Supporting Information

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

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Experimental section

ILs synthesis. GB-ILs were synthesized through the neutralization of cholinium hydroxide with Good's buffers, as described elsewhere.[1] Briefly, a slight excess of equimolar buffer aqueous solution was added drop-wise to the cholinium hydroxide solution. The mixture was stirred continuously for at least 12 h at ambient conditions. The mixture was then evaporated at 60 °C under reduced pressure, yielding viscous liquids. A mixture of acetonitrile and methanol (1:1, v/v) was added and then vigorously stirred at room temperature for 1 h to precipitate any excess of buffer. The solutions were filtrated to remove the precipitated solids, and further dried under vacuum (10 Pa) at room temperature.

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. Growth was carried out in shake-flasks of 1 L, containing 250 mL 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) supplemented with 30 µg/mL of kanamycin, in a rotary shaker at 37 °C and 250 rpm. Cell growth was evaluated by optical density of the culture medium, carried out at a wavelength of 600 nm. The cultivated cells of *E. coli* DH5α were then recovered by centrifugation at 4500 g for 20 min at 4 °C, and the pellets stored at -20 °C. The isolation of sRNA was carried out according to the acid guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and co-workers[2], with some modifications. Bacterial pellets were resuspended in 5 mL of denaturing cell lysis solutions (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 4.0; 0.5% (w/v) N-laurosylsarcosine and 0.1 M β-mercaptoethanol) to perform the lysis. After incubating on

ice for 10 min, cellular debris, genomic DNA and proteins were precipitated by gently adding and 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 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, 260 and 280 nm absorbance of the samples was measured using a Nanodrop spectrophotometer to assess the RNA quantity, and an agarose gel electrophoresis was performed to assess the RNA purity.

Electrophoretic analysis. 20 µL of sRNA corresponding to 48 µg was analyzed by horizontal electrophoresis using 0.8% of agarose gel (Hoefer, San Francisco, CA, USA). Agarose electrophoresis was 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). 10 µL corresponding to 24 µg of sRNA was also analyzed by vertical electrophoresis using an Amersham Biosciences system (GE Healthcare, Uppsala, Sweden) with 10% polyacrylamide gel. Polyacrylamide electrophoresis was carried out at 125 V for 90 min with TBE buffer (0.84 M Tris base, 0.89 M boric acid and 0.01 M EDTA, pH 8.3). sRNA samples were previously denatured with 97.5% formamide, and denatured conditions were kept in the gel owing to the presence of 8 M urea. In both electrophoresis, the bands corresponding to sRNA molecules were visualized in the gel using the UVItec FireReader system (UVItec,

Cambridge, UK) after staining with 0.01% GreenSafe Premium (NZYtech, Lisbon, Portugal).

Cell viability. The cellular viability of the different IL-RNA formulations was assessed by the MTS assay using the Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, USA), 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 passages of each cell line were seeded at a density of 1×10^4 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, 10 µL of a mixture containing 200 µg/mL sRNA and 20% (w/w) or 50% (w/w) IL was used to transfect each well, corresponding to a final concentration of 20 µg/mL sRNA and 2% (w/w) or 5% (w/w). Transfection was carried out for 24 h, and subsequently 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, California, USA) at 490 nm. Moreover, cells incubated with absolute ethanol were used as positive control for cytotoxicity, while untreated cells were used as negative control. The values given correspond to the average percentage values relative to the untreated cells and standard error in 3 independent experiments (ANOVA, average ± SD).

Figures/Tables

Table S1. pH values (25 °C) of aqueous solutions of 20 and 50 % (w/w) of the ionic liquids used in this study.

