



## Improved ionic-liquid-functionalized macroporous supports able to purify nucleic acids in one step



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### ABSTRACT

Nucleic acids are relevant biopolymers in therapy and diagnosis, for which their purity and biological activity are of crucial relevance. However, these features are difficult to achieve by cost-effective methods. Herein, we report the functionalization of a macroporous chromatographic support functionalized with an ionic liquid (IL) with remarkable performance to purify nucleic acids. An initial screening with distinct IL chemical structures supported in silica was carried out, allowing to identify the IL 1-methyl-3-propylimidazolium chloride as the most promising ligand. A chromatographic macroporous matrix able to be used in preparative liquid chromatography was then functionalized and binding/elution studies were performed. The IL 1-methyl-3-propylimidazolium chloride acts as a multimodal ligand with a remarkable dynamic binding capacity. This macroporous support allows the (one-step) purification of nucleic acids, namely small RNAs, ribosomal RNA, and genomic DNA, from a bacterial lysate, and can be regenerated and reused without compromising its separation performance.

### 1. Introduction

Nucleic acids play relevant roles in the fields of biochemistry, biology, and medicine, and as biological materials they manage the information of life for life [1,2]. For many decades, RNA was thought to behave as a mere intermediate between DNA and proteins [3]. However, this paradigm has changed since the discoveries on the RNA biological functions [3], headed by the RNA interference mechanism proposed by Fire and coworkers [4,5]. Currently, RNA is regarded as a dynamic and versatile biopolymer, regulating the expression of genes that are involved in numerous cellular processes [6,7]. This development in basic RNA biology has spawned a revolution in RNA-based strategies to generate new therapeutics [8], often requiring the development of appropriate drug delivery systems to reinforce their efficacy for cancer [9] or neurodegenerative diseases [10]. Regardless of the application, e.g., studies on the structure and function of RNAs, in diagnosis or as therapeutics, high quantities of RNA with adequate integrity, purity, and biological activity are always required [11]. In addition to RNA, functional biodevices employing DNA have been constructed, either

integrated into spider silk nanohydrogels for the bioselective binding of proteins [12] or as DNA-based nanotechnology architectures, in sensors, photonics, or interfaces between technical systems [13].

Therefore, and independently of the RNAs sources (*in vitro* transcription, chemical synthesis, or recombinant production), there is a crucial need on cost-effective nucleic acids purification protocols to remove process-associated impurities [3]. Furthermore, if these methodologies allow the simultaneous separation of distinct biomolecules from the same cellular matrix, tissue heterogeneity issues will be avoided, and systems biology approaches relying on a coordinated analysis of the genome, transcriptome, and proteome will be highly improved [14]. In addition to its range of inherent biological activities, molecular simplicity and ease of modification have also accelerated the progress of RNA nanotechnology.

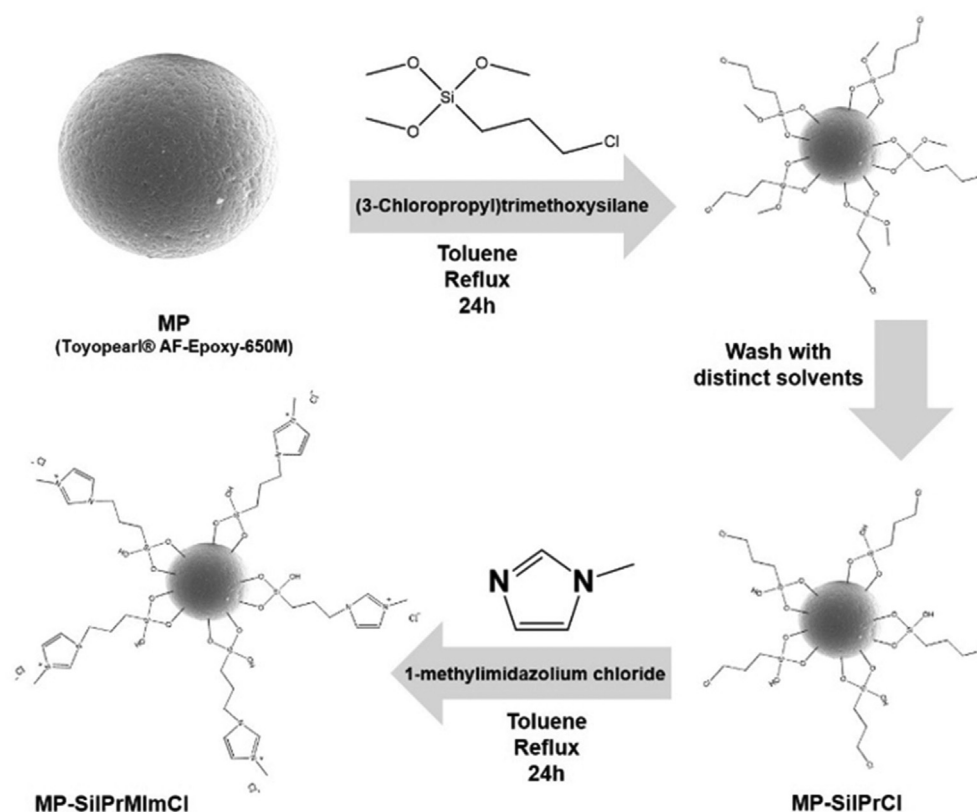
The commonly used methods for nucleic acids purification include denaturing polyacrylamide gel electrophoresis and chromatographic strategies (such as ion-exchange, ion-pairing, reversed-phase, and gel filtration), which are laborious and time-consuming, and still result in RNA and DNA samples with low yields and purity levels [1,15,16]. Affinity chromatography with amino acids as immobilized ligands,

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**Fig. 1.** Schematic representation of the preparation of the IL-functionalized macroporous support (MP). The MP was functionalized with 3-(chloropropyl)trimethoxysilane and 1-methylimidazole, generating the macroporous support with the 1-methyl-3-propylimidazolium chloride IL as ligand (MP-SiPrMImCl).

including histidine or arginine, allows overcoming some of these drawbacks for RNA [6]. Yet, the search for chromatographic ligands for nucleic acids purification endowed of superior selectivity, robustness, and reproducibility is still in high demand [3], for which ionic liquids (ILs) could be investigated as alternative and effective ligands.

ILs are salts, usually described as liquid salts below 100 °C, which can be tailored in terms of their properties, such as polarity, density, viscosity, conductivity, among others [17,18]. ILs have been used in liquid chromatography in distinct ways [19,20]: as additives of mobile phases [21], as ligands of stationary phases [22,23], or acting themselves as stationary phases [24]. In most of these works, it was demonstrated the high performance of ILs achieved by the effective design of their chemical structures. Moreover, both non-magnetic ILs [25–27] and magnetic ILs [28–30] can stabilize the conformational structures of nucleic acids, being therefore projected as proper solvents and preservation media.

