Cholinium-based Good's buffers ionic liquids as remarkable stabilizers and recyclable preservation media for recombinant small RNAs

Augusto Q. Pedro[†a], Patrícia Pereira[‡§a], Maria J. Quental[†], André P. Carvalho[†], Sérgio M. Santos[†], João A. Queiroz[‡], Fani Sousa[‡], Mara G. Freire[†*]*

† CICECO – Aveiro Institute of Materials, Chemistry Department, University of Aveiro, Complexo de Laboratórios Tecnológicos, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal.

‡ CICS-UBI – Health Sciences Research Centre, Universidade da Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal.

§ Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal

[a] The authors contributed equally in the work.

[*] Email adresses: maragfreire@ua.pt; fani.sousa@fcsaude.ubi.pt

KEYWORDS: Nucleic acids, Recombinant RNA, Ionic liquids, Good's Buffers, Stability, Recyclable media

ABSTRACT: RNA is a biopolymer of high relevance in the biopharmaceuticals field and in fundamental and applied research; however, the preservation of the RNA stability is still a remarkable challenge. Herein, we demonstrate the enhanced potential of aqueous solutions of self-buffering cholinium-based Good's buffers ionic liquids (GB-ILs), at 20 and 50 % (w/w), as alternative preservation media of recombinant small RNAs. The thermal stability of RNA is highly enhanced by GB-ILs, with an increase of 14 °C in the biopolymer melting temperature - the highest increase observed up to date with ILs. Most GB-ILs investigated improve the stability of RNA at least up to 30-days, both at 25 °C and at 4 °C, without requiring the typical samples freezing. Molecular dynamics simulations were applied to better understand the molecular-level mechanisms responsible for the observed RNA improved stability. The number of IL cations surrounding the RNA chain is similar, yet with differences found for the IL anions, which are responsible for the overall charge of the biopolymer first solvation sphere. No cytotoxicity of the studied solutions containing RNA and ILs at 20 % (w/w) was observed onto two distinct human cell lines, reinforcing their potential to act as preservation media when foreseeing biopharmaceutical applications. Finally, RNA was successfully recovered from the ILs aqueous solutions, without changes in its structural integrity, and the ILs successfully recycled and reused.

INTRODUCTION

For a long time, RNA has been considered a biopolymer of high interest, regarded as an intermediate between DNA and proteins. However, the milestones discoveries of catalytic RNAs in the early 80's and RNA interference (RNAi) in the late 90's changed the RNA landscape to a dynamic and versatile biopolymer that regulates many functions related with gene expression and cellular mechanisms.[1] The RNAi technology[2] involves some small RNAs – microRNAs (miRNAs) and small interfering RNAs (siRNAs) - that act as regulators of the messenger RNA (mRNA) expression. This fact led to the development of novel therapeutic strategies based on RNA, where siRNAs and miRNAs have shown promising outcomes in clinical trials, streamline drug discovery and development beyond protein therapeutics.[3,4] However, the understanding of the biological role of RNAs or their use as biotherapeutic agents require pure and intact RNA with adequate integrity, stability and biological activity.[5] It is known that the stability of nucleic acids depends on a balance of features, such as base pairing, electrostatic interactions, and hydration capacity, with impact on their structure and function, in which the decrease of their lifetime occurs by the bases hydrolysis (deamination, depurination).[6-9]

The RNA required for most biomolecular applications may be obtained by *in vitro* transcription and chemical synthesis.[10] Still, natural RNAs obtained by *in vivo* procedures are better in retaining their structure and function, and have improved safety characteristics. Therefore, their recombinant production using bacterial-adapted cells has been a hot topic of research.[10] Regardless of the approach used to produce RNA, the final product has always to be purified, mainly to remove contaminants (plasmid DNA template, enzymes, free nucleotides, salts or buffers, chemicals, genomic DNA or proteins)[10-12], which is traditionally

achieved using highly-selective chromatographic techniques.[13] Nevertheless, the peculiar three-dimensional compaction and structural instability of RNA are still crucial challenges, as the biological activity and integrity are easily compromised during the extraction and purification procedures.[5] To overcome this drawback, general approaches to work with RNA, mostly focusing on the inactivation of ribonucleases, have been reported.[5,12] In addition to the purification and recovery steps, the RNA short-term storage is usually accomplished using high purity water or sterile phosphate buffered saline (PBS) solutions at temperatures below 4 °C, while lyophilization and temperatures below -80 °C are required for the long-term storage of RNA;[14] at this temperature, RNA is stable when stored as an ethanol precipitate.[15] Prior to any application, RNA may be dissolved in aqueous solutions of buffers, either sterile water or modified Tris buffer (10 mM Tris, 0.1 mM EDTA, overall pH 7.5).[15] Highly purified 100% formamide may also be used for RNA long-term storage,[16] although it limits the subsequent applications of this biopolymer. In general, divalent cations such as Mg^{2+}, monovalent cations (e.g. K^{+}, Na^{+}), and polycationic amines (polyamines such as spermidine) increase the RNA stability by shielding negative phosphates in the backbone and coordinating 2' hydroxy groups.[17] Alternative strategies focused on enhancing the RNA stability comprise chemical modifications,[18] and the application of the tRNA scaffold approach for bacterial RNA constructs.[19]

Based on the exposed, it is of paramount relevance to develop simple and sustainable strategies and protocols to maintain and/or increase the RNA stability and biological activity during the manufacturing and storage steps, ideally using less extreme temperatures and more benign solvents, and for which aqueous solutions of ionic liquids (ILs) may be considered as potential solvents for both the RNA purification and preservation steps.

ILs, commonly described as molten salts at temperatures below 100 °C, and composed of large and unsymmetrical organic cations and organic or inorganic anions, have been reported as appropriate stabilizing media for proteins and enzymes.[20-22] During the last years, ILs have also been applied to improve some properties of nucleic acids, mainly of DNA, such as solubility, stability and bioavailability.[23-29] Despite its high importance, few reports dealing with ILs and RNA are available.[14,23] In these works, cholinium dihydrogen phosphate and cholinium chloride were investigated. However, it is known that the stability, structure and functionality of RNA depend on the concentration and chemical identity of the ions in solution[7], justifying a more intensive search on adequate IL ions, which is even of higher relevance given the ILs designer ability.

Recently, we proposed a novel class of self-buffering imidazolium- and tetraalkylammonium-based ILs containing anions derived from biological buffers (Good's buffers) – GB-ILs.[30] These ILs display high stabilizing effects over proteins and allow their complete extraction from aqueous media when used as phase-forming components of liquid-liquid systems.[31] Cholinium chloride is a water-soluble nutrient, and appropriate cholinium-based ILs usually present high biodegradability, low toxicity, and are able to maintain the structure and function of proteins and/or other labile biomolecules.[31] Based on the exposed, and considering that nucleic acids are not stable in extreme ranges of pH values[12], aqueous solutions of GB-ILs comprising the cholinium cation seem thus to be an attractive and biocompatible milieu to maintain the integrity and improve the stability of RNA. Furthermore, the use of self-buffering ILs can avoid the need of adding an additional buffer. Accordingly, in this work, we synthesized several cholinium-based GB-ILs and evaluated their performance to stabilize a recombinant *Escherichia coli* small RNAs fraction (sRNAs) containing a microRNA precursor (pre-miR-29). The integrity, structural stability,

cytotoxicity, and superficial charge of RNA in aqueous solutions comprising 20 and 50 % (w/w) of each IL were evaluated, as well as the medium-term storage stability of RNA in these aqueous solutions. Given the remarkable results obtained, zeta potential measurements and molecular dynamics simulations were used to appraise the ILs effects on the RNA stability. Finally, it was demonstrated that the dissolved RNA can be successfully recovered from the aqueous solutions of ILs, further allowing the IL recycling and reuse.

EXPERIMENTAL SECTION

Materials. All chemicals used in the lysis buffer were obtained from Sigma-Aldrich (St Louis, MO, USA). Cholinium hydroxide solution (46% (w/w in methanol), 2-(N-morpholino) ethanesulfonic acid (MES, purity >99% (w/w), 2-[(2-hydroxy-1,1-[bis(hydroxymethyl)ethyl)amino]ethane sulfonic acid (TES, purity >99% (w/w), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES, purity >99.5% (w/w), and N-[tris(hydroxymethyl)methyl]glycine (Tricine, purity >99% (w/w) were supplied by Sigma-Aldrich (St Louis, MO, USA). Sodium hydroxide was purchased from Eka Chemicals. Methanol (HPLC grade, purity >99.9%) was supplied from Fisher Scientific, and acetonitrile (purity >99.7%) was from LabScan. Hyper Ladder I (Bioline, London, UK) was used as DNA molecular weight marker. All materials used in the experimental assays were RNase-free. Ultrapure reagent-grade water (Mili-Q system, Milipore/Waters) was treated with 0.05% diethyl pyrocarbonate (DEPC, Sigma-Aldrich, St Louis, MO, USA).

