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Sustainable strategies based on glycine-betaine analogues ionic liquids for the recovery of monoclonal antibodies from cell culture supernatants

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Monoclonal antibodies (mAbs) are of crucial interest for therapeutic purposes, particularly in vaccination, immunization, and in the treatment of life-threatening diseases. However, their downstream processing from the complex cell culture media in which they are produced still requires multiple steps, rendering mAbs as extremely high-cost products. Therefore, the development of cost-effective, sustainable and biocompatible purification strategies for mAbs is in high demand to decrease the associated economic, environmental and health burdens. Herein, novel aqueous biphasic systems (ABS) composed of glycine-betaine analogues ionic liquids (AGB-ILs) and K$_2$HPO$_4$/KH$_2$PO$_4$ at pH 7.0, the respective three-phase partitioning (TPP) systems, and hybrid processes combining ultrafiltration, were investigated and compared in terms of performance as alternative strategies for the purification and recovery of anti-interleukin-8 (anti-IL-8) mAbs, which are specific therapeutics in the treatment of inflammatory diseases, from Chinese Hamster Ovary (CHO) cell culture supernatants. With the studied ABS, mAbs preferentially partition to the IL-rich phase, with recovery yields up to 100% and purification factors up to 1.6. The best systems were optimized in what concerns the IL concentration, allowing to take advantage of IL-based three-phase partitioning approaches where a precipitate enriched in mAbs is obtained at the ABS interface, yielding 41.0% of IgG with a purification factor of 2.7 (purity of 60.9%). Hybrid processes combining the two previous techniques and an ultrafiltration step were finally applied, allowing the recovery of mAbs from the different fractions in an appropriate buffer solution for further biopharmaceuticals formulation, while allowing the simultaneous IL removal and reuse. The best results were obtained with the hybrid process combining TPP and ultrafiltration, allowing to obtain mAbs with a purity greater than 60%. The recyclability of the IL was additionally demonstrated, revealing no losses on the purification and recovery performance of these systems for mAbs. The biological activity of anti-IL-8 mAbs is maintained after the several purification and recovery steps, indicating that the novel ABS, three-phase partitioning and hybrid processes comprising AGB-ILs are promising and sustainable strategies in mAbs downstream processing.

Introduction

Despite all significant advances that have been accomplished in the development of effective therapies, biopharmaceuticals are in many cases the unique option in the treatment of particular diseases.\textsuperscript{1,2} Amongst biopharmaceuticals, monoclonal antibodies (mAbs) are widely applied for therapeutic purposes, namely in vaccination and immunization, but also in the treatment of oncologic, autoimmune, cardiovascular, inflammatory and neurological diseases.\textsuperscript{1,3} The first therapeutic monoclonal antibody approved, in 1986 by the US Food and Drug Administration (FDA), was Muromonab (Orthoclone OKT3), which is an in vivo produced mAb by hybridoma cells for the prevention of kidney transplant rejection.\textsuperscript{4} By the end of 2017, 57 therapeutic mAbs were approved by FDA and the European Medicines Agency (EMA) for therapeutic purposes.\textsuperscript{5} In 2017, the worldwide mAbs market exceeded US$98 billion in sales, aiming to reach US$ 137-200 billion in 2022.\textsuperscript{6,7} Despite all advantages of mAbs, the high quantities of pure mAbs required and their extremely high manufacturing costs, mainly derived from the multiple downstream processing steps, are the most challenging features limiting their widespread use.\textsuperscript{6} The bioprocessing of mAbs comprises 2 steps: (i) upstream processing, based on biological processes where mAbs production occurs through cell cultures derived from
mammalian cells; and (ii) downstream processing, where the recovery, purification and isolation of mAbs from the complex medium takes place. The upstream processing suffered significant improvements in recent years and the main current bottleneck in mAbs production conveys in the downstream processing. The current downstream platform includes several steps: (i) clarification of the supernatant, (ii) capture of mAbs, (iii) viral inactivation, (iv) mAbs polishing, (v) viral removal, and (vi) concentration/formulation of the final mAb-product. 

Protein A (ProA) affinity chromatography is the “gold standard” approach in the capture and purification steps since it is highly selective. However, it presents several limitations, namely the low pH values necessary to elute mAb-products that can cause their aggregation, the presence of some impurities in the final formulation since the ProA ligand can be degraded by proteases present in the supernatants, and the high cost of the resin (between US$5,000 and US$15,000/L; industrial columns require volumes up to 1000 L). Accordingly, ProA affinity chromatography is the main cost of the mAbs manufacturing process, with the overall downstream processing contributing up to 80% of the mAbs global production costs. Therefore, it is imperative to develop sustainable and cost-effective downstream processes for mAbs to decrease their cost and to allow their widespread use.

