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Enhanced performance of polymer-polymer aqueous two-phase systems using ionic liquids as adjuvants towards the purification of recombinant proteins

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ABSTRACT:

Protein biopharmaceuticals, among which interferon alpha-2b (IFNα-2b) that can be used in the treatment of chronic hepatitis C and hairy cell leukemia, have become an indispensable product of current medicine. However, their current high costs derived from the lack of cost-effective downstream strategies still limits their widespread use. Polymer-based aqueous two-phase systems (ATPS) or aqueous biphasic systems (ABS) can be used in biopharmaceuticals purification. This work investigates the application of ionic liquids (ILs) as adjuvants (at 5 wt%) in ATPS constituted by polyethylene glycol with a molecular weight of 600 g.mol^{-1} (PEG 600) and polypropylene glycol with a molecular weight of 400 g.mol^{-1} (PPG 400) at constant pH (8) to purify the recombinant protein IFNα-2b from *Escherichia coli* lysates. IFNα-2b was produced from isopropyl β-d-1-thiogalactopyranoside (1 mM)-induced *Escherichia coli* BL21 (DE3) cultures and recovered from inclusion bodies after mechanical lysis, involving glass beads and a solubilization step with urea (8M) in alkaline pH (12.5). PEG-PPG-based ATPS involving ILs as adjuvants were subsequently applied for IFNα-2b purification, in which the target protein tends to migrate to the PEG-rich phase (being the phase also enriched in IL) and the remaining proteins tend to precipitate at the interface (fitting within the three-phase partitioning approach). In comparison with the ATPS without adjuvant, most systems comprising ILs as adjuvants lead to enhancements in the purification factors of IFNα-2b, namely from 2.28 ± 0.06 up to 6.77 ± 0.49. The purity of IFNα-2b is maximized using ILs composed of aromatic cations and anions with high hydrogen-bond basicity. The secondary structure of IFNα-2b is preserved during the purification step, as appraised by circular dichroism and western-blot studies. Overall, the obtained results demonstrate the ILs ability to tune the characteristics of the ATPS coexisting phases towards improved

purification processes, paving the way for their investigation in the purification of other

high-value biological products.

1. INTRODUCTION:

The advent of biopharmaceuticals in modern medicine for the treatment of numerous diseases brought a significant impact on the improvement of global health. Proteins (including antibodies and/or other recombinant proteins) currently dominate the biopharmaceuticals market, with over 300 drug products approved and many more undergoing preclinical and clinical trials [1, 2], and with global sales reaching $100 billion in 2017 [1]. Among these, interferon alpha-2b (INFα-2b) has become widely applied in the treatment of cancer, hepatitis, and hairy cell leukemia [3], being marketed under the trademarks of Intron A® (Merck Sharp & Dohme), Rebetron® (Schering Plough) and distinct pegylated forms: PEG-Intron/Rebetol® (Schering Plough), Pegasys® (Roche/Genentech), PEG-intron® (Merck Sharp & Dohme) and ViraferonPeg® (Merck Sharp & Dohme) [1]. Owing to the wide spectrum of biological activities displayed by INFα-2b, namely in terms of antiproliferation, immunomodulation and antiviral properties [3], there has been an increased demand for the development of cost-effective manufacturing processes of recombinant human INFα-2b.

The INFα-2b upstream phase has been typically accomplished through recombinant protein production in a host microorganism, such as *Escherichia coli* (*E. coli*) [4, 5]. Within the upstream stage, the major advantage of protein expression strategies in the form of inclusion bodies is that they can be produced in high concentrations, so that the amount of generated product often outweighs the additional downstream steps, which can boost time/space yields for recombinant protein production [6]. Although inclusion bodies have been seen as inactive protein aggregates, a new term of non-classical inclusion bodies has emerged in more recent years, which are believed to contain reasonable amounts of correctly folded protein, while exceeding 40% of total recombinant protein produced in cells [7, 8].

The downstream processing of biopharmaceuticals comprises the removal of the process- and product-associated impurities, as well as other contaminants [9], allowing to obtain a purified protein sample. Currently, liquid chromatography is the key technique of most purification processes resorting to protein biopharmaceuticals [10], and has been applied to the purification of IFNα-2b comprising ion-exchange/size exclusion matrices [5, 11] or immobilized metal-affinity matrices/reverse-phase HPLC [12]. However, chromatography may present some limitations in terms of the high cost derived from the resin's low stability [13]. To overcome such limitations, alternative downstream processes have been investigated, including non-chromatographic operations, namely membrane-based procedures [14], high-performance tangential flow filtration [15], high gradient magnetic fishing [16], precipitation [17], crystallization [17] and aqueous two-phase systems (ATPS) [18]. Initially proposed by Albertsson as a separation technique [19], ATPS are liquid-liquid systems formed when two aqueous solutions of two polymers, or a polymer and a salt, are mixed above given concentrations [20]. Some of the advantages displayed by ATPS include their biocompatibility, mostly due to the high-water content in both phases. Polymer-polymer and polymer-salt ATPS have been applied in the separation, recovery, and purification of several (bio)molecules, including antibiotics, proteins, DNA, and enzymes [21]. In particular, ATPS formed by PEG 8000 and the dibasic potassium phosphate salt were investigated for the purification of inclusion bodies resulting from the overexpression of external genes in prokaryotes [22]. Most polymer-polymer-based ATPS investigated are composed of PEG and dextran [23-25]. However, due to the high cost of some dextran polymers, research has evolved towards the application of less expensive polymers, among which polypropylene glycol (PPG) [26]. In general, PEG and PPG present low toxicity and are approved by the Food and Drug

Administration (FDA), being both listed in the FDA's Inactive Ingredient Guide for uses in topical, oral and other formulations [27, 28].

A common drawback associated to polymer-polymer-based ATPS is that they exhibit a small range of polarities between the two phases [20], thus hampering their effective application in the purification of target proteins from complex samples. To overcome such limitation, it has been proposed the functionalization of PEG with glutaric acid to improve the recovery yields of immunoglobulins [29, 30]. More recently, and with the same goal of improving the ATPS separation performance, ionic liquids (ILs) were proposed as adjuvants in polymer-salt ATPS [31-35]. This strategy has been successfully applied to increase the target proteins purification performance of PEG-citrate salt [31, 33, 34], PEG-potassium phosphate [32], and PEG-ammonium sulphate [35] systems, although to the best of our knowledge was never attempted with polymer-polymer ATPS. Up to date, ATPS composed of alcohol/salt [36] and PEG/salt [37] were investigated for the purification of IFNα-2b.

In addition to ATPS, three-phase partitioning (TPP) approaches based on ammonium sulfate aqueous solutions and tert-butanol can be used to concentrate and purify proteins, involving the recovery of the target protein as a precipitate at the interface of the two liquid phases [38]. In particular, TPP has been used for the refolding of proteins from urea-solubilized inclusion bodies [39]. However, TPP based on ATPS can be applied as well for the purification of target proteins by inducing their precipitation at the ATPS interface, while avoiding the use of tert-butanol. Recently, IL-based TPP have been proposed by Alvarez et al [40, 41] to recover model food proteins at the ATPS interface.

Herein it is described the production and purification of IFNα-2b from *E. coli* BL21 (DE3) inclusion bodies using PEG -600-PPG-400-based ATPS/TPP with ILs as adjuvants (at 5 wt%). Previous reports [31-35] investigated the use of ILs as adjuvants in polymer-

salt systems, which are composed of salts presenting a high salting-out ability and a high ionic strength [42]. Distinctly, the current work focuses on two polymers as phase-forming components, thus providing a low ionic strength, and hence a more amenable environment to address the relevance of the ILs chemical structure to tailor the phases characteristics, which is beneficial when envisioning the purification of a target protein from a complex medium rich in other proteins. The ATPS process was combined with TPP, in which the purification of the target protein was aimed at the PEG-rich phase with the simultaneous precipitation of the remaining proteins at the ATPS interface.

2. EXPERIMENTAL SECTION:

2.1. Materials, strain, and media:

The plasmid pET-3a containing a codon-optimized version of the gene coding for human IFNα-2b (NCBI accession KY780371.1, full nucleotide sequence given in the Supplementary Information) – pET-3a_IFNα-2b – was acquired from Genscript (Piscataway, New Jersey, USA) and used for the heat-shock transformation of *E. coli* BL21 (DE3) chemically competent cells (Merck/Sigma-Aldrich, St. Louis, USA). The reagents used in the preparation of *E. coli* culture media, including tryptone and yeast extract, were acquired from Biokar Diagnostics (Cedex, France), while agar and NaCl (purity >99.5%) were bought from PanReac (Barcelona, Spain). The salts $MgCl_2$ (purity >99%) and $MgSO_4$ (purity >99%) were obtained from Prolab (São Paulo, Brazil). Reagents required for bacterial fermentation, cell lysis and protein recovery, such as isopropyl β-d-1-thiogalactopyranoside (IPTG), ampicillin sodium salt, and phenylmethylsulfonyl fluoride (PMSF), were provided by NZYtech (Lisbon, Portugal), Pierce protease inhibitor was obtained from Fisher Scientific (Massachusetts, USA) and Triton X-100, urea (purity 99%) and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)

were acquired from Acros Organics (New Jersey, USA). Polymers used as ATPS phase-forming components were purchased from Alfa Aesar, Massachusetts, USA (PEG 600) and Merck/Sigma-Aldrich, St. Louis, USA (PPG 400). The list of names, acronyms, chemical structures, purities and suppliers of the ILs investigated as adjuvants in this work as well as of the polymers used in ATPS are shown in Table 1.

Reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were stained with BlueSafe from NZYtech (Lisbon, Portugal). Total protein quantification was performed using the Pierce BCA protein assay from Thermo Fisher Scientific (Massachusetts, USA), while the quantification of IFNα-2b in ATPS phases was achieved with the human IFN-α pan ELISA[PRO] kit (MabTech, Stockholm, Sweden - Fig. S1 in Supplementary Information). The IFNα-2b standard (Recombinant human Interferon alpha-2b protein) and the Anti-IFNα-2b antibody were acquired from Abcam (Cambridge, United Kingdom), while the secondary antibody Anti-Chicken IgY (IgG) was obtained from Sigma-Aldrich (St. Louis, USA).

2.2. Biosynthesis and recovery of IFNα-2b:

Recombinant production of IFNα-2b was performed based on the protocol described by Ling and co-workers [36], yet with minor modifications: *E. coli* BL21 (DE3) was cultivated in shake-flasks containing SOB medium, and induction was performed with 1 mM IPTG when the optical density (600 nm) reached 0.2. Cell lysis was performed using glass beads according to the protocol previously reported by Passarinha and co-workers [43], while the subsequent recovery of IFNα-2b from inclusion bodies was achieved using a buffer containing urea in an alkaline pH, based on the works developed by Prazeres [4] and Mukherjee [5] research groups and general guidelines provided by Palmer and Wingfield [44]. Additional details on the recombinant production and recovery of IFNα-2b are given in the Supplementary Information.

