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Original paper**Design of ionic liquids as versatile chromatographic ligands for the purification of the recombinant interferon alpha-2b biopharmaceutical**

Leonor S. Castro¹, Guilherme S. Lobo¹, Márcia C. Neves¹, Augusto Q. Pedro^{1,*}, and Mara G. Freire^{1,*}

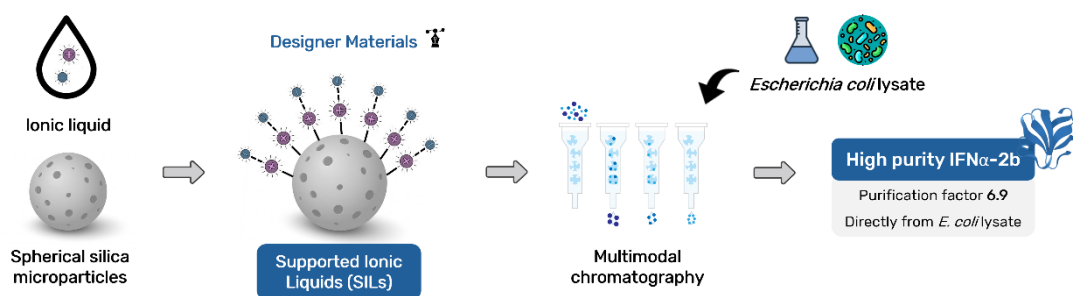
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Abstract

Biopharmaceuticals, which include a wide array of recombinant proteins, are effective therapeutic agents and essential in modern healthcare systems. However, the high cost of chromatographic biological ligands often applied in their downstream processing still limits the widespread use of biopharmaceuticals. To overcome these bottlenecks, ionic liquids (ILs) covalently immobilized in spherical silica microparticles – supported ionic liquids, SILs – are herein proposed as novel synthetic ligands in preparative liquid chromatography of interferon alpha-2b (IFN α -2b), a recombinant protein used in the treatment of disorders such as hepatitis C and multiple sclerosis. Three chloride-based ILs, comprising the cations 1-methyl-3-propylimidazolium, dimethylbutylpropylammonium, and dimethylhexylpropylammonium, were synthesized, characterized, and investigated as potential ligands of chromatographic supports. Column chromatographic experiments confirmed the suitability of the designed SILs to be packed into gravity flow columns, and demonstrated their ability to bind the target protein in conditions favoring ionic or hydrophobic interactions. IL synthetic ligands immobilized onto SILs exhibit a multimodal behavior, which can be tuned to the target application. Under chromatographic conditions in which ionic conditions are applied, the SIL comprising the 1-methyl-3-propylimidazolium cation exhibited the highest selectivity, allowing a remarkable purification factor of 6.9 of the target protein IFN α -2b, being directly purified from *Escherichia coli* lysates.

Graphical Abstract



Keywords: supported ionic liquid; interferon alpha 2b; downstream processing; chromatographic ligand; multimodal/mixed-mode chromatography; selectivity.

1. Introduction

In the last decades, advances in technology and medicine improved human lifespan and general health, with life expectancy increasing worldwide. This was in part due to the development of innovative medicines, as is the case of biopharmaceuticals. Biopharmaceuticals, *e.g.* therapeutic proteins, antibodies, and nucleic acids and their derivatives, are biologic-based products manufactured from living cells or organisms by biotechnological processes [1, 2]. To improve the widespread application of biopharmaceuticals while ensuring their continuous application, it is imperative to decrease their costs and time release to the market, which can only be accomplished by improvements in their manufacturing processes [3]. Despite remarkable advances achieved in the upstream stage, the main current bottleneck is the downstream processing, accounting for up to 80% of the manufacturing costs [4]. This high cost is generally derived from the capture and purification steps, usually carried out by packed-bed chromatography with expensive biological ligands [5]. Furthermore, the biopharmaceutical industry currently faces new purification challenges due to the increased molecular diversity of novel therapeutic modalities, which bring new and difficult-to-remove product-related impurities [6]. Using chromatography, different molecular and physicochemical properties of therapeutic proteins, such as size and shape, charge, or hydrophobicity can be explored for their separation and isolation from complex samples [7]. In this field, affinity chromatography has been widely applied due to the high amount of active sites per volume, low non-specific interactions, pores large enough to allow accessibility, and easiness of modification [5, 8, 9]. In the early days of affinity chromatography, natural ligands such as enzymes, coenzymes, inhibitors, effectors, or antigens were mostly used; however, recent efforts are aimed to develop

low-cost and more stable synthetic compounds that can mimic the properties of natural ligands, such as short peptides or protein A mimetic ligands [10]. However, these approaches still entail some drawbacks, which may include low efficiency, poor resolution, selectivity and specificity, and harsh elution conditions [11].

In the search for improved chromatographic performance for biopharmaceuticals, multimodal (or mixed-mode) chromatographic ligands capable of establishing more than one type of non-covalent molecular interactions (ionic, hydrophobic, hydrogen-bond, van der Waals, among others) are becoming increasingly important [6, 12]. Although significant advances in the downstream processing of therapeutic proteins have been demonstrated using new supports and/or synthetic ligands in affinity and multimodal chromatography [13, 14], innovative and cost-effective chromatographic ligands able to simultaneously provide high efficiency, high purity, and high robustness with mild elution conditions are still scarce. The range of possible synthetic ligands for application in multimodal chromatography can, however, be expanded using ionic liquids (ILs) [15], while envisioning the design of more efficient chromatographic separations of biopharmaceuticals.

ILs are molten salts comprising an organic cation (*e.g.*, imidazolium, pyridinium, tetraalkylammonium, and tetraalkylphosphonium) paired with an organic or inorganic anion. As a result of the remarkable structural diversity of ILs, their physicochemical properties can be adjusted to meet the requirements of specific applications, being for this reason recognized as designer solvents [16]. Although ILs have been mainly investigated for biopharmaceuticals purification in liquid-liquid extraction as phase-forming components of aqueous biphasic systems [17-19], ILs immobilized onto solid supports – supported ionic liquids, SILs – may be considered a relevant alternative. Since the designer solvent character of ILs is transferable to SILs upon immobilization, high-performance materials can be prepared for the extraction and purification of a wide range of compounds from aqueous media, previously demonstrated with anti-inflammatory drugs [20], nucleic acids [15, 21], and the protein ovalbumin [22].

From the exposed, this work aims to develop SILs based on spherical silica with improved packing and flow properties, and to carry out their application as stationary phases in column liquid chromatography for the purification of a recombinant therapeutic protein, *i.e.* interferon alpha-2b (IFN α -2b), directly from lysates of *Escherichia coli* (*E. coli*) cultures. The uniform particle shape from spherical silica microparticles promotes more regular and reproducible packing and reduces flow

resistance than heterogeneous silica gel previously reported [20,21], providing better permeability, more stable backpressure, and improved chromatographic performance.

2. Experimental

2.1. Materials and reagents

1-methylimidazole and 3-chloropropyltrimetoxysilane were purchased at Acros Organics (New Jersey, USA), with purities above 98%. The remaining compounds for the synthesis of SILs were acquired at Fischer Scientific (New Hampshire, USA), namely ethanol, methanol, and toluene, or Sigma-Aldrich (Missouri, United States) for hydrochloric Acid, N,N-Dimethylbutylamine, N,N-Dimethylhexylamine, and spherical silica (75-200 μm). The salts used for the preparation of the chromatographic mobile phases were purchased at Merck (Darmstadt, Germany), LabKem (Barcelona, Spain), and Alfa-Aesar (Massachusetts, USA), respectively, for ammonium sulfate, NaCl, and Tris(hydroxymethyl)aminomethane hydrochloride (Tris). The Econo-Pac gravity-flow columns were acquired at Bio-Rad (California, USA). The ELISA kit used for IFN α -2b quantification was purchased at MabTech (Nacka Strand, Sweden).

2.1. Modification of spherical silica microparticles with ionic liquids

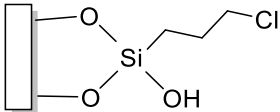
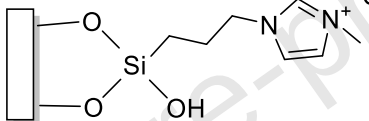
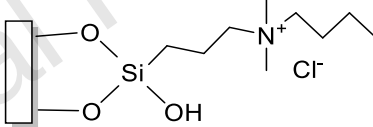
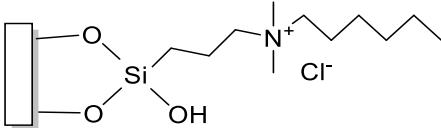
Materials were prepared by the chemical modification of spherical silica microparticles, here shown for the first time with IL ligands, with particle sizes ranging between 75 and 200 μm . This material was selected since the spherical particle shape may result in more homogeneous packing characteristics, providing sharper peak adsorption and elution curves. The silica functionalization process was adapted from procedures previously reported by us [20, 21] and others [23] for the functionalization of heterogeneous silica gel, yet with two relevant differences: i) the starting material of the current work has a different morphology, spherical over heterogeneous powder as used in previous works [20, 21]; and ii) equimolar amounts of the cation source (imidazole and several tertiary amines) were used in the second reaction step, as described below. The use of spherical silica allows improved flow conditions under preparative liquid chromatography, whereas equimolar amounts of the cation source allow a more controlled investigation of the SIL multimodal character.

