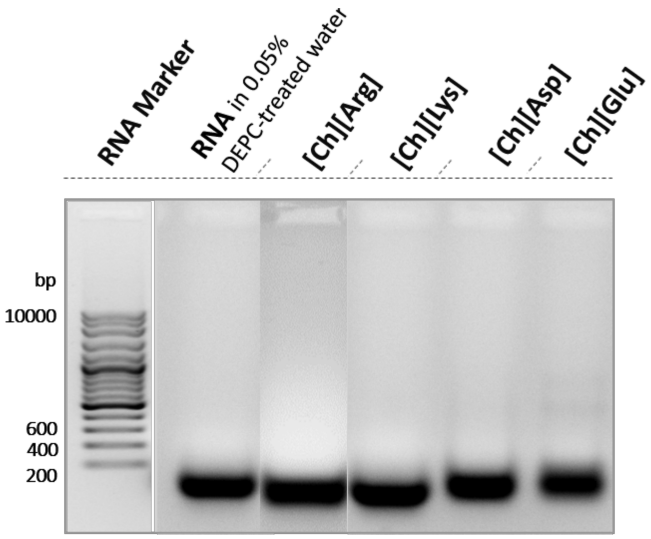


**Figure 1.** Electrophoretic analysis of the integrity of RNA in 0.05% DEPC-treated water and RNA in the presence of aqueous solutions of 20 wt % of the studied ILs. RNA markers are included.

molecular weight is shown, corresponding to the recombinant RNA fraction recovered from *E. coli*. To avoid interferences of ILs in the electrophoretic run, alcohol-induced precipitation of RNA was applied in all samples. Different alcohols (propan-2-ol, ethanol, propanol, and butanol) were tested in what concerns to their ability to precipitate RNA, and distinct behaviors were observed (shown in Figure S6 in the Supporting Information). Although discussed here with a different purpose, the identification of appropriate solvents for the induced precipitation of RNA is valuable when considering the RNA and ILs recovery proposed below.

The results shown in Figure 1 correspond to RNA precipitated with the best identified alcohols, namely, butanol for the [Ch][Arg] aqueous solution and ethanol for the remaining ILs aqueous solutions. The first lane of the agarose gel corresponds to sRNA recovered from *E. coli*, including a well-defined band with a low molecular weight, and in agreement with a previous work.[39] Depending on the original media, either ethanol or isopropanol have been reported for the precipitation of nucleic acids.[40] Ethanol and butanol are however more benign and less hazardous alcohols than isopropanol;[41] therefore, the efficient recovery of RNA from ILs aqueous solutions with these alcohols further reinforces the sustainability of the proposed recovery strategy in comparison with processes based on isopropanol.

It is well-known that RNA secondary structure elements and global folding patterns are temperature dependent.[42] Accordingly, RNA melting studies were performed in the absence and presence of different aqueous solutions of 20 wt % of ILs. The typical CD spectrum of RNA includes a minimum ellipticity peak at 215 nm and a maximum ellipticity peak at 265 nm; however, the investigated ILs interfere with the CD spectrum


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of RNA at lower wavelengths (cf. the representative CD spectrum of RNA in the presence of [Ch][Arg] in Figure S7 in the Supporting Information). The CD melting temperatures ($T_m$) of RNA were thus determined by addressing the changes in the RNA ellipticity at 265 nm in the temperature range from 10 to 110 °C. The obtained results corresponding to 1 h of incubation with each AA-ILs are given in Table 1 (the respective melting curves are depicted in Figure S8 in the Supporting Information). $T_m$ is defined as the temperature at which half of the molecules of a double-stranded species become single-stranded, where higher $T_m$ values are an indicator of a higher RNA thermostability.[43] The $T_m$ of RNA dissolved in high-purity water is 45.8 °C, but it is lower in the presence of [Ch][Lys] and [Ch][Arg] aqueous solutions (39.9 and 43.3 °C, respectively). Contrarily, a remarkable enhancement of ca. 15 °C (60.0 and 60.3 °C, respectively) in the $T_m$ of RNA was observed in the presence of aqueous solutions of [Ch][Glu] and [Ch][Asp], indicating that the thermal stability of RNA during 1 h of incubation in AA-ILs is significantly enhanced with these two ILs. In addition to the high stabilizing effect and preservation of RNA afforded by the studied ILs for short-term periods, the results shown in Table 1 also demonstrate the potential of these two ILs as preservation agents even after 15 days of incubation. These results are in agreement with a previous work,[17] showing an increase of the RNA stability with buffered aqueous solutions of [Ch][DHP], although a lower enhancement, ca. 8.5 °C, was achieved.