2.3. Phase Diagrams:

The phase diagrams of ATPS composed of PEG 600 + PPG 400 + 10 mM Tris pH 8 were determined in presence of 5 wt% of the following ILs: [C$_2$mim]Cl, [C$_4$mim]Cl, [C$_6$mim]Cl, [C$_4$mim]Br, [C$_4$mim][CH$_3$COO], [N$_{4444}$]Cl, and [Ch]Cl - cf. Fig. 1. Each ATPS binodal curve was determined by the cloud point titration method at 25 ($\pm$ 1) °C and atmospheric pressure, as previously described [45]. For the determination of the liquid-liquid phase diagrams with ILs as adjuvants, 10 mM Tris (pH 8) aqueous solutions and buffered solutions of PEG 600 and PPG 400 containing 5 wt% of the respective IL were used, allowing to keep the IL concentration constant along all the phase diagram regions. Buffered solutions (10 mM Tris pH 8) of PEG were drop-wise added to buffered solutions of PPG until a cloudy biphasic solution was obtained; subsequently, drop-wise addition of water proceeded until a monophasic region was observed. This procedure was carried out under constant stirring and the system's composition was determined by weight ($\pm10^{-4}$ g). The experimental binodal curves were fitted using equation 1:

$$[PEG] = A\exp\left[\left(B[PPG]^{0.5}\right) - \left(C[PPG]^3\right)\right] \quad (Eq.1)$$

where [PEG] and [PPG] represent the weight percentage (wt%) of each polymer, and *A*, *B* and *C* are fitting parameters obtained by regression of the experimental data.

2.4. Purification of IFNα-2b in polymer-polymer aqueous two-phase systems:

The purification of IFNα-2b in PEG-PPG-based ATPS was evaluated in a common mixture point (30 wt% of PEG 600 + 30 wt% of PPG 400 + 5 wt% of IL). After careful weighing each ATPS component (including IL if present), systems were homogenized, 22.5 wt% of dialyzed inclusion body fraction was added (corresponding to a total protein mass of ca. 400 µg), and 10 mM Tris (pH 8) was added to make up 100 wt%. Subsequently, the mixture was stirred for 5 min at room temperature (25 ($\pm$ 1) °C) and was then centrifuged (3500 rpm for 2 min at room temperature) to enhance phase

separation. Phases (PEG-rich bottom and PPG-rich top phases) were isolated using a syringe and their weight determined. The precipitate was also isolated and dissolved in 10 mM Tris (pH 8). Both phases and the dissolved precipitate were analyzed for total proteins and IFNα-2b contents. Total protein concentration was determined by the Pierce BCA protein assay using convenient dilutions of ATPS phases, yielding typically concentrations below 3 wt% of each polymer that avoid polymer interferences in the determination of the proteins content, in agreement with the recommendations of Beutel and co-workers [46]. The migration pattern of IFNα-2b and remaining proteins between the two phases and precipitation at the interface were evaluated by SDS-PAGE; additionally, the levels of IFNα-2b were quantified by enzyme-linked immunosorbent assay (ELISA) (additional details are given in the Supplementary Information). Using the values obtained for the concentration of IFNα-2b and/or total proteins in each phase and at the precipitate, both the purification factor and extraction efficiency were calculated to evaluate the purification performance of the studied systems.

The overall purification performance of the ATPS was evaluated through the determination of the purification factor (PF) and equation 2:

$$\text{PF}_{\text{IFN}\alpha-2b} = \frac{([\text{IFN}\alpha\text{-}2b]_{\text{PEG}-\text{rich phase}} \div [\text{Total proteins}]_{\text{PEG}-\text{rich phase}})}{([\text{IFN}\alpha\text{-}2b]_{\text{initial sample}} \div [\text{Total proteins}]_{\text{initial sample}})} \text{ (Eq. 2)}$$

where [IFNα-2b] represents the concentration of IFNα-2b (μg/ml) in the PEG-rich phase and in the initial solubilized sample, and [total proteins] represents the concentration of total proteins (including IFNα-2b) in the PEG-rich phase and initial solubilized sample. Finally, IFNα-2b extraction efficiencies (EE) were calculated using equation 3:

$$\text{EE (\%)} = \frac{w\,\text{IFN}\alpha\text{-}2b_{\text{PEG}}}{(w\,\text{IFN}\alpha\text{-}2b_{\text{PEG}} + w\,\text{IFN}\alpha\text{-}2b_{\text{PP}} + w\,\text{IFN}\alpha\text{-}2b_{\text{PPG}})} \times 100 \text{ (Eq.3)}$$

where w IFNα-2b represents the weight of IFNα-2b (μg) in the PEG-rich phase (PEG), PPG-rich phase (PPG) and the resulting precipitate (PP) of each ATPS.

The migration pattern or precipitation of total proteins in each sample was analyzed by SDS-PAGE according to the method of Laemmli [47]. To remove interferences caused by polymers and/or ILs, samples were precipitated using acetone (1:4 (v/v)) and the mixture was incubated at -20 ˚C overnight, followed by centrifugation at 13000 rpm (4 ˚C for 15 min) [48]. The resulting pellet from each ATPS phase was then dissolved in 50 μL of 10 mM Tris pH 8. The precipitates at the ATPS interface were recovered and dissolved in 100 μL of 10 mM Tris pH 8. Samples were diluted 1:1 in Laemmli loading buffer (4% (w/v) of SDS, 20% (w/v) of glycerol, 120 mM of Tris-HCl pH 6.8, 0.02% (w/v) bromophenol blue) and denatured by incubation at 95 °C for 5 min, and were then injected in the polyacrylamide gel. Lastly, protein staining was achieved by incubation with BlueSafe under mild agitation for 1 h.

Western blot was performed to identify immunologically active IFNα-2b. To this end, proteins were transferred from an unstained SDS-PAGE gel (obtained as described above) to a nitrocellulose membrane (Bio-Rad, Hercules, USA) over 30 min at 200 mA and 4 °C in a specific buffer [50 mM Tris, 380 mM glycine and 20% (v/v) methanol]. Membranes were blocked with TBS-T (pH 7.4) supplemented with 5% (v/v) non-fat milk during 1 h at room temperature and were then probed with primary antibodies recognizing human IFN-α2b [1:2000 in TBS-T and 0.5% (w/v) non-fat milk] overnight at 4 °C. After three consecutive washes with TBS-T, membranes were incubated with HRP-labeled anti-chicken/rabbit IgG secondary antibody diluted 1:10000 in TBS-T containing 5% (w/v) non-fat milk. Signal detection was performed with ECL substrate (Advansta Inc., CA, USA) according to the manufacturer's instructions, and images were acquired with the ChemiDoc™ XRS system (Bio-Rad, Hercules, USA) and analyzed with Image Lab (Bio-Rad, Hercules, USA).

Circular dichroism (CD) was used to address the secondary structure of IFNα-2b and to infer possible changes induced during the ATPS purification step. Analyses were performed in a JASCO J-815 spectropolarimeter (JASCO, Easton, MD, USA). To remove interferences caused by polymers and/or ILs, the target ATPS phase was subjected to dialysis against PBS buffer (Sigma Aldrich, Missouri, USA). CD spectra were recorded at 25 °C in the interval of 195-280 nm in quartz Suprasil CD cuvettes (0.1 cm), using the following acquisition parameters: data pitch, 0.5 nm; bandwidth, 1.0 nm; response, 4 s; and scanning speed, 50 nm/min.
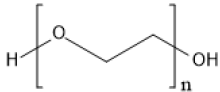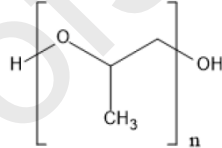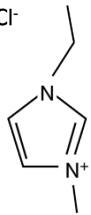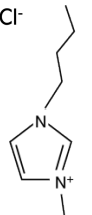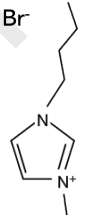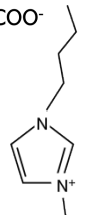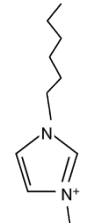
2.5. Partition coefficients of ionic liquids:

The partition coefficients of each IL (K_{IL}) in ATPS were determined to evaluate their influence on the IFNα-2b partitioning between the two phases following the protocol previously reported by Coutinho and co-workers [45]. To accomplish this, ATPS were prepared with the following composition: 30 wt% of PEG 600, 30 wt% of PPG 400, 35 wt% of an aqueous solution with 10 mM Tris pH 8 and 5 wt% of IL. The mixture point herein selected for the determination of K_{IL} was the same from that employed in the purification experiments of IFNα-2b, allowing to infer if the partition of IFNα-2b is governed by the quantity of IL present in each ATPS phase. The concentration of the chloride-based ILs in each phase was determined using a Metrohm 904 Titrando ion chloride electrode. A stock solution of NaCl 0.2 M was prepared and diluted in concentrations ranging from 0.1 to 100 mM to establish a calibration curve. A total ionic strength adjustment buffer (TISAB) composed of $NaNO_3$ 2 M was added to all standard solutions and samples to keep the ionic strength. Each ATPS phase was diluted 250 times before analysis, guaranteeing that ILs are dissociated in aqueous media. The partition coefficient (K_{IL}) of each IL was determined using equation 4:

$$K_{IL} = \frac{[IL]_{PPG}}{[IL]_{PEG}} \quad (Eq. 4)$$

where $[IL]_{PPG}$ and $[IL]_{PEG}$ represent, respectively, the concentrations (M) of each IL in the top and bottom phases. Top and bottom phases correspond, respectively, to the PPG- and PEG-rich phases in all investigated systems. The K_{IL} values result from the average of two independent measurements with the respective standard deviations.

Table 1 - List of names, acronyms, chemical structures and suppliers of ILs and polymers investigated in this work.