Aqueous biphasic systems (ABS) were first proposed as an extraction technique by Albertsson, being typically composed of two polymers, a polymer and a salt or two salts dissolved in aqueous media (ternary systems), which above given concentrations undergo phase separation. Their physicochemical characteristics can be tailored by changing the phase-forming components and their concentration, pH and temperature to profit the selective partitioning of a target compound. One of the major advantages of ABS over other techniques commonly applied to the recovery of mAbs is that the clarification, extraction, purification and concentration steps can be integrated in a single step. Furthermore, ABS are rich in water, do not require the use of volatile organic solvents, and represent a technique easy to scale up and able to operate in continuous mode. Thus, if properly designed, ABS are effective, sustainable and low-cost strategies when compared to the applied chromatographic methods. Traditional ABS formed by polymers have been largely investigated for the purification of IgG (including mAbs). However, the restricted polarity of the coexisting phases presented by this type of systems compromises enhanced recovery yields and purity factors to be achieved, thus encouraging the development of other strategies to overcome this bottleneck. The addition of salts, affinity ligands and the use of modified phase-forming components are some examples in this field. In addition to more traditional polymer-based ABS, ionic-liquid-(IL)-based ABS emerged in more recent years with the pioneering work of Rogers and co-workers, and since then have been investigated as extraction/purification platforms for a plethora of (bio)molecules. By using ILs as phase-forming components of ABS it is possible to tailor the phases’ polarities and affinities to specific bioproducts, overcoming the limited polarity difference presented by traditional polymer-based systems. Given the designer solvents ability of ILs, they can be foreseen as promising phase-forming components to carry out the purification and recovery of mAbs. To the best of our knowledge, there are no reports in the literature considering the mAbs recovery from CHO cell culture supernatants using ILs as phase-forming components of ABS.

Within the field of IL-based ABS, ionic-liquid-based three-phase partitioning (ILTPP) have been investigated for the recovery of proteins, yet never considered for the purification of antibodies. Typically, three-phase partitioning (TPP) approaches involve the recovery of the target protein in an enriched precipitate at the interface of two liquid phases. Alvarez et al. demonstrated the feasibility of this approach using IL-based ABS, proposing the concept of ionic-liquid-based TPP (ILTPP), but still only applied to food proteins and less complex matrices.

Even though ILs present interesting environmental features compared to volatile organic compounds, mainly due to their negligible vapor pressure at ambient conditions, their toxicity and biodegradability should be always considered. In order to overcome these drawbacks, glycine-betaine analogues ILs (AGB-ILs) were here synthesized and investigated as phase-forming components of ABS and TPP approaches. Glycine-betaine and its analogous are part of the mammalian diet, being present in fruits, vegetables, and coffee beans. In particular, novel ABS and TPP systems formed by AGB-ILs and ionic-liquid-based systems were investigated for the recovery of mAbs from Chinese Hamster Ovary (CHO) cell culture supernatants. These antibodies are potential therapeutics to treat inflammatory diseases. Given their novelty, the ABS phase diagrams were determined to ascertain the compositions required to form two-phase and TPP systems able to act as separation techniques. Then, their potential in the extraction and purification of mAbs was evaluated and optimized, either as one-step platforms, as three-phase partitioning systems, or as hybrid processes combined with ultrafiltration, followed by the evaluation of the mAbs specific activity. The IL recyclability was additionally demonstrated.