**Table 1. Melting Temperature of RNA in 0.05% DEPC-Treated Water and in AA-ILs (20 wt %) Aqueous Solutions after 1 h and 15 days of Incubation at 25 °C**

| | $T_m$/°C after 1 h | $T_m$/°C after 15 days |
|---|---|---|
| RNA in 0.05% DEPC-treated water | 45.8 ± 0.5 | 46.0 ± 0.5 |
| RNA in 20 wt % [Ch][Lys] | 39.9 ± 0.5 | 29.5 ± 2.0 |
| RNA in 20 wt % [Ch][Arg] | 43.3 ± 0.7 | 32.4 ± 5.5 |
| RNA in 20 wt % [Ch][Glu] | 60.0 ± 0.3 | 56.4 ± 0.4 |
| RNA in 20 wt % [Ch][Asp] | 60.3 ± 0.3 | 56.2 ± 0.4 |

Overall, this set of results carried out with pure RNA demonstrate that RNA is more stable in aqueous solutions of [Ch][Glu] and [Ch][Asp] than in 0.05% DEPC-treated water, both for short-term and medium-term storage periods (1 h and 15 days). Thus, it seems that the anionic counterpart of ILs has a significant influence on the RNA structure and stability, which is discussed in more detail below.

Aiming at better understanding the mechanisms behind the preservation and enhanced stability promoted by some of the investigated ILs, zeta potential measurements of RNA in pure water and in aqueous solutions of 20 wt % of ILs were carried out. Zeta potential corresponds to the electrostatic potential generated in an applied electric field due to the attraction between charged species and the oppositely charged electrode, thereby allowing to draw some conclusions on the solution effective charge density. Nucleotides are the building blocks of RNA, which comprise phosphate groups that confer to RNA a negative charge, resulting in a zeta potential of approximately −40 mV, as shown in Figure 2. In all assays, it was observed that when dissolved in aqueous solutions of ILs, RNA displays a less negative value, confirming that all ILs employed in this study interact with RNA. Since less negative zeta potential values are obtained when in the presence of ILs, it is plausible

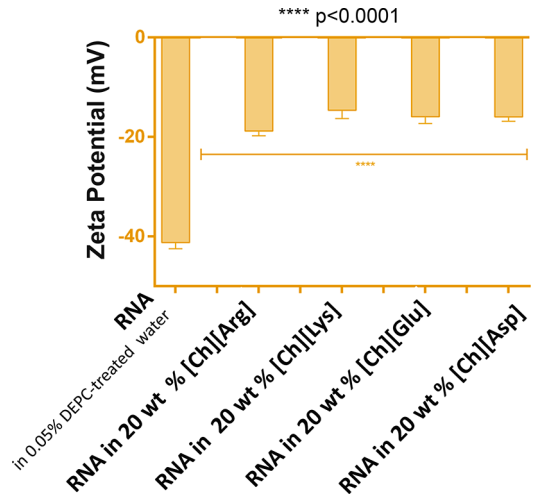


**Figure 2.** Average zeta potential (mV) of RNA dissolved in 0.05% DEPC-treated water and in 20 wt % AA-ILs aqueous solutions.

to admit that when in aqueous solution the cholinium cation forms an outer shell around RNA, in agreement with previously reported molecular dynamics simulations results.[33]

Considering the application of AA-ILs as efficient preservation and stabilization media of RNA for biological assays or for its use as biopharmaceuticals, it is crucial to ascertain the RNA safety profile. Different studies already approached the ecotoxicity[30] and biodegradability[44] of AA-ILs. These studies demonstrated that AA-ILs display low ecotoxicity and high biodegradable character. In this work, we addressed their impact onto different cell lines by cytotoxicity studies. Figure 3 depicts the effect of mixtures of RNA and ILs at 20 wt % in the viability of two human cell lines, namely, fibroblasts and a cervical cancer cell line (HeLa). These two distinct cell lines were chosen since they are physiologically distinct entities and, therefore, present different reactivity to external stimulus. According to the results obtained, aqueous solutions of [Ch][Lys] impair the viability of both cell lines. In contrast, the remaining AA-ILs tested ([Ch][Arg], [Ch][Glu], and [Ch][Asp] at 20 wt %) are harmless to both tested human cell lines, thereby confirming their noncytotoxic character at the concentrations used.