	Polymers	
Name/Acronym	Polyethylene glycol (PEG) 600	Polypropylene glycol (PPG) 400
Chemical Structure		
Supplier	Alfa Aesar (Massachusetts, USA)	Merck/Sigma-Aldrich (St. Louis, Missouri, USA)

	Imidazolium-based ILs				
Name/Acronym	[C$_2$mim]Cl 1-Ethyl-3-methylimidazolium chloride	[C$_4$mim]Cl 1-Butyl-3-methylimidazolium chloride	[C$_4$mim]Br 1-Butyl-3-methylimidazolium bromide	[C$_4$mim][CH$_3$COO] 1-Butyl-3-methylimidazolium acetate	[C$_6$mim]Cl 1-Hexyl-3-methylimidazolium chloride
Chemical Structure					
Purity (wt%)	98	99	98	97	98
Supplier	Iolitec® (Ionic Liquid Technologies, Germany)	Iolitec® (Ionic Liquid Technologies, Germany)	Iolitec® (Ionic Liquid Technologies, Germany)	Iolitec® (Ionic Liquid Technologies, Germany)	Iolitec® (Ionic Liquid Technologies, Germany)

	Cholinium-based IL	Ammonium-based IL	Pyrrolidinium-based IL	Piperidinium-based IL
Name/Acronym	[Ch]Cl Cholinium chloride	[N$_{4444}$]Cl Tetrabutylammonium chloride	[C$_4$mpyr]Cl 1-Butyl-1-methylpyrrolidinium chloride	[C$_4$mpip]Cl 1-Butyl-1-methylpiperidinium chloride
Chemical Structure				
Purity (wt%)	98	98	99	99
Supplier	Acros Organics (New Jersey, USA)	Merck/Sigma-Aldrich (St. Louis, Missouri, USA)	Iolitec® (Ionic Liquid Technologies, Germany)	Iolitec® (Ionic Liquid Technologies, Germany)

3. RESULTS AND DISCUSSION:

3.1. Production and recovery of IFNα-2b:

The purification of IFNα-2b from recombinant bacterial lysates is herein addressed due to this protein potential as a major biopharmaceutical currently employed in the treatment of several human diseases [1]. Considering that *E. coli* continues to be a major host for manufacturing biopharmaceutical proteins [1], and the high product titers delivered by *E. coli* inclusion bodies [7], it is of utmost importance to develop protein purification strategies from inclusion bodies.

The manufacture of IFNα-2b is commonly performed using *E. coli*, for which distinct operational parameters such as medium composition, inducer concentration and period of induction influencing the production of IFNα-2b should be initially optimized. After testing different culture media, it was observed that the production of monomeric IFNα-2b was maximized using the SOB medium, mainly in the form of inclusion bodies at 37 °C and 250 rpm. The time growth profile of IFNα-2b in the SOB medium was evaluated by SDS-PAGE (Fig. S2 in the Supplementary Information), whereas the densitometric analysis of the band corresponding to the target protein (molecular weight of approximately 19 kDa) revealed that higher IFNα-2b levels are achieved after 3 h of induction. Accordingly, the production of IFNα-2b was then performed in SOB medium with 1 mM IPTG and the induction carried out during 3 h, conditions that were kept constant in all further investigations.

Fractionation of recombinant *E. coli* into soluble and insoluble fractions after lysis with glass beads showed that IFNα-2b preferentially accumulates as inclusion bodies; this profile is not significantly changed using distinct concentrations of inducer or temperatures (data not shown). The preferential accumulation of IFNα-2b as inclusion bodies in *E. coli*-based systems is in good agreement with previous reports [4, 5] and

requires additional processing steps envisaging the isolation and solubilization of the target protein. Considering the works developed by Prazeres [4] and Mukherjee [5] research groups on the solubilization of IFNα-2b from inclusion bodies, and general guidelines provided by Palmer and Wingfield [44], an optimized strategy towards the efficient solubilization of IFNα-2b was here adopted. The designed protocol includes two sequential steps with 1% (v/v) Triton X-100 and 4 M urea (in 10 mM Tris, pH 8), followed by solubilization with 8 M urea pH 12.5. Each of these fractions was subsequently analysed by SDS-PAGE and western blot to evaluate if there are losses of the target protein in the washing steps and if IFNα-2b is efficiently solubilized. These results are shown in Fig. 2. Some protein impurities are removed in the designed washing steps, but no losses of IFNα-2b occur. Moreover, immunologically active IFNα-2b was identified in the fraction solubilized with the alkaline urea buffer, with a molecular weight of approximately 19 kDa.

Based on the obtained results, the production of IFNα-2b was set up in the following conditions: *E. coli* cultivation at 37 °C and 250 rpm in shake-flasks containing SOB medium using 1 mM IPTG, and an induction period of 3 h. The inclusion body fraction was solubilized with 8 M urea in an alkaline buffer, which was subsequently dialyzed to remove urea against Tris buffer pH 8, constituting this sample the one used in ATPS.

3.2. ATPS phase diagrams:

To use ATPS as an extraction/purification strategy it is of utmost importance to define their monophasic and biphasic regions by the determination of the respective phase diagrams. Considering that the addition of ILs as adjuvants in ATPS can influence not only the partition of proteins but also the extent of the biphasic region, the binodal curves for quaternary (PEG 600 + PPG 400 + 10mM Tris pH 8 + 5 wt% IL) and ternary (same

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phase-forming components but without IL) systems were first determined at 25 (± 1) °C and atmospheric pressure. The respective phase diagrams in an orthogonal representation are shown in Fig. 1. Detailed experimental data and the corresponding regression parameters obtained by Eq. (1) are reported in Table S1 in the Supplementary Information.

All mixture compositions above each binodal curve shown in Fig. 1 result in the formation of a biphasic regime, in which the closest to the axes the curve is, the lower the amount of phase-forming components required for two-phase formation. The ability of ILs as adjuvants to induce the formation of two-phase systems follows the trend: [Ch]Cl > [C$_2$mim]Cl ≈ [C$_4$mim][CH$_3$COO] > [C$_6$mim]Cl ≈ [C$_4$mim]Cl > [C$_4$mim]Br > [N$_{4444}$]Cl > No IL adjuvant. Overall, the presence of all ILs increase the two-phase formation ability. The predicted ILs hydrogen bond acidity (α) of [N$_{4444}$]Cl, [C$_4$mim]Cl and [Ch]Cl are, respectively, 0.423, 0.986 and 1.555 [45, 49, 50], which are grouped in increasing order of hydrophilicity. According to the data shown in Fig. 1A, there is a good correlation between the ability of each IL cation to donate protons and to establish hydrogen-bonds and their ability to induce the formation of two phases. On the other hand, the predicted hydrogen-bond basicity (β) of [C$_4$mim$^+$]-based ILs paired with bromide, chloride, and acetate anions are, respectively, 0.87, 0.95 and 1.20 [50]. By being the most hydrophilic IL, the acetate-based IL is the one with the highest impact in the formation of two phases. As verified with the IL cation effect, the higher the ability of the IL anion to accept protons and establish hydrogen-bonds, the higher the IL salting-out ability and capacity to create two-phase systems (Fig. 1B). Although with a different anion, the predicted hydrogen bond acidity (α) of bis(trifluoromethylsulfonyl)imide-([NTf2]$^-$)-based ILs paired with 1-ethyl-3-methylimidazolium-, 1-butyl-3-methylimidazolium- and 1-hexyl-3-methylimidazolium-cations was reported to be, respectively, 0.750, 0.692 and 0.659 [49],

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allowing to address the IL cation effect to donate protons. As discussed above, also when analysing the IL cation alkyl side chain length, there is a common trend between the IL cation hydrogen-bond acidity and ability to create ABS (Fig. 1C).

Based on the exposed, the effect of ILs on the PEG-PPG-water binodal curves follows the IL ions capacity to accept or donate protons (measured as a function of α and β values), and thus to establish hydrogen-bonds, which may occur with water or the –OH terminal groups of both polymers. An increase of the ILs' capacity to accept or donate protons also correlates with the ILs partitioning to the PEG-rich phase, the phase where ILs are preferentially enriched (data given in the Fig. S3 in Supplementary Information). As a consequence, the differences in hydrophobicity of both ATPS phases are enhanced by using ILs with higher hydrogen-bond donor and acceptor characteristics, resulting in increased phase separation of the PEG-PPG systems herein investigated.

Previously, it was reported that ILs are preferentially partitioned towards the PEG-rich phase when applied as adjuvants in PEG 600-Na$_2$SO$_4$ [51] and PEG 400-(NH$_4$)$_2$SO$_4$ ATPS [45], the most hydrophobic phase in such systems. In these reports, an increase of the ILs' hydrophobic nature leads to a lower affinity for water, thus enhancing phase separation [45, 51]. The results obtained in this work are in good agreement with previous reports since ILs preferentially partition to the PEG-rich phase, and the higher the partition extent the higher is the phase separation ability. However, in the current work, the PEG-rich phase is the most hydrophilic (when compared to the PPG-rich phase and not with a salt-rich phase as shown in the literature [45, 51]), and the opposite was observed. ILs with a higher hydrophilic nature, reflected by higher hydrogen-bond basicity and hydrogen-bond acidity values, are those that lead to an easier phase separation, i.e. requiring lower amounts of phase-forming components (PEG and PPG) to create ATPS.

The feasibility of PPG 400 and PEG 600 to form ATPS with some ILs used in this work was previously investigated [52-55]. Such studies demonstrated that more hydrophobic ILs such as [N$_{4444}$]Cl or [C$_6$mim]Cl present a low capability to dehydrate moderately hydrophobic polymers such as PPG 400, and hence no ATPS could be obtained using such ILs and PPG 400. Moreover, and although [C$_4$mim]Cl, [C$_4$mim]Br, and [C$_4$mim][CH$_3$COO] are able to form ATPS with PPG 400, no ATPS are formed at the concentrations applied in the current work (5 wt%) [55]. With respect to PEG and at the concentrations of ILs applied in this work (5 wt%), a monophasic region will be obtained with PEG 1000-[C$_4$mim]Cl [52], PEG 1500-[C$_2$mim]Cl [53], and PEG 600-[Ch]Cl [54]. It has been additionally shown that PEGs with a molecular weight lower than 3400 g mol^{-1} are not able to form two-phases at 25 °C with protic ILs [56]. Overall, the results and trends reported in the literature point out that at the concentrations of ILs applied (5 wt%) in the current work, such compounds will not form two-phases with PPG 400 or PEG 600.

3.3. IFNα-2b purification using polymer-polymer aqueous two-phase systems using ILs as adjuvants:

PEG and PPG were investigated as phase-forming components of ATPS aiming the purification of IFNα-2b from *E. coli* inclusion bodies. The biocompatible behaviour displayed by such polymers coupled with their low molecular weight (600 and 400 g mol^{-1}, respectively for PEG and PPG) allows to have relatively low viscous phases. On the other hand, the use of ILs as adjuvants in polymer-polymer ATPS represents a more amenable environment in which the effect of the ILs is less masked by salting-out effects occurring in the previously studied salt-polymer-based ATPS [31-35].

The purification of IFNα-2b was investigated using a common mixture point in ATPS: PEG 600 at 30 wt% + PPG at 30 wt% + IL at 5 wt%. In general, an interfacial

precipitate corresponding to precipitated proteins was formed in all systems (Fig. S4 in the Supplementary Information), fitting within the three-phase partitioning approach previously applied for the purification of proteins [57] and antibodies [58]. Contrarily to these reports, the interfacial precipitate in the current work is not preferentially enriched in the target protein, but instead in the remaining proteins.