Herein we report on the preparation and characterization of a novel multimodal chromatographic matrix, namely a macroporous resin modified with the IL 1-methyl-3-propylimidazolium chloride (MP-SiPrMImCl), and on its remarkable ability to effectively separate nucleic acids from bacterial lysates in one step. According to the chemical structure of 1-methyl-3-propylimidazolium chloride, encompassing an aromatic imidazolium cation and positive charge centers, it is expected the establishment of hydrophobic and electrostatic interactions (among others) with nucleic acids, which besides the negative charge conferred by phosphate groups, also contain aromatic moieties in the nitrogenous bases. Accordingly, the effect of different salts and binding/elution strategies on RNA binding onto the modified supports was investigated to address these possible types of interactions.

## 2. Materials and methods

### 2.1. Materials

All chemicals used in sample processing were acquired from Sigma-Aldrich (St Louis, MO, USA), except for  $\beta$ -mercaptoethanol that was obtained from Merck (Whitehouse Station, USA). Methanol (HPLC grade, purity >99.9%) and toluene (HPLC grade, purity >99.7%) were supplied from Fischer Chemical (Thermo Fisher Scientific, Waltham, USA). Ammonium sulfate  $[(\text{NH}_4)_2\text{SO}_4]$  and sodium chloride (NaCl) were used in chromatography and supplied by Panreac Quimica SLU (Castellar del Vallès, Spain), while tris(hydroxymethyl) aminomethane (Tris) was from Merck (Darmstadt, Germany). 1-Methylimidazole (MIm) was purchased from IoLITEC (Heilbronn, Germany), while (3-chloropropyl)trimethoxysilane was acquired from Acros Organics (Thermo Fisher Scientific, Geel, Belgium). The macroporous matrix Toyopearl® AF-Epoxy-650M was bought from Tosoh Biosciences (GmbH, Greisheim, Germany). Disposable polypropylene columns (Econo-Pac®) were acquired from BioRad (California, USA). All buffers used in chromatographic experiments were freshly prepared with ultra-pure grade deionized water purified in Milli-Q system from Millipore (Billerica, MA, USA) treated with 0.05% diethylpyrocarbonate (DEPC) from Sigma-Aldrich (St. Louis, MO, USA). Buffers were filtered through a 0.20  $\mu\text{m}$  pore size membrane (Whatman, Dassel, Germany) and degassed ultrasonically before use. All materials used in the experimental assays were RNase-free. GreenSafe Premium stain for nucleic acid gel electrophoresis and NZYtech Maxi Prep Kit were purchased from NZYTech (Lisbon, Portugal).

## 2.2. Preparation of IL-functionalized supports

The MP chromatographic matrix with the IL 1-methyl-3-propylimidazolium chloride as the chromatographic ligand was prepared via a two-step procedure, according to Fig. 1: (i) Covalent attachment of (3-chloropropyl)trimethoxysilane to commercial MP. A MP 5 g suspension was prepared in 60 mL toluene, and 5 mL of (3-chloropropyl)trimethoxysilane were added. This suspension was kept in reflux during 24 h under mechanical agitation (550 rpm), and then cooled down to room temperature, filtered and washed with distinct solvents, namely toluene, ethanol-water (1:1), distilled water, and methanol. The matrix was vacuum-dried at 60 °C for 24 h, thereby yielding the matrix MP with the spacer arm (MP-SilPrCl); and (ii) Reaction of MP-SilPrCl with 1-methylimidazole (MIm) to obtain the MP matrix functionalized with the IL 3-methyl-1-propylimidazolium chloride. Five grams of MP-SilPrCl was suspended in 60 mL toluene, and 5 mL of MIm was added. The suspension was refluxed during 24 h at 550 rpm, and further recovered by filtration and washed with ethanol, distilled water, and methanol. The functionalized chromatographic matrix—MP-SilPrMImCl—was finally dried at 60 °C. Similar procedures (detailed in the Supplementary Data) were applied to other functionalized supports with other IL ligands. These supports were used in the initial screening and their chemical structures are shown in the Supplementary Data.

The MP chromatographic matrix Toyopearl® AF-Epoxy-650M with 1-methylimidazole as the chromatographic ligand was obtained by mixing MP with MIm, mainly to address the performance of a macroporous support without the spacer arm. Five grams of MP was suspended in 60 mL toluene and 5 mL of MIm was added. The suspension was refluxed during 24 h at 550 rpm, and the functionalized support was recovered by filtration and washed with methanol, distilled water, and methanol. The support MP-MIm was finally dried at 60 °C.

## 2.3. Characterization of ionic-liquid-functionalized supports

The IL-modified supports were thoroughly characterized using distinct techniques to verify if the functionalization process induces any morphological changes and to confirm the successful immobilization of the IL onto the support.

### 2.3.1. <sup>13</sup>C nuclear magnetic resonance (NMR) spectroscopy

The analysis of the prepared materials by solid-state <sup>13</sup>C NMR was performed at room temperature using a Bruker Avance III - 400 MHz spectrometer (DSX model) with 4 mm BL cross-polarization magic angle spinning (CPMAS) VTN probes at 100.6 MHz. A sample of 100 mg was placed in a ZrO<sub>2</sub> rotator of 4 mm diameter. In order to increase the signal-to-noise ratio of the solid-state spectra, the used CPMAS NMR <sup>13</sup>C settings were the following:  $V_1^{13}C = 55$  kHz; recycle delay, 4 s; contact time, 1–2 ms; NS = 1 k;  $V^R = 12$  kHz. SPINAL-64 decoupling was used during data acquisition and Bruker Topspin 3.2 applied for spectra processing.

### 2.3.2. Fourier Transform Infrared (FTIR) spectroscopy

Fourier Transform Infrared spectroscopy (FTIR) was applied to the MP, MP-SilPrCl, and MP-SilPrMImCl samples. The data were acquired with a spectral width ranging from 4000 to 500 cm<sup>-1</sup> and a spectral resolution of 1 cm<sup>-1</sup>. The spectra were recorded on a Perkin Elmer FTIR System Spectrum BX equipped with a horizontal Golden Gate attenuated reflectance (ATR) cell.

### 2.3.3. Elemental analysis

The content in carbon, hydrogen, and nitrogen (in weight percentage) was determined using 2 mg of the prepared chromatographic supports using a TruSpec 630-200-200. The combustion furnace temperature was set up at 1075 °C, and the afterburner temperature was 850 °C. Carbon and hydrogen were detected by infrared absorption, while thermal conductivity was applied for nitrogen.

### 2.3.4. Scanning electron microscopy

Scanning electron microscopy (SEM) was carried out to ascertain the effect of the immobilization process in the macroporous matrix morphology. The samples were evaluated, before and after the immobilization process, using a Hitachi S-3400 N (Tokyo, Japan), operating at an accelerating voltage of 20 kV, at variable magnifications. The samples were fixed on a brass stub using double-sided tape and then made electrically conductive by coating with gold using a Quorum Q150R ES sputter coater.