Ionic Liquid	Ionic liquid concentration	
	20% (w/w)	**50% (w/w)**
[Ch][HEPES]	9.26 ± 0.09	9.53 ± 0.03
[Ch][MES]	11.29 ± 0.10	12.38 ± 0.03
[Ch][Tricine]	9.76 ± 0.03	10.13 ± 0.03
[Ch][TES]	8.62 ± 0.02	8.92 ± 0.04
Non-Buffered [Ch][DHP]	3.54 ± 0.04	4.17 ± 0.09
Buffered [Ch][DHP]	7.00 ± 0.04	7.10 ± 0.06

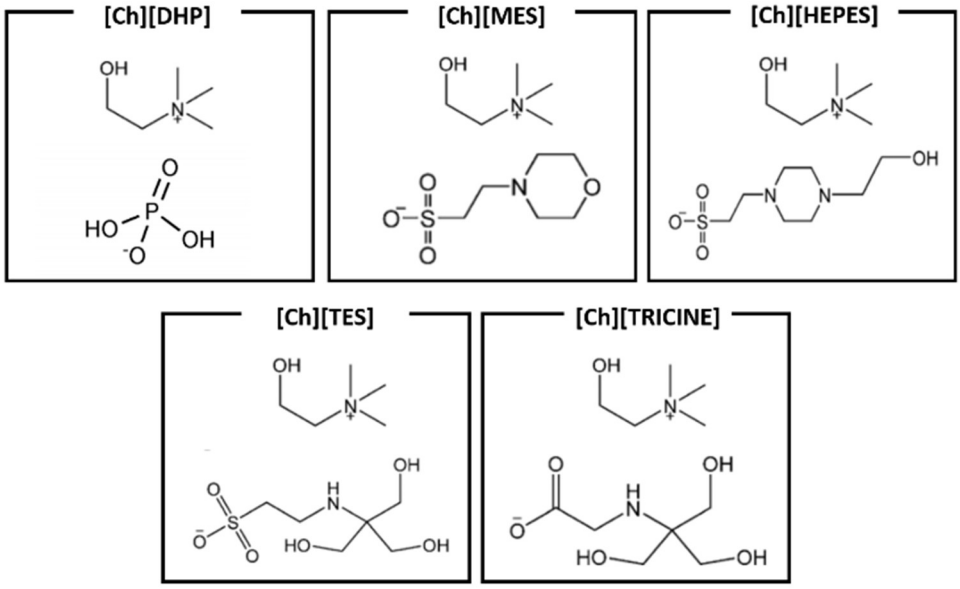

Figure S1. Chemical structure of GB-ILs.