ILs synthesis. GB-ILs were synthesized through the neutralization of cholinium hydroxide with Good's buffers, as described elsewhere.[31] The following ILs have been synthesized: cholinium 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonate ([Ch][HEPES]), cholinium 2-(N-morpholino)ethanesulfonate ([Ch][MES]), cholinium N-[tris(hydroxymethyl)methyl]glycinate ([Ch][Tricine]), and cholinium 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonate ([Ch][TES]). Further details on the ILs preparation are provided in the Supporting Information. The chemical structure of each GB-IL was confirmed by ^{1}H and ^{13}C NMR spectroscopy, showing to be > 98 % (w/wt) pure. Their chemical structures are shown in the Supporting Information, Figure S1. Cholinium dihydrogen phosphate ([Ch][DHP]) acquired from Iolitec was also used, as well as buffered

cholinium dihydrogen phosphate (Buff[Ch][DHP]), which was obtained by adjusting the pH of [Ch][DHP] to 7.0 with cholinium hydroxide.

Small RNA biosynthesis and isolation. A cell culture of *Escherichia coli* (*E. coli*) DH5α strain modified with plasmid pBHSR1-RM containing the sequence of human pre-miR-29b was used for the sRNA production, namely transfer RNA (tRNA), pre-miR-29b and 6S RNA. The isolation of sRNA was carried out according to the acid guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and co-workers.[32] Details on sRNA recombinant production and subsequent isolation are given in the Supporting Information.

ILs aqueous solutions as solvent media of RNA. The sRNA samples were dissolved in aqueous solutions of the different ILs, at 20 and 50 % (w/w). These concentrations were chosen based on the results previously reported by Mazid *et al.*[14] with buffered [Ch][DHP]. The mixtures were incubated for different periods, namely 1 h, and 15 and 30 days at room temperature (25 ± 1 °C) and at 4 °C. After the incubation period, RNA was precipitated by adding 2 volumes of pure ethanol, incubated for 2 h at -80 °C, and recovered by centrifugation at 15,000 g for 30 min at 4 °C. Finally, the nucleic acids pellets were air-dried for 10 min at room temperature and solubilized in 0.05% DEPC (diethyl pyrocarbonate)-treated water. The pH of the aqueous solutions at 20 and 50 % (w/w) of ILs was determined from at least two repeated measurements performed at room temperature (25 ± 1) and using a pH glass electrode (Metrohm, Switzerland).

Electrophoretic analysis. The integrity and identification of sRNAs recovered after incubation with ILs for 1 h at 4 °C were evaluated using denaturating urea polyacrylamide

gel electrophoresis and agarose gel electrophoresis. Further details can be found in the Supporting Information.

Molecular Dynamics simulations. All molecular dynamics simulations were performed with GROMACS 2016.[33] Parameters for RNA were taken from the Amber99SB forcefield[34] and partial charges for [DHP]- and $[MES]^-$ were calculated by the Restrained Electrostatic Potential (RESP) method[35], using optimized geometries at the B3LYP/6-31G* level, using Gaussian-09[36]; remaining parameters were taken from the GAFF (General Amber Force Field) forcefield[37]; the description of $[Ch]^+$ was carried out according to Gontrani *et al.*[38]. Simulations started with the insertion of a linear single random RNA chain (AGCGAACGCAUCUCGAGUUC) into a box of water containing *ca.* 430 [Ch][DHP] or [Ch][MES] pairs and 18k water molecules, leading to a final IL concentration of 20% (w/w), in simulation boxes with volumes *ca.* 85x85x85 $Å^3$. The overall negative charge of the simulation box was neutralized upon addition of 19 Na^+ cations. Prior to all MD simulations, the overall configurations were geometry optimized to remove bad contacts using a combination of steepest-descent and conjugate-gradient minimizers.

A short 1 ns equilibration in the NVT (constant Number of particles, Volume and Temperature) ensemble at 300 K, followed by 1 ns of NPT equilibration at 1 atm, were used to ensure proper temperature and pressure equilibration, respectively. Both thermodynamic quantities remained stable at the reference value during the last 500 ps of the equilibration stages. Finally, 20 ns NPT production runs were obtained. Simulations used a 12 Å nonbonded cutoff with long range electrostatic interactions calculated using the PME (Particle Mesh Ewald) method; the integration timestep was 2 fs; hydrogen involving bonds were constrained using LINCS (Linear Constraint Solver); temperature was controlled using

the v-rescale and Berendsen thermostats in the equilibration and production stages, respectively; pressure was controlled with the isotropic Berendsen barostat.

Circular Dichroism (CD) spectroscopy. CD experiments were performed in a Jasco J-815 spectropolarimeter (Jasco, Easton, MD, USA), 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. 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 (Θ) plots using the following equation:

$$\theta = \frac{CD_\lambda - CD_\lambda^{min}}{CD_\lambda^{max} - CD_\lambda^{min}} \quad (1)$$

where CD is the ellipticity of the monitored wavelength at each temperature, and CD^{min} and CD^{max} are the lowest and highest ellipticity, respectively. Data points where then fitted to a Boltzmann distribution (OriginPro 2015) and the melting temperatures (T_m) determined from the two-state transition model used, using the first derivative method. Total RNA concentration was 150 μg/mL, and distinct incubation periods and temperatures of sRNA with ILs were performed.

Zeta potential. The zeta potential of RNA samples, with and without ILs, were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS particle analyzer (Malvern Instruments, Worcestershire, UK), equipped with a He-Ne laser, at 25 °C. Zeta potential

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 v7.03. Experiments were performed in triplicate and an average of 30 measurements was acquired for each sample. sRNA was incubated with ILs during 1 h at 4 °C, and the total RNA concentration was 200 µg/mL. The given values represent the average and associated standard deviation of three independent samples.

Cell viability. The cellular viability of the different IL-RNA formulations was assessed by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay using the Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, USA), according to the manufacturer's instructions and as described in the Supporting Information.

RESULTS AND DISCUSSION

The milestone discoveries related with catalytic RNAs and RNA interference mechanism[1] changed the RNA landscape to a versatile biopolymer, leading to the development of novel RNA-based therapeutics, and to their widespread use in fundamental and applied research. However, and in contrast with DNA, the higher flexibility and folding heterogeneity of RNAs, coupled with their increased susceptibility to degradation by naturally occurring nucleases,[11] demand for improved protocols for their manipulation and adequate formulations for their storage. ILs have been described as biocompatible media, being able to enhance the stability of DNA, proteins and siRNA;[39] however, limited attention has been given to RNA,[14,23] which may be due to its poor stability and inherent complex manipulation. Hitherto, the majority of the published works dealing with nucleic acids have focused on cholinium dihydrogen phosphate[14] and cholinium chloride[23] and, to the best of our knowledge, no studies were reported concerning the stability of recombinant sRNA fractions in aqueous solutions of ILs, nor the effect of the IL anion was ascertained with the same RNA type.

Small RNAs integrity and structure in aqueous solutions of ILs. The sRNA fraction profile obtained from *E. coli* DH5α by the phenol-chloroform method includes a well-defined band with a low molecular weight visualized by agarose electrophoresis (*cf.* the Supporting Information, Figure S2). Furthermore, the increased resolution offered by polyacrylamide electrophoresis allows the identification of three bands corresponding to three classes of RNAs, namely 6S RNA, pre-miR-29 and tRNA, in a decreasing order of molecular weight (Figure 1).

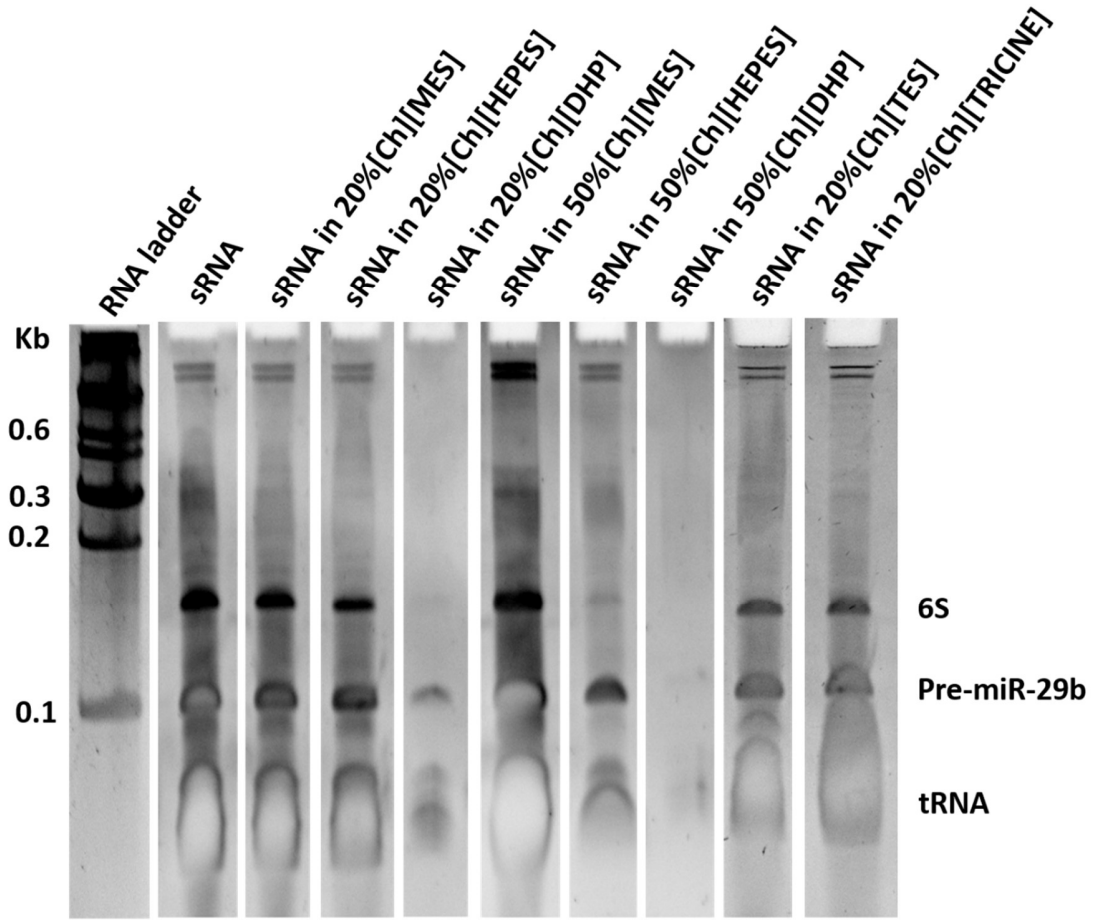


Figure 1. Polyacrylamide gel electrophoresis of sRNA in presence of aqueous solutions of 20 and 50% (w/w) of the following ILs: [Ch][DHP], [Ch][MES], [Ch][HEPES], [Ch][TES], and [Ch][TRICINE]. RNA control samples without ILs are also included.