The migration patterns and precipitation of the target protein and protein impurities were evaluated by SDS-PAGE, whose results are shown in Fig. 3 and Figs. S4 and S5 in the Supplementary information. In general, no proteins are found in the top PPG-rich phase, which is the most hydrophobic ATPS phase. Instead, both IFNα-2b and protein impurities preferentially migrate to the bottom PEG-rich phase or precipitate at the interface, in which the latter is most enriched in protein impurities. The SDS-PAGE profile of the initial sample (Fig. 3) shows that IFNα-2b appears as a 19 kDa band (identity confirmed by western-blot in Fig. 2). Moreover, multiple bands with higher molecular weights corresponding to *E. coli* endogenous proteins demonstrate the evident complexity of the starting sample and the inherent difficulty to separate IFNα-2b from proteins with similar properties.

Without the addition of an adjuvant, IFNα-2b migrates to the PEG-rich phase (Fig. 3), although some target protein precipitates at the interface (Fig. S4 in the Supplementary Information). When ILs are added as adjuvants, it is seen that more hydrophobic and higher volume ILs, such as [N$_{4444}$]Cl, lead to a lower recovery yield of IFNα-2b in the bottom phase (Fig. 3). However, good recoveries of IFNα-2b in the PEG-rich phase were observed using the investigated imidazolium-, ammonium-, piperidinium-, and pyrrolidinium-based ILs, outperforming the system without the addition of adjuvant (Fig. 3). Overall and in all systems, the PEG-rich phase was identified as the ATPS phase promoting the highest recoveries of IFNα-2b in a more purified form.

The SDS-PAGE analysis carried out provided useful insights about the purification performance of the investigated PEG-PPG systems. However, the need of an acetone precipitation step and subsequent dissolution in a specific buffer may not guarantee that all proteins are equally precipitated, hence preventing an accurate evaluation of the proteins partition behavior and precipitation. Therefore, ELISA was used to quantify the levels of biologically active IFNα-2b in each ATPS phase and BCA was used to determine the total proteins content in all samples. Based on these assays, the IFNα-2b purification factors and extraction efficiencies in the PEG-rich phase were determined, allowing to evaluate the purification performance of ATPS with distinct ILs as adjuvants. The overall performance of PEG-PPG ATPS towards the purification of IFNα-2b is shown in Fig. 4, being represented the purification factors and extraction efficiencies of the target protein in the bottom (PEG-rich) phase. In general, good extraction efficiencies (EE, %) towards the PEG-rich phase were obtained ranging from 87% (for the ATPS constituted by [N$_{4444}$]Cl) to 95% (for the ATPS composed of [C$_4$mim]Cl or [Ch]Cl), indicating that the majority of IFNα-2b is present in the PEG-rich phase.

The obtained results show that the addition of adjuvants significantly improves the purity of IFNα-2b recovered in the bottom phase, which is mainly due to the selective precipitation of the remaining proteins at the ATPS interface. Furthermore, the trend in increased purification performance closely follows the ILs enrichment in the PEG-rich phase (cf. Fig. S3 in the Supplementary Information with the partition coefficients of chloride-based ILs). In summary, the higher the IL content in the PEG-rich phase, the higher the purification factor obtained for IFNα-2b. Overall, ILs composed of an aromatic cation, such as imidazolium-based, are those that lead to a higher separation performance, i.e. by inducing the selective precipitation of the remaining proteins at the interface. On

the other hand, higher purification factors are obtained with ILs containing ethyl and butyl side chains at the imidazolium cation, indicating that an increase in the alkyl side chain length of the IL cation slightly decreases the IFNα-2b purification factor (Fig. 4). Finally, ILs containing anions with higher hydrogen-bond basicity, namely [C$_4$mim][CH$_3$COO] and [C$_4$mim]Cl, are those that lead to higher purification factors. As shown in Fig. 4, the application of 5 wt% of [C$_4$mim][CH$_3$COO] as an adjuvant in PEG-PPG ATPS leads to the highest purification factor obtained, representing an increase by a factor of 7 over the initial sample, and allowing an improvement of the purification factor of 3.5 in comparison with the system without IL as adjuvant. These results clearly support the ILs designer solvents aptitude and capacity to tailor the phase's polarities and characteristics aiming at improving the separation of proteins from biological media.

Given the best results obtained with [C$_4$mim][CH$_3$COO] as an adjuvant at 5 wt% in PEG-PPG ATPS, distinct concentrations (2.5 and 12.5 wt%) of this IL were further evaluated. However, no improvements in the purification factors were accomplished (Fig. S6 in the Supplementary Information). Accordingly, in addition to the quantity of IL in the bottom phase which seems to play a relevant effect as appraised by the IL partition coefficients discussed above, the chemical structure of each IL seems to play a more significant role to improve the studied ATPS separation performance.

All ILs have a positive charge center, allowing the establishment of electrostatic interactions with the target protein, which is negatively charged at pH (8) (IFNα-2b pI = 5.9 [5]). Although it has been reported that NaCl increases the purification factor of IFNα-2b in alcohol-salt [36] and polymer-salt [37] ATPS, it should be remarked that ILs additionally allow a range of non-covalent interactions to be established with the target protein, including electrostatic, hydrophobic, π-π, cation-π, and hydrogen-bonding interactions that can be exploited towards the development of enhanced IFNα-2b

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purification processes. Taking into account the results obtained and ILs chemical structures, and in addition to electrostatic interactions common to all ILs, the enrichment of IFNα-2b in the PEG-phase containing ILs as adjuvants seems to be driven by aromatic interactions between the aromatic residues of IFNα-2b and aromatic IL cations and hydrogen bonding interactions established between IL anions and IFNα-2b.

3.4. Stability of purified IFNα-2b:

Western blot analysis of each phase of representative ATPS in the absence and presence of 5 wt% of [C$_4$mim][CH$_3$COO] allowed the identification of a single band with a molecular weight of approximately 19 KDa (Fig. 5), corresponding to immunologically active IFNα-2b, being in good agreement with the IFNα-2b standard. Moreover, no dimers of the target protein were detected, indicating that with the purification strategy herein optimized, no protein aggregation was observed. Concerning the PPG-rich phases of both ATPS, no IFNα-2b was detected, as with the SDS-PAGE profiles of these phases (Fig. S5 in the Supplementary Information). On the other hand, IFNα-2b was detected in the precipitates of both systems, indicating that some losses of the target protein occur in the precipitate, in agreement with the SDS-PAGE profile (Fig. S4 in the Supplementary Information).

Circular dichroism (CD) spectroscopy was used to infer if the purification process impacts the secondary structure of the target protein by comparison with a commercial IFNα-2b standard. The CD spectra of the bottom phase (dialyzed against PBS) isolated from an ATPS composed of 30 wt% PEG 600, 30 wt% PPG 400 and 5 wt% [C$_4$mim]Cl and that of the standard are shown in Fig. S7 in the Supplementary Information. Considering the CD spectra of IFNα-2b previously reported in the literature [59] and of the protein standard, two minimum ellipticity peaks are observed at 208 and 222 nm, which are typical of alpha-helix proteins as is the case of IFNα-2b. In general, no major

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differences were obtained between the dialyzed bottom phase of the ATPS investigated and the IFNα-2b standard, indicating that the secondary structure of the target recombinant protein is not significantly changed and remains stable during the ATPS/TPP purification step.

Although previous reports have shown that imidazolium-based ILs could destabilize and/or impair the biological activity of enzymes and proteins [60, 61], the imidazolium-based ILs herein investigated, at least when used as adjuvants at 5 wt% in PEG-PPG ATPS, do not lead to losses of stability of the target protein. Unlike the application of ILs as phase-forming components in polymer/salt-IL ATPS, the strategy herein adopted with ILs acting as adjuvants employ lower concentrations of ILs, thus contributing to more amenable and less expensive strategies.

4. CONCLUSIONS:

The purification of IFNα-2b as a major protein biopharmaceutical using polymer-polymer ATPS and ILs as adjuvants was attempted in this work. The IFNα-2b upstream stage consisted of *E. coli* BL21 (DE3) cultivation with the target protein accumulated as inclusion bodies, followed by *E. coli* lysis with glass beads and inclusion bodies solubilization with a buffer containing urea and at alkaline pH. After the phase diagrams determination, ATPS were then investigated for IFNα-2b purification by enriching IFNα-2b in the PEG-rich phase, with the simultaneous precipitation of the remaining proteins at the interface (fitting within the three-phase partitioning approach). Overall, ILs present in higher quantities in the PEG-rich phase, being those composed of aromatic cations and anions with high hydrogen-bond basicity, allow to obtain higher IFNα-2b purification factors. Accordingly, electrostatic, π-···π and hydrogen-bond interactions seem to be the main forces ruling the target protein selective partitioning to the PEG-rich phase, where ILs are preferentially partitioned. The IFNα-2b recovered in the bottom PEG-rich phase was shown to be immunologically active, with its secondary structure preserved. In summary, this work reinforces the potential effect brought by the use of ILs in small concentrations as adjuvants in PEG-PPG ATPS, by enhancing the purity of high-value proteins such as IFNα-2b.

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6. FIGURES LEGENDS:

Fig. 1 - Phase diagrams at 25 (± 1) °C and atmospheric pressure for systems composed of PPG 400, PEG 600 and 10 mM Tris pH 8 in the absence and presence of 5 wt% of the following ILs as adjuvants: [C$_4$mim]Cl; [C$_4$mim]Br; [C$_4$mim][CH$_3$COO]; [C$_2$mim]Cl; [C$_6$mim]Cl; [Ch]Cl; [N$_{4444}$]Cl. Phase diagrams were organized according to the chemical structure of IL, namely according to the type of IL cation, the type of IL anion and the impact of the length of the alkyl side chain, respectively, A, B, and C.

Fig. 2 - SDS-PAGE and western-blot analysis of fractions obtained during the solubilization of inclusion bodies from recombinant *E. coli* BL21 (DE3) harboring pET-3a_ IFNα-2b.

Fig. 3 - SDS-PAGE analysis of representative bottom phases from ATPS composed of PEG 600 at 30 wt% + PPG 400 at 30 wt% in the presence and absence of 5 wt% of IL.

Fig. 4 - Purification factors (blue bars) and extraction efficiencies (black dots) of IFNα-2b recovered in the bottom phase of ATPS composed of 30 wt% of PEG 600 and 30 wt% of PPG 400 in the absence and presence of 5 wt% of ILs.

Fig. 5 - Western blot analysis of the phases of representative ATPS (30 wt% of PEG 600 and 30 wt% of PPG 400) investigated in the absence (A) and presence of 5 wt% of [C$_4$mim][CH$_3$COO] (B). A commercial IFNα-2b standard is included for comparison.

7. REFERENCES:

[1] G. Walsh, Biopharmaceutical benchmarks 2018, Nat. Biotechnol. 36 (2018) 1136–1145. https://doi.org/10.1038/nbt.4305.