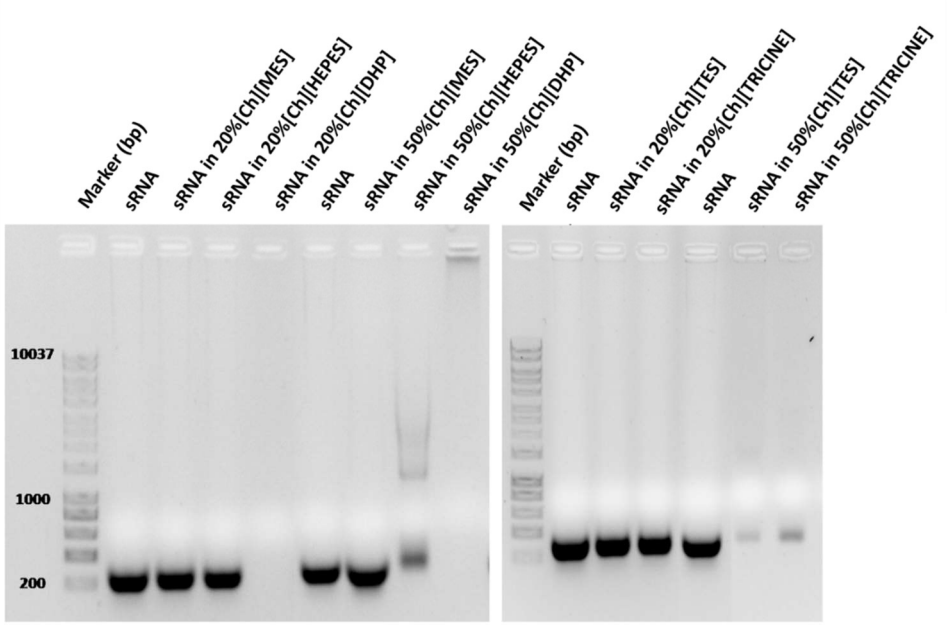

Figure S2. Agarose gel electrophoresis analysis of the structural integrity of a sRNA fraction from *E. coli* DH5α obtained by the phenol-chloroform method in presence of aqueous solutions of 20 and 50% (w/w) of the following ILs: [Ch][DHP], [Ch][MES], [Ch][HEPES], [Ch][TES], and [Ch][TRICINE]. RNA control samples without ILs are also included.

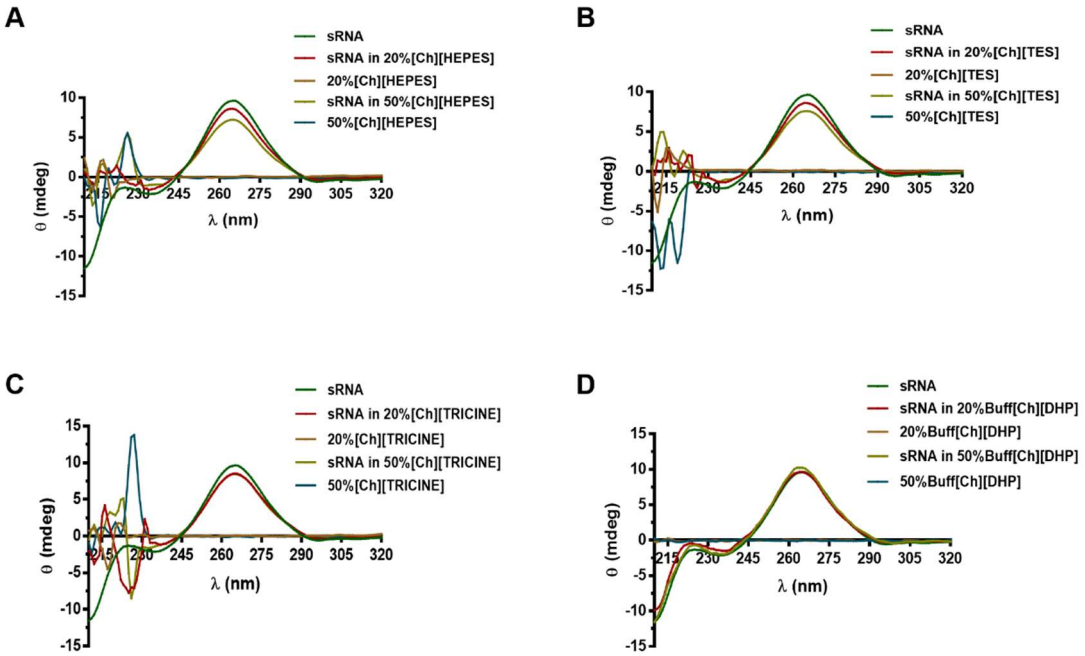

Figure S3. CD spectra (210 – 320 nm) of a sRNA fraction from *E. coli* DH5α in the absence and presence of ILs: A) 20 and 50% (w/w) of [Ch][HEPES]; B) 20 and 50% (w/w) of [Ch][TES]; C) 20 and 50% (w/w) [Ch][TRICINE]; D) 20 and 50% (w/w) Buff[Ch][DHP]. For each set, the green line represents RNA, the red line RNA in 20% (w/w) IL, the brown line 20% (w/w) IL, the light green line RNA in 50% (w/w) IL, and the blue line 50% (w/w) IL. Total RNA concentration was 150 µg/mL. sRNA was incubated with ILs for 1 h at 4 °C.