Several cholinium-based GB-ILs were synthesized, by neutralization of cholinium hydroxide with Good's buffers (MES, TES, HEPES and TRICINE). Aqueous solutions of these ILs and of non- and buffered cholinium dihydrogen phosphate, at 20 and 50% (w/w), were then used to appraise their potential as preserving media of RNA. To this end, the sRNA fraction was mixed with the several aqueous solutions of ILs, incubated for 1 h at room temperature (25 ± 1 °C), and further characterized in terms of integrity, structural stability, and charge.

To avoid interferences of ILs in the eletrophoretic runs, RNA was precipitated from the ILs aqueous solutions and further solubilized in DEPC (diethyl pyrocarbonate)-treated water. The results obtained are depicted in Figure 1, and in Figure S2 in the Supporting Information. The agarose gel electrophoresis results (Figure S2 in the Supporting Information) reveal that for the lowest concentration of IL (20 % (w/w)), the structural integrity of RNA is maintained in all investigated ILs, with the exception of the non-buffered [Ch][DHP]. Although no RNA was observed in agarose electrophoresis with [Ch][DHP], the higher detection limit of polyacrylamide electrophoresis allowed to confirm the presence of RNA and to demonstrate that the biopolymer suffers degradation when incubated with this IL, as shown in Figure 1. On the other hand, for higher concentrations of ILs (50% (w/w)), different trends were observed. The structural integrity of RNA is maintained with [Ch][MES], but highly impaired in presence of non-buffered [Ch][DHP]. In this set of results, the pH values of the respective aqueous solutions play a significant role. According to the data shown in Table S1 in the Supporting Information, aqueous solutions of the non-buffered [Ch][DHP] are acidic (pH ranging between 3.54 and 4.17) whereas the pH values of aqueous solutions of GB-ILs range between 8.62 and 12.38, demonstrating that alkaline solutions are beneficial to maintain the RNA integrity.

In particular, [Ch][HEPES] selectively stabilizes pre-miR-29 over the other host RNAs - Figure 1. This behavior may be due to the stem-loop structure of pre-miR-29,[11] contrasting with the linear structure of other host RNAs. These hairpin loops represent short secondary structural folds, which are formed in single-stranded nucleic acids and consist of a base-paired stem and a loop sequence with unpaired nucleotide bases.[40] Hydrogen-bonding is relevant in base pairing, while base stacking between adjacent bases through van der Waals interactions may contribute to the stabilization of RNA structural motifs. Amongst the

diverse ILs anions investigated, $[HEPES]^-$ has been described as the most polar anion according to their dipole moment values,[31] which can further justify its higher polarity and ability to establish hydrogen bonds with the RNA nitrogen bases and that may be responsible for the enhanced stabilization of stem-loop RNAs.

At higher IL concentrations we were unable to efficiently remove [Ch][TES] and [Ch][TRICINE] and their analysis by electrophoresis could not be performed correctly. Even so, the best results in terms of RNA integrity were obtained with aqueous solutions of cholinium-based GB-ILs at 20% (w/w).

To address the sRNA structural stability, CD spectra were recorded for all mixtures of sRNA and aqueous solutions of ILs - shown in Figure 2, and Figure S3 in the Supporting Information.

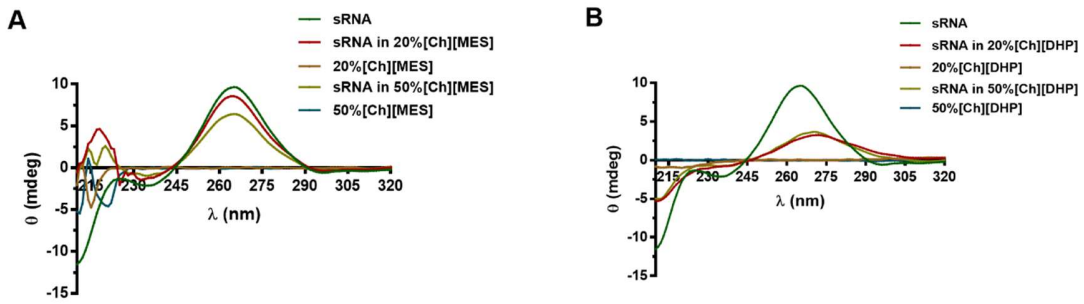


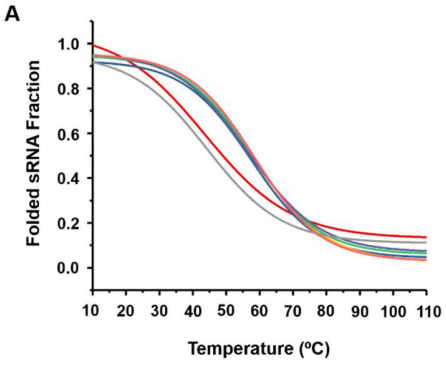
Figure 2. CD spectra (200 – 320 nm) of sRNA in the absence and presence of ILs: (A) 20 and 50% (w/w) of [Ch][MES]; (B) 20 and 50% (w/w) of [Ch][DHP]. For each set, the dark green line represents RNA, the red line corresponds to RNA in 20% (w/w) IL, the brown line to 20% (w/w) IL, the light green line to RNA in 50% (w/w) IL, and the blue line to 50% (w/w) IL. sRNA was incubated for 1 h at 4 °C.

The typical CD spectrum of the studied sRNA sample (dark green line) comprises two major peaks of ellipticity: a maximum at 265 nm (positive band) and a minimum at 215 nm (negative band). From the analysis of the CD spectra of all aqueous solutions and sRNA, it is clear that ILs largely interfere with the CD signal below 230 nm. However, through the analysis of the CD spectra of sRNA-IL at 20% (w/w) (red line in Figure 2 and in Figure S2 in the Supporting Information) and 50% (w/w) (light green line in Figure 2 and in Figure S2 in the Supporting Information) for wavelengths above 230 nm, it is shown that there is a shift in the maximum ellipticity with the non-buffered [Ch][DHP], at both IL concentrations, indicating that this IL causes the destabilization of the RNA structure. On the other hand, the structure of sRNA is maintained in all GB-ILs investigated, thus reinforcing their ability to stabilize RNAs. As discussed above, and at least for an incubation period of 1h, the pH value of the aqueous solutions plays a significant role over the RNA stability. Specifically, and as shown in Table S1 (Supporting Information), the pH of 20 and 50 % (w/w) aqueous solutions of unbuffered [Ch][DHP] is highly acidic (below 4). As RNA tends to hydrolyze at room temperature and at pH values below 5,[12] we further explored if the adjustment of the pH of the [Ch][DHP] aqueous solution with cholinium hydroxide up to 7 – Buff[Ch][DHP] – would enhance the stability of RNA. Cholinium hydroxide was added to [Ch][DHP] up to pH 7 and was chosen to maintain the same species in solution. As shown in Figure S3 in the Supporting Information, the sRNA structure is also maintained with 20 and 50 % (w/w) of Buff[Ch][DHP]. Although no results were yet reported on a recombinant sRNA fraction and ILs, Mazid *et al.*[14] previously studied the influence of aqueous solutions of buffered [Ch][DHP] on a siRNA sample. Buffered [Ch][DHP] was indeed identified as an efficient stabilizer of a double-stranded RNA,[14] and it is here additionally proved that this buffered IL is also able to stabilize *E. coli* sRNAs. In general, and at least for an incubation period of 1h,

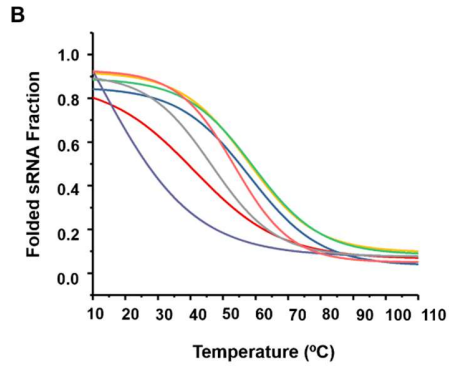
the RNA stability in the selected ILs strongly depends on the pH and is enhanced at neutral to alkaline pH values.