Materials and methods

Material, chemicals and biologicals. AGB-ILs were synthesized by us according to previously reported protocols, corresponding to the following ILs: triethyl(4-ethoxy-4-oxobutyl)ammonium bromide ([Et₃NC₅]Br), tri(propyl)(4-ethoxy-4-oxobutyl)ammonium bromide ([Pr₃NC₅]Br), tri(n-butyl)(4-ethoxy-4-oxobutyl)ammonium bromide ([Bu₃NC₅]Br) and N-[1-methylpyrrolidyl-4-ethoxy-4-oxobutyl]ammonium bromide ([MeηpyrNC₅]Br). ILs with a common anion, Br⁻, were used since these correspond to ILs with low toxicity, as previously demonstrated. Furthermore, bromide has a low hydrogen-bond basicity when compared to other common IL anions, thus requiring lower amounts of salt to create ABS while contributing to the process sustainability. ILs were synthesized by the reaction of 4-bromobutyrate acid ethyl ester and
triethylamine, tri(n-propyl)amine, tri(n-butyl)amine or 1-methylpyrrolidine, respectively. All ILs were dried under vacuum for at least 72 h at 45°C. After this procedure, the purity of each IL was checked by $^1$H and $^{13}$C nuclear magnetic resonance (NMR), being > 97%. All ILs synthesized are solid at room temperature, yet with melting points below 100°C, and are water soluble at 25°C.\(^ {34}\) The commercial ILs studied for comparison purposes are the following: tetra(n-butyl)ammonium bromide ([N$_{4444}$]Br, 98% purity) and 1-butyl-3-methylimidazolium bromide ([C$_4$ mim]Br, 99% purity), acquired from Fluka and lolitec, respectively. The chemical structures of the synthesized and commercial ILs are depicted in Figure 1.

**Figure 1.** Chemical structures of the investigated ILs: (i) [Et$_3$ NC$_4$]Br; (ii) [Pr$_3$ NC$_4$]Br; (iii) [Bu$_3$ NC$_4$]Br; (iv) [Mepry NC$_4$]Br; (v) [N$_{4444}$]Br; (vi) [C$_4$ mim]Br.

A mixture of the salts K$_2$HPO$_4$ and KH$_2$PO$_4$ at pH 7.0 was used for the formation of ABS and TPP systems with AGB-ILs. K$_2$HPO$_4$-3H$_2$O extra pure was acquired from Scharlau, and KH$_2$PO$_4$ extra pure was acquired from Roic Farma, S.A. Phosphate buffered saline solution (PBS, pH = 7.4) pellets were acquired from Sigma-Aldrich.

Human IL-8 (77aa) (CXCL8) standard (98% purity) was acquired from Sigma-Aldrich. Commercial human immunoglobulin G (IgG) for therapeutic administration (trade name: Gammanorm\(^ \text{®} \)) was obtained from Octapharma (Lachen, Switzerland), as a 165 g L$^{-1}$ solution containing 95% of IgG (of which 59% correspond to IgG1, 36% to IgG2, 4.9% to IgG3 and 0.5% to IgG4). Bovine serum albumin (BSA) standards (2 g L$^{-1}$) were purchased from Thermo Scientific Pierce.

Anti-human interleukin-8 (anti-IL-8) monoclonal antibodies were produced in-house by a CHO DP-12 clone#1934 (ATCC CRL-12445) from the American Type Culture Collection (LGC Standards, Middlesex, UK), and grown in a mixture of 75% (v/v) of serum-free media and 25% (v/v) of Dulbecco’s modified Eagle’s medium (DMEM). This culture was maintained for several months, with the mAbs concentration varying between 40.5 and 99.4 mg L$^{-1}$. The produced anti-IL-8 mAb has an isoelectric point (pI) of 9.3.$^{36}$ Additional information on the mAbs upstream processing is provided in the ESI$^\dagger$.

**Determination of ABS phase diagrams, TLs and TLLs.** The ternary phase diagrams of the investigated ABS were initially determined to identify the mixture compositions where two phases can be formed, so that the extraction conditions could be defined for the recovery of mAbs. The determination of the binodal curves was performed using the cloud point titration method at 25 (± 1) °C and atmospheric pressure, where the mixture compositions were gravimetrically determined.$^{37}$ The experimental binodal curves were adjusted by the equation proposed by Merchuk \textit{et al.}$^{38}$ (given in the ESI$^\dagger$). The tie-lines (TLs), which give the coexisting phases compositions for a given mixture point, and respective lengths (tie-line lengths, TLLs), were determined according to lever-arm rule originally proposed by Merchuk \textit{et al.}$^{38}$ For each ternary phase diagram, 3 TLs were determined, including the mixture compositions at which the extractions of IgG were carried out. Further details for the determination of TLs and TLLs are given in the ESI$^\dagger$. In all studied systems, the top phase is majorly enriched in the IL, whereas the bottom phase is mainly composed of the salt and water.