In the first step, the commercially acquired spherical silica microparticles were submitted to an activation process in the presence of HCl at 37% (w/w) for 24h. The activated spherical silica was then washed with distilled water until a neutral pH was achieved and dried at 55 °C in an oven for 24 h. Following this step, the previously activated spherical silica was subjected to the chemical modification with 3-

chloropropyltrimethylsilane in a reflux reaction, using toluene as solvent at 93 °C for 24 h without magnetic stirring (to avoid destruction of the spherical morphology). The resulting solid was then vacuum-dried and washed four times with distinct solvents in the following order: toluene; ethanol/H₂O (1:1); H₂O; methanol. The solid, *i.e.*, chloropropyl silanized silica ([SSi][C₃]Cl), was dried once again before the addition of the cation source. In the second reaction step, the intermediate material, [SSi][C₃]Cl, reacted with the respective cation source of IL, namely 1-methylimidazole, N,N-dimethylbutylamine, and N,N-dimethylhexylamine, all added in equimolar amounts (0.05 mol). The reaction was refluxed for 24h at 115 °C in toluene. This two-step synthesis strategy, using [SSi][C₃]Cl as the intermediate material, also guarantees that all SILs have a propyl moiety, which will act as the spacer arm and generally ensures that the molecules to be purified can interact with the immobilized ligands.

The name of the cation source, the functionalized moiety on silica, the chemical structure and abbreviation of all SILs synthesized in this work are shown in Table 1. Different cation sources were investigated, keeping however chloride as the counterion in all SILs. Chloride was chosen to lower the cost of materials and reduce their environmental impact by avoiding the use of more complex organic anions [20, 24]. Adding to the cost efficiency, selecting an anion other than chloride will require an extra step in the synthesis process for anion exchange [20], which will have a negative environmental impact. After drying, all the prepared materials were characterized.

Table 1. Cation source, chemical structure, functionalized moiety on silica, and abbreviations of synthesized SILs and the intermediate material.

SIL Abbreviation	Cation Source	SIL chemical structure	Functionalized moiety on silica
[SSi][C ₃]Cl	3-chloropropyltrimethylsilane		chloropropyl
[SSi][C ₃ C ₁ Im]Cl	1-methylimidazole		1-methyl-3-propylimidazolium chloride
[SSi][N ₃₁₁₄]Cl	N,N-dimethylbutylamine		dimethylbutylpropylammonium chloride
[SSi][N ₃₁₁₆]Cl	N,N-dimethylhexylamine		dimethylhexylpropylammonium chloride

2.2. Characterization of spherical silica-based supported ionic liquids

SILs were chemically characterized by distinct techniques, namely solid-state carbon-13 nuclear magnetic resonance (^{13}C solid-state NMR), elemental analysis, Fourier-transform infrared spectroscopy (FTIR), and zeta potential measurements, allowing to confirm the correct functionalization of spherical silica with different ILs and to determine the degree of functionalization. Additionally, scanning electron microscopy (SEM) was applied to appraise any changes in the spherical silica morphology caused by the functionalization process.

2.2.1. Solid-state ^{13}C NMR

The NMR analysis was performed at room temperature using a Bruker Avance III-400 MHz spectrometer (DSX model). A small amount of the material was added to a ZrO_2 rotator 4 mm in diameter and the spectra recorder at 100.63 MHz in 4 mm BL cross-polarization magic angle spinning (CPMAS) VTN probes. Spectra were determined using Bruker Topspin 3.2.

2.2.2. Elemental Analysis

A small sample of 2 mg of the synthesized materials was employed for elemental analysis resorting to a TruSpec 630-200-200 and using a combustion furnace temperature of 1075 °C and afterburner temperature of 850 °C. Carbon, hydrogen, and nitrogen were detected by infrared absorption, and thermal conductivity was applied to determine the nitrogen content.

2.2.3. Surface area determination

The surface area (SBET) for spherical silica was determined using the method of Brunauer, Emmett, and Teller, being performed using nitrogen adsorption in a Micrometrics Gemini V-2380 equipment.

2.2.3.1 Bonding amount determination

Considering that variable ligand densities impact the chromatographic performance of the prepared supports, the degree of functionalization of each SIL, measured as the bonding amount (BA) of each IL on spherical silica microparticles, was determined. Spherical silica microparticles SBET and elemental analysis data were used for the determination of BA according to equations (1) and (2) [24]. The BA for the intermediate material $[\text{SSi}][\text{C}_3]\text{Cl}$ was determined using equation (1):

$$BA = \frac{\frac{\%C}{Y \times Mw(C)}}{SBET} \quad (\text{Eq. 1})$$

where %C represents the carbon content percentage (wt%), determined by elemental

analysis, $M_w(C)$ is the atomic weight of carbon, Y represents the number of atoms of carbon present in the intermediate material, and spherical silica $SBET$ was determined to be $288.59 \text{ m}^2/\text{g}$ of the silica material.

Unlike the intermediate material, all cations used to prepare SILs contain nitrogen in their composition, which was considered to calculate the BA for SILs according to equation (2):

$$BA = \frac{\frac{\%N}{Z \times M_w(N)}}{SBET} \quad (\text{Eq. 2})$$

where $\%N$ represents the nitrogen content percentage (wt%), determined by elemental analysis, $M_w(N)$ is the atomic weight of nitrogen, Z represents the number of atoms of nitrogen present in each SIL, and spherical silica $SBET$ was determined to be $288.59 \text{ m}^2 \text{ g}^{-1}$ of the silica material.

The reaction efficiency (RE) of the second immobilization step was additionally estimated by comparing the bonding amount (BA) values obtained for the final SILs with the BA value determined for the intermediate chloropropyl-functionalized silica material ($[SSi][C_3]Cl$), according to Equation (3):

$$RE (\%) = \frac{BA_{SIL}}{BA_{[SSi][C_3]Cl}} \times 100 \quad (\text{Eq. 3})$$

where BA_{SIL} corresponds to the bonding amount of the final supported ionic liquid material, and $BA_{[SSi][C_3]Cl}$ corresponds to the bonding amount of the chloropropyl-functionalized intermediate silica. This parameter provides an estimation of the fraction of chloropropyl groups that successfully reacted during the nucleophilic substitution step leading to the immobilized ionic liquid functionalities.

2.2.4. Fourier-transform infrared spectroscopy

The activated spherical silica, $[SSi][C_3]Cl$, and the functionalized materials, were analyzed by FTIR using a Perkin Elmer FT-IR System Spectrum BX spectrophotometer, with a resolution of 5 cm^{-1} and equipped with a horizontal Golden Gate ATR cell, in the range of $4000\text{-}500 \text{ cm}^{-1}$.

2.2.5. Determination of the point of zero charge

The zeta potential of synthesized SILs was determined as a function of pH using suspensions of SILs in water at different pH values. To adjust the pH values, 0.01 M solutions of NaOH and HCl were used, and measurements were carried out at $25 \text{ }^\circ\text{C}$ using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK).

2.2.6. Scanning Electron Microscopy

SEM was performed to appraise if the functionalization process induced any morphological changes in spherical silica. Samples before and after the functionalization process, namely activated spherical silica and each SIL, as well as the intermediate material [SSi][C₃]Cl were analyzed using a Hitachi SU70 microscope. A carbon thin film was used to increase the conductivity of the samples, and samples were coated by carbon evaporation (Emitech K950X, France).

2.3. Biosynthesis and recovery of IFN α -2b

Recombinant production of IFN α -2b was performed based on the protocol described by Castro et al [25] using *E. coli* BL21 (DE3) cultivated in super optimal broth medium (cf. Section 1.1. in the Supplementary Material for detailed information). Briefly, recombinant protein production was achieved by inducing cultures with 1 mM isopropyl β -D-1-thiogalactopyranoside when the optical density (600 nm) reached 0.6. The target therapeutic protein was expressed in the form of inclusion bodies, being recovered after the application of a mechanical lysis process involving glass beads, and a solubilization procedure involving a buffer at alkaline pH in the presence of high concentrations of urea. Finally, the buffer was exchanged for the corresponding chromatographic binding buffer.