Overall, [Ch][Glu] and [Ch][Asp] can increase the thermal stability of RNA and do not display cytotoxicity onto two human cell lines. However, the major factor affecting the RNA stability, and thereby causing its degradation, is the ubiquitous presence of RNases (active nucleases).[45] By mimicking conditions often observed in *in vitro* assays wherein culture media are usually supplemented with fetal bovine serum (FBS) as a source of essential growth factors, we further appraised the protective effect of both [Ch][Glu] and [Ch][Asp] aqueous solutions against FBS-derived ribonucleases. The CD ellipticity at 265 nm of RNA dissolved in high-purity water was monitored during different incubation periods (6, 12, 24, 36, and 48 h) and compared with the ellipticity of RNA in aqueous solutions of 20 wt % of [Ch][Glu] and [Ch][Asp], supplemented with 1% (v/v) FBS. The results depicted in Figure S9 in the Supporting Information show that the RNA ellipticity decreases with the increase of the incubation period, indicating that the biopolymer is naturally degraded by RNases. In comparison with RNA in high-purity water, the ellipticity of RNA is slightly higher in the presence of [Ch][Asp], meaning that this IL is more effective on RNA


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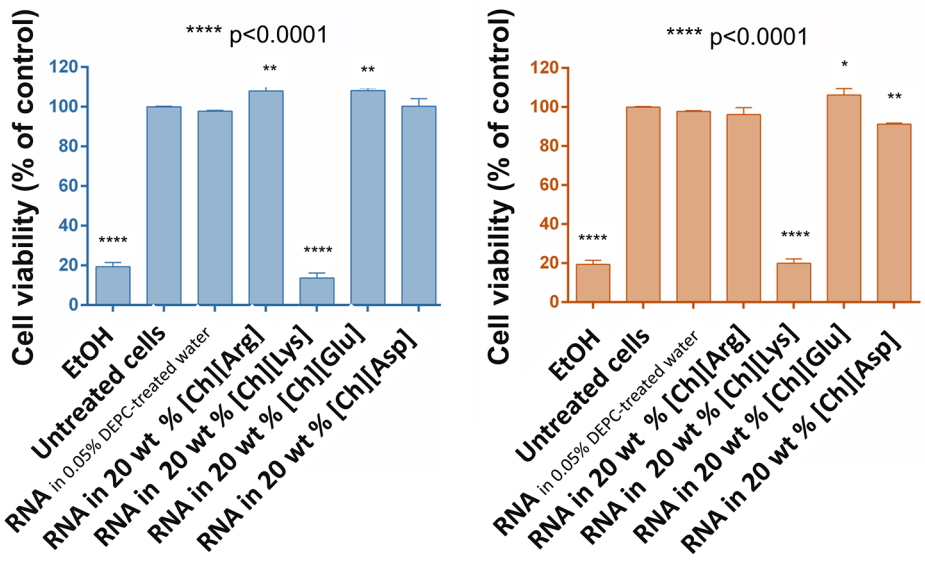


**Figure 3.** Cytotoxic effect of AA-ILs in two different human cell lines: Fibroblasts (left) and HeLa (right).

protection from degradation by RNases. The beneficial role of [Ch][Asp] reinforces the idea that this IL is particularly relevant to be used as preservation and stabilization media for RNA, for instance, for storage or to be used as an additive in biological assays.

**Extraction of RNA Using IL-Based ABS.** The set of results presented above with pure RNA samples demonstrate that, among the ILs studied, [Ch][Glu] and [Ch][Asp] are the most promising ones to be employed in RNA bioprocessing. These AA-ILs do not disturb the RNA secondary structure, but instead, they increase their thermal stability and offer protection against RNase activity without signs of cytotoxicity. With the purpose of creating an integrated extraction-preservation process with AA-ILs, their ability to form ABS with PPG 400 for the extraction of RNA was investigated. In addition to these promising AA-ILs ([Ch][Glu] and [Ch][Asp]), the remaining studied AA-ILs were also tested as ABS phase-forming agents, whose results are shown in Figures S10 and S11 in the Supporting Information. To the best of our knowledge, only the ABS formed by PPG 400 + [Ch][Lys] + water was previously reported.[27] The ternary phase diagram of the ABS formed by PPG 400 + [Ch][Lys] + water is in agreement with literature data,[27] as depicted in Figure S11 in the Supporting Information. The experimental weight fraction data and corresponding regression parameters[38] of the respective binodal curves of each ABS are reported in the Supporting Information (Figure S10 and Tables S1−S2). The TLs, along with their TLL, were also determined and are given in the Supporting Information (Table S3). These data and parameters are useful to characterize the monophasic/biphasic regimes of each ABS, being determined by the method originally proposed by Merchuk et al.[38] In particular, the TL data give the composition of the coexisting phases at the mixture point at which the extraction of RNA was carried out, whereas the TLL data provide information on the difference of compositions between the two phases. In addition, TL data are crucial in the recovery and reuse of the IL in order to create a new ABS with the same mixture composition, as addressed below.