[2] T.W. Overton, Recombinant protein production in bacterial hosts, Drug Discov. Today. 19 (2014) 590–601. https://doi.org/10.1016/j.drudis.2013.11.008.

[3] R. Asmana Ningrum, Human Interferon Alpha-2b: A Therapeutic Protein for Cancer Treatment, Scientifica (Cairo). 2014 (2014) 1–8. https://doi.org/10.1155/2014/970315.

[4] C.A. Valente, G.A. Monteiro, J.M.S. Cabral, M. Fevereiro, D.M.F. Prazeres, Optimization of the primary recovery of human interferon α2b from Escherichia coli inclusion bodies, Protein Expr. Purif. 45 (2006) 226–234. https://doi.org/10.1016/j.pep.2005.06.014.

[5] P. Srivastava, P. Bhattacharaya, G. Pandey, K.J. Mukherjee, Overexpression and purification of recombinant human interferon alpha2b in Escherichia coli, Protein Expr. Purif. 41 (2005) 313–322. https://doi.org/10.1016/j.pep.2004.12.018.

[6] C. Slouka, J. Kopp, S. Hutwimmer, M. Strahammer, D. Strohmer, E. Eitenberger, A. Schwaighofer, C. Herwig, Custom made inclusion bodies: Impact of classical process parameters and physiological parameters on inclusion body quality attributes, Microb. Cell Fact. 17 (2018) 1–15. https://doi.org/10.1186/s12934-018-0997-5.

[7] C. Slouka, J. Kopp, O. Spadiut, C. Herwig, Perspectives of inclusion bodies for bio-based products: curse or blessing?, Appl. Microbiol. Biotechnol. 103 (2019) 1143–1153. https://doi.org/10.1007/s00253-018-9569-1.

[8] S. Jevševar, V. Gaberc-Porekar, I. Fonda, B. Podobnik, J. Grdadolnik, V. Menart, Production of nonclassical inclusion bodies from which correctly folded protein can be extracted, Biotechnol. Prog. 21 (2005) 632–639. https://doi.org/10.1021/bp0497839.

[9] D.G. Bracewell, C.M. Smales, The challenges of product- and process-related impurities to an evolving biopharmaceutical industry, Bioanalysis. 5 (2013) 123–126. https://doi.org/10.4155/bio.12.314.

[10] A.T. Hanke, M. Ottens, Purifying biopharmaceuticals: Knowledge-based chromatographic process development, Trends Biotechnol. 32 (2014) 210–220. https://doi.org/10.1016/j.tibtech.2014.02.001.

[11] L. Shi, D. Wang, W. Chan, L. Cheng, Efficient expression and purification of human interferon alpha2b in the methylotrophic yeast, Pichia pastoris, Protein Expr. Purif. 54 (2007) 220–226. https://doi.org/10.1016/j.pep.2007.03.005.

[12] A. Beldarraín, Y. Cruz, O. Cruz, M. Navarro, M. Gil, Purification and conformational properties of a human interferon α2b produced in Escherichia coli, Biotechnol. Appl. Biochem. 33 (2001) 173. https://doi.org/10.1042/BA20010001.

[13] P.A.J. Rosa, A.M. Azevedo, S. Sommerfeld, W. Bäcker, M.R. Aires-Barros, Aqueous two-phase extraction as a platform in the biomanufacturing industry: Economical and environmental sustainability, Biotechnol. Adv. 29 (2011) 559–567. https://doi.org/10.1016/j.biotechadv.2011.03.006.

[14] A.S. Rathore, A. Shirke, Recent Developments in Membrane-Based Separations in Biotechnology Processes: REVIEW, Prep. Biochem. Biotechnol. 41 (2011) 398–421. https://doi.org/10.1080/10826068.2011.613976.

[15] R. van Reis, A. Zydney, Membrane separations in biotechnology, Curr. Opin. Biotechnol. 12 (2001) 208–211. https://doi.org/10.1016/S0958-1669(00)00201-9.

[16] C.S.G. Gomes, A. Fashina, A. Fernández-Castané, T.W. Overton, T.J. Hobley, E. Theodosiou, O.R.T. Thomas, Magnetic hydrophobic-charge induction adsorbents for the recovery of immunoglobulins from antiserum feedstocks by high-gradient magnetic fishing, J. Chem. Technol. Biotechnol. 93 (2018) 1901–1915. https://doi.org/10.1002/jctb.5599.

[17] R. dos Santos, A.L. Carvalho, A.C.A. Roque, Renaissance of protein crystallization and precipitation in biopharmaceuticals purification, Biotechnol. Adv. 35 (2017) 41–50. https://doi.org/10.1016/j.biotechadv.2016.11.005.

[18] A.M. Azevedo, P.A.J. Rosa, I.F. Ferreira, M.R. Aires-Barros, Chromatography-free recovery of biopharmaceuticals through aqueous two-phase processing, Trends Biotechnol. 27 (2009) 240–247. https://doi.org/10.1016/j.tibtech.2009.01.004.

[19] P.-Å. Albertsson, Separation of particles and macromolecules by phase partition, Endeavour. 1 (1977) 69–74. https://doi.org/10.1016/0160-9327(77)90109-0.

[20] M.G. Freire, A.F.M. Cláudio, J.M.M. Araújo, J.A.P. Coutinho, I.M. Marrucho, J.N.C. Lopes, L.P.N. Rebelo, Aqueous biphasic systems: A boost brought about by using ionic liquids, Chem. Soc. Rev. 41 (2012) 4966–4995. https://doi.org/10.1039/c2cs35151j.

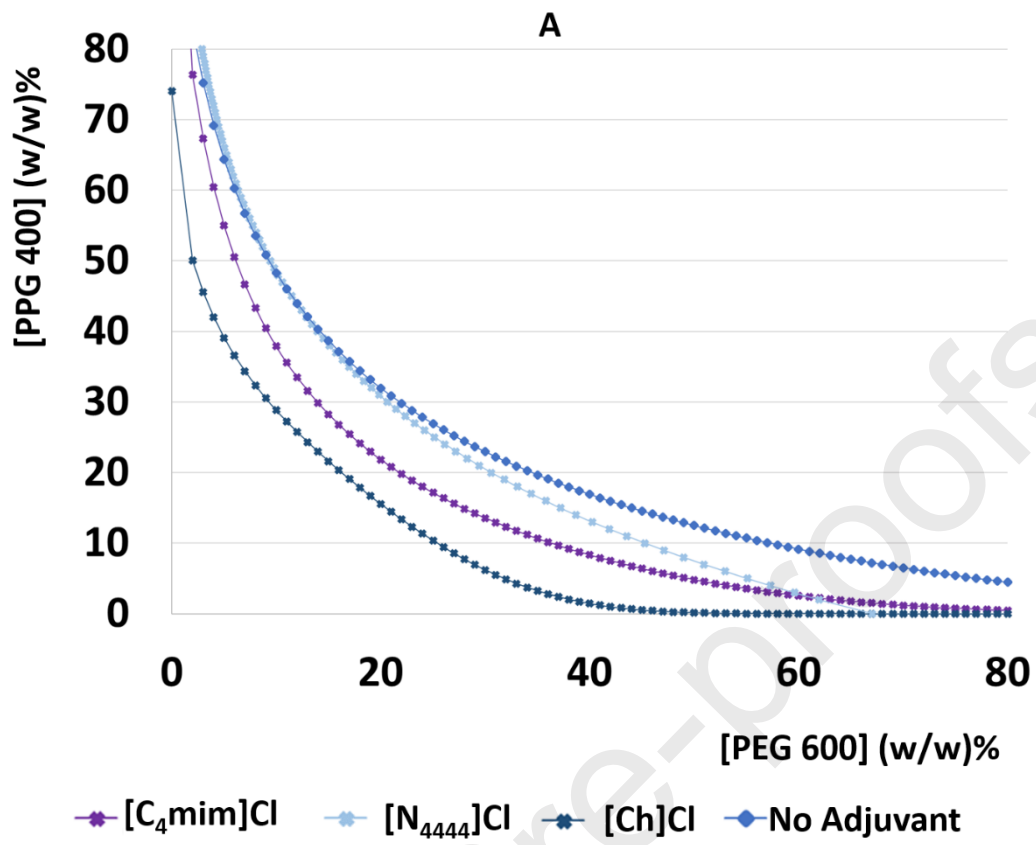[21] M.A. Torres-Acosta, J.F.B. Pereira, M.G. Freire, J.M. Aguilar-Yáñez, J.A.P.

Coutinho, N.J. Titchener-Hooker, M. Rito-Palomares, Economic evaluation of the primary recovery of tetracycline with traditional and novel aqueous two-phase systems, Sep. Purif. Technol. 203 (2018) 178–184. https://doi.org/10.1016/j.seppur.2018.04.041.

[22] S.G. Walker, A. Lyddiatt, Aqueous two-phase systems as an alternative process route for the fractionation of small inclusion bodies, J. Chromatogr. B Biomed. Sci. Appl. 711 (1998) 185–194. https://doi.org/10.1016/S0378-4347(97)00604-X.

[23] J.P. Chen, M.S. Lee, Enhanced production of Serratia marcescens chitinase in PEG/dextran aqueous two-phase systems, Enzyme Microb. Technol. 17 (1995) 1021–1027. https://doi.org/10.1016/0141-0229(95)00030-5.

[24] G. Johansson, K. Reczey, Concentration and purification of β-glucosidase from Aspergillus niger by using aqueous two-phase partitioning, J. Chromatogr. B Biomed. Sci. Appl. 711 (1998) 161–172. https://doi.org/10.1016/S0378-4347(97)00601-4.

[25] A.M. Azevedo, P.A.J. Rosa, I.F. Ferreira, J. de Vries, T.J. Visser, M.R. Aires-Barros, Downstream processing of human antibodies integrating an extraction capture step and cation exchange chromatography, J. Chromatogr. B. 877 (2009) 50–58. https://doi.org/10.1016/j.jchromb.2008.11.014.

[26] A. Salabat, R. Sadeghi, S.T. Moghadam, B. Jamehbozorg, Partitioning of l-methionine in aqueous two-phase systems containing poly(propylene glycol) and sodium phosphate salts, J. Chem. Thermodyn. 43 (2011) 1525–1529. https://doi.org/10.1016/j.jct.2011.05.001.

[27] Food and Drug Administration (FDA) Inactive Ingredient Database (IID), (n.d.). https://www.accessdata.fda.gov/scripts/cder/iig/ (Accessed 28 January 2020) (accessed January 28, 2020).

[28] A.A. D'souza, R. Shegokar, Polyethylene glycol (PEG): a versatile polymer for pharmaceutical applications, Expert Opin. Drug Deliv. 13 (2016) 1257–1275. https://doi.org/10.1080/17425247.2016.1182485.