2.4. Preparative liquid chromatography

2.4.1. Binding-elution studies in a batch mode

The performance of the synthesized materials was initially evaluated in a batch mode, thus allowing the identification of the appropriate mobile phase compositions. These experiments were performed with the materials [SSi][C₃C₁Im]Cl and [SSi]C₃]Cl, respectively, representative of a material functionalized with an IL, and the intermediate and non-functionalized material. To this end, and since no previous information regarding the interaction behavior of these new materials with the target protein was available, several chromatographic buffers were evaluated: 10 mM Tris-HCl pH 8 and 1.5 M NaCl (in 10 mM Tris-HCl pH 8) to test preferably ionic interactions, and 2.0 M (NH₄)₂SO₄ (in 10 mM Tris-HCl pH 8) and Tris-HCl 10 mM pH 8 to explore mostly the establishment of hydrophobic interactions.

Experiments in a batch mode were performed by weighing 150 mg of the corresponding material in a 2.0 mL tube, after which the following procedure was applied: 1) washing the material with 1 mL of distilled water; 2) equilibration of the chromatographic support with 1 mL of binding buffer – 10 mM Tris-HCl buffer pH 8 or 2.0 M (NH₄)₂SO₄ –, respectively, for studying the preferential establishment of ionic or

hydrophobic interactions; 3) addition of 250 μ L of the recombinant sample containing IFN α -2b (inclusion bodies solubilization buffer was exchanged to binding buffer); 4) addition of 1.0 mL of binding buffer for the removal of proteins that did not interact with the material; 5) elution with one or multiple steps using 1.0 mL of the elution buffer: for ionic interactions, increasing concentrations of NaCl (in 10 mM Tris-HCl pH 8) were applied, while for hydrophobic interactions, decreasing concentrations of (NH $_4$) $_2$ SO $_4$ (in 10 mM Tris-Cl pH 8) were used. Between each of the abovementioned steps, samples were centrifuged (1,800 $\times$ g for 15 min at room temperature), and each supernatant was collected for subsequent spectrophotometric analysis at 280 nm. Finally, the samples were concentrated and desalted with Vivaspin[®] concentrators (10,000 MWCO, Sartorius, Gottingen, Germany), and analyzed using sodium dodecyl electrophoresis analysis (SDS-PAGE).

2.4.2. Column liquid chromatography

Based on the binding/elution studies performed in a batch mode, the most promising mobile phase compositions were identified, and the SILs were further evaluated in preparative column liquid chromatography using gravity-flow columns. Herein, the utilization of spherical silica microparticles proved to be crucial for efficient packing of the chromatographic support, further allowing a constant flow of the mobile phase.

Approximately 2 mL of hydrated SIL material was packed into Econo-Pac gravity-flow chromatography columns (Bio-Rad, California, USA) with an internal diameter of 1.5 cm, and a bed height of approximately 1.13 cm, corresponding to a bed volume of approximately 2 mL. The packing was performed under gravity-flow conditions, allowing homogeneous packing and continuous mobile phase flow throughout the experiments. The role of the modified silica-based materials and the type of interaction established with the therapeutic recombinant protein were explored using two different approaches based on the studies of Sousa and co-workers [15, 21]. In the first study, the preferential establishment of ionic interactions was appraised using the following procedure: 1) equilibration with 5 CV (10 mL) of binding buffer – 10 mM Tris-HCl pH 8; 2) injection of the solubilized recombinant samples containing IFN α -2b (inclusion bodies solubilization buffer was exchanged to binding buffer); 3) two stepwise gradient elution of retained proteins was accomplished by an increase in the ionic strength: the first step was performed with 5 CV of 0.35 M NaCl (in 10 mM Tris-HCl pH 8), while the second was accomplished with 5 CV of 1.5 M NaCl (in 10 mM Tris-HCl pH 8). In the second approach, the establishment of mainly hydrophobic interactions was

promoted using a similar procedure: 1) equilibration with 5 CV of binding buffer – 3 M $(\text{NH}_4)_2\text{SO}_4$ (in 10 mM Tris-HCl pH 8); 2) injection of the solubilized recombinant protein fraction (in binding buffer); 3) two stepwise gradient elution of retained proteins: the first step was performed with 5 CV 10 mM Tris-HCl pH 8, while the second step was accomplished using 1.5 M NaCl (in 10 mM Tris-HCl pH 8). In the experiments designed to explore the establishment of mainly ionic or hydrophobic interactions, other binding buffers were studied, respectively: 10 mM Tris-HCl pH 7 and 9, and 2.5 and 3.0 M $(\text{NH}_4)_2\text{SO}_4$ (in 10 mM Tris-HCl pH 8). For both strategies, samples of 1.0 mL were continuously taken, and the absorbance of the eluate was monitored at 280 nm, allowing the respective chromatograms to be drawn. Finally, fractions were pooled according to the chromatograms obtained, concentrated, and desalted with Vivaspin[®] concentrators (10,000 MWCO, Sartorius, Gottingen, Germany), and stored at $-20\text{ }^\circ\text{C}$ until further analysis.

2.4.3. Qualitative and quantitative analysis of total proteins and IFN α -2b elution patterns

Samples corresponding to the chromatographic peaks of interest were analyzed concerning their content in total proteins, achieved by measuring the absorbance at 280 nm using bovine serum albumin (BSA) as a model protein for establishing the standard curve. IFN α -2b was quantified using an enzyme-linked immunosorbent assay (ELISA). From the determination of IFN α -2b/total protein concentrations in SILs chromatographic assays designed to evaluate conditions mainly favoring ionic or hydrophobic interactions, IFN α -2b purification factors in each chromatographic peak were calculated using equation (4):

$$\text{Purification Factor} = \frac{\frac{[\text{IFN}] \text{ in the chromatographic peak}}{[\text{Total protein}] \text{ in the chromatographic peak}}}{\frac{[\text{IFN}] \text{ in the initial sample}}{[\text{Total protein}] \text{ in the initial sample}}} \quad (\text{Eq. 4})$$

To gather information on the elution profile of IFN α -2b and host cell proteins, the chromatographic samples were also analyzed using SDS-PAGE, following the procedure previously reported [25]. Additional details are provided in the Supplementary Material (Section 1.2 and Fig. S1).

3. Results and discussion

This work describes the preparation and application of spherical silica microparticles functionalized with ionic liquid ligands as multimodal chromatographic supports for the purification of the therapeutic protein IFN α -2b. The materials were synthesized through

a two-step functionalization strategy and characterized using complementary techniques to assess their morphology, surface properties, and degree of ligand immobilization. The prepared supported ionic liquid materials were first evaluated in batch assays to investigate their interaction with the target protein. They were then packed into gravity-flow columns and tested in column liquid chromatography using recombinant *E. coli* lysates containing IFN α -2b. Different chromatographic conditions were finally explored to assess the contribution of ionic and hydrophobic interactions to protein binding and elution.

3.1. Preparation and characterization of supported ionic liquids

The functionalization of spherical silica microparticles with ILs, namely imidazolium and quaternary ammonium cations combined with chloride anions, was carried out. The prepared materials were packed onto gravity columns and their pioneering application in preparative column liquid chromatography of a therapeutic protein was investigated. Intending to ascertain the correct functionalization of the prepared SILs and to address relevant physicochemical properties, we proceeded with their characterization by several techniques. The original silica material and the intermediate material [SSi][C₃]Cl were also characterized. The successful modification of silica was first confirmed using FTIR, whose spectra are presented in Fig. 1. A band around 1052 cm⁻¹ is present in all materials (activated spherical silica, intermediate material, and SILs), which is attributed to the asymmetric and symmetric vibration of Si-O-Si. Additionally, changes at 970 cm⁻¹ are observed, being related to changes in the silanol group and indicating that silica is functionalized with silane. In general, these slight, yet visible, differences between the spectrum of the intermediate material, and the spectra of the prepared SILs, reveal that the materials were correctly functionalized with ILs. In the particular case of [SSi][C₃C₁Im]Cl, there is the appearance of a weak band around 1570 cm⁻¹, which is attributed to the imidazolium ring.