### 2.3.5. Zeta potential analysis

The zeta potential of the chromatographic supports was determined as a function of pH, using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) equipped with an autotitrator (Malvern Instruments Ltd, UK). This enables measurement of electrophoretic mobility, and indirectly of the zeta potential, based on laser Doppler velocimetry. The electrokinetic potential was determined in the presence of HCl or NaOH over the whole considered pH range (2–10), and the electrokinetic curves were plotted. The zeta potential was calculated from the Smoluchowski equation. The apparatus measures 30 times single zeta potential at defined pH, providing an average value.

## 2.4. Biological sample

The biological samples used in this study were obtained from a cell culture of *E. coli* DH5 $\alpha$ . Cell growth was carried out in shake flasks at 37 °C and 250 rpm containing 250 mL of Terrific Broth medium (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH<sub>2</sub>PO<sub>4</sub>, 0.072M K<sub>2</sub>HPO<sub>4</sub>). The bacterial growth was suspended at different time-points: after 2 h of fermentation (OD<sub>600</sub>  $\pm$  2) to obtain a nucleic acids sample enriched in genomic DNA, small RNAs, and ribosomal RNAs; and on the early exponential phase (OD<sub>600</sub>  $\pm$  6), for samples mainly composed of genomic DNA and small RNAs. Cells were recovered by centrifugation (4000 g, 10 min, 4 °C), and the bacterial pellets were stored at -20 °C. The biological samples were extracted using the acid guanidinium thiocyanate-phenol-chloroform method based on the protocol described by Chomczynski [31].

To obtain small RNA sample, the bacterial cells were lysed using denaturing cell lysis solution (4 M guanidine thiocyanate; 25 mM sodium citrate, pH 7; 0.5% N-lauroylsarcosine and 0.1 M  $\beta$ -mercaptoethanol) and the small RNA fraction obtained was precipitated with ice-cold isopropanol. Precipitated molecules were recovered by centrifugation at 10,000 g, for 20 min, at 4 °C. After centrifuging, the small RNA pellets were washed with 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 small RNA pellets were solubilized in 1 mL of 0.05% DEPC-treated water. To evaluate the quantity and purity of the extracted RNA, spectrophotometry analysis (nanophotometer) was conducted to measure the absorbance of the samples at the wavelengths of 260 and 280 nm. The reading at 260 nm allows calculation of the concentration of RNA in the sample, as an optical density of one corresponds to approximately 40  $\mu$ g/mL of single-stranded RNA. The ratio of the absorbance at 260 nm and 280 nm ( $A_{260}/A_{280}$ ) will provide an estimation of the RNA purity. Pure preparations of RNA have an  $A_{260}/A_{280}$  ratio of between 1.8 and 2.0. If there is some contamination with proteins or phenol, the  $A_{260}/A_{280}$  ratio will be much lower, hampering the accurate quantification of RNA [32].

To obtain lysate sample, the bacterial pellets were resuspended using denatured cell lysis solution and were then incubated on ice for 10 min. After incubation, the suspension was centrifuged at 19,000 g and 4 °C, for 30 min at 4 °C, and the soluble nucleic acids (genomic DNA, small RNAs, and ribosomal RNAs) present in the supernatant were concentrated by adding 5 mL of ice-cold isopropanol [33]. After centrifugation (16,000 g for 20 min at 4 °C), the resulting pellets were washed with 75% ethanol and incubated at room temperature for 10 min. After new centrifugation

(16,000 g for 5 min at 4 °C), the air-dried pellets were dissolved in 2 mL of 0.05% DEPC-treated water and incubated for 10 min at 60 °C to ensure complete solubilization [33].

The pcDNA3-FLAG-p53 plasmid (Plasmid 10,838, Addgene, Cambridge, USA) was amplified from a cell culture of *E. coli* DH5 $\alpha$  grown at 37 °C in an Erlenmeyer containing 250 mL of Terrific Broth medium supplemented with 30  $\mu$ g/mL ampicillin. The bacteria growth was suspended in the advanced log phase (OD<sub>600</sub>  $\approx$  9), the cells recovered by centrifugation, and native plasmid DNA (supercoiled + open circular) was isolated with the NZYTech Maxi Prep Kit, according the manufacturer's instructions.

## 2.5. Binding/elution studies of small RNA

To ascertain the role of 1-methyl-3-propylimidazolium chloride in the retention of small RNA, and therefore its usefulness as a chromatographic ligand, the initial chromatographic experiments were performed using disposable polypropylene columns that were packed with 1 mL of hydrated MP, MP-MIm, or MP-SilPrMImCl. To preferentially explore electrostatic interactions, the following procedure was performed: (i) equilibration with 5 CV (column volume) of 10 mM Tris-HCl (pH 8); (ii) injection of a small RNA sample (see details on small RNA isolation procedure from *Escherichia coli* DH5 $\alpha$  cultivations) in the same equilibrium buffer; (iii) elution of retained nucleic acids by an increase in the ionic strength with 5 CV of 1 M NaCl (in 10 mM Tris-HCl, pH 8); (iv) measurement of absorbance at 260 nm. On the other hand, to preferentially explore hydrophobic interactions, a similar procedure was adopted with the following changes: 2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (in 10 mM Tris-HCl, pH 8) was applied as the equilibrium buffer, and the elution of retained species was carried out with Tris-HCl 10 mM, pH 8.

## 2.6. Automated column liquid chromatography

The chromatographic experiments were performed in an ÄKTA Avant system with UNICORN 6 software (GE Healthcare, Sweden). A 10 mm diameter x 20 mm long (about 2 mL) column was packed with MP-SilPrMImCl. The temperature was set up at 20 °C using a water circulating bath, and a flowrate of 1 mL/min was used. Foreseeing the separation of genomic DNA from small RNA, the support was equilibrated with 0.38 M NaCl (in 10 mM Tris-HCl, pH 8). The bacterial lysate sample containing genomic DNA and small RNA (see bacterial lysate preparation from *E. coli* DH5 $\alpha$  cultivations for further details [2.4 Biological sample](#)) with approximately 30  $\mu$ g was injected using a 100  $\mu$ L loop. After washing out the unbound species, the ionic strength of the buffer was stepwise increased to 1 M NaCl (in 10 mM Tris-HCl, pH 8). For the separation of genomic DNA, small RNA, and ribosomal RNA, the support was equilibrated with 0.38 M NaCl, and 30  $\mu$ g of a nucleic acids sample was then injected. After the elution of unbound species under this condition, the concentration of NaCl (in 10 mM Tris-HCl, pH 8) was increased to 0.41 M and then to 0.50M in a stepwise gradient mode to elute the most retained nucleic acids species. For both strategies, the absorbance of the eluate was continuously monitored at 260 nm. Fractions were pooled according to the chromatograms obtained and following concentration and desalting with Vivaspin® concentrators (10,000 MWCO, Sartorius, Gottingen, Germany), the samples were kept for quantification and further analysis.

## 2.7. Quality control analysis of nucleic acids

The integrity, identification, and purity of nucleic acids eluting species from chromatographic experiments were evaluated by horizontal electrophoresis using 1% agarose gels. Electrophoresis was carried out at 110 V, for 30 min, with TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8) in DEPC-treated water. The bands corresponding to nucleic acid molecules were visualized in the gel using the UVItc Fire-Reader system after staining with greenSafe Premium.