Thermal denaturation experiments were additionally performed to infer the stability of the secondary structure of RNA in the presence/absence of ILs, assessed by the RNA melting temperature (T_m). The CD T_m was determined at 265 nm since ILs do not interfere with the RNA ellipticity at this wavelength. According to Figure 3, the melting temperature of RNA dissolved in high-purity water is 43.7 °C. Nevertheless, a remarkable enhancement in the T_m of sRNA was observed in presence of GB-ILs. For the lowest concentration of IL (20% (w/w)), [Ch][MES], [Ch][TES], [Ch][HEPES], [Ch][TRICINE], and Buff[Ch][DHP] lead to an increase of *ca.* 14 °C in the sRNA T_m, indicating that it is significantly more stable in aqueous solutions of appropriate ILs than in high-purity water. The same protection effect was observed with 50% (w/w) of GB-ILs, increasing the sRNA T_m values up to 58.1, 57.8 and 59.3 °C for [Ch][MES], [Ch][HEPES] and [Ch][TES], respectively. In general, and taking into account the associated standard deviations, no significant differences are observed between the GB-ILs comprising the $[MES]^-$, $[TES]^-$, and $[HEPES]^-$ anions. The T_m obtained with [Ch][TRICINE] is lower for both concentrations of IL, and for 50% (w/w) it leads to a decrease of 11.5 °C when compared with the RNA in high-purity water, indicating that RNA samples are not stable in high amounts of this particular IL. This result is in accordance with the electrophoresis data, where the RNA integrity is compromised in presence of [Ch][TRICINE] at higher concentrations (Figure 1). According to the chemical structures of the ILs shown in Figure S1 in the Supporting Information, and with the exception of $[TRICINE]^-$ that contains a terminal carboxylic group, all the remaining GB-ILs present a negatively charged sulphonate group which seems to be of high relevance to improve the RNA stability. For [Ch][DHP], at 20 and 50% (w/w), a sRNA T_m similar to sRNA in high-

purity water was obtained (44.1 and 46.5 °C, respectively), being this a main effect of the acidic pH afforded by non-buffered [Ch][DHP] aqueous solutions. These results correlate with those obtained from the CD spectra, where the RNA destabilization was identified in non-buffered [Ch][DHP] (Figure 2 B).



	Melting Temperature (°C)
sRNA	43.67±0.65
sRNA in 20%[Ch][MES]	57.42±0.33
sRNA in 20%[Ch][HEPES]	58.05±0.28
sRNA in 20%[Ch][TES]	57.50±0.32
sRNA in 20%[Ch][TRICINE]	56.66±0.37
sRNA in 20%[Ch][DHP]	44.12±0.63
sRNA in 20%Buff[Ch][DHP]	58.48±0.27



	Melting Temperature (°C)
sRNA	43.30±0.51
sRNA in 50%[Ch][MES]	58.14±0.42
sRNA in 50%[Ch][HEPES]	57.78±0.35
sRNA in 50%[Ch][TES]	59.29±0.36
sRNA in 50%[Ch][TRICINE]	11.46±4.97
sRNA in 50%[Ch][DHP]	46.52±0.41
sRNA in 50%Buff[Ch][DHP]	53.59±0.23

Figure 3. CD melting curves (265 nm) of a sRNA fraction from *E. coli* DH5α. sRNA was incubated for 1 h at 4 °C with (A) 20% (w/w) IL, and (B) 50% (w/w) IL: absence of IL (red); [Ch][MES], blue; [Ch][TES], green; [Ch][HEPES], yellow; [Ch][TRICINE], purple; [Ch][DHP], grey; and Buff[Ch][DHP], pink.

Aqueous solutions of GB-ILs and Buff[Ch][DHP] at 20% (w/w) are outstanding solvents for preserving the RNA integrity and to enhance its stability. Contrarily to most ILs investigated (including Buff[Ch][DHP]), GB-ILs display self-buffering characteristics[30,31] and there is no need to add additional buffers to control the pH, as carried out with [Ch][DHP]. Other

advantages of GB-ILs include their high solubility in water, their inability to chelate with metal-ions, and their high chemical stability and resistance to enzymatic degradation.[30,39]

Superficial charge and molecular dynamics simulations. Aiming at better understanding the overall enhanced performance afforded by GB-ILs to preserve the sRNA integrity and enhance its stability, zeta potential measurements were performed, allowing us to conclude on the solution effective charge density. The results obtained are depicted in Figure 4. As polyanionic biopolymers, the typical zeta potential of sRNA at 200 µg/mL is approximately – 40 mV. All mixtures of sRNA and aqueous solutions at 20 or 50% (w/w) of ILs also display negative zeta potential values, although less negative than the isolated RNA. This trend may be attributed to the presence of cholinium cations in the outer shell of RNA – also discussed below based on molecular dynamics simulations. On the other hand, the charge of the IL alone is less negative than when complexed with RNA. Altogether, these observations confirm that all ILs employed in this study effectively surround sRNA. Furthermore, sRNA in presence of [Ch][TRICINE] displays the most negative zeta potential values. The presence of a higher number of $[TRICINE]^-$ anions in close proximity to RNA may account for this behavior, thus justifying the poorer performance of this GB-IL to keep the RNA integrity. Our results agree with those previously reported by Satpathi *et al.*[41] using calf thymus DNA and a guanidinium-based IL (Gua-IL). The authors[41] reported that DNA presents a negative charge in the presence of distinct concentrations of Gua-IL (0.1 – 100 mM), becoming less negative as the IL concentration increases.

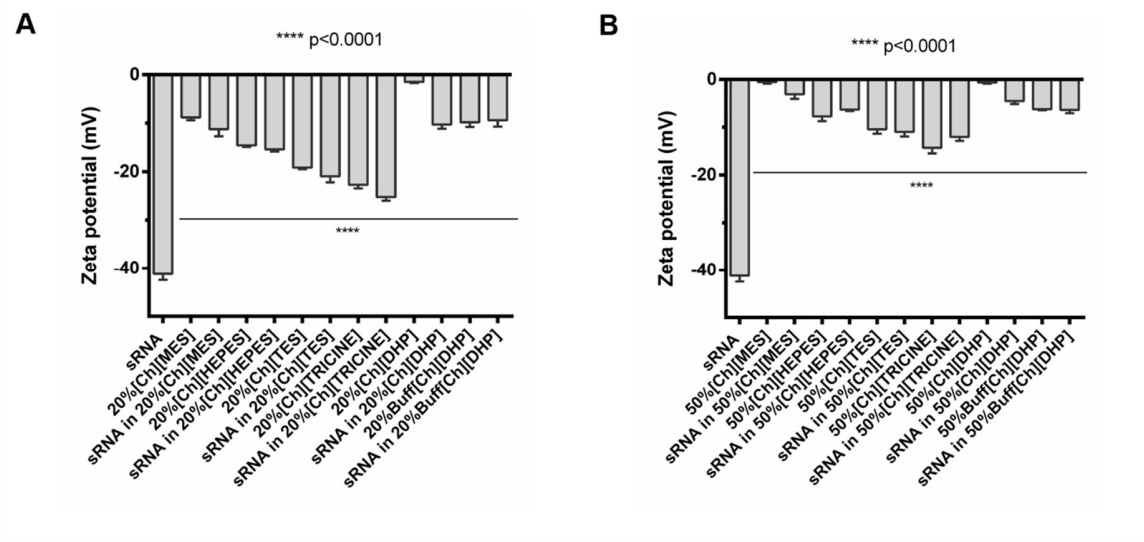


Figure 4. Average zeta potential (mV) of sRNA, ILs in aqueous solution, and ILs with the sRNA fraction from *E. coli*, at distinct IL concentrations: (A) 20% (w/w), and (B) 50% (w/w).

Molecular dynamics simulations were performed to gain insights into the molecular-level mechanisms ruling the outstanding performance of GB-ILs to stabilize and maintain the integrity of sRNA. A random 20-residue long single chain of RNA was assembled and simulated, in presence of aqueous solutions containing 20% (w/w) of IL. To this end, [Ch][DHP] and [Ch][MES] were considered: the first as a well-studied IL and the latter as a GB-IL representative. According to the snapshots provided in Figure 5 (see also Figures S4-S6 in the Supporting Information for a full depiction of the simulation boxes), which are representative of the overall dynamical behavior of the systems, the number of IL anions near the sRNA chain (consistent with the first IL solvation-like layer) is lower in [Ch][MES] than in [Ch][DHP]. This means that the chain first solvation layer has an overall more negative charge in presence of [Ch][DHP] than in presence of [Ch][MES]. The number of cations ($[Ch]^+$) is however similar in both environments (Figures S4-S6 in the Supporting

Information), implying that electrostatic interactions between the negatively charged RNA chain and $[Ch]^+$ do not depend on the IL anion type. As highlighted, differences are however observed for the number of IL anions at the RNA first hydration layer. Taking 4 Å as the cutoff (*i.e.* the distance between any atom of the RNA chain and a representative atom of the ion: N for $[Ch]^+$, S for $[MES]^-$ and P for $[DHP]^-$), the average number of cations obeying this threshold is 14.16 and 14.50 for [Ch][MES] and [Ch][DHP], respectively, whereas for the anions the averages are 8.92 and 12.14. The smaller size and higher charge density of $[DHP]^-$ suggests that it is more likely to be found closer to the IL cation (stronger IL cation-anion interactions) and to the nucleic acid chain than $[MES]^-$, facing thus more repulsive electrostatic interactions with the negatively charged nucleic acid. Similar trends are observed for cutoff distances up to 8 Å (see Figures S4-S6 in the Supporting Information). It is interesting to note that the $-SO_3$ group of $[MES]^-$, which bears most of the negative charge of this anion, has a size similar to that of $[DHP]^-$, but yet fails to be accommodated in an analogous fashion. Therefore, the stability of the sRNA chain is higher in presence of [Ch][MES] because the global charge of the overall complex (RNA chain + first solvation layer) is less negative than in presence of [Ch][DHP].