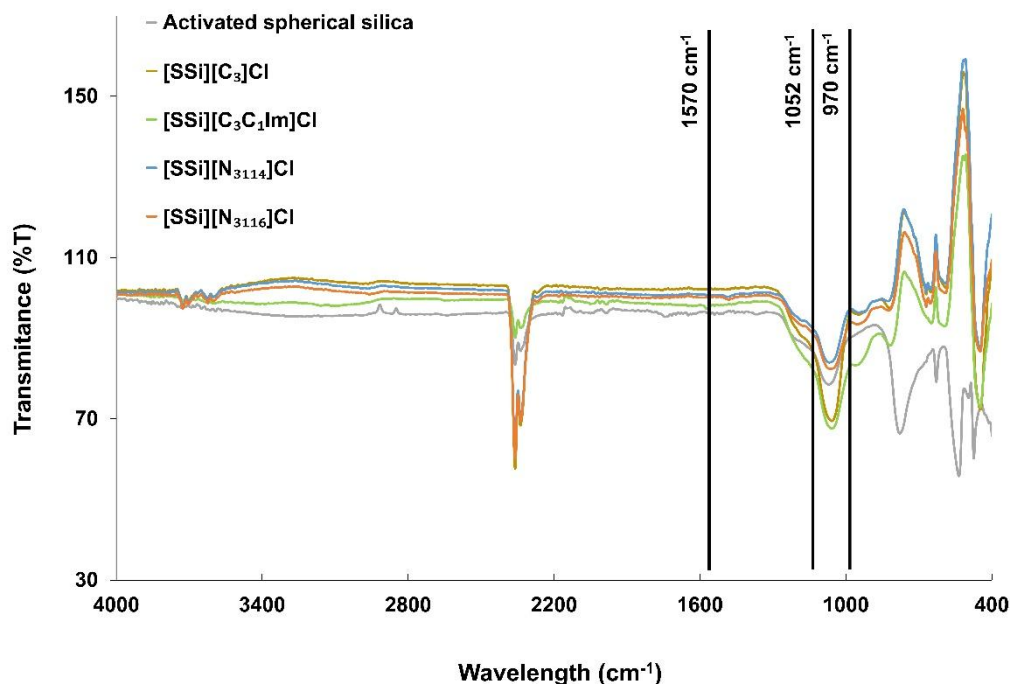


Fig. 1. FTIR spectra of activated silica, chloropropyl silica, and synthesized SILs. Black lines indicate wavelengths where major differences in the spectra of $[\text{SSi}][\text{C}_3]\text{Cl}$ and SILs are observed.

The successful preparation of SILs was additionally confirmed by ^{13}C solid-state NMR spectroscopy, and the corresponding spectra are shown in Fig. 2. The spectrum recorded for the intermediate material $[\text{SSi}][\text{C}_3]\text{Cl}$ displays three characteristic resonances at chemical shifts of 10, 27, and 47 ppm, assigned to the three carbon atoms of the chloropropyl chain covalently attached to the silica surface. The resonance at higher chemical shifts is attributed to the methylene group adjacent to the chloride atom, whereas the remaining signals correspond to the other methylene carbons of the propyl spacer linking the silica surface to the terminal chloride functionality.

For the material $[\text{SSi}][\text{C}_3\text{C}_1\text{Im}]\text{Cl}$, additional resonances are observed in the aromatic region, namely at 122 and 137 ppm, which are assigned to the carbon atoms of the imidazolium ring, confirming the successful formation of the imidazolium-based supported ionic liquid. Moreover, the signals detected at 37 and 52 ppm are attributed to the methyl group attached to the imidazolium nitrogen and to the methylene carbon of the propyl spacer directly connected to the imidazolium ring, respectively. The persistence of the propyl-chain resonances further supports the covalent linkage between the silica surface and the immobilized ionic liquid moiety.

Regarding the materials modified with tetraalkylammonium-based ionic liquids, the

spectra are mainly dominated by resonances in the aliphatic region, consistent with the presence of long alkyl chains in the quaternary ammonium cations. Since both $[N_{3114}]Cl$ and $[N_{3116}]Cl$ contain methyl groups bonded to the quaternary nitrogen atom, the resonance observed at approximately 50 ppm is attributed to these carbons, confirming the successful functionalization of the silica materials. The peaks detected at approximately 67 and 65 ppm are assigned to the methylene carbons directly bonded to the quaternary nitrogen atom from the propyl and butyl/hexyl chains, respectively, while the resonances at lower chemical shifts are associated with the remaining aliphatic carbons of the butyl and hexyl chains. Furthermore, the spectrum corresponding to $[SSi][N_{3116}]Cl$ exhibits a more pronounced contribution in the methylene region due to the presence of the longer hexyl alkyl chain.

Importantly, the ^{13}C solid-state NMR spectra of the materials modified with quaternary ammonium cations still display resonances associated with the chloropropyl-functionalized intermediate $[SSi][C_3]Cl$, suggesting that not all chloropropyl groups reacted during the nucleophilic substitution step. This observation is consistent with the functionalization degree values presented below. Additionally, the relatively broad resonances observed in all spectra are typical of solid-state materials and reflect the heterogeneous chemical environments and restricted molecular mobility resulting from covalent immobilization onto the silica surface.

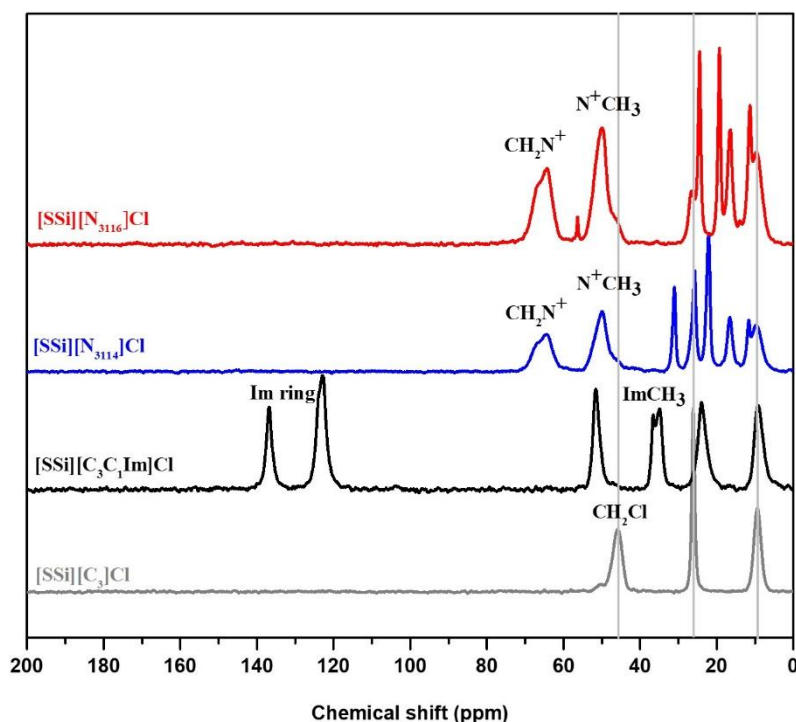


Fig. 2. ^{13}C NMR solid-state spectra of pristine silica, $[\text{SSi}][\text{C}_3]\text{Cl}$, and the prepared SILs. Characteristic resonances assigned to the CH_2Cl group, imidazolium ring carbons, and quaternary ammonium carbon atoms are indicated in the corresponding spectra.

Elemental analysis was also performed, further allowing to quantitatively determine the functionalization degree. The content of carbon, hydrogen, and nitrogen for all the prepared silica materials and the chloropropyl silica ($[\text{SSi}][\text{C}_3]\text{Cl}$) material is shown in Table 2. In comparison with $[\text{SSi}][\text{C}_3]\text{Cl}$, the elemental analysis of SILs shows changes in their relative atomic composition. The carbon content (wt%) in $[\text{SSi}][\text{C}_3]\text{Cl}$ is 3.72 and no nitrogen was detected, thereby supporting the absence of IL functional moieties in the intermediate material. On the other hand, for the IL-functionalized silica supports with quaternary ammonium- and imidazolium-based ILs, there is the presence of nitrogen, as well as changes in the percentage of carbon when compared with $[\text{SSi}][\text{C}_3]\text{Cl}$, disclosing the presence of the IL.

Table 2. Elemental analysis (C, H, and N weight fraction percentage) of the synthesized materials, and IL bonding amount on SILs.

Supported ionic liquid	%C	%H	%N	Bonding amount (BA, mmol m ⁻²)	RE (%)
------------------------	----	----	----	--	--------

[SSi][C ₃]Cl	3.72	1.16	----	3.58	---
[SSi][C ₃ C ₁ Im]Cl	6.05	1.38	1.95	2.41	67.32
[SSi][N ₃₁₁₄]Cl	8.51	2.16	0.83	2.05	57.26
[SSi]N ₃₁₁₆]Cl	8.41	2.00	0.85	2.10	58.66

As appraised from Table 2, the synthesized materials present distinct contents (wt%) of nitrogen, which is correlated with distinct levels of immobilized ligands in each material. These results demonstrate that the material with a higher amount of nitrogen is [SSi][C₃C₁Im]Cl, which was expected considering that this IL has a higher amount of nitrogen *per* molecule of IL, but also confirms the higher efficiency of the immobilization process in this SIL. On the other hand, spherical silica-based materials prepared with tertiary amines ([SSi][N₃₁₁₄]Cl and [SSi][N₃₁₁₆]Cl), leading thus to quaternary ammoniums, also exhibited high percentages of nitrogen, namely 0.83 to 0.85%. To have a quantitative measure of the degree of functionalization, the BA for each SIL was determined using Eqs. (1) and (2) considering the value of SBET (288.59 m² g⁻¹) of the silica material.