Protein residual contamination in the nucleic acid samples collected was measured using the micro-BCA (micro-bicinchoninic) assay. A calibration curve was prepared using Bovine serum albumin (BSA) (0.01–0.25 mg/mL). A total of 25  $\mu$ L of each standard or nucleic acids samples were added to 200  $\mu$ L of BCA reagent in a microplate and incubated for 30 min at 60 °C. The absorbance was measured at 570 nm in a microplate reader.

The levels of genomic DNA were evaluated using the samples pooled from chromatographic experiments in a CFX Connect™ Real-Time PCR Detection System using the Maxima® SYBR Green/Fluorescein qPCR Master Mix by real-time qPCR. Selective amplification of a 181 bp DNA-fragment was performed using specific primer oligonucleotides for 16S rRNA (5'-ACACGGTCCAGAACTCCTACG-3' and 5'-CCGGTGCTTCTTGCGGGTAACGTCA-3', respectively, for forward and reverse primers), and according to the following conditions: denaturation at 95 °C for 3 min, 40 cycles consisting of an initial denaturation at 95 °C for 30 s, annealing at 60 °C during 30 s, and final elongation at 72 °C for 30 s [34]. The amplified PCR fragments were checked by melting curves: reactions were heated from 55 °C to 95 °C with 10 s holds at each temperature (0.05 °C/s). To confirm the presence and purity of amplicons, PCR products were analyzed using 1% agarose gel electrophoresis. *E. coli* DH5 $\alpha$  genomic DNA obtained with Wizard SV genomic DNA purification kit (Promega, Madison, USA) was used to evaluate the qPCR efficiency of 16S primer oligonucleotides, and to construct a standard curve in the concentration range of 0.5–500 ng/ $\mu$ L. Real-time qPCR efficiencies were calculated from the given slopes with MyIQ 2.0 software (Bio, Hercules, CA, USA). The relative quantification of the genomic DNA was calculated by applying the comparative threshold cycle (Ct) method. Each sample was run in triplicate, and Ct values were averaged from the triplicate. The final data are an average of three independent experiments.

Complementary DNA was synthesized from 1  $\mu$ g of rRNA purified by MP-SilPrMImCl and reverse transcribed using the RevertAid First Strand complementary DNA Synthesis Kit (Thermo Fisher Scientific Inc.), according to the manufacturer's instructions. Then, complementary DNA was PCR-amplified using specific primer oligonucleotides for 16S ribosomal RNA (5'-ACACGGTCCAGAACTCCTACG-3' and 5'-CCGGTGCTTCTTGCGGGTAACGTCA-3', respectively, for forward and reverse primers) and according to the following program: an initial denaturation at 95 °C for 5 min, 35 cycles consisting of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 15 s, and a final elongation step of 5 min at 72 °C. Finally, the PCR products were analyzed by agarose gel electrophoresis.

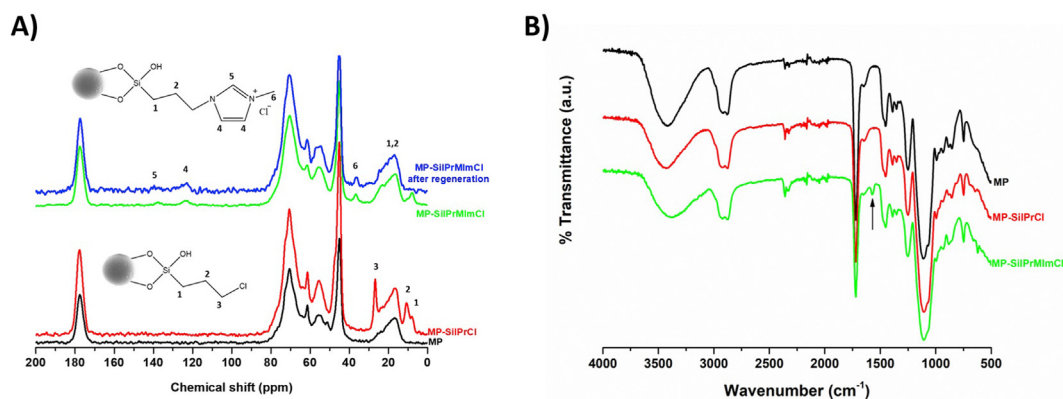
## 2.8. Assessment of dynamic binding capacity (DBC)

The DBC of MP-SilPrMImCl was addressed toward BSA, pcDNA3-FLAG-p53 (Addgene, plasmid 10,838) and recombinant *E. coli* DH5 $\alpha$  small RNA (see sample preparation of small RNA and plasmid DNA), being representative of three major classes of biomolecules present in lysate media. The typical chromatographic process for the determination of DBC was performed with a flow-rate of 1 mL/min and includes: (i) MP-SilPrMImCl equilibrium with 10 mM Tris-HCl, pH 8 to allow the binding of small RNA; (ii) overloading of 50  $\mu$ g/mL small RNA solution; (iii) elution of the retained small RNA through an increase of the NaCl concentration to 1 M (in 10 mM Tris-HCl, pH 8) using a stepwise gradient. MP-SilPrMImCl DBC for BSA and pcDNA3-FLAG-p53 were determined using 2.0 mg/mL and 50.0  $\mu$ g/mL aqueous solutions, respectively. The DBC values (mg/mL) are represented at 10 and 50 of the breakthrough, and correspond to 10% and 50% of maximum capacity.

## 3. Results and discussion

An initial screening with distinct IL chemical structures (viz., 1-methyl-3-propylimidazolium chloride, triethylpropylammonium chloride, butyldimethylpropylammonium chloride, and trioctylpropylammonium chloride; results shown in [Figs. S1 and S2](#) in the





**Fig. 2.** (A) <sup>13</sup>C CP-MAS NMR spectra of MP, MP-SilPrCl, MP-SilPrMImCl, and regenerated MP-SilPrMImCl support. (B) ATR-FTIR spectra of MP, MP-SilPrCl, and MP-SilPrMImCl support.

Supplementary Data) supported in silica was carried out. These materials were prepared according to previously established protocols [35] and in addition to an imidazolium-based IL, other ammonium-based ILs were also studied to better address the effect of non-aromatic groups in the cation and size of the cation alkyl side chain to differently interact and isolate nucleic acids. In all experiments, chloride was selected as the counter-ion due to its lower toxicity, low cost, and easiness in regenerating column (addressed in more detail below). Based on these initial screening assays, the IL 1-methyl-3-propylimidazolium chloride was identified as the most relevant chromatographic ligand given its ability to retain small RNAs.