[29] P.A.J. Rosa, A.M. Azevedo, I.F. Ferreira, J. de Vries, R. Korporaal, H.J. Verhoef, T.J. Visser, M.R. Aires-Barros, Affinity partitioning of human antibodies in aqueous two-phase systems, J. Chromatogr. A. 1162 (2007) 103–113. https://doi.org/10.1016/j.chroma.2007.03.067.

[30] A. Azevedo, P. Rosa, I.F. Ferreira, A. Oliveira Pisco, J. Vries, R. Korporaal, T. Visser, M. Aires-Barros, Affinity-enhanced purification of human antibodies by
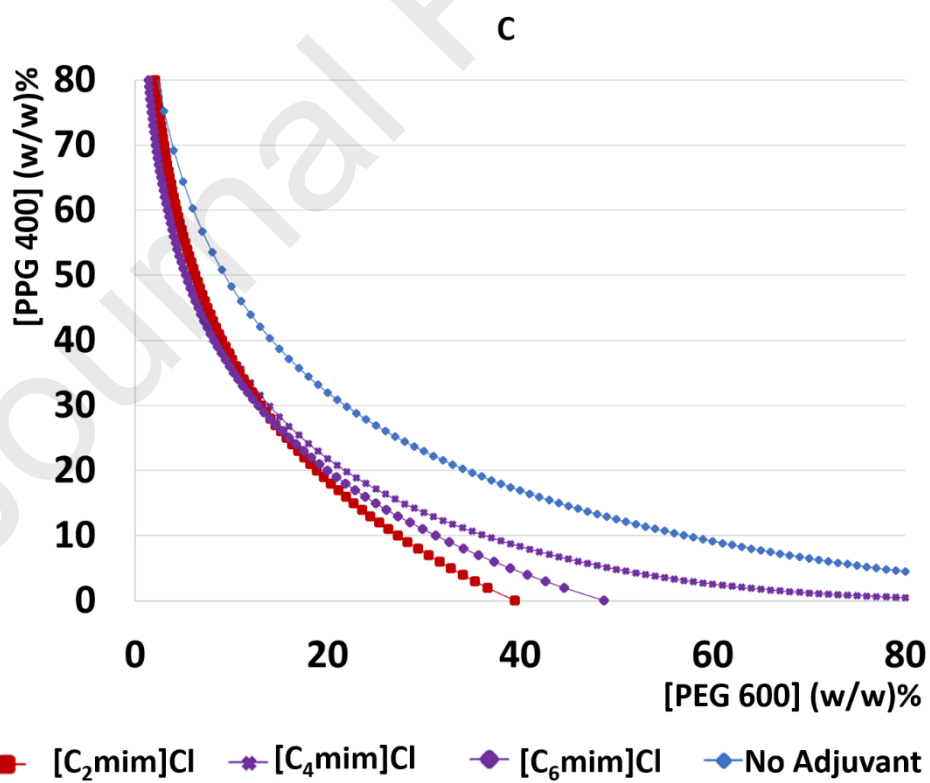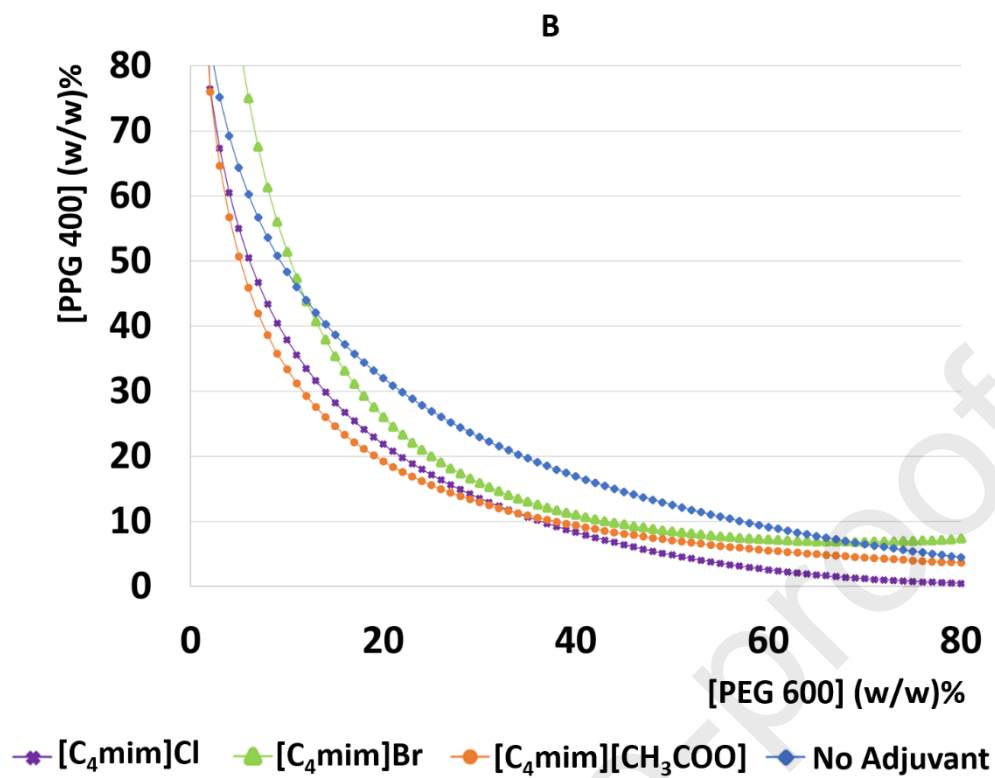
aqueous two-phase extraction, Sep. Purif. Technol. 65 (2009) 31–39. https://doi.org/10.1016/j.seppur.2008.03.006.

[31]   J.H.P.M. Santos, J.C. Flores-Santos, G.P. Meneguetti, C.O. Rangel-Yagui, J.A.P. Coutinho, M. Vitolo, S.P.M. Ventura, A. Pessoa, In situ purification of periplasmatic L-asparaginase by aqueous two phase systems with ionic liquids (ILs) as adjuvants, J. Chem. Technol. Biotechnol. 93 (2018) 1871–1880. https://doi.org/10.1002/jctb.5455.

[32]   R.L. Souza, S.P.M. Ventura, C.M.F. Soares, J.A.P. Coutinho, Á.S. Lima, Lipase purification using ionic liquids as adjuvants in aqueous two-phase systems, Green Chem. 17 (2015) 3026–3034. https://doi.org/10.1039/C5GC00262A.

[33]   R. de C.S. Sousa, M.M. Pereira, M.G. Freire, J.A.P. Coutinho, Evaluation of the effect of ionic liquids as adjuvants in polymer-based aqueous biphasic systems using biomolecules as molecular probes, Sep. Purif. Technol. 196 (2018) 244–253. https://doi.org/10.1016/j.seppur.2017.07.018.

[34]   A.M. Ferreira, V.F.M. Faustino, D. Mondal, J.A.P. Coutinho, M.G. Freire, Improving the extraction and purification of immunoglobulin G by the use of ionic liquids as adjuvants in aqueous biphasic systems, J. Biotechnol. 236 (2016) 166–175. https://doi.org/10.1016/j.jbiotec.2016.08.015.

[35]   M. Marchel, K.G. João, I.M. Marrucho, On the use of ionic liquids as adjuvants in PEG-(NH4)2SO4 aqueous biphasic systems: Phase diagrams behavior and the effect of IL concentration on myoglobin partition, Sep. Purif. Technol. 210 (2019) 710–718. https://doi.org/10.1016/j.seppur.2018.08.058.

[36]   Y.K. Lin, C.W. Ooi, J.S. Tan, P.L. Show, A. Ariff, T.C. Ling, Recovery of human interferon alpha-2b from recombinant Escherichia coli using alcohol/salt-based aqueous two-phase systems, Sep. Purif. Technol. 120 (2013) 362–366. https://doi.org/10.1016/j.seppur.2013.09.038.

[37]   Y.K. Lin, C.W. Ooi, R.N. Ramanan, A. Ariff, T.C. Ling, Recovery of Human Interferon Alpha-2b from Recombinant Escherichia coli by Aqueous Two-Phase System, Sep. Sci. Technol. 47 (2012) 1023–1030. https://doi.org/10.1080/01496395.2011.644018.

[38]   K.W. Chew, T.C. Ling, P.L. Show, Recent Developments and Applications of Three-Phase Partitioning for the Recovery of Proteins, Sep. Purif. Rev. 48 (2019) 52–64. https://doi.org/10.1080/15422119.2018.1427596.