The BA of each SIL is displayed in Table 2, where the degree of functionalization of the synthesized materials decreases in the following order: [SSi][C₃C₁Im]Cl > [SSi][N₃₁₁₆]Cl > [SSi][N₃₁₁₄]Cl. Independent of the content of nitrogen atoms per molecule of IL, and considering that equimolar amounts of base were used in the synthesis, [SSi][C₃C₁Im]Cl is the SIL with the highest amount of immobilized IL ligands.

The bonding amount (BA) determined for the intermediate chloropropyl-functionalized silica material [SSi][C₃]Cl was 3.58 mmol m⁻², confirming the successful grafting of chloropropylsilane onto the silica surface. Based on this value, the reaction efficiency (RE) of the second immobilization step was estimated for the prepared SILs by comparing the BA values of the final materials with that of the intermediate support, according to Eq (3).

The obtained RE values were 67.32% for [SSi][C₃C₁Im]Cl, 57.26% for [SSi][N₃₁₁₄]Cl, and 58.66% for [SSi][N₃₁₁₆]Cl. These results indicate that only a fraction of the chloropropyl groups initially introduced onto the silica surface underwent nucleophilic substitution to form the corresponding immobilized ionic liquid functionalities. Such incomplete conversion may be associated with steric hindrance effects, restricted accessibility of some chloropropyl groups within the porous structure, and diffusion

limitations during the functionalization step.

Among the prepared materials, [SSi][C₃C₁Im]Cl exhibited the highest reaction efficiency, suggesting that 1-methylimidazole presents higher accessibility and/or reactivity towards the chloropropyl-functionalized surface under the experimental conditions employed. In contrast, the slightly lower RE values obtained for the tetraalkylammonium-based SILs may be related to the bulkier nature of the tertiary amines, which can hinder the nucleophilic substitution reaction on the silica surface.

For a more complete SIL characterization, changes in zeta potential values as a function of pH were also appraised, allowing to determine their point of zero charge (PZC). The graphical representations obtained for each material are shown in Supplementary Material (see Fig. S2), whereas the corresponding PZC, as well as the variation of the superficial charge of each material with pH, are depicted in Fig. 3.

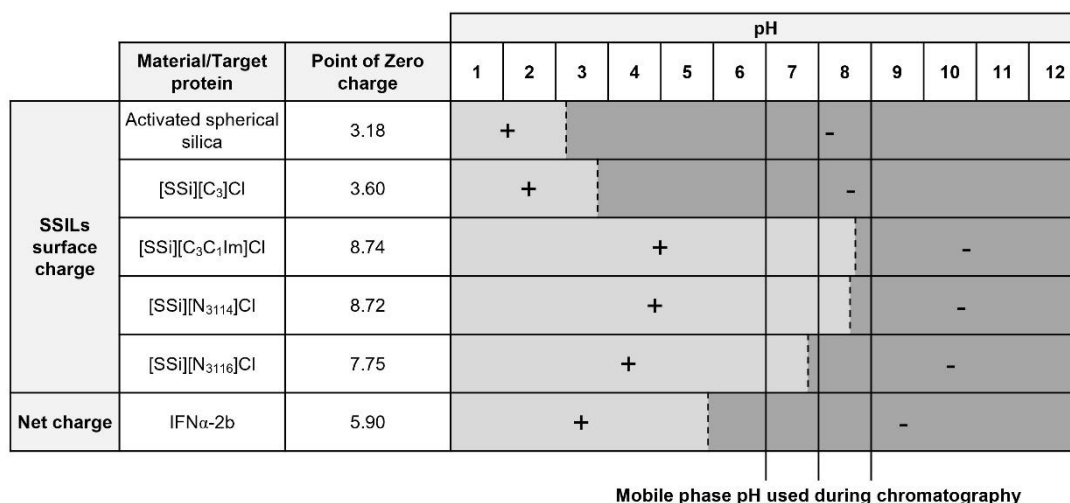


Fig. 3. Overall surface charge of IFNα-2b, activated spherical silica microparticles, the intermediate material [SSi][C₃]Cl, and SILs at different pH values. Dashed lines indicate the point of zero charge (PZC) for each material and the isoelectric point of IFNα-2b, while solid lines represent the mobile phase pH investigated during chromatography.

The PCZ of all SILs is above the PZC of activated spherical silica and of the intermediate material [SSi][C₃]Cl, thus confirming that the materials were functionalized with IL moieties. The PZC values obtained for the prepared SILs (Fig. 3) followed the order: [SSi][C₃C₁Im]Cl > [SSi][N₃₁₁₄]Cl > [SSi][N₃₁₁₆]Cl. These differences are likely influenced by both the chemical nature of the immobilized ionic liquid moieties and the corresponding bonding amount (Table 2). Since the SILs exhibit different BA values, a direct correlation between PZC and degree of immobilization cannot be unequivocally established from the present data. Nevertheless, the results

suggest that both the extent of surface functionalization and the structure of the immobilized ionic liquid contribute to the surface charge properties of the obtained materials. The determination of PZC confirms not only the successful immobilization of ILs but also the superficial charge exhibited by each SIL at different pH values. Considering that the isoelectric point of IFN α -2b is around 5.9 [26], the determination of PZC values allows selecting the mobile phase pH at which the target protein and the material can have the same superficial charge or opposite charges, which is relevant to explore the ion-exchange character of the synthesized chromatographic supports.

In addition to the previous analyses, which were able to confirm the successful modification of silica-based materials with ILs, SEM analyses were performed to determine if there were any changes in the morphology of spherical silica microparticles resulting from the chemical immobilization process. These results are provided in Fig. 4, overall demonstrating that the spherical morphology of silica microparticles is maintained after IL immobilization. As discussed below in Section 3.2.2, this feature was crucial for efficient packing of the SIL materials, enabling their use in column liquid chromatography for the purification of IFN α -2b.

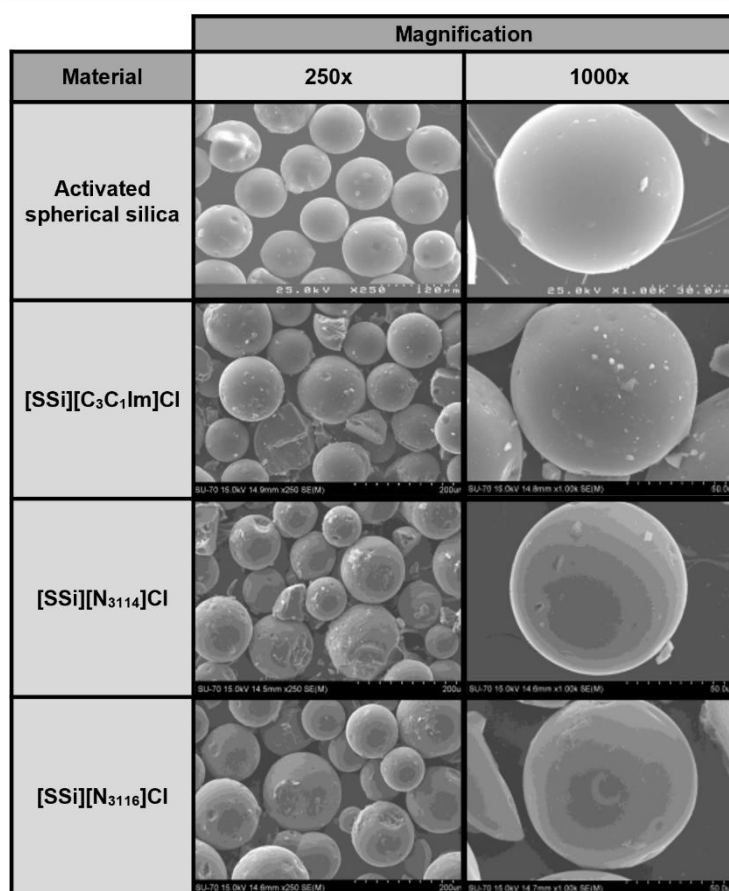


Fig. 4. SEM analysis of activated spherical silica, and synthesized SIL materials. Images were

acquired at 15 kV and taken at 250x and 1000x magnification, left and right, respectively.

3.2. Chromatographic experiments

Previous results on SILs characterization confirmed that silica was successfully modified with ILs, reinforcing the suitability of the proposed methodology for the covalent attachment of IL moieties in spherical silica microparticles. Overall, materials exhibiting different physicochemical properties were prepared, which were then explored as chromatographic supports for the purification of IFN α -2b. However, before evaluating their application as chromatographic supports, an initial screening in a batch mode was carried out and deemed fundamental to identify suitable binding and elution conditions.