Accordingly, a new support based on a macroporous resin modified with this IL, and able to be used in preparative liquid chromatography, was prepared. The modification of the macroporous resin consists on the immobilization of 1-methylimidazole (MIm) using 3-(chloropropyl)trimethoxysilane as the spacer arm, and with the IL ligand 1-methyl-3-propylimidazolium chloride. Fig. 1 summarizes the steps required to prepare the chromatographic support with the IL ligand. Aiming to characterize the mechanisms of interaction between nucleic acids and the prepared support, binding/elution studies with small RNAs were initially performed, demonstrating that the immobilized IL behaves as a multimodal ligand. This novel chromatographic macroporous support was then applied in the selective separation of nucleic acids (genomic DNA from small RNAs and from ribosomal RNA) from highly complex bacterial mixtures, leading to remarkable results in terms of DBC, selectivity, robustness, reproducibility, regeneration, and reuse.

### 3.1. Preparation and characterization of IL-functionalized macroporous supports

Previously published works on the modification of materials with ILs have mainly focused on silica materials functionalization [36–38], whose silanol functional groups can be easily modified with ILs (Fig. S1, Supplementary Data). In this work, we describe the modification of a macroporous chromatographic support with ILs, namely with the IL 1-methyl-3-propylimidazolium chloride (PrMImCl), while demonstrating its effectiveness on the purification of three types of nucleic acids (in one step) from complex bacterial lysates. This multimodal macroporous support, MP-SilPrMImCl, was prepared according to the procedure described in Fig. 1, which initially includes the covalent attachment of the spacer arm in the macroporous (MP) matrix, followed by the immobilization of 1-methylimidazole (MIm) (*cf.* details given in the experimental procedure section). To prove the relevance of the IL chemical structure in the retention behavior of distinct nucleic acids, an additional support was prepared, namely MP-MIm. This support was manufactured via the immobilization of MIm directly in the MP matrix, *i.e.*, without the presence of the spacer arm.

Both functionalized supports were characterized by several techniques, including <sup>13</sup>C solid state NMR spectroscopy, elemental analysis, zeta potential measurements, and SEM, in comparison with the original sample (MP). The solid-state <sup>13</sup>C cross-polarization/magic angle spinning NMR (<sup>13</sup>C CP-MAS) spectrum of MP-SilPrMImCl (Fig. 2A) shows chemical shifts in the range between 120–130 and 35–40 ppm, which are not present in the NMR <sup>13</sup>C CP-MAS spectrum of the original MP support. The chemical shifts between 120 and 130 ppm correspond to carbon atoms present in the aromatic imidazolium ring, while the methyl group of the side chain of the imidazolium ring is responsible for the chemical shift at 37 ppm. While these chemical shifts also are detected in the spectrum of MP-MIm support (*cf.* additional information in Fig. S3, Supplementary Data), the peak at 26.7 ppm corresponding to the C bonded to the Cl atom was observed only in the spectrum of MP-SilPrCl, indicating the presence of the silane group.

ATR-FTIR was applied to gather additional evidences on the successful modification of the support with the IL (Fig. 2B). The ATR-FTIR spectra of the MP, MP-SilPrCl, and MP-SilPrMImCl supports are very similar, showing that the chemical integrity of the matrix is maintained. Moreover, an additional band at 1571 cm<sup>-1</sup> is observed in the MP-SilPrMImCl spectrum, being attributed to the stretching vibrations of the imidazole ring skeleton [39,40], which therefore reinforces the success of the immobilization process.

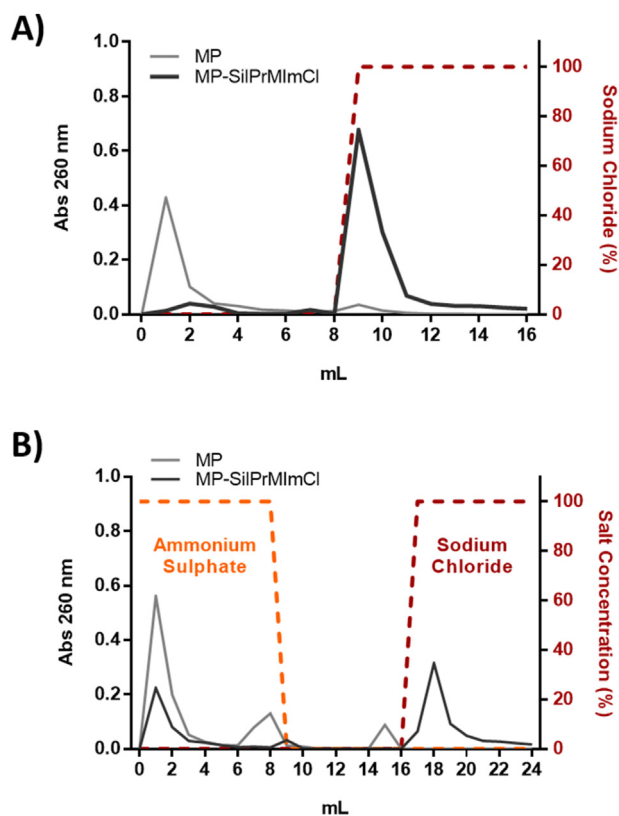
The elemental analysis results given in Table 1 show no significant differences in the carbon and hydrogen content (*wt%*) for both matrices, namely MP and MP-SilPrMImCl supports; however, nitrogen (2.03 *wt%*) was detected in the final modified support, as well as in MP-MIm support, confirming the presence of the imidazolium ring. Based on the nitrogen content provided by elemental analysis, the ligand density [41] of MP-SilPrMImCl (given in Table 1) corresponds to 0.725 mmol of IL per gram of support, a higher value than that present in supports modified

**Table 1**  
Elemental analysis (EA): carbon, hydrogen, and nitrogen content (*wt%*) in MP, MP-MIm, MP-SilPrMImCl, and regenerated MP-SilPrMImCl supports. The ligand densities were calculated from the corresponding nitrogen content.

	% Carbon	% Hydrogen	% Nitrogen	Ligand Density (mmol immobilized MImCl per g of matrix) <sup>a</sup>
MP	53.17	6.63	–	<sup>b</sup>
MP-MIm	48.30	6.27	1.05	0.375
MP-SilPrMImCl	46.27	6.85	2.03	0.725
MP-SilPrMImCl—after regeneration	49.08	6.48	1.75	0.625

<sup>a</sup> Determined by  $Q = ((\% \text{ Nitrogen}) / (1.4 * 2))$ .

<sup>b</sup> Below nitrogen detection limit of the EA apparatus.



**Fig. 3.** Chromatographic profiles of a small RNA sample loaded onto the MP and MP-SilPrMImCl supports. A) increasing the concentration of NaCl from 0 M to 1 M, represented by the red line. B) decreasing the concentration of  $(\text{NH}_4)_2\text{SO}_4$  from 2 M to 0 M, represented by the red line. A second elution step was carried out by an increase in the NaCl concentration up to 1 M, represented in orange. Salt aqueous buffers were prepared in 10 mM Tris-HCl, pH 8.

with common ligands used in the separation of nucleic acids (naphthalene tripodal: 0.32 mmol/g sepharose CL-6B [41], and 3,8-diamino-6-phenylphenanthridine: 0.15 mmol/g derivatized sepharose [42]). Taking all characterization results into consideration, it is shown that the IL is efficiently and covalently bound to the support.