In all studied ABS, the top phase corresponds to the PPG-rich phase, while the bottom phase is enriched in the IL (as confirmed by conductivity measurements, whose details and results are given in the Supporting Information). All ILs share a common cation (cholinium); thus, differences in the solubility curves shown in Figure S10 in the Supporting Information, that is, the amount of each phase-forming component required to form two-phase systems, is a result of the IL anion nature. As PPG 400 is a hydrophobic polymer, and given the trend obtained, it is plausible to admit that ILs act as salting-out agents over PPG 400 in aqueous media. More hydrophobic ILs, such as [Ch][Asp],[46] require higher amounts of IL and/or PPG 400 to undergo liquid−liquid demixing and create two phases.

After determining the ABS phase diagrams, extraction studies for pure RNA with ABS formed by [Ch][Glu] and [Ch][Asp] were carried out. ABS with fixed compositions of 20 wt % of PPG 400 + 20 wt % of IL + 60 wt % of RNA in DEPC-treated water (at 400 $\mu$g $L^{-1}$) were used. The partitioning of RNA in the ABS phases was evaluated by agarose gel electrophoresis—results shown in Figure 4.

In the systems investigated, RNA completely partitions to the IL-rich phase (bottom phase)—complete extraction in one-step. No RNA was detected at the PPG 400-rich phase (top phase) and no losses of RNA by precipitation were perceived. Furthermore, according to the agarose gel electrophoresis profile, the structural integrity of RNA is preserved after the extraction step. Some studies already reported the use of ABS formed by ILs and PPG 400 for the extraction of other biomolecules, for example, BSA,[27] IgG[47] and IgY,[48] demonstrating their preferential partitioning to the IL-rich phase. However, and contrary to what is typically observed in the extraction of proteins using systems with PPG where significant losses in yield are observed, in this work, no losses of RNA were detected. In amino-acid-based affinity chromatography, multiple interactions, such as electrostatic interactions, van de Waals forces, hydrogen bonding, and dispersive forces between amino acids and RNA occur.[29] In the same line, and since no salting-out effects by PPG dominate the partition behavior, these favorable interactions must be responsible for the complete partition of RNA to the IL-rich phase.

After confirming the potential of AA-IL-based ABS to extract pure RNA, the same systems were employed in the extraction


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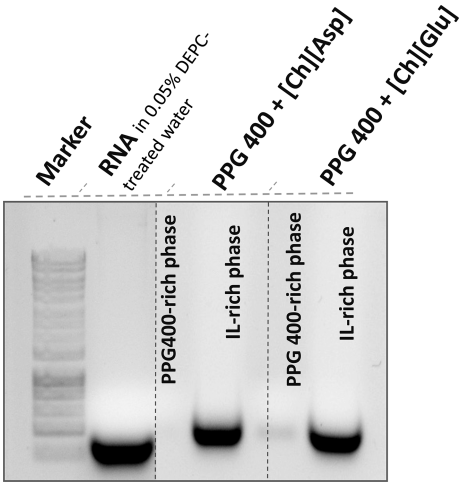


**Figure 4.** Agarose gel electrophoresis of the ABS coexisting phases at the mixture point: 20 wt % of PPG 400 + 20 wt % of AA-IL + 60 wt % of RNA in DEPC-treated water (400 $\mu$g $L^{-1}$). RNA in 0.05% DEPC-treated water was used as control sample. RNA markers are included.