It should be noted that the correlation of these results with the measured zeta potential is not straightforward because the former is a macroscopic measure of the electrostatic potential at the interface of the solvated complex (with its multiple solvation spheres in which the solvent molecules are not fully mobile) and bulk solvent. Still, the second solvation shell of the complex should accommodate more cations in the case of [Ch][DHP] because of its higher negative charge, in contrast with [Ch][MES], which ultimately should lead to a more neutral total charge of the full complex in the former case, and consistent with the zeta potential results. The solvation structure of the RNA chain would be visible through analysis of spatial

distribution functions, although not possible because of the large size and mobility of the RNA chain.

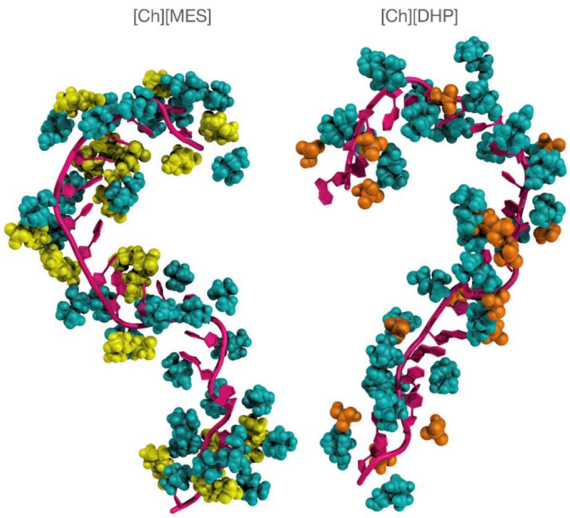
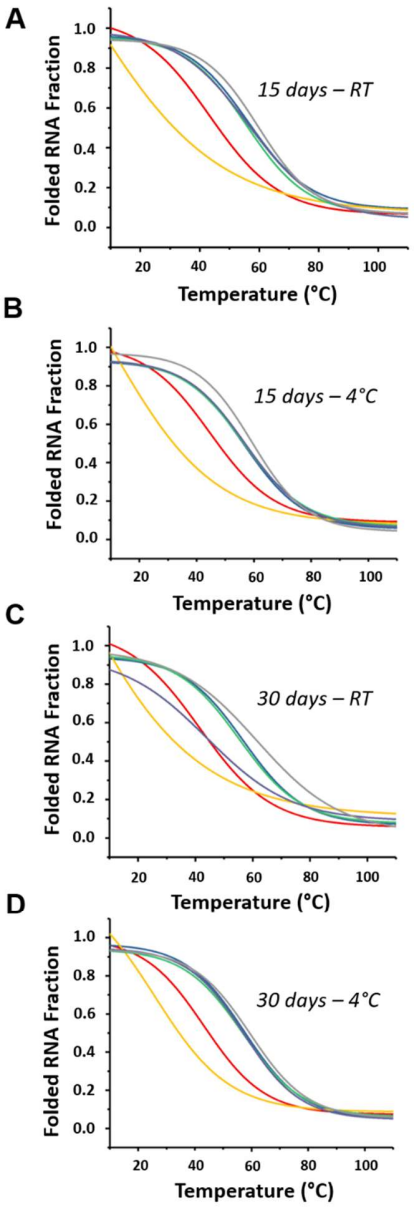


Figure 5. Snapshot of a 20-residue long linear sRNA chain in [Ch][MES] and [Ch][DHP] (left and right, respectively). The RNA chain is depicted as purple cartoon and the IL components as teal, yellow and orange spheres for $[Ch]^+$, $[MES]^-$ and $[DHP]^-$, respectively. Only ions at a distance up to 4 Å from the chain surface are shown.

Medium-term stability of small RNAs in ILs aqueous solutions, their cytotoxicity, and IL recyclability. Following the previously described assays with 1 h of incubation at 4 °C, and to ascertain if GB-ILs are suitable to be used as preservation media for RNAs storage, experiments with longer incubation periods - 15 and 30 days – at temperatures at which RNA is only marginally stable (room temperature and 4 °C) were performed. These studies were carried out with aqueous solutions of ILs at 20% (w/w), a concentration chosen given the results discussed before. This lower concentration of IL also results in a more economical and sustainable approach. The obtained results are summarized in Figure 6.



	Melting Temperature (°C)
sRNA	43.30±0.51
sRNA in 20%[Ch][MES]	56.30±0.33
sRNA in 20%[Ch][HEPES]	55.68±0.31
sRNA in 20%[Ch][TES]	56.37±0.36
sRNA in 20%[Ch][TRICINE]	-
sRNA in 20%Buff][Ch][DHP]	59.62±0.26

	Melting Temperature (°C)
sRNA	44.65±0.50
sRNA in 20%[Ch][MES]	56.65±0.33
sRNA in 20%[Ch][HEPES]	56.45±0.34
sRNA in 20%[Ch][TES]	57.00±0.35
sRNA in 20%[Ch][TRICINE]	-
sRNA in 20%Buff[Ch][DHP]	59.35±0.25

	Melting Temperature (°C)
sRNA	41.51±0.58
sRNA in 20%[Ch][MES]	56.76±0.31
sRNA in 20%[Ch][HEPES]	55.23±0.33
sRNA in 20%[Ch][TES]	43.94±1.50
sRNA in 20%[Ch][TRICINE]	-
sRNA in 20%Buff[Ch][DHP]	61.57±0.57

	Melting Temperature (°C)
sRNA	43.25±0.47
sRNA in 20%[Ch][MES]	57.40±0.30
sRNA in 20%[Ch][HEPES]	56.88±0.36
sRNA in 20%[Ch][TES]	57.28±0.29
sRNA in 20%[Ch][TRICINE]	-
sRNA in 20%Buff[Ch][DHP]	59.51±0.29

Figure 6. CD melting curves (265 nm) of a sRNA fraction from *E. coli* DH5α at different incubation periods and distinct temperatures. (A) sRNA incubated with 20% (w/w) of ILs for 15 days at room temperature (25 ± 1 °C); (B) for 15 days at 4 °C; (C) for 30 days at room temperature (25 ± 1 °C); and (D) for 30 days at 4 °C: absence of IL (red); [Ch][MES], blue; [Ch][TES], purple; [Ch][HEPES], green; [Ch][TRICINE], yellow; and Buff[Ch][DHP], grey.

The behavior and stability of sRNA in the absence and presence of GB-ILs is quite different, being their stability particularly enhanced in aqueous solutions of [Ch][MES], [Ch][HEPES], and Buff[Ch][DHP]. The sRNA T_m in aqueous solutions of [Ch][TES] is 57 °C after 15 days of storage at room temperature and at 4 °C. However, after 30 days at room temperature, it decreases down to 44 °C, similarly to the result obtained with RNA in high-purity water. This trend indicates that for longer storage periods at room temperature, [Ch][TES] is not an effective stabilizer for sRNA. Even so, based on the RNA melting temperature experiments, [Ch][MES], [Ch][HEPES], and Buff[Ch][DHP] are remarkable sRNA stabilizers, at least up to 30 days. The chemical structures of the selected ILs are shown in Figure S1 in the Supporting information. If on one hand all ILs share the same cholinium cation, they are composed of different anions. $[TES]^-$ and $[TRICINE]^-$ anions present multiple hydroxyl groups, which may be responsible for the lower RNA stability due to a stronger hydrogen-bonding with RNA nitrogen bases, thus resulting in a higher number of IL anions at the RNA first hydration layer as discussed above with the MD simulations, and in base-pairing disruption mediated by intramolecular hydrogen. This mechanism may be similar to that reported for the destabilization of RNA by urea, attributed to the disruption of base-pair interactions by direct hydrogen-bonding of urea with the RNA bases.[42] Moreover, it has also been shown that polyols, containing multiple hydroxyl groups, induce the thermal destabilization of double stranded DNA.[43] On the other hand, RNA is stable after one-month storage at room temperature (see Figure 6) in Buff[Ch][DHP], [Ch][MES], and [Ch][HEPES]. The observed enhanced stability in presence of these GB-ILs is, at least in part, due to the shielding of negative phosphates in the backbone and coordinating 2' hydroxy groups by the cholinium cation, similar to what was previously reported for divalent and monovalent cations and polycationic amines.[17]

To ensure the sustainable character of GB-ILs aqueous solutions as stabilizing and preservation media of sRNA, both the safety profile of the complexes sRNA-IL and the ILs reusability are important criteria. Previously, Taha *et al*.[31] determined the ecotoxicity of several GB-ILs towards *Vibrio fischeri* and found that the investigated ILs present non-toxic character as indicated by their high EC_{50} values. According to the same standard assay, [Ch][DHP] was described as "practically harmless".[44] However, no cytotoxicity studies involving human cell lines have yet been performed for GB-ILs, essential assays given the RNA biological role or when used as a therapeutic. Figure 7 shows the viability of human cell lines, namely human fibroblasts and a cervical cancer cell line (HeLa), which represents prototypical cells of the human epithelium,[45] in presence of sRNA in aqueous solutions of ILs at 20 and 50% (w/w). These cell lines were selected based on the rationale that epithelial cells are generally the site of first contact of an organism with toxic compounds,[45] and since they are physiologically distinct entities they display different reactivity to external stimulus. Moreover, both are human cell lines, a relevant factor when envisaging the use of sRNA as alternative biopharmaceuticals.