Additional characterization of the prepared IL-modified MP included the determination of their zeta potential values and the evaluation of their morphology by SEM. Zeta potential values were determined by electrophoretic measurements using aqueous suspensions of MP, MP-SilPrMImCl, and regenerated MP-SilPrMImCl at different pH values (adjusted with 0.1 M HCl and 0.1 M NaOH aqueous solutions). From these data, it was determined the point of zero charge (pzc), which is the pH value at which a solid particle in suspension exhibits zero net electrical charge on its surface (Fig. S4, Supplementary Data). MP support has the lowest pzc value (3.4), while the MP-SilPrMImCl and regenerated MP-SilPrMImCl display higher pzc values (8.7). These results indicate that the MP-SilPrMImCl surface is more positively charged when compared to the MP. This change in the pzc from MP to MP-SilPrMImCl is due to the IL binding, further confirming the presence of imidazolium cations in the surface of the MP support and the successful support functionalization.

Given the relevance of cleaning in place and regeneration procedures in preparative liquid chromatography from a sustainable point of view, as well as to remove highly bound species and to avoid fouling of packed chromatography matrices and increased back-pressure [43], a regeneration protocol of the prepared chromatographic support was developed. This regeneration protocol involves consecutive washing steps with aqueous solutions of 0.2 M NaOH, 0.5 M HCl, and distilled water, being essential to keep the levels of the chloride counter-ion in MP-SilPrMImCl

after using the prepared chromatographic macroporous support. This is particularly relevant for separations resulting from an interplay of electrostatic interactions or ion exchange.  $^{13}\text{C}$  CP-MAS NMR spectra and EA of the original and regenerated MP-SilPrMImCl show no relevant differences (Fig. 2 and Table 1). The evaluation of zeta potential for the regenerated support also resulted in equivalent profile and pzc, demonstrating the stability and robustness of the prepared chromatographic support.

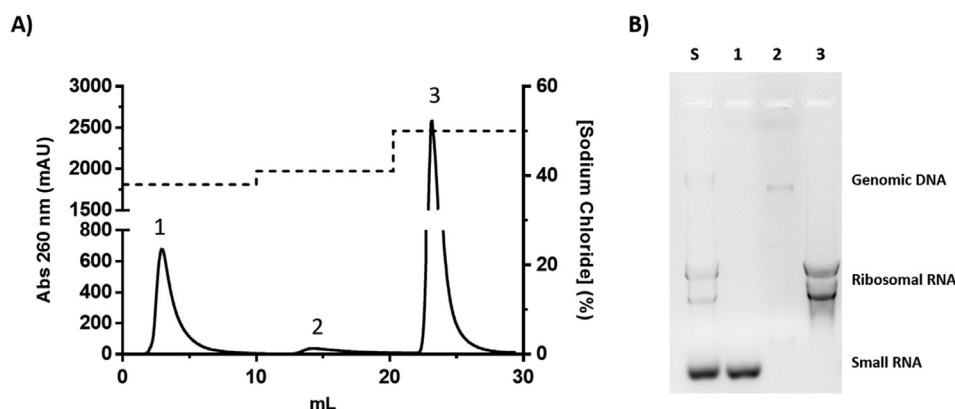
Finally, SEM was used to address the morphology of the prepared materials. Fig. S5 in the Supplementary Data depicts the SEM images of non-functionalized MP support, and the functionalized MP-SilPrMImCl support before/after a regeneration process that includes consecutive washes with NaOH, HCl, and distilled water. The results revealed slight changes on the morphology of some particles, probably resulting from the process of synthesis, but those were not influencing the performance of the chromatographic support regarding the separation efficiency.

### 3.2. Binding-elution studies of recombinant small RNAs

The performance of MP, MP-MIm, and MP-SilPrMImCl to retain a recombinant small RNA sample was addressed. The recombinant small RNA sample was obtained from *E. coli* fermentation, followed by the application of the thiocyanate guanidine lysis method [31]. Polypropylene columns (Econo-Pac®, BioRad, California, USA) were packed with 2 mL of hydrated MP or MP-SilPrMImCl, and preliminary experiments were performed in conditions that promote electrostatic interactions. These conditions include the equilibrium of the chromatographic matrix with 10 mM Tris-HCl (pH 8), injection of small RNA in the same equilibrium buffer, and elution of the retained RNA species through an increase of the ionic strength, using a stepwise gradient with an aqueous solution of 1 M NaCl (in 10 mM Tris-HCl, pH 8). Eluted samples (1 mL) were collected, and the absorbance at 260 nm ( $A_{260}$ ) was measured for each sample to identify and quantify RNA.

According to the chromatograms shown in Fig. 3A, most RNA is retained with the functionalized support MP-SilPrMImCl, and is only eluted with the increase in the ionic strength (as indicated by the null  $A_{260}$  observed in the binding step and the subsequent increase in  $A_{260}$  with 1 M NaCl). It should be noted that the non-functionalized material—MP—has a negligible ability to bind RNA, being immediately eluted in the flowthrough (almost null  $A_{260}$  in the elution step with 1 M NaCl in Tris-HCl 10 mM pH 8). Complementary experiments were performed using MP-MIm samples (given in Fig. S6, Supplementary Data), but the results were similar to those obtained with MP, thus demonstrating that at least in conditions that favor electrostatic interactions MP-MIm is not a suitable material for nucleic acids separation/purification. The uncharged heterocyclic aromatic compound 1-methylimidazole acts as the chromatographic ligand in MP-MIm, whereas the IL 1-methyl-3-propylimidazolium chloride is present in MP-SilPrMImCl. Therefore, the enhanced performance of this type of support to retain nucleic acids in experimental conditions that favor electrostatic interactions is dependent on the presence of a positive charge center, which is only present in the IL immobilized onto MP-SilPrMImCl. Overall, these results prove that the IL is the ligand responsible for the interactions occurring between the novel chromatographic material and RNA, responsible for its high binding capacity as discussed below.