of RNA directly from a complex real sample—a recombinant bacterial lysate sample that contains RNA, genomic DNA, proteins, and other impurities (cf. the Supporting Information for the protocol on the lysate sample preparation and further information). In this set of experiments, the liquid lysate sample was directly introduced in the ABS composition. Similarly to what occurred in the previous assays with pure RNA, the preferential partition of RNA to the IL-rich phase is also observed when using the bacterial lysate sample. However, some RNA is also identified in the polymer-rich phase (Figure S12 in the Supporting Information), indicating that there is a competition by the other molecules present in the lysate medium to partition to the IL-rich phase. Both gDNA and gDNA fragments (the first two bands with higher molecular weight shown in Figure S12 in the Supporting Information) also preferentially partition to the IL-rich phase. Although additional studies are still required to improve the selectivity of the investigated ABS for RNA, for example, by studying different mixture compositions and other operational conditions such as pH and temperature, the structural integrity of RNA is preserved after the extraction step from the original complex sample. These results reinforce the potential of IL-based ABS to be used as integrated extraction-preservation processes, in which RNA can be preserved up to use and recovered by alcohol precipitation. In addition to these conditions, the ABS purification performance can be improved by continuous processes, such as centrifugal partitioning chromatography (CPC). In CPC, both the stationary and mobile phases are liquid, where the stationary phase is kept by centrifugation.[49] It was already demonstrated that ABS can be applied in CPC, being effective in the fractionation of various biomolecules by enhancing the separation resolution.[50] Furthermore, CPC allows the technology scale-up as demonstrated by Sutherland et al.[50] They carried out optimization tests at the laboratory scale (500 mL) and then applied the best systems in a pilot scale (6.25 L) using CPC, where the resolution increased from 1.28 to 1.88.

**ABS Phase-Forming Components Recovery/Recyclability and Integrated Extraction-Preservation Process.** The sustainability of IL-based ABS processes can be improved not only by using more benign (low-toxicity and high biodegradability) ABS phase-forming components, such as amino-acid-ILs and polymers, but also through their recycling and reuse.

We first evaluated the integrity of RNA in the IL-rich phase, followed by its recovery from the IL-rich-phase and the IL/PPG recycle and reuse. The ABS formed by PPG 400 and [Ch][Glu] was used since it was one of the best identified systems according to the stability, extraction, and cytotoxicity data discussed before. The proposed integrated extraction-preservation strategy includes four main steps: (1) RNA extraction directly from a bacterial lysate sample (or other complex samples containing RNA) using ABS and RNA preservation in the IL-rich phase; (2) RNA recovery from the IL-rich phase by alcohol precipitation; (3) evaporation under vacuum of the ABS phase-forming components at room

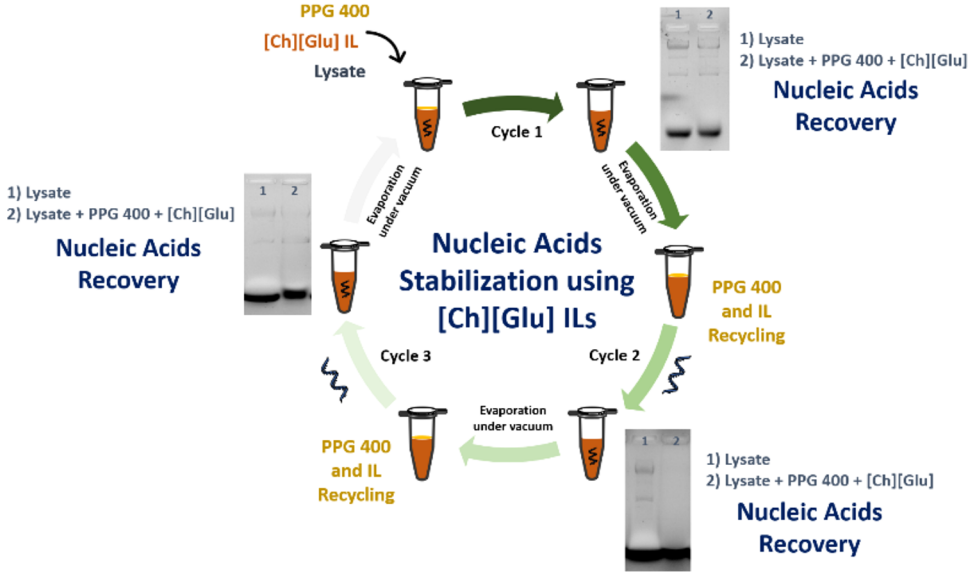


**Figure 5.** Agarose gel electrophoresis of the ABS coexisting phases at the mixture point: 20 wt % of PPG 400 + 20 wt % of AA-IL + 60 wt % of bacterial lysate. RNA in 0.05% DEPC-treated water was used as control sample and a marker is also included.