As depicted in Figure 7 A, and by comparison with untreated cells, aqueous solutions of both non- and buffered-[Ch][DHP] highly impair the viability of fibroblasts, with only *ca.* 20% of viable cells. On the other hand, non- and buffered [Ch][DHP] at 20 and 50% (w/w) and [Ch][TRICINE] at 50% (w/w) affect the HeLa viability, with *ca.* 50, 25, 20 and 30% of viable cells, respectively. Previously, Weaver *et al*.[46] determined the [Ch][DHP] EC_{50} for a J774 murine macrophage cell line and observed that this IL exceeds the buffer capacity of the complemented media at high concentrations (≥50 mM), but it is not clearly apparent whether toxicity can be attributed to pH effects. These effects were however observed in this work for [Ch][DHP] and Buff[Ch][DHP] ILs. Therefore, the use of this IL, at the described

concentrations and at least in biological assays or for biologics storage purposes, is not advisable, unless it could be previously removed. The same applies to [Ch][TRICINE] at 50% (w/w). Despite these results, promising ILs were however identified, namely [Ch][MES], [Ch][TES], [Ch][TRICINE] and [Ch][HEPES] at 20% (w/w). Their non-cytotoxic profile combined with their ability to maintain the integrity and to enhance the thermal stability of sRNA allow us to conclude that these self-buffering ILs are appropriate solvents for RNA preservation.

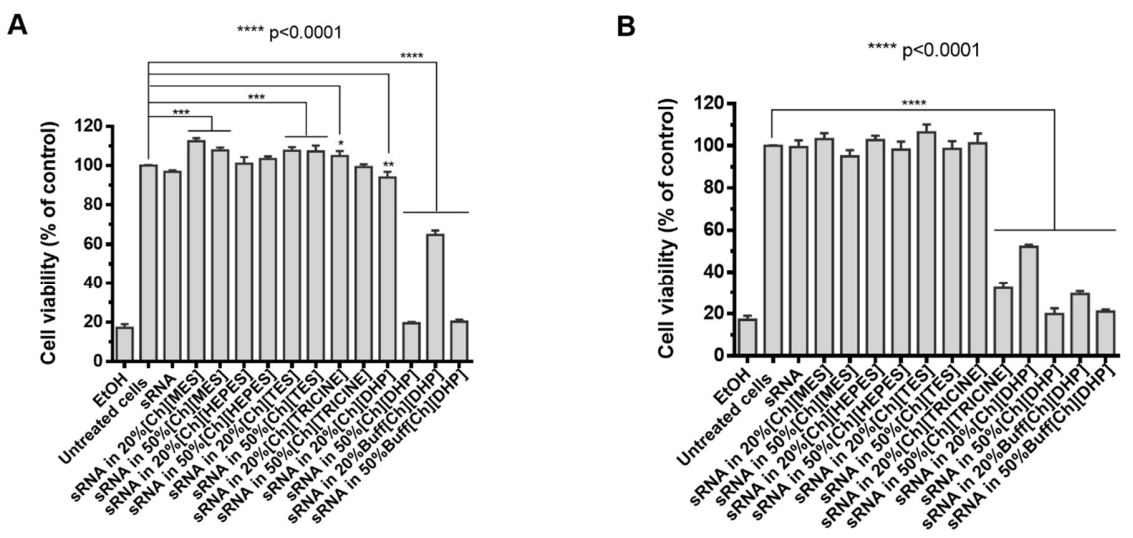


Figure 7. Viability of two human cell lines in presence of sRNA-IL aqueous solutions: (A) Fibroblasts, and (B) HeLa cells. Cell viability was measured using the MTS assay, and is shown as percentage of control (%).

In the pursuit of developing sustainable processes and solvent media for preserving RNA, we finally evaluated the possibility of recycling the ILs used, after the proper RNA recovery from the IL aqueous solution. A scheme of the overall process is depicted in Figure 8, in

which the RNA was incubated during 1 h with the corresponding IL. Before any application, sRNA can be successfully recovered from the IL aqueous solutions by precipitation with ethanol (after a 2h incubation period at -80 °C, followed by a centrifugation step at 15,000 g). Then, the IL can be recovered and reused again as preservation media of sRNA. This possibility was experimentally addressed with two ILs, namely [Ch][MES] and [Ch][HEPES], and repeated for 3 times. At the laboratory scale, a maximum loss of 3 (w/w) % of IL in each cycle of IL recovery/reuse was registered , which is mainly due to the transference process between different vials. Since ethanol (well-known anti-solvent for RNA) was used for RNA precipitation and no degraded RNA was observed in the agarose gel electrophoresis (Figure 8), it is safe to admit that there are no significant losses of RNA among the different cycles. In all cycles, RNA was recovered with high-integrity, and the IL was reused. This strategy, comprising both the RNA recovery and the IL reuse, is a step forward in the demand for effective RNA stabilizing and preservation solvents, which not only leads to less hazardous solvent wastes, but from an economical point of view is also less expensive.

Overall, aqueous solutions of adequate GB-ILs are enhanced solvents for the stabilization of recombinant sRNA, being able to maintain the sRNA integrity and to promote an increase of *ca.* 14 °C in its melting temperature, even after a medium-term storage of 1 month. In comparison with volatile organic solvents, the use of non-volatile ILs as RNA stabilizers eliminates organic solvent losses to the atmosphere[34], decreasing both the environmental footprint and the cost of the process. The sustainability of this strategy is also reinforced not only by the low environmental impact of appropriate GB-ILs (low toxicity to *Vibrio fischeri* and low cytotoxicity to human cell lines), as well as by the ILs recycling possibility without compromising the sRNA integrity.

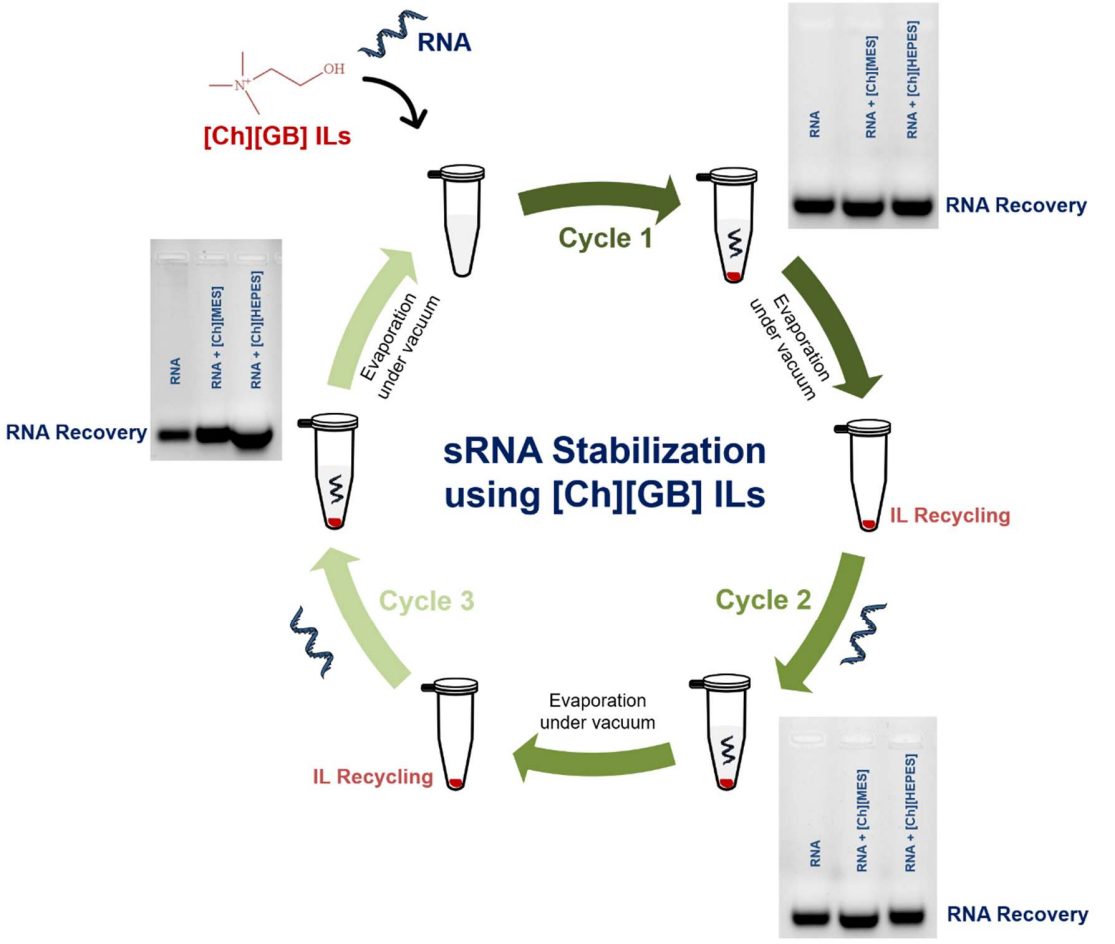


Figure 8. Scheme describing the recovery of recombinant *E. coli* sRNAs from aqueous solutions of GB-ILs and the IL recycling. Three cycles were successfully carried out with [Ch][MES] and [Ch][HEPES], for mixtures incubated during 1 h.