To investigate which non-covalent interactions are mainly responsible for retaining RNA onto MP-SilPrMImCl, the nucleic acid adsorption behavior was studied by applying conditions that favor hydrophobic interactions. The matrices MP and MP-SilPrMImCl were equilibrated with 2 M  $(\text{NH}_4)_2\text{SO}_4$  (in 10 mM Tris-HCl, pH 8), the small RNA sample was injected in the same equilibrium buffer, and the elution of RNA species was promoted by stepwise decreasing the ionic strength using 10 mM Tris-HCl (pH 8). As depicted in Fig. 3B, a decrease in the ionic strength to 10 mM Tris-HCl (pH 8) does not promote the elution of the retained RNA species. This suggests that other interactions rather than hydrophobic interactions are involved. In order to validate this



**Fig. 4.** A) Chromatographic profile corresponding to the separation of ribosomal RNA, small RNA, and genomic DNA from a complex bacterial lysate with the MP-SilPrMimCl support. Elution performed at 1 mL/min using an increasing NaCl concentration gradient (in 10 mM Tris-HCl, pH 8) in a stepwise mode, as represented by the dashed line. B) Agarose gel electrophoresis of the different fractions recovered from the chromatographic assay performed in MP-SilPrMimCl. S—Initial bacterial lysate sample; 1, 2, and 3—Chromatographic peaks 1, 2, and 3, respectively.

hypothesis, a second elution step with an aqueous solution of 1 M NaCl (in 10 mM Tris-HCl, pH 8) was performed. RNA is effectively eluted under these conditions, as shown in Fig. 3B. This chromatographic behavior where more than one form of non-covalent chemical interactions occurs between the stationary phase and the target molecules is typical of multimodal ligands [44], where two main types of interactions, namely hydrophobic and electrostatic interactions, occur between RNA and MP-SilPrMimCl. These interactions are expected given the IL chemical structure, which has an aromatic ring and an aliphatic moiety, and it is positively charged. As nucleic acids are negatively charged due to their phosphate groups and are moderately hydrophobic due to the presence of nitrogen bases, it is likely that both electrostatic and hydrophobic interactions could be explored for their interaction with chromatographic supports and even useful for the separation of different classes of nucleic acids.

Regarding the selection of the best sustainable strategy, i.e., to use conditions that favor hydrophobic or electrostatic interactions, it should be taking into account that ammonium sulfate not only derives from non-renewable sources, as it presents a high eutrophication potential [45]. Even so, it is usually applied in nucleic acids purification processes at high concentrations (usually in the range 2–3 M salt) to allow the binding of nucleic acids in conditions that favor hydrophobic interactions. Therefore, if multimodal ligands are found, it is desirable to separate nucleic acids using conditions that favor electrostatic interactions as these are milder conditions (use of sodium chloride instead of ammonium sulfate) [46]. Accordingly, the separation/purification performance of the developed IL-functionalized chromatographic support was further evaluated under conditions that favor electrostatic interactions.

### 3.3. Separation of nucleic acids from complex bacterial samples

The first set of results discussed above proved that small RNA retention onto MP-SilPrMimCl is due to the presence of the IL ligand. Accordingly, the versatility and wider applicability of this support was evaluated in the purification of nucleic acids from complex matrices, namely from recombinant bacterial lysates. MP-SilPrMimCl was packed in a chromatographic column (GE Healthcare, Uppsala, Sweden) and the experiments were conducted in an AKTA Avant® system under the control of Unicorn 6 software (GE Healthcare, Uppsala, Sweden) at room temperature (ca. 25 °C). The retention profile of a sample containing small RNA and genomic DNA derived from *E. coli* fermentations was initially investigated, which was obtained by literature protocols [33]. After several optimization steps, it was found that the optimized strategy for the separation of small RNA from genomic DNA consists in the equilibrium of the MP-SilPrMimCl matrix with aqueous solutions at 0.38 M NaCl (in 10 mM Tris-HCl, pH 8), followed by a stepwise increase up to 1 M NaCl (in 10 mM Tris-HCl, pH 8). Under these conditions, two chromatographic peaks were obtained (cf. the chromatograms shown in Fig. S7, Supplementary Data). These chromatographic conditions provide

the required selectivity of the IL-based support to separate small RNA from genomic DNA, being the small RNA eluted in the binding step and genomic DNA with aqueous solutions at 1 M NaCl.

The recovery of the small RNA from the IL-functionalized matrix in the binding step is highly advantageous because it elutes with a low salt concentration, as well as with a low residence time within the chromatographic system—two crucial factors for maintaining the RNA stability given its high susceptibility to degradation by ribonucleases. It should be remarked that these factors are not always obeyed in conventional strategies to separate RNA, such as lysine affinity chromatography [47] and agmatine affinity chromatography [48] where small RNA is recovered with aqueous solutions of  $(\text{NH}_4)_2\text{SO}_4$  at 1.5 M and NaCl at 1.75 M, respectively.

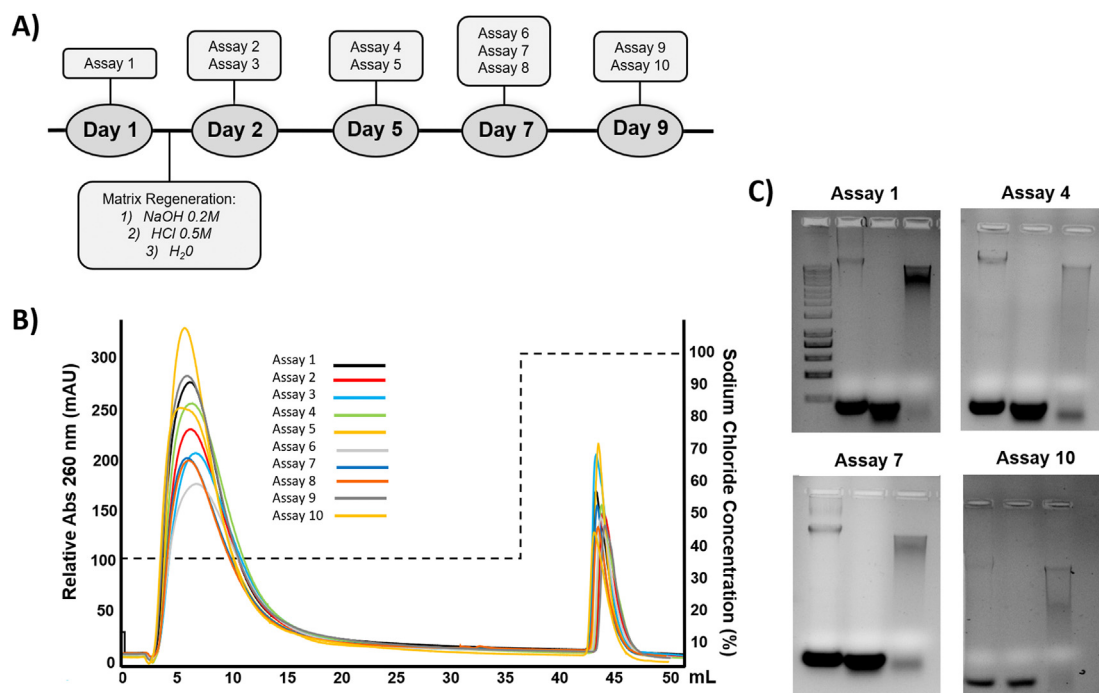
The remarkable performance of MP-SilPrMimCl in the separation of small RNA and genomic DNA prompted us to investigate its application using a more complex sample containing small RNA, genomic DNA, and ribosomal RNA. These experiments were performed using increasing NaCl stepwise gradients: injection of the bacterial lysate sample with 0.38 M NaCl (in 10 mM Tris-HCl, pH 8), followed by two stepwise gradient steps with increasing NaCl concentrations at 0.41 and 0.5 M, as shown in Fig. 4A. The support MP-SilPrMimCl again demonstrated a remarkable selectivity, being able to effectively separate different classes of nucleic acids despite their similar physicochemical properties. The samples corresponding to peaks 1, 2, and 3 were collected, concentrated, then buffer-exchanged to 10 mM Tris-HCl, pH 8, using Vivaspin® concentrators (10,000 MWCO, Sartorius, Gottingen, Germany), and finally analyzed by agarose gel electrophoresis, allowing to conclude that small RNA, genomic DNA, and ribosomal RNA are preferentially eluted, respectively, in peaks 1, 2, and 3, with high purity and high integrity (Fig. 4B).