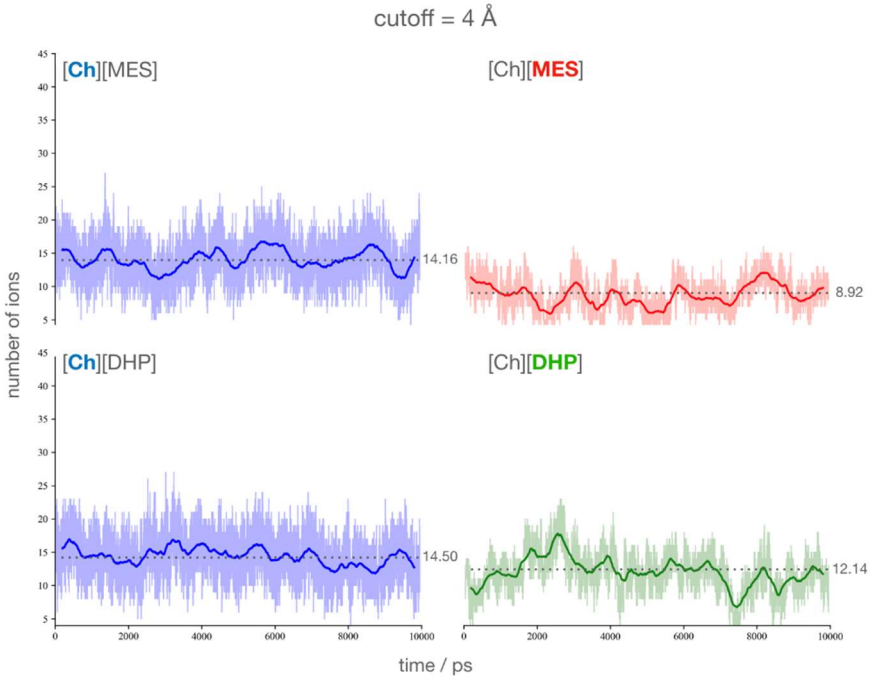

Figure S4. Time evolution of the number of contacts below 4 Å between the representative atom of the IL component (highlighted in bold) and the surface (represented by any atom of the chain) of the sRNA chain, at an IL concentration of 20% (w/w) in water. The representative atom of each IL was taken as N for $[Ch]^+$, S for $[MES]^-$ and P for $[DHP]^-$. The faded curves correspond to data points collect every 0.1 ps, whereas the bold line corresponds to the associated running average obtained using a sliding window of 0.01 ps; the average contact number is depicted by the horizontal dashed line. The number of $[Ch]^+$ cations (approx. 14) surrounding the sRNA chain is similar in both [Ch][MES] and [Ch][DHP]; however, the number of $[DHP]^-$ anions (approx. 12) in direct contact with the RNA chain is *ca.* 33% higher than the number of [MES]- anions (approx. 9) under similar conditions.