**Recovery of anti-IL-8 mAbs from CHO cell culture supernatants.** For the recovery of anti-IL-8 mAbs directly from CHO cell culture supernatants, ABS and TPP systems with 2.0 g of total weight were prepared with a fixed concentration of phosphate salt (15 wt%), combined with (i) 25 wt%, (ii) 30 wt% and (iii) 40 wt% of IL. CHO cell culture supernatant was loaded at 37.5 wt% in all systems, with the remaining amount to complete each composition corresponding to water. Each ABS and TPP was prepared at least in duplicate. Each mixture was stirred, centrifuged for 3 min at 112 × g, and left to equilibrate for 30 min at 25°C in order to achieve the separation of mAbs from the remaining proteins. The volume and weight of the phases and the macroscopic aspect of each extraction system were registered, and both phases were carefully separated. In the cases in which a precipitate was observed in the ABS interface, i.e. for the TPP systems, the precipitate was completely isolated from the remaining phases and resuspended in PBS aqueous solutions for further analysis. The pH values of each phase at 25 (± 1) °C were determined using a Mettler Toledo U402-M3-S7/200 micro electrode, showing that a pH 7.0 ± 0.2 was maintained in all systems.

The best identified systems to purify mAbs from the cell culture supernatant were exposed to an additional ultrafiltration step aiming at recovering mAbs in PBS aqueous solution, while allowing the IL removal and recycling. The IL-rich phase and the precipitate of the ABS composed of 40 wt% of [Bu$_3$ NC$_4$]Br + 15 wt% of K$_2$HPO$_4$/KH$_2$PO$_4$ (pH 7) + 37.5 wt% of CHO cell culture supernatant + 7.5 wt% of water were placed in a microcentrifuge tube with an Amicon$^\text{®}$ Ultra-0.5 device containing a cut-off filter of 100 kDa (aiming the simultaneous IL recovery, buffer exchange, and to improve the purification factor by separating lower molecular weight proteins), and centrifuged at 14000 x g for 15 min. The filtrated solution was...
collected and phosphate buffer aqueous solution was added. This procedure was repeated for two times to assure the maximum removal of the IL. To recover the retentate concentrated solution, the Amicon Ultra-0.5 filter device was placed upside down in a clean microcentrifuge tube and centrifuged at 1000 × g for 2 min, and then PBS aqueous solution was added. Both the retentate and the filtrate were analyzed.

IgG and protein impurities were quantified in all feeds and in each ABS phase by size-exclusion high-performance liquid chromatography (SE-HPLC). Samples were diluted at a 1:2 (v:v) ratio in an aqueous potassium phosphate buffer solution (100 mmol·L⁻¹, pH 7.0, with NaCl 0.3 mol·L⁻¹) used as the mobile phase. The equipment used was a Chromaster HPLC system (VWR Hitachi) equipped with a binary pump, column oven (operating at 40 °C), temperature controlled auto-sampler (operating at 10 °C), DAD detector and a column Shodex Protein KW-802.5 (8 mm × 300 mm). The mobile phase was run isocratically with a flow rate of 0.5 mL·min⁻¹ and the injection volume was 25 μL. The wavelength was set at 280 nm. The calibration curve was established with commercial human IgG, from 5 to 200 mg·L⁻¹. Blank systems without biological sample were also prepared to address the interference of the ABS phase-forming compounds.

The ABS performance was evaluated by the recovery yield and purification factor or purity level for IgG. The recovery yield (%YieldIgG) in the top (IL-rich) phase was determined according to the following equation:

\[
\%\text{Yield}_{\text{IgG}} = \frac{[\text{IgG}]_{\text{TOP}} \times V_{\text{TOP}}}{[\text{IgG}]_{\text{INITIAL}} \times V_{\text{INITIAL}}} \times 100 
\]  

(1)

where \([\text{IgG}]_{\text{TOP}}\) and \([\text{IgG}]_{\text{INITIAL}}\) represent the IgG concentration in the top phase and the initial concentration of IgG in the CHO cell culture supernatant, respectively, and \(V_{\text{TOP}}\) and \(V_{\text{INITIAL}}\) correspond to the volumes of the top phase and cell culture supernatant loaded in the system, respectively.

In the cases where a precipitate of proteins at the ABS interface occurs, corresponding to the ILTPP approach, the \(\%\text{Yield}_{\text{IgG}}\) in the precipitate was determined according to the following equation:

\[
\%\text{Yield}_{\text{IgG}} = \frac{[\text{IgG}]_{\text{PP}} \times V_{\text{FINAL}}}{[\text{IgG}]_{\text{INITIAL}} \times V_{\text{INITIAL}}} \times 100 
\]  

(2)

where \([\text{IgG}]_{\text{PP}}\) and \(V_{\text{FINAL}}\) represent the IgG concentration and the final volume of the solution after resuspension, respectively.