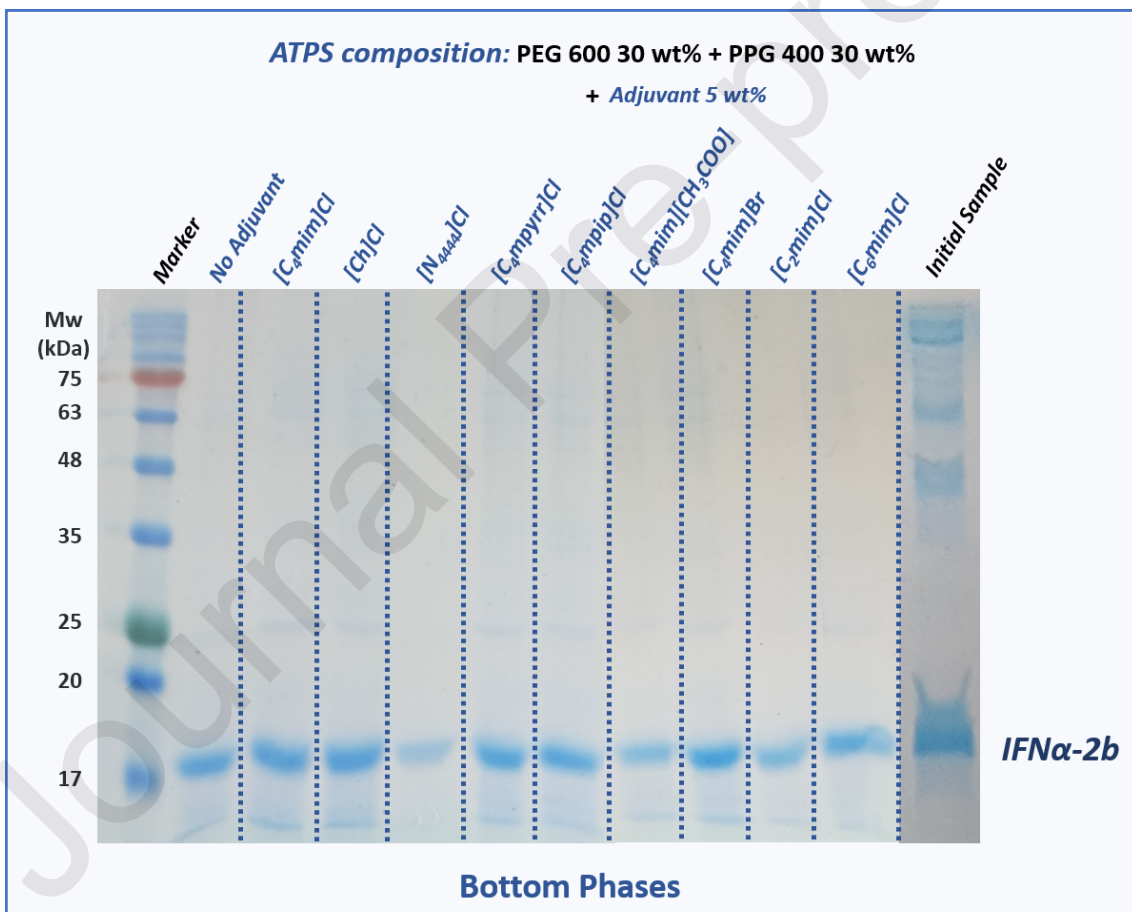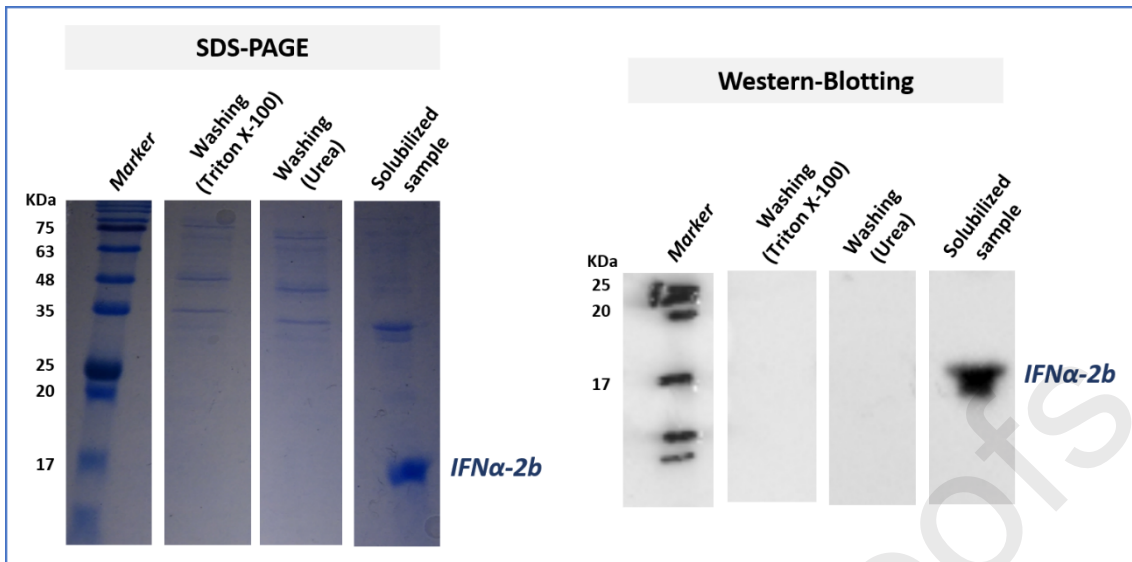[39]   S. Raghava, B. Barua, P.K. Singh, M. Das, L. Madan, S. Bhattacharyya, K. Bajaj,

B. Gopal, R. Varadarajan, M.N. Gupta, Refolding and simultaneous purification by three-phase partitioning of recombinant proteins from inclusion bodies, Protein Sci. 17 (2008) 1987–1997. https://doi.org/10.1110/ps.036939.108.

[40]   E. Alvarez-Guerra, A. Irabien, Ionic Liquid-Based Three Phase Partitioning (ILTPP) for Lactoferrin Recovery, Sep. Sci. Technol. 49 (2014) 957–965. https://doi.org/10.1080/01496395.2013.878722.

[41]   E. Alvarez-Guerra, A. Irabien, Ionic liquid-based three phase partitioning (ILTPP) systems for whey protein recovery: Ionic liquid selection, J. Chem. Technol. Biotechnol. 90 (2015) 939–946. https://doi.org/10.1002/jctb.4401.

[42]   M. Iqbal, Y. Tao, S. Xie, Y. Zhu, D. Chen, X. Wang, L. Huang, D. Peng, A. Sattar, M.A.B. Shabbir, H.I. Hussain, S. Ahmed, Z. Yuan, Aqueous two-phase system (ATPS): an overview and advances in its applications, Biol. Proced. Online. 18 (2016) 18. https://doi.org/10.1186/s12575-016-0048-8.

[43]   A.Q. Pedro, D. Oppolzer, M.J. Bonifácio, C.J. Maia, J.A. Queiroz, L.A. Passarinha, Evaluation of MutS and Mut+ Pichia pastoris Strains for Membrane-Bound Catechol-O-Methyltransferase Biosynthesis, Appl. Biochem. Biotechnol. 175 (2015) 3840–3855. https://doi.org/10.1007/s12010-015-1551-0.

[44]   I. Palmer, P.T. Wingfield, Preparation and extraction of insoluble (Inclusion-body) proteins from Escherichia coli, Curr. Protoc. Protein Sci. 1 (2012) 1–18. https://doi.org/10.1002/0471140864.ps0603s70.

[45]   C.M.S.S. Neves, R. de Cássia S. Sousa, M.M. Pereira, M.G. Freire, J.A.P. Coutinho, Understanding the effect of ionic liquids as adjuvants in the partition of biomolecules in aqueous two-phase systems formed by polymers and weak salting-out agents, Biochem. Eng. J. 141 (2019) 239–246. https://doi.org/10.1016/j.bej.2018.10.022.

[46]   A. Glyk, S.L. Heinisch, T. Scheper, S. Beutel, Comparison of colorimetric m ethods for the quantification of model proteins in aqueous two-phase systems, Anal. Biochem. 477 (2015) 35–37. https://doi.org/10.1016/j.ab.2015.02.007.

[47]   U.K. Laemmli, Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, Nature. 227 (1970) 680–685. https://doi.org/10.1038/227680a0.

[48]   D. Botelho, M.J. Wall, D.B. Vieira, S. Fitzsimmons, F. Liu, A. Doucette, Top-down and bottom-up proteomics of sds-containing solutions following mass-based separation, J. Proteome Res. 9 (2010) 2863–2870.
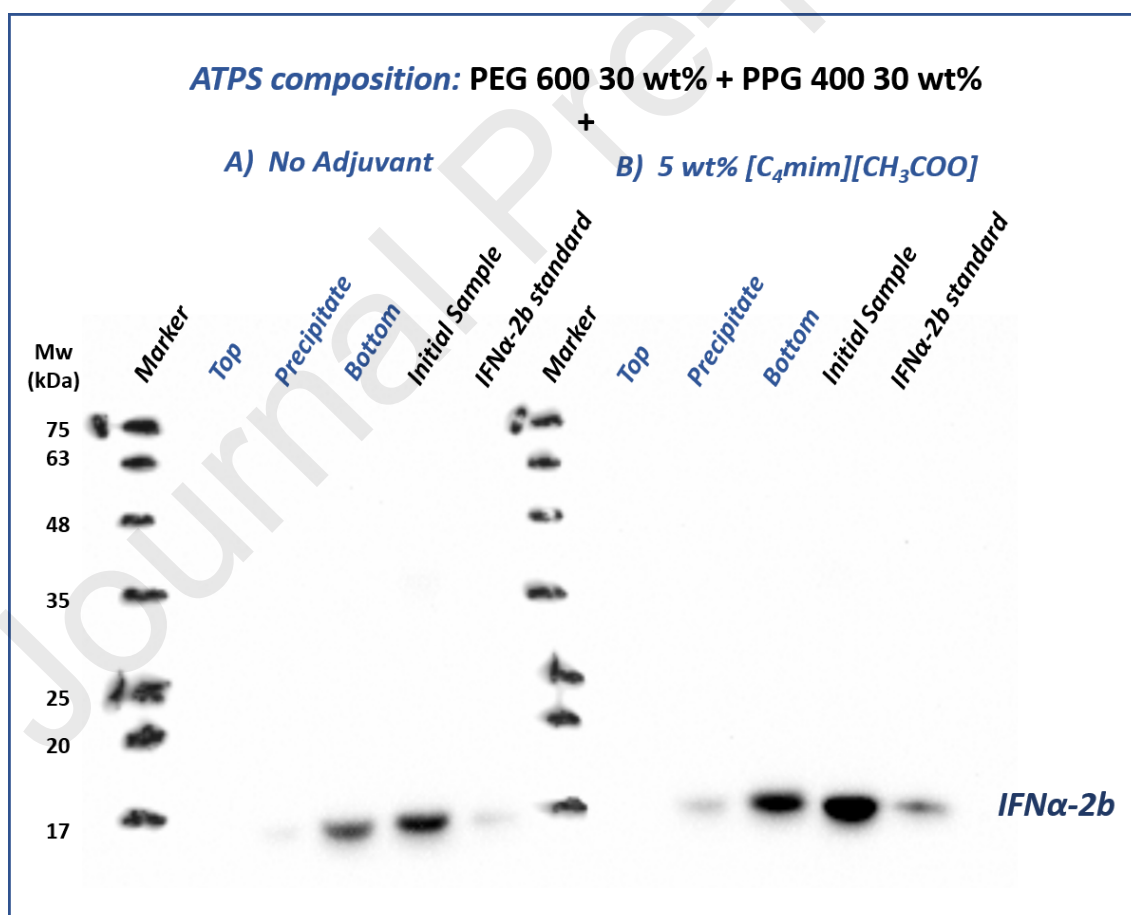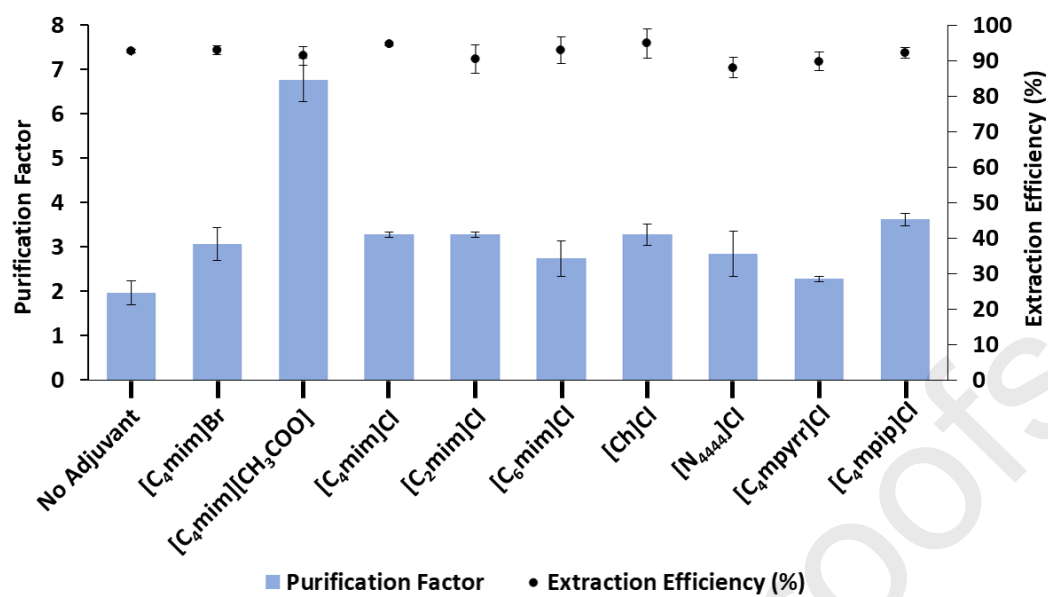
33

https://doi.org/10.1021/pr900949p.