3.2.1. Batch experiments

In a batch mode set of experiments, different mobile phase compositions with varying buffer components, salts, and their concentrations, were assessed to better understand the role of the IL ligand and which type of interactions would be favored between the ligand and the biomolecule. To this end, the materials [SSi][C₃C₁Im]Cl and [SSi][C₃]Cl were selected as model SILs of a functionalized spherical silica-based material and of a non-functionalized material, respectively, thus studying the role of the immobilized ligand in the purification performance. Considering that IFN α -2b isoelectric point is 5.9 [26], and that at pH 8 [SSi][C₃C₁Im]Cl has an overall net positive superficial charge (Fig. 3), the preferential establishment of ionic interactions between the ligand and the protein was evaluated. Two mobile phase buffers were studied: Tris-HCl 10 mM pH 8 and phosphate 200 mM pH 7. After promoting the binding of the target protein, the next step is to proceed to the elution of retained proteins, which can be achieved by increasing the concentration of NaCl, ultimately disrupting the interactions between the target protein and the chromatographic ligand. In this sense, it was expected to promote noncovalent ionic interactions between the ligands and the target protein, and that the SIL would behave as a typical anion-exchange chromatographic resin.

Not involving SILs, Mukherjee and co-authors [26] investigated the purification of recombinant IFN α -2b using anion-exchange chromatography based on the Q-Sepharose column. The protein was eluted using a linear gradient of NaCl at a concentration of approximately 0.250 M [26]. Since no previous information was available regarding which NaCl concentrations would be ideal to promote IFN α -2b elution, and based on this reported work [26], a two-step elution using 0.4 M and 1.5 M of NaCl (in the respective buffer component) was initially evaluated. The application of these

concentrations ensured that both the target protein and host cell proteins would be eluted.

Considering the electrophoretic profile provided in Fig. 5 A), a buffer with low ionic strength, such as Tris-HCl 10 mM pH 8, promotes the interaction of the proteins with the IL synthetic ligands immobilized in the material. In comparison with Tris-HCl 10 mM pH 8, the application of phosphate 200 mM renders the interaction of the target protein with the support less effective, being confirmed by the SDS-PAGE analysis, whereby the presence of higher amounts of the target protein (around 17-20 kDa) in the unretained fraction was observed.

Following these results and after the binding step resorting to Tris-HCl 10 mM pH 8, a plethora of other proteins elute with 1.5 M of NaCl, while an IFN α -2b-enriched fraction is obtained with 0.4 M NaCl. This indicates that the process is selective for the target protein by controlling the ionic interaction between the ligand and the biomolecule. Further assays were attempted to optimize the selective elution of IFN α -2b by using different concentrations of NaCl. In this sense, by changing the salt concentration in the first elution step to 0.35 M (results shown in Fig. 5 B), it is possible to detect that the target protein was recovered with 0.35 M NaCl in a more purified form, with the remaining proteins eluting with 1.5 M of NaCl. Therefore, these experimental conditions were selected for the subsequent column assays.

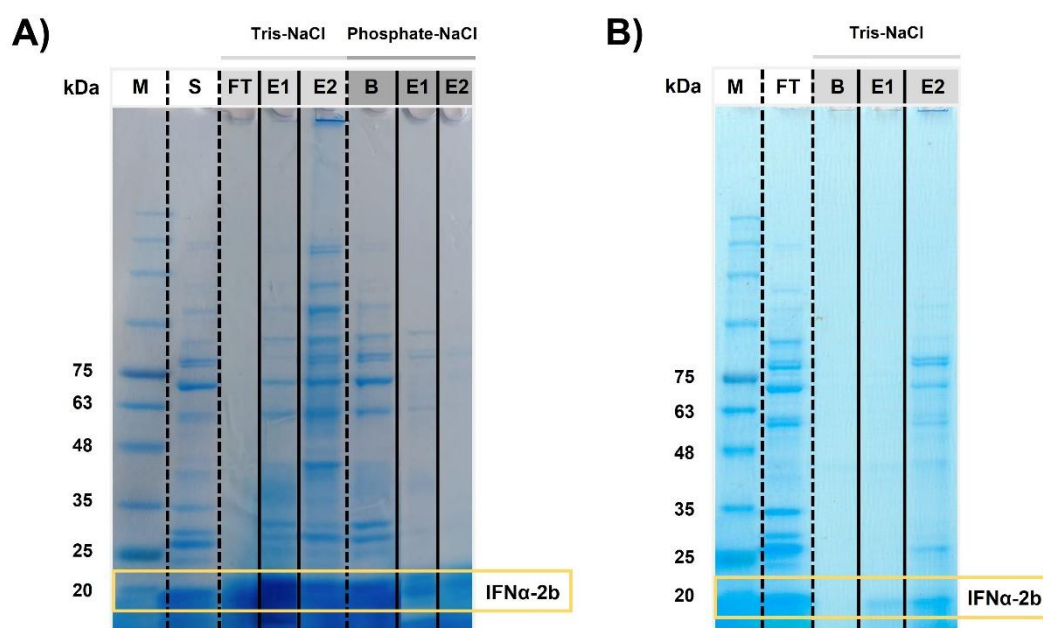


Fig. 5. SDS-PAGE analysis under denaturing and reducing conditions of the chromatographic fractions obtained using the material [SSi][C₃C₁Im]Cl. Assays carried out in a batch mode under

the following conditions: **A)** binding with 10 mM Tris pH 8 or 200 mM phosphate pH 8 (denoted as flowthrough - FT), followed by elution with 0.4 and 1.5 M NaCl (in 10 mM Tris pH 8 or 200 mM phosphate pH 8), respectively, denoted as E1 and E2; **B)** binding with 10 mM Tris pH 8 (denoted as FT), followed by elution with 0.35 and 1.5 M NaCl (in 10 mM Tris pH 8), respectively, denoted as E1 and E2. M and S represent, respectively, the molecular weight marker and the solubilized sample.

According to the chemical structure of the immobilized ILs (Table 1), in addition to ionic interactions studied by varying NaCl concentrations, it is conceivable that they can also establish hydrophobic interactions with the target proteins. Therefore, experiments were designed to appraise if the target protein is retained in conditions favoring mainly hydrophobic interactions, *i.e.*, using a decreasing $(\text{NH}_4)_2\text{SO}_4$ gradient. Herein, an initial concentration of $(\text{NH}_4)_2\text{SO}_4$ of 1.8 M was selected to promote protein binding, a concentration that is higher than that applied by Zhou and co-authors [27] to retain IFN α -2b using phenyl Sepharose HP – 0.5 M. However, the selected concentration is within the order of magnitude necessary to promote the binding of other biopharmaceuticals in heterogenous silica-based SILs [21]. The SDS-PAGE analysis of the samples from these assays is reported in Fig. S3 in the Supplementary Material. It is demonstrated that IFN α -2b binds to the materials and is capable of establishing hydrophobic interactions with the ligand, despite a fraction of the target protein being found in the flowthrough.

After establishing the described initial experiments with a spherical silica-based material functionalized with an imidazolium IL, we aimed to investigate if the promising chromatographic behavior exhibited by this SIL was due to the presence of the IL moiety. Therefore, the next assays were performed with the intermediate material $[\text{SSi}][\text{C}_3]\text{Cl}$ in the experimental conditions previously optimized for $[\text{SSi}]\text{C}_3\text{C}_1\text{Im}]\text{Cl}$. The SDS-PAGE analysis of the assays performed in conditions favoring ionic or hydrophobic interactions using $[\text{SSi}][\text{C}_3]\text{Cl}$ are shown in the Supplementary Material, respectively, in Fig. S4 A) and B). Based on the results obtained, the protein does not interact with $[\text{SSi}][\text{C}_3]\text{Cl}$, which contains only the spacer arm and no IL ligands. Under both binding conditions – 10 mM Tris-HCl (pH 8) and 1.8 M $(\text{NH}_4)_2\text{SO}_4$, designed to mainly promote ionic and hydrophobic interactions, respectively – the protein was not retained and was instead detected in the flowthrough. Therefore, the interaction between the studied materials and the protein is mediated through the covalently attached IL. This observation applies to all other functionalized materials, which will be discussed

below.

Overall, the batch chromatographic assays performed with [SSi][C₃C₁Im]Cl disclose the ability of spherical silica-based materials functionalized with ILs to interact with target therapeutic proteins. Accordingly, the suitability of these novel SILs for packing into gravity-flow columns was investigated, along with their chromatographic performance in column liquid chromatography.