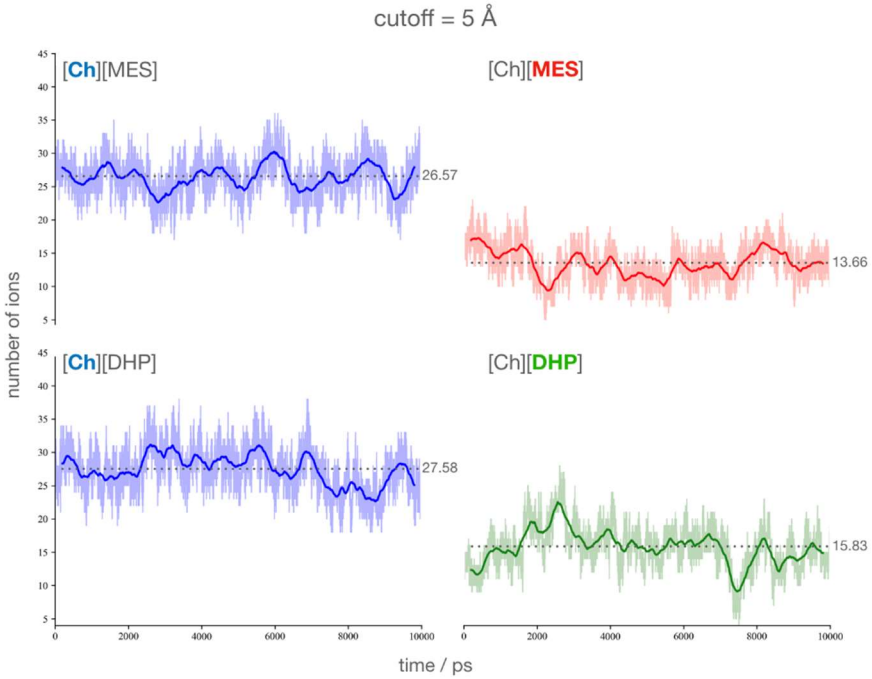

Figure S5. Time evolution of the number of contacts below 5 Å between the representative atom of the IL component (highlighted in bold) and the surface (represented by any atom of the chain) of the sRNA chain, at an IL concentration of 20% (w/w) in water. Remaining details as in Supplementary Figure 3. At a cutoff distance of 5 Å, the number of $[DHP]^-$ anions (*ca.* 16) surrounding the sRNA chain remains higher than the equivalent number of $[MES]^-$ anions (*ca.* 14).

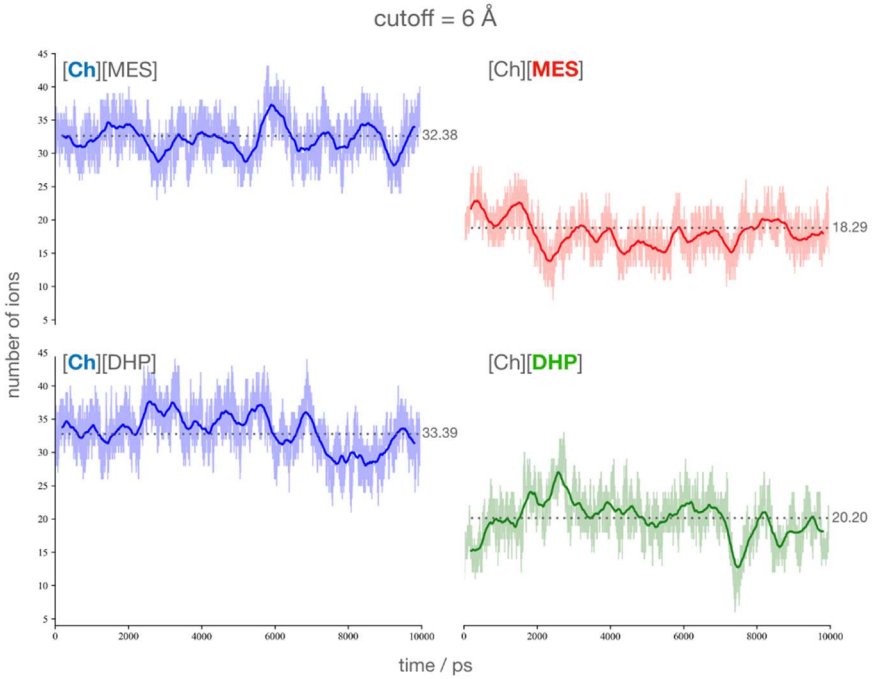

Figure S6. Time evolution of the number of contacts below 6 Å between the representative atom of the IL component (highlighted in bold) and the surface (represented by any atom of the chain) of the sRNA chain, at an IL concentration of 20% (w/w) in water. Remaining details as in Figure S3.

References

1. 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.

2. 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.