[49]   K.A. Kurnia, F. Lima, A.F.M. Cláudio, J.A.P. Coutinho, M.G. Freire, Hydrogen-bond acidity of ionic liquids: an extended scale, Phys. Chem. Chem. Phys. 17 (2015) 18980–18990. https://doi.org/10.1039/C5CP03094C.

[50]   A.F.M. Cláudio, L. Swift, J.P. Hallett, T. Welton, J.A.P. Coutinho, M.G. Freire, Extended scale for the hydrogen-bond basicity of ionic liquids, Phys. Chem. Chem. Phys. 16 (2014) 6593–6601. https://doi.org/10.1039/c3cp55285c.

[51]   J.F.B. Pereira, Á.S. Lima, M.G. Freire, J.A.P. Coutinho, Ionic liquids as adjuvants for the tailored extraction of biomolecules in aqueous biphasic systems, Green Chem. 12 (2010) 1661. https://doi.org/10.1039/c003578e.

[52]   M.G. Freire, J.F.B. Pereira, M. Francisco, H. Rodríguez, L.P.N. Rebelo, R.D. Rogers, J.A.P. Coutinho, Insight into the interactions that control the phase behaviour of new aqueous biphasic systems composed of polyethylene glycol polymers and ionic liquids, Chem. - A Eur. J. 18 (2012) 1831–1839. https://doi.org/10.1002/chem.201101780.

[53]   J.F.B. Pereira, L.P.N. Rebelo, R.D. Rogers, J.A.P. Coutinho, M.G. Freire, Combining ionic liquids and polyethylene glycols to boost the hydrophobic-hydrophilic range of aqueous biphasic systems, Phys. Chem. Chem. Phys. 15 (2013) 19580–19583. https://doi.org/10.1039/c3cp53701c.

[54]   J.F.B. Pereira, K.A. Kurnia, O.A. Cojocaru, G. Gurau, L.P.N. Rebelo, R.D. Rogers, M.G. Freire, J.A.P. Coutinho, Molecular interactions in aqueous biphasic systems composed of polyethylene glycol and crystalline vs. liquid cholinium-based salts, Phys. Chem. Chem. Phys. 16 (2014) 5723–5731. https://doi.org/10.1039/c3cp54907k.

[55]   C.M.S.S. Neves, S. Shahriari, J. Lemus, J.F.B. Pereira, M.G. Freire, J.A.P. Coutinho, Aqueous biphasic systems composed of ionic liquids and polypropylene glycol: Insights into their liquid-liquid demixing mechanisms, Phys. Chem. Chem. Phys. 18 (2016) 20571–20582. https://doi.org/10.1039/c6cp04023c.

[56]   A.F.M. Cláudio, J.F.B. Pereira, P.D. McCrary, M.G. Freire, J.A.P. Coutinho, R.D. Rogers, A critical assessment of the mechanisms governing the formation of aqueous biphasic systems composed of protic ionic liquids and polyethylene glycol, Phys. Chem. Chem. Phys. 18 (2016) 30009–30019. https://doi.org/10.1039/c6cp06289j.

[57]   R. Garg, B.N. Thorat, Nattokinase purification by three phase partitioning and

impact of t-butanol on freeze drying, Sep. Purif. Technol. 131 (2014) 19–26. https://doi.org/10.1016/j.seppur.2014.04.011.

[58] E. V. Capela, A.E. Santiago, A.F.C.S. Rufino, A.P.M. Tavares, M.M. Pereira, A. Mohamadou, M.R. Aires-Barros, J.A.P. Coutinho, A.M. Azevedo, M.G. Freire, Sustainable strategies based on glycine–betaine analogue ionic liquids for the recovery of monoclonal antibodies from cell culture supernatants, Green Chem. 21 (2019) 5671–5682. https://doi.org/10.1039/C9GC02733E.

[59] M.J.W. Johnston, K. Nemr, M.A. Hefford, Influence of bovine serum albumin on the secondary structure of interferon alpha 2b as determined by far UV circular dichroism spectropolarimetry, Biologicals. 38 (2010) 314–320. https://doi.org/10.1016/j.biologicals.2009.11.010.

[60] R. Madeira Lau, M.J. Sorgedrager, G. Carrea, F. van Rantwijk, F. Secundo, R.A. Sheldon, Dissolution of Candida antarctica lipase B in ionic liquids: effects on structure and activity, Green Chem. 6 (2004) 483. https://doi.org/10.1039/b405693k

[61] P.K. Kumar, I. Jha, P. Venkatesu, I. Bahadur, E.E. Ebenso, A comparative study of the stability of stem bromelain based on the variation of anions of imidazolium-based ionic liquids, J. Mol. Liq. 246 (2017) 178–186. https://doi.org/10.1016/j.molliq.2017.09.037.

B

C

ATPS composition: **PEG 600 30 wt% + PPG 400 30 wt%**
+
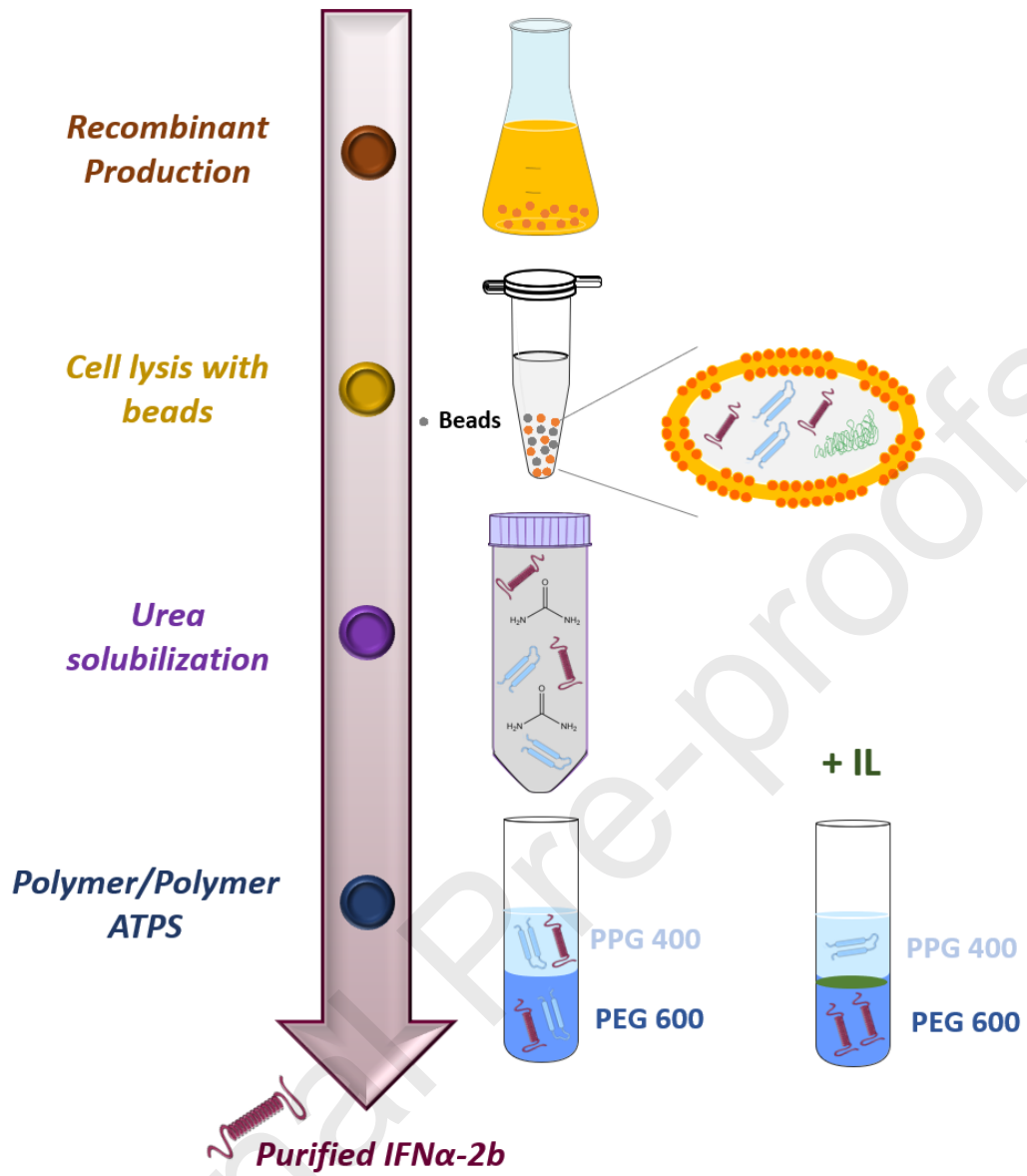**A) No Adjuvant**   **B) 5 wt% [C₄mim][CH₃COO]**

HIGHLIGHTS:

1. IFNα-2b purification improvements are achieved using polymer-polymer ATPS with ILs as adjuvants.

2. The biphasic region of investigated ATPS can be tailored with the ionic liquid chemical structure.

3. IFNα-2b is mainly enriched in the PEG-rich phase, whereas the remaining proteins tend to precipitate at the ATPS interface.

4. ILs with aromatic cations and anions with high hydrogen-bond basicity enhance the IFNα-2b purity.

5. The stability and immunological activity of IFNα-2b is preserved during purification.

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Recombinant Production

Cell lysis with beads

• Beads

Urea solubilization

H₂N—NH₂

Polymer/Polymer ATPS

+ IL

PPG 400

PEG 600

PPG 400

PEG 600

Purified IFNα-2b

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Declaration of interests

☒ 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 paper.

Leonor S. Castro: Investigation, Visualization, Writing - Original draft preparation; **Patrícia Pereira:** Investigation, Validation; **Luís A. Passarinha:** Writing - Reviewing and Editing; **Mara G. Freire:** Conceptualization, Methodology, Supervision, Writing - Reviewing and Editing; **Augusto Q. Pedro:** Conceptualization, Methodology, Investigation, Writing - Reviewing and Editing.