CONCLUSIONS

Nucleic-acid-based products have been identified as promising biopharmaceuticals and as relevant biomolecules in biological assays. Nevertheless, nucleic acids are highly susceptible to nuclease cleavage, and although RNA is more prone to degradation than DNA, most studies aiming at finding adequate preservation media focused on improving the stability of DNA. Based on this lacuna, we evaluated the potential of self-buffering and biological-based ILs for extending the shelf life of a recombinant sRNA fraction derived from *E. coli*, containing the therapeutically relevant pre-miR-29. To the best of our knowledge, this is the first study that successfully reports the enhanced stability of a recombinant *E. coli* sRNA fraction containing a pre-miRNA in aqueous solutions of ILs.

It was found that ILs comprising the cholinium cation combined with anions derived from Good's buffers are able to maintain the sRNA stability, at least up to 30 days, thereby increasing the RNA shelf life. From the ILs investigated, [Ch][MES], [Ch][HEPES] and Buff[Ch][DHP] are the most suitable for maintaining the integrity and stability of sRNA. Molecular dynamics simulations allowed to better understand the molecular-level phenomena responsible for the observed improved stability of RNA, in which the overall charge of the biopolymer first solvation sphere plays a primary role.

Amongst the most suitable ILs that improve the sRNA stability, [Ch][HEPES] and [Ch][MES] were additionally identified as non-cytotoxic to human cell lines, which is particularly relevant when envisaging the use of RNA as therapeutics. The sustainability of GB-ILs solvents and preservation approach to RNA is reinforced not only by their low environmental impact and low cytotoxicity, but also by the possibility of the ILs recycling without compromising the RNA integrity, as demonstrated.

In summary, aqueous solutions of [Ch][MES] and [Ch][HEPES] are remarkable stabilizing and storage media of recombinant sRNAs at room temperature, without requiring the typically carried out samples freezing, thus reinforcing their effectiveness in RNA bioprocessing.

SUPPORTING INFORMATION

Experimental details on ILs chemical structures and synthesis, small RNAs biosynthesis and isolation, eletrophoretic analysis and cell viability, pH values of aqueous solutions of ILs, agarose gel electrophoresis results, CD spectra, and time evolution of the number of contacts below 4 Å, 5 Å, and 6 Å between the representative IL atom and the surface of sRNA.

AUTHOR INFORMATION

Corresponding Authors

*E-mail address: maragfreire@ua.pt; Tel: +351-234-401422; Fax: +351-234-370084;
*E-mail address: fani.sousa@fcsaude.ubi.pt; Tel: +351-275-329074;

ACKNOWLEDGEMENTS

The authors acknowledge to the CICS-UBI projects Pest-OE/SAU/UI0709/2014, UID/Multi/00709/2013 and the program COMPETE, Pest-C/SAU/UI0709/2011, and the CICECO-Aveiro Institute of Materials projects, POCI-01-0145-FEDER-007679 (UID/CTM/50011/2013), financed by national funds through the FCT/MEC and when appropriate co-financed by FEDER under the PT2020 Partnership Agreement. CICS-UBI was also supported by FEDER funds through the POCI – COMPETE 2020 – Operational Programme Competitiveness and Internationalisation in Axis I – Strengthening research, technological development and innovation (Project POCI-01-0145-FEDER-007491). The authors acknowledge the financial support from the European Union Framework Programme for Research and Innovation HORIZON 2020, under the TEAMING Grant agreement No 739572 - The Discoveries CTR. P. Pereira acknowledges a post-doctoral fellowship from a FCT-funded project (Ref: PTDC/1399/2014), and M. V. Quental the FCT PhD grant

SFRH/BD/100155/2014. M. G. Freire acknowledges the European Research Council (ERC) for the Starting Grant ERC-2013-StG-337753.

REFERENCES

(1) Burnett, J. C.; Rossi, J. J. RNA-based therapeutics: current progress and future prospects. *Chem. Biol.* **2012**, *19* (1), 60 – 71, DOI 10.1016/j.chembiol.2011.12.008.

(2) Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. **1998**, *391* (6669), 806 – 811, DOI 10.1038/35888.

(3) Singh, M. S.; Peer, D. RNA nanomedicines: the next generation drugs? *Curr. Opin. Biotechnol.* **2016**, *39*, 28 – 34, DOI 10.1016/j.copbio.2015.12.011.

(4) Ho, S. V.; McLaughlin, J. M.; Cue, B. W.; Dunn, P. J. Environmental considerations in biologics manufacturing. *Green. Chem.* **2010**, *12*, 755 – 766, DOI 10.1039/B927443J.

(5) Martins, R.; Queiroz, J. A.; Sousa, F. Ribonucleic acid purification. *J. Chromatogr. A*. **2014**, *1355*, 1 – 14, DOI 10.1016/j.chroma.2014.05.075.

(6) Sugimoto, N. Effects of Ionic Liquid and Liposomes on the structure, stability, and function of nucleic acids. In Erdmann, V., Markiewicz, W. and Barciszewski, J. (eds.), *Chemical biology of nucleic acids*. RNA technologies, Springer, Berlin, Heidelberg, **2014**, 57 – 74, DOI 10.1007/978-3-642-54452-1_4.

(7) Benedetto, A.; Ballone, P. Room temperature ionic liquids interacting with bio-molecules: an overview of experimental and computational studies. *Philos. Mag.* **2016**, *96* (7-9), 870 – 894, DOI 10.1080/14786435.2015.1119323.

(8) Sugimoto, N. Noncanonical structures and their thermodynamics of DNA and RNA under molecular crowding: beyond the Watson-Crick double helix. *Int. Rev. Cell. Mol. Biol.* **2014**, *307*, 205 – 273, DOI 10.1016/B978-0-12-800046-5.00008-4.

(9) Singh, N.; Sharma, M.; Mondal, D.; Pereira, M. M.; Prasad, K. Very high concentration solubility and long-term stability of DNA in an ammonium-based ionic liquid: a suitable medium for nucleic packaging and preservation. *ACS. Sustainable. Chem. Eng.* **2017**, *5* (2), 1998 – 2005, DOI 10.1021/acssuschemeng.6b02842.

(10) Pereira, P.; Pedro, A. Q.; Queiroz, J. A.; Figueiras, A. R.; Sousa, F. New insights for therapeutic recombinant human miRNAs heterologous production: *Rhodovolum sulfidophilum* vs *Escherichia coli. Bioengineered*. **2017**, *8* (5), 670 – 677, DOI 10.1080/21655979.2017.1284710.

(11) Pereira, P. A.; Tomás, J. F.; Queiroz, J. A.; Figueiras, A. R.; Sousa, F. Recombinant pre-miR-29b for Alzheimer's disease therapeutics. *Sci. Rep.* **2016**, *6*, 19946, DOI 10.1038/srep19946.

(12) Edelmman, F. T.; Niedner, A; Niessing, D. Production of pure and functional RNA for *in vitro* reconstitution experiments. *Methods*. **2014**, *65*, 333 – 341, DOI 10.1016/j.ymeth.2013.08.034.

(13) Pereira, P.; Sousa, A.; Queiroz, J. A.; Correia, I.; Figueiras, A; Sousa, F. Purification of pre-miR-29 by arginine-affinity chromatography. *J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci.* **2014**, *951-952*, 16 – 23, DOI 10.1016/j.jchromb.2014.01.020.

(14) Mazid, R. R.; Divisekera, U.; Yang, W.; Ranganathan, V.; MacFarlane, D. R.; Cortez-Jugo, C.; Cheng, W. Biological stability and activity of siRNA in ionic liquids. *Chem. Commun.* **2014**, *50* (88), 13457 – 13460, DOI 10.1039/c4cc05086j.

(15) Farrell, R. E. Jr., Ed. RNA methodologies: A laboratory guide for isolation and characterization, 4th ed.; Academic Press New York, **2010**., DOI 10.1016/C2009-0-01850-9.

(16) Chomczynski, P. Solubilization in formamide protects RNA from degradation. *Nucleic. Acids. Res.* **1992**, *20*, 3791 – 3792, DOI 10.1093/nar/20.14.3791.

(17) Silvers, R.; Keller, H.; Schwalbe, H.; Hengesbach, M. Differential scanning fluorimetry for monitoring RNA stability. *Chembiochem*. **2015**, *16*, 1109 – 1114, DOI 10.1002/cbic.201500046.

(18) Deleavey, G. F.; Damha, M. J. Designing chemically modified oligonucleotides for targeted gene silencing. *Chem. Biol.* **2012**, *19*, 937 – 954, DOI 10.1016/j.chembiol.2012.07.011.

(19) Ponchon, L.; Dardel, F. Large scale expression and purification of recombinant RNA in *Escherichia coli*. *Methods*. **2011**, *54*, 267 – 273, 10.1016/j.ymeth.2011.02.007.