3.2.2. Column Liquid chromatography

Considering the degrees of functionalization reported in Table 2, as well as the PZC values (Fig. 3), the synthesized materials will be predominantly negatively charged within the range of the mobile phase pH (7-9) commonly selected for biomolecules chromatographic purification [15]. Since the materials [SSi][C₃C₁Im]Cl, [SSi][N₃₁₁₄]Cl, and [SSi][N₃₁₁₆]Cl maintain their morphology after the functionalization process (See Fig. 4), they were packed for column liquid chromatography and then applied for IFN α -2b purification.

In chromatography, the ion-exchange character of the resins is commonly explored for biopharmaceuticals' purification. To evaluate the establishment of ionic interactions, column chromatographic assays were performed with the following steps: 1) equilibration with 5 CV of 10 mM Tris-HCl (pH 7-9); 2) injection of the solubilized recombinant protein in the same equilibrium buffer; 3) elution of retained proteins by an increase in the ionic strength with 5 CV of 1.5 M NaCl (in 10 mM Tris-HCl, pH 7, pH 8 or pH 9). After performing the chromatographic assays, the concentrations of IFN α -2b and total proteins in the elution peaks were determined for the samples obtained with NaCl concentrations of 0.35 M and 1.5 M. The assays were conducted at pH 7 and 9, representing the experimental conditions aimed at addressing scenarios where SILs are positively (pH 7) or negatively charged (pH 9). Under this pH range, IFN α -2b will present an overall negative surface charge. Chromatograms and the calculated IFN α -2b purification factors at pH 7 and 9 are shown in Fig. 6 A) and B), respectively. The chromatograms corresponding to the assays at pH 8 and the replicates of those shown in Fig. 6 are depicted in Fig. S5 (Supplementary Material). IFN α -2b purification factors were calculated using Eq. (4), and the detailed data for all the SILs purification assays can be found in the Supplementary Material (*cf.* Tables S1 and S2).

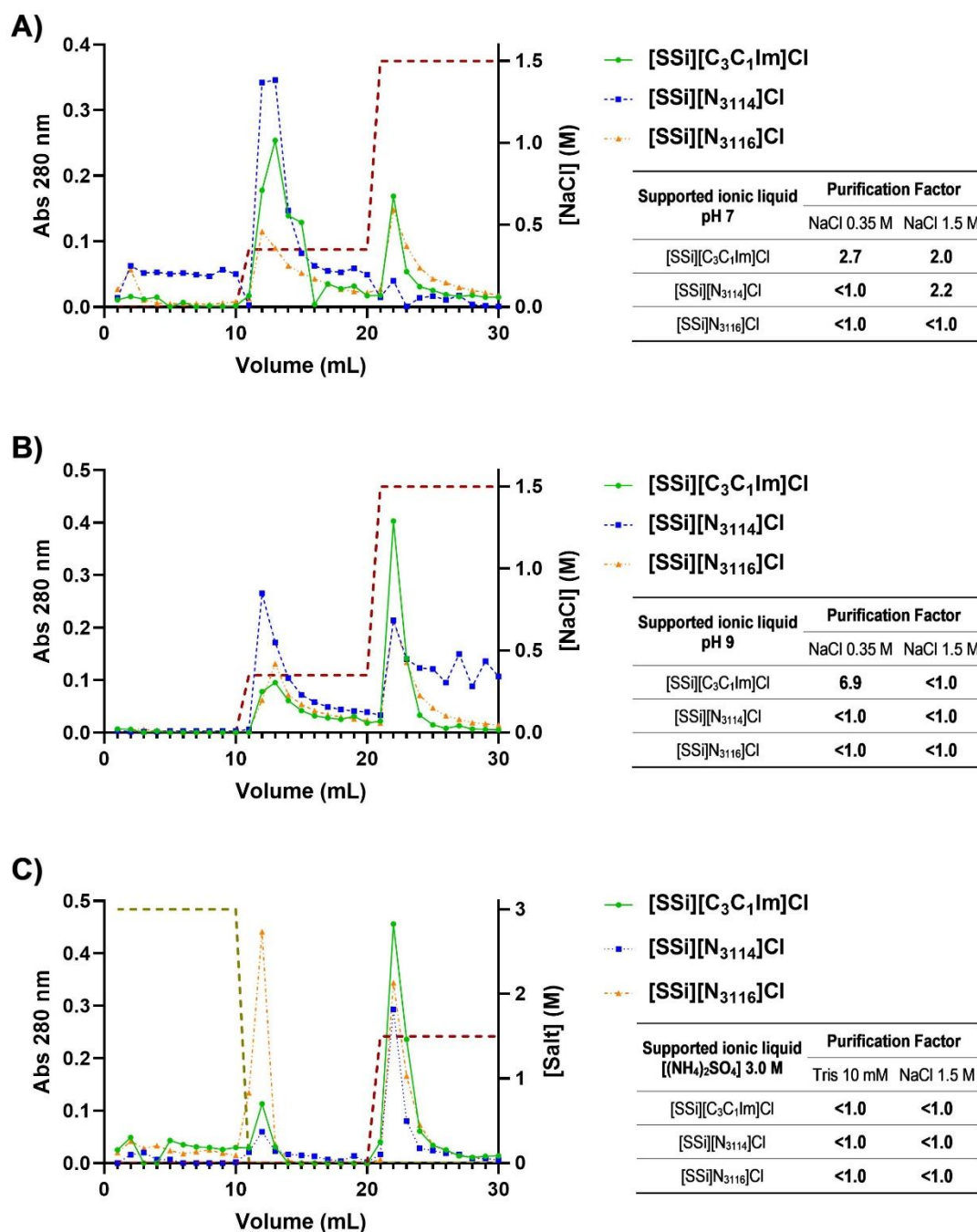


Fig. 6. Chromatographic purification of the recombinant sample containing IFN α -2b loaded onto different spherical silica-based materials (green circle [SSi][C₃C₁Im]Cl, blue square [SSi][N₃₁₁₄]Cl, orange triangle [SSi][N₃₁₁₆]Cl). **A)** Chromatographic profile and IFN α -2b purification factors in elution peaks (NaCl 0.35 M and NaCl 1.5 M (in Tris-HCl 10 mM pH 7)) using an increasing NaCl gradient at pH 7, represented by the dashed line. **B)** Chromatographic profile and IFN α -2b purification factors in elution peaks (NaCl 0.35 M and NaCl 1.5 M (in Tris-HCl 10 mM pH 7)) using an increasing NaCl gradient at pH 9, represented by the dashed line. **C)** Chromatographic profile and IFN α -2b purification factors in elution peaks (Tris 10 mM and NaCl 1.5 M (in Tris-HCl 10 mM pH 8)) using a two-step elution strategy based on a decreasing

(NH₄)₂SO₄ 3 M gradient in Tris-HCl 10 mM, followed by an increasing NaCl gradient represented by the dashed line.

According to the chromatograms represented in Fig. 6 A) and for a mobile phase pH of 7, the majority of proteins present in the lysate bind to all studied SILs by using a low ionic strength buffer (Tris-HCl 10 mM). However, an increase in the ionic strength of the elution buffer by increasing the concentration of NaCl to 0.35 M and 1.5 M leads to the elution of the proteins previously adsorbed onto each functionalized material, indicating that these SILs behave as typical anion-exchange chromatographic supports.

It is noticeable that IFN α -2b was retained in all the functionalized supports, as shown in Table S1 (Supplementary Material). Moreover, [SSi][C₃C₁Im]Cl and [SSi][N₃₁₁₄]Cl were able to selectively isolate IFN α -2b from *E. coli* host cell proteins, achieving, respectively, IFN α -2b purification factors of 2.7 and 2.2 for the samples eluted with 0.35M NaCl and 1.5M NaCl (Fig. 6A). [SSi][N₃₁₁₆]Cl exhibits a lower PZC value than that of [SSi][N₃₁₁₄]Cl (Fig. 3) indicating that at pH 7 it will be less positively charged, rendering the ionic interaction with IFN α -2b comparatively weaker, and thus providing a possible explanation to why the target protein was not purified with the support [SSi][N₃₁₁₆]Cl. At pH 7, both [SSi][C₃C₁Im]Cl and [SSi][N₃₁₁₄]Cl show purification factors above 1 and hence an improvement in the purity of the target protein. At this point, one can assume that the interaction between the synthetic IL ligand immobilized in [SSi][N₃₁₁₄]Cl and the protein is mainly mediated by ionic interactions. Herein, IFN α -2b was mainly eluted with 1.5 M NaCl, indicating that the strength of interactions was higher, probably because at pH 7 the SIL has a higher positive superficial charge (the PZC is 8.72).

Considering the differences of PCZ of the prepared SILs, the chromatographic behavior of the synthesized materials was also studied at a pH higher than their PZC by adjusting the pH of the mobile phase to 9. The corresponding results are displayed in Fig. 6 B), whereas replicates are shown in the Supplementary Material (Fig. S5). Detailed data are provided in Table S1 in the Supplementary Material.