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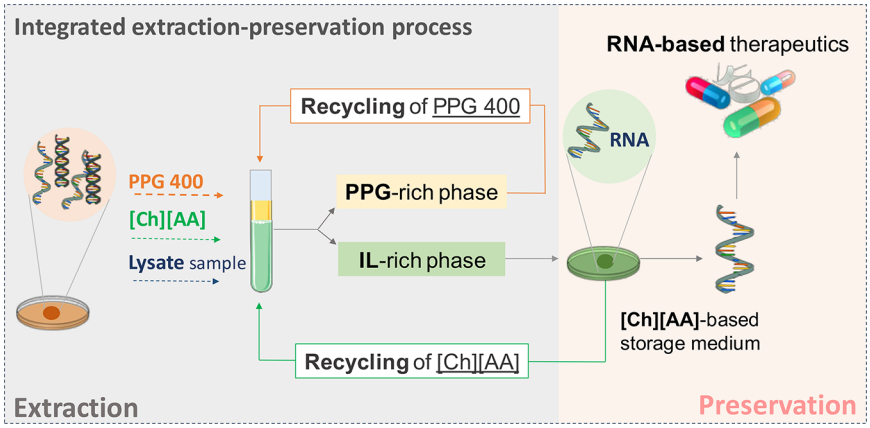


**Figure 6.** Schematic diagram of the proposed integrated extraction-preservation process of RNA, where all steps occur at ca. 25 °C, while foreseeing its use as biotherapeutic.

temperature up to constant weight; and (4) formation of new ABS with the recovered nonvolatile (IL and PPG) solvents for RNA extraction and preservation. Three cycles of this combined strategy were tested herein as a proof of concept, which is summarized in Figure 5. A maximum of 5 wt % of IL and PPG was lost in each cycle, mainly due to the transfer of solvents between vials. This value could be reduced by scaling-up the proposed process and by improving the experimental assays. The ABS phase-forming components were successfully recovered and reused, without losses on the ABS extraction and RNA stabilization performance, as shown by the agarose gel electrophoresis results given in Figure 5.

Figure 6 depicts a schematic overview of the proposed integrated extraction-preservation process for RNA, targeting its use as biopharmaceuticals. To the best of our knowledge, this is the first study that reports the proposal of an integrated extraction-preservation platform for RNA bioprocessing resorting to IL-based ABS.

## CONCLUSIONS

In this work, several studies were carried out to validate the hypothesis of using IL-based ABS as extraction-preservation strategies of RNA. It was initially shown that the stability of RNA is preserved in aqueous solutions of the studied AA-ILs, even in the presence of ribonucleases (RNases). Furthermore, and with the exception of [Ch][Lys], the studied ILs display low cytotoxicity to human cell lines. After identifying the most favorable ILs for RNA bioprocessing, ABS formed by AA-ILs and PPG 400 were investigated as potential extraction and purification platforms for RNA. Both with pure RNA and bacterial lysate samples, RNA is majorly extracted to the IL-rich phase, without compromising its integrity and stability. Finally, the envisioned integrated extraction-preservation process for RNA was demonstrated. It was shown that RNA can be extracted from a bacterial lysate sample using ABS, after which the IL-rich phase acts as the preservation medium of RNA up to its use. RNA can be then recovered from the IL-rich phase by ethanol precipitation, and the ABS phase-forming components recovered and reused. The IL-rich phase allows to keep the RNA integrity and stability at temperatures close to room temperature, thus avoiding the need for freezing RNA-containing samples. Furthermore, the use of aqueous solutions of nonvolatile ILs as RNA preservation media eliminates volatile organic solvent losses to the atmosphere, decreasing both the environmental footprint and the cost of the process. Both features are also improved by the demonstrated possibility of recycling the ABS phase-forming components. Even though improvements in the purity of RNA are still required, IL-based ABS can be considered as novel, integrated, and sustainable extraction-preservation processes for nucleic acids and may positively contribute to overcome the current critical demand of high-quality/high-purity biotherapeutics.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.9b00688.

Detailed experimental procedure, ionic liquids chemical structure and respective RMN spectra; RNA recovery tests with different alcohols; RNA stability addressed by CD spectra and RNA average ellipticity at 265 nm; experimental data of each ternary phase diagram and extraction parameters of the systems applied in the RNA extraction studies; and agarose gel electrophoresis results (PDF)

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### Notes

The authors declare no competing financial interest.

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