Additional quality control of the recovered nucleic acids corresponding to each chromatographic peak was performed by analyzing the content of endogenous *E. coli* proteins and genomic DNA. This was performed, respectively, by the micro-BCA method and by qPCR using specific primers for 16S ribosomal RNA gene. The results shown in Table 2 reveal that proteins elute in all peaks; however, the prepared

**Table 2**

Quality control analysis of the purified nucleic acids by MP-SilPrMimCl support. Quantitative analysis of major contaminants—genomic DNA and total protein; Peaks 1, 2, and 3 correspond, respectively, to the fractions recovered with 0.38 M, 0.41 M, and 0.5 M NaCl in 10 mM Tris-HCl, pH 8.0.

Chromatographic peak	Contaminants	
	Total protein (ng/ $\mu\text{L}$ )	Genomic DNA (ng/ $\mu\text{L}$ )
Initial lysate sample	0.360 $\pm$ 0.132	0.310 $\pm$ 0.046
Peak 1	0.085 $\pm$ 0.035	Not detectable
Peak 2	0.100 $\pm$ 0.011	0.077 $\pm$ 0.007
Peak 3	0.144 $\pm$ 0.021	0.021 $\pm$ 0.002



**Fig. 5.** Evaluation of the reproducibility of MP-SilPrMimCl in the separation of small RNA from genomic DNA. **A)** General procedure for evaluation of the reproducibility (10 chromatographic assays performed in five non-consecutive days). **B)** Chromatographic profiles of the 10 assays, represented by distinct colors. **C)** Agarose gel electrophoresis of the different fractions recovered in the chromatographic assays 1, 4, 7, and 10 performed in MP-SilPrMimCl support.

support is able to separate distinct classes of nucleic acids and the proteins removal can be easily achieved if required, e.g., by digestion with proteinase K [49], treatment with sodium dodecyl sulfate in an alkaline medium [16], or using commercially available resins [50].

The feasibility of using ribosomal RNAs purified by the MP-SilPrMimCl in molecular biology procedures was finally addressed. These tools have been employed for bacterial community identification, and involve an initial step of RNA extraction, followed by sequencing of 16S ribosomal RNA amplicons generated by reverse transcription polymerase chain reaction (RT-PCR) [51]. Aiming to infer the integrity and applicability of ribosomal RNA isolated by MP-SilPrMimCl, complementary DNA was reverse-transcribed from the sample recovered with 0.5 M NaCl (peak 3 in Fig. 4A) and analyzed by PCR using specific oligonucleotide primers for 16S ribosomal RNA (experimental details are given in the experimental procedure section). According to the agarose gel electrophoresis of PCR products, depicted in Fig. S8, a single band was obtained, which corresponds to the PCR product of the complementary DNA reverse-transcribed from ribosomal RNA isolated using the novel developed microporous support, but it is feasible for molecular biology analysis.

#### 3.4. Regeneration, reproducibility, and dynamic binding capacity of MP-SilPrMimCl

The reproducibility of the novel material in the separation of small RNA from genomic DNA was evaluated by conducting 10 chromatographic experiments in five non-consecutive days, and by regenerating the support with aqueous solutions of NaOH and HCl, and distilled water, as described before. Fig. 5 shows the schematic diagram of the process, the corresponding chromatograms of the 10 assays, and the respective agarose gel electrophoresis results. Overall, the results show that the MP-SilPrMimCl macroporous support has a high robustness and

reproducibility, allowing the separation of small RNA from genomic DNA, and the recovery of highly pure small RNA. Furthermore, the support can be regenerated with slightly acidic and alkaline aqueous solutions, which are amenable and do not correspond to the typically used volatile organic solvents. The dynamic binding capacity (DBC) of MP-SilPrMimCl to small RNA, pcDNA3-FLAG, and BSA was finally determined. These biomolecules were chosen as representative of three major classes of biomolecules (RNA, DNA, and proteins) typically present in bacterial lysates. The MP-SilPrMimCl DBC for BSA, plasmid DNA, and small RNA at 10% of maximum capacity is, respectively, 37.0, 0.60, and 3.68 mg/mL, while at 50% of maximum capacity it is, respectively, 59.1, 0.83, and 12.0 mg/mL. The values of the MP-SilPrMimCl binding capacity toward RNA are particularly remarkable, especially when compared to the agmatine-based monolith, where 0.71 and 1.37 mg/mL of DBC, at 10% and 50% of maximum capacity, respectively, have been reported [48], the HPLC 21.2 mm × 100 mm RNASep™ Semi-Prep Cartridge (ADS Biotec, Omaha, USA) wherein a load capacity of 600 µg total RNA is achievable, or the loading capacities ranging from 0.6 to 8.0 mg double stranded RNA/mL of resin using distinct anion-exchange columns [52].

#### 4. Conclusion

In this work it was described the functionalization of a macroporous chromatographic support with an IL that acts as a multimodal ligand. This material can be used in preparative liquid chromatography and allows the purification of three types of nucleic acids, namely ribosomal RNA, small RNA, and genomic DNA, from complex bacterial lysates in one step. Nucleic acids are obtained with a high purity level, with high integrity, and are compatible with biological applications. In addition to the offered high selectivity, the new functionalized macroporous support has a high dynamic binding capacity. Furthermore, NaCl aqueous solutions are used in the elution assays, representing a salt with a low eutrophication potential. The chromatographic support can be easily



regenerated with NaOH and HCl aqueous solutions and reused without compromising its separation performance. Overall, all these results and conditions contribute toward the design of more sustainable purification techniques of nucleic acids. Since the IL acts a multimodal ligand, this type of chromatographic support deserves to be investigated in the purification of other high-value biomolecules from complex media.

### Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study. The required information can be asked directly to the authors.

### Credit author statement

**Márcia C. Neves:** Conceptualization, Methodology, Investigation, Validation, Writing - original draft; **Patrícia Pereira:** Conceptualization, Methodology, Investigation, Validation, Writing - original draft; **Augusto Q. Pedro:** Conceptualization, Methodology, Investigation, Validation, Writing - original draft; **João C. Martins:** Investigation, Validation; **Tito Trindade:** Methodology, Writing - review & editing; **João A. Queiroz:** Methodology, Writing - review & editing; **Mara G. Freire:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition; **Fani Sousa:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition

### Declaration of competing interest

The authors declare that they have no financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A Supplementary data

Supplementary Data to this article can be found online at <https://doi.org/10.1016/j.mtbio.2020.100086>.

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