In conditions mainly favoring ionic interactions, and even when the superficial charge exhibited by the materials is the same as the target protein, IFN α -2b is adsorbed in all supports. The obtained chromatograms indicate that most of the protein interacts with the SIL, and are once again capable of selectively eluting IFN α -2b by changing the concentration of NaCl. However, the chromatographic performance exhibited by the

supports was distinct, indicating that the selectivity of this chromatographic process can be controlled by the pH of the mobile phase. As it is observable in Table S1 (Supplementary Material) and Fig 6 B), the elution of IFN α -2b is more favorable in [SSi][C₃C₁Im]Cl with 0.35 M of NaCl, where IFN α -2b was recovered with a purification factor of 6.9. The observed results at pH 7 and pH 9 seem to indicate that, in addition to ionic interactions, the synthetic IL ligand can establish multiple non-covalent interactions with proteins, including hydrophobic and van der Waals interactions due to the presence of the alkyl chain, as well as hydrogen bonding and π - π interactions due to the aromatic ring, pointing out that the synthetic IL chromatographic ligands exhibit a multimodal behavior. This pattern is more evident for [SSi][C₃C₁Im]Cl, which is in agreement with the wider type of interactions that its chemical structure allows.

Along with an increasing NaCl gradient, protein adsorption in conditions favoring mainly hydrophobic interactions was also evaluated. This behavior was also expected to be established with proteins, since all the selected materials present alkyl chains in their chemical structures (Table 1). To reach this goal, (NH₄)₂SO₄ was added to the sample and the chromatographic mobile phases, enhancing hydrophobic interactions between IL ligands and proteins [28]. The following steps were considered in this set of experiments: 1) a mobile phase composed of (NH₄)₂SO₄ in the range of 2-3 M (NH₄)₂SO₄ (in 10 mM Tris-HCl, pH 8) was applied as the equilibrium buffer, 2) injection of the solubilized recombinant protein in the same equilibrium buffer, 3) two elution steps, the first with 10 mM Tris-HCl, pH 8, and the second resorting to 1.5 M NaCl (in 10 mM Tris-HCl pH 8).

The concentration of IFN α -2b and total proteins were determined in the samples from the elution peaks, namely Tris-HCl 10 mM and NaCl 1.5 M (in Tris-HCl 10 mM pH 8), being provided in Table S2 (Supplementary Material). The results for the assays where 3 M of (NH₄)₂SO₄ was applied as the binding buffer are presented in Fig. 6 C); the corresponding replicate and the assays where the concentration of (NH₄)₂SO₄ in the binding buffer was 2.5 or 2.0 M are shown in Fig. S6 in the Supplementary Material.

The obtained chromatograms, *cf.* Fig. 6 C), and Fig. S6 in the Supplementary Material, confirm that the immobilized ligand and proteins are indeed able to interact via hydrophobic interactions. Among the concentrations of (NH₄)₂SO₄ applied to retain proteins in the different SILs, as the concentration becomes lower, the higher the content of proteins that do not interact with the support and hence are detected in the

binding step. In this sense, higher concentrations of salt seem to be necessary to increase the hydrophobic interactions between the ligand and the protein, and thus to promote their adsorption. No significant differences were observed between the elution patterns of IFN α -2b obtained with [SSi][C₃C₁Im]Cl and [SSi][N₃₁₁₄]Cl, being thus assumed that these materials have similar behavior.

Considering the chromatographic profiles displayed in Fig. 6, and Fig. S6 in the Supplementary Material, a decrease in the concentration of (NH₄)₂SO₄, leads to the elution of a small amount of proteins, being most of the proteins eluted afterwards in the second elution step with 1.5 M NaCl (in 10 mM Tris-HCl pH 8). Regarding the levels of IFN α -2b and for all SILs investigated, it can be observed that the target protein is detected in residual amounts in both eluted fractions. Considering these results, it means that high amounts of salt promote hydrophobic interactions between IFN α -2b and the ligand. However, after removing the salt (NH₄)₂SO₄, the proteins are still able to interact with the ligand by ionic interactions rather than hydrophobic interactions. These results once again favor the hypothesis that the synthetic IL ligands immobilized in spherical silica-based materials behave as multimodal chromatographic matrices, whereby a multitude of non-covalent interactions, including ionic, hydrophobic, van der Waals, and hydrogen-bond interactions, mediate the interaction of IFN α -2b with the ligand.

Finally, the stability of SIL materials in the chromatographic mobile phase was addressed by elemental analysis before and after an incubation period of 72h. These studies were conducted with [SSi][C₃C₁Im]Cl and a mobile phase composed of Tris 10 mM pH 9, which are representative of the best-performing experimental conditions, and the obtained results are shown in the Supplementary Material (Table S3). In general, no significant changes were observed in the macroscopic appearance of [C₃C₁Im]Cl up to a contact time of 72h in the mobile phase. However, the elemental analysis revealed that the percentage of nitrogen [SSi][C₃C₁Im]Cl was 1.60, 1.58, and 1.20%, respectively, before and after the 6h and 72h incubation periods. From these results, the calculated BA of the SIL material was 1.98 mmol m⁻², followed by a negligible decrease to 1.96 mmol m⁻² after the 6h incubation period, and a more significant decrease to 1.49 mmol m⁻² at 72h. Overall, these data indicate that the BA relative to the starting point was 98.95% (6h), and drops to 75.14% after the 72h incubation period. For short-term periods, up to 6h, the collected evidence rules out concerns regarding the stability of SIL materials in mobile phases with alkaline character (pH 9), thus supporting the

application of these materials for single use and one-batch purification processes. Moreover, the lower cost of the starting material applied in this work (spherical silica microparticles) renders the resulting SIL materials more cost-effective than polymeric macroporous-based materials. The SILs here proposed follow a trend on the field of biopharmaceuticals since single-use strategies may eliminate cleaning validation and reduce cross-contamination risks, offering enhanced efficiency, flexibility, and sustainability [29-31].

Overall, spherical silica-based materials functionalized with ILs were successfully prepared and, unlike the heterogeneous silica gel previously reported [20,21], can be efficiently packed into gravity-flow columns and used in column liquid chromatography. Gathered evidence demonstrates that IL synthetic ligands immobilized in SILs exhibit a multimodal behavior, being thus envisaged as efficient and versatile chromatographic supports for the purification of therapeutic proteins from complex recombinant lysates. By avoiding the use of tags, protein stability is preserved, and the number of processing steps may be reduced (*e.g.*, enzymatic digestion steps to remove tags).

Moreover and despite SILs can retain IFN α -2b in different binding conditions, the purity of the target protein was enhanced in conditions mainly promoting ionic interactions. Among the investigated materials, [SSi][C₃C₁Im]Cl was found to be most selective towards IFN α -2b, which is recovered in an immunologically active form, as confirmed by ELISA. Future work should focus on evaluating the performance of supported ionic liquids in medium- and high-pressure systems, as well as elucidating their applicability and effectiveness at preparative industrial scale.

4. Conclusions

Overall, this work demonstrates the application of ILs as versatile synthetic multimodal chromatographic ligands immobilized onto highly functionalized spherical silica microparticles for the purification of the therapeutic protein IFN α -2b. The prepared SILs were able to adsorb the target protein under conditions mainly favoring either ionic or hydrophobic interactions, confirming the multimodal character of IL-based ligands, which can be tuned to the target application. Among the investigated materials, the SIL comprising the 1-methyl-3-propylimidazolium chloride cation exhibited the highest selectivity, enabling an IFN α -2b purification factor of 6.9 using a pH 9 binding buffer and elution with 0.35 M NaCl in 10 mM Tris-HCl buffer at pH 9. These findings demonstrate the potential of IL-functionalized spherical silica microparticles as effective chromatographic supports for the purification of recombinant therapeutic

proteins, opening new perspectives for their broader application in biopharmaceutical downstream processing.

Declaration of Competing Interest

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

CRedit authorship contribution statement

Leonor S. Castro – Investigation, Data curation, Formal Analysis, Validation, Visualization, Writing – Original draft; **Guilherme S. Lobo** – Investigation, Data Curation, Formal Analysis; **Márcia C. Neves** – Conceptualization, Methodology, Data curation, Supervision, Writing – review & Editing; **Augusto Q. Pedro** – Conceptualization, Methodology, Data curation, Supervision, Project administration, Funding acquisition, Writing – review & Editing; **Mara G. Freire** – Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Writing – review & Editing.

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Highlights

- Protein–support interactions are driven by immobilized IL, not the backbone;
- Ionic liquid structure was tailored to modulate protein–material interactions;
- Interaction supported ionic liquid-protein is governed by multiple non-covalent forces;
- Supported ionic liquids display a multimodal chromatographic behavior;
- Selective isolation of IFN α -2b was achieved from a complex biological sample.