(20) Gutowski, K. E.; Broker, G. A.; Willauer, H. D.; Huddleston, J. G.; Swatloski, R. P.; Holbrey, J. D.; Rogers, R. D. Controlling the aqueous miscibility of ionic liquids: aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations. *J. Am. Chem. Soc.* **2003**, *125*, 6632 – 6633, DOI 10.1021/ja0351802.

(21) Lee, S. Y.; Vicente, F. A.; Silva, F. A.; Sintra, T. E.; Taha, M.; Khoiroh, I.; Coutinho, J. A. P.; Show, P. L.; Ventura, S. P. M. Evaluating self-buffering ionic liquids for biotechnological applications. *ACS. Sustainable. Chem. Eng.* **2015**, *3* (12), 3420 – 3428, DOI 10.1021/acssuschemeng.5b01155.

(22) Song, C. P.; Ramanan, R. N.; Vijayaraghavan, R.; MacFarlane, D. R.; Chan, E-S.; Ooi, C-W. Green, aqueous two-phase systems based on cholinium aminoate ionic liquids with tunable hydrophobicity and charge density. *ACS. Sustainable. Chem. Eng.* **2015**, *3* (12), 3291 – 3298, DOI 10.1021/acssuschemeng.5b00881.

(23) Mamajanov, I.; Engelhart, A. E.; Bean, H. D; Hud, N. V. DNA and RNA in anhydrous media: duplex, triplex, and G-quadruplex secondary structures in a deep eutectic solvent. *Angew. Chem. Int.* **2010**, *49* (36), 6310 – 6314, DOI 10.1002/anie.201001561.

(24) Tateishi-Karimata, H.; Sugimoto, N. A-T base pairs are more stable than G-C base pairs in a hydrated ionic liquid. *Angew. Chem. Int.* **2012**, *51*, 1416 – 1419, DOI 10.1002/anie.201106423.

(25) Tateishi-Karimata, H.; Sugimoto, N. Structure, stability and behaviour of nucleic acids in ionic liquids. *Nucleic. Acids. Res.* **2014**, *42*, (14), 8831 – 8844, DOI 10.1093/nar/gku499.

(26) Vijayaraghavan, R.; Izgorodin, A.; Ganesh, V.; Surianarayanan, M.; MacFarlane, D. R. Long-term structural and chemical stability of DNA in hydrated ionic liquids. *Angew. Chem. Int.* **2010**, *49*, 1631 – 1633, DOI 10.1002/anie.200906610.

(27) Jumbri, K.; Abdul Rahman, M. B.; Abdulmalek, E.; Ahmadab, H.; Micaelo, N. M. An insight into structure and stability of DNA in ionic liquids from molecular

dynamics simulation and experimental studies. *Phys. Chem. Chem. Phys.* **2014**, *16*, 14036 – 14046, DOI 10.1039/c4cp01159g.

(28) Marušič, M.; Tateishi-Karimata, H.; Sugimoto, N.; Plavec, J. Structural foundation for DNA behavior in hydrated ionic liquid: An NMR study. *Biochimie*. **2015**, *108*, 169 – 177, DOI 10.1016/j.biochi.2014.11.015.

(29) Haque, A.; Khan, I.; Hassan, S. I.; Khan, M. S. Interaction studies of cholinium-based ionic liquids with calf thymus DNA: Spectrophotometric and computational methods. *J. Mol. Liq.* **2017**, *237*, 201 – 207, DOI 10.1016/j.molliq.2017.04.068.

(30) Taha, M.; Silva, F. A.; Quental, M. V.; Ventura, S. P. M.; Freire, M. G.; Coutinho, J. A. P. Good's buffers as a basis for developing self-buffering and biocompatible ionic liquids for biological research. *Green. Chem.* **2014**, *16*, 3149 – 3159, DOI 10.1039/C4GC00328D.

(31) Taha, M.; Almeida, M. R.; Silva, F. A.; Domingues, P.; Ventura, S. P.; Coutinho, J. A.; Freire, M. G. Novel biocompatible and self-buffering ionic liquids for biopharmaceutical applications. *Chem. Eur. J.* **2015**, *21* (12), 4781 – 4788, DOI 10.1002/chem.201405693.

(32) Chomczynski, P.; Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chlorophorm extraction. *Anal. Biochem.* **1987**, *162* (1), 156 – 159, DOI 10.1006/abio.1987.9999.

(33) Abraham, M. J.; Murtola, T.; Schulz, R.; Páll, S.; Smith, J. C.; Hess, B.; Lindahl, E. GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX*. **2015**, *1*, 19 – 25, DOI 10.1016/j.softx.2015.06.001.

(34) Hornak, V.; Abel, R.; Okur, A.; Strockbine, B.; Roitberg, A.; Simmerling, C. Comparison of multiple Amber force fields and development of improved protein backbone parameters. *Proteins*. **2006**, *65*, 712 – 725, DOI 10.1002/prot.21123.

(35) Bayly, C. I.; Cieplak, P.; Cornell, W.; Kollman, P. A. A well-behaved electrostatic potential based method using charge restraints for deriving atomic charges: the RESP model. *J. Phys. Chem.* **1993**, *97* (40), 10269 – 10280, DOI 10.1021/j100142a004.

(36) Gaussian 09, Revision A.02, Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; *et al.,* Gaussian, Inc., Wallingford CT, **2009**.

(37) Wang, J.; Wolf, R. M.; Caldwell, J.; Kollman, P. A.; Case, D. A. Development and testing of a general amber force field. *J. Comput. Chem.* **2004**, *25*, 1157 – 1174, DOI 10.1002/jcc.20035.

(38) Campetella, M.; Bodo, E.; Caminiti, R.; Martino, A.; D'Apuzzo, F.; Lupi, S.; Gontrani, L. Interaction and dynamics of ionic liquids based on choline and amino acid anions. *J. Chem. Phys.* **2015**, *142* (23), 234502, DOI 10.1063/1.4922442.

(39) Ventura, S. P. M.; Silva, F. A.; Quental, M. V.; Mondal, D.; Freire, M. G.; Coutinho, J. A. P. Ionic-liquid-mediated extraction and separation processes for bioactive compounds: past, present, and future trends. *Chem. Rev.* **2017**, *117* (10), 6984 – 7052, DOI 10.1021/acs.chemrev.6b00550.

(40) Swadling, J. B.; Ishii, K.; Tahara, T.; Kitao, A. Origins of biological function in DNA and RNA hairpin loop motifs from replica exchange molecular dynamics simulation. *Phys. Chem. Chem. Phys.* **2018**, *20*, 2990 – 3001, DOI 10.1039/c7cp06355e.

(41) Satpathi, S.; Sengupta, A.; Hridya, V. M.; Gavvala, K.; Koninti, R. K.; Roy, B.; Hazra, P. A green solvent induced DNA package. *Sci. Rep.* **2015**, *5*, 9137, DOI 10.1038/srep09137.

(42) Priyakumar, U. D.; Hyeon, C.; Thirumalai, D.; Mackerell Jr., A. D. Urea destabilizes RNA by forming stacking interactions and multiple hydrogen bonds with nucleic acid bases. *J. Am. Chem. Soc.* **2009**, *131*, 17759 – 17761, DOI 10.1021/ja905795v.

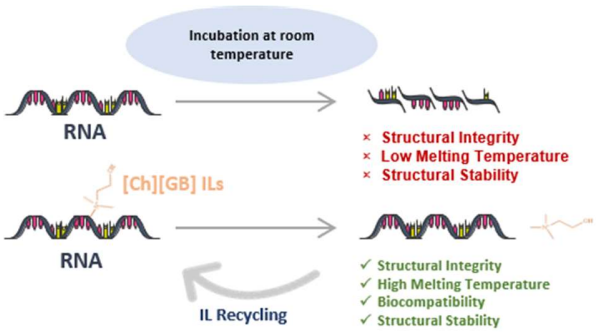
(43) Del Vecchio, P.; Esposito, D.; Ricchi, L.; Barone, G. The effects of polyols on the thermal stability of calf thymus DNA. *Int. J. Biol. Macromol.* **1999**, *24*, 361 – 369, DOI 10.1016/S0141-8130(99)00058-6.

(44) Ventura, S. P.; Silva, F. A.; Gonçalves, A. M.; Pereira, J. L.; Gonçalves, F.; Coutinho, J. A. Ecotoxicity analysis of cholinium-based ionic liquids to *Vibrio fischeri* marine bacteria. *Ecotoxicol. Environ. Saf.* **2014**, *102*, 48 – 54, DOI 10.1016/j.ecoenv.2014.01.003.

(45) Stepnowski, P.; Skladanowski, A. C.; Ludwiczak, A.; Laczynska, E. Evaluating the cytotoxicity of ionic liquids using human cell line HeLa. *Hum. Exp. Toxicol.* **2004**, *23*, 513 – 517, DOI 10.1191/0960327104ht480oa.

(46) Weaver, K. D.; Kim, H. J.; Sun, J.; MacFarlane, D. R.; Elliott, G. D. Cyto-toxicity and biocompatibility of a family of choline phosphate ionic liquids designed for pharmaceutical applications. *Green. Chem.* **2010**, *12*, 507 – 513, DOI 10.1039/B918726J.

TOC graphic:



SYNOPSIS: Aqueous solutions of cholinium-based ionic liquids comprising Good's buffers anions are improved recyclable solvents for the stabilization of recombinant RNAs.