The percentage purity level of IgG (%PurityIgG) was calculated by dividing the HPLC peak area of IgG \(A_{\text{IgG}}\) by the total area of the peaks corresponding to all proteins present in the respective sample \(A_{\text{TOTAL}}\):

\[
\%\text{Purity}_{\text{IgG}} = \frac{A_{\text{IgG}}}{A_{\text{TOTAL}}} \times 100 
\]  

(3)

The purification factor of IgG \(PF_{\text{IgG}}\) was calculated according to Equation 4:

\[
PF_{\text{IgG}} = \frac{\%\text{Purity}_{\text{IgG phase/precipitate}}}{\%\text{Purity}_{\text{IgG initial}}} 
\]  

(4)

where %Purity_{IgG phase/precipitate} and %Purity_{IgG initial} correspond to the purity level of IgG in the top phase of each system or in the precipitate (when the ILTPP studies are being carried out) and the purity level of IgG in the cell culture supernatant, respectively.

The same type of analysis, comprising the determination of the recovery yield, purity level and purification factor, was performed after the ultrafiltration step (hybrid process). The overall yield of the two-step process was calculated according to Equation 5:

\[
\%\text{Yield}_{\text{overall}} = \frac{\%\text{Yield}_{\text{ABS/TPP}} \times \%\text{Yield}_{\text{UF}}}{100} 
\]  

(5)

where %Yield_{ABS/TPP} and %Yield_{UF} represent the recovery yield of IgG in the ABS or in the TPP approach and ultrafiltration step, respectively.

IL recycling. The IL recycling possibility was investigated in the best identified system, composed of 40 wt% of [Bu₄N]Br + 15 wt% of K₂HPO₄/KH₂PO₄ (pH 7) + 37.5 wt% of CHO cell culture supernatant + 7.5 wt% of water, in which the first separation step was performed according to the procedure previously described. After the IL recovery in the ultrafiltration filtrate fraction of the top phase, biological contaminants present in the sample were precipitated with cold ethanol, and removed after centrifugation. Ethanol was chosen due to its greener credentials compared to other organic solvents that can be used for the same purpose. The ethanol present in the IL-rich sample was removed and recovered using a rotary evaporator. However, in order to assure the minimal presence of volatile solvents (including water) aiming the preparation of accurate mixture compositions to address the process performance using the recycled IL, the IL sample was further subjected to high vacuum (0.1 Pa) at 60 °C for 72h. It should be however remarked that when foreseeing the process scale-up, less energetic-intensive conditions need to be optimized and applied. Prior to the IL reuse in a new ABS and TPP system, the water content (< 200 ppm) of the dried IL sample was determined using a Metrohm 831 Karl Fischer coulometer, with the anlyte Hydranal® – Coulomat AG from Riedel-de Haën, and considered in the mixture composition. This procedure was repeated for 2 times. The IL stability was confirmed by ¹H and ¹³C NMR, showing no evidences of degradation.

Proteins profile and anti-IL-8 mAbs integrity and activity. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) assays were performed to infer the proteins profile of each fraction, and to address the anti-IL-8 mAbs...
The qualitative and quantitative characterization of the CHO cell culture supernatants was initially performed to appraise the complexity of the medium from which anti-IL-8 mAbs aimed to be purified. The SDS-PAGE under reducing conditions and SE-HPLC characterization results of the CHO cell culture supernatant are provided in Figure 3. Under the chromatographic conditions used, pure IgG samples present 2 chromatographic peaks: one corresponding to the IgG...
monomer and the other to IgG aggregates, with a retention time of ca. 15.0 min and 13.8 min, respectively. IgG is composed of 2 heavy chains with a molecular weight of 50 kDa, and 2 light chains with a molecular weight of 25 kDa, visible in the reduced SDS-PAGE results (Figure 3 (b)). The major protein impurity in the feed is bovine serum albumin (BSA), with a retention time of ca. 16.8 min and a molecular weight of 66.5 kDa. Apart from IgG and BSA, CHO cell culture supernatants also present other protein impurities resulting from the medium used in the cell proliferation and growth, and also from their metabolism (e.g. insulin, transferrin and other CHO host cell proteins), which may correspond to the lighter protein corresponding bands in Figure 3.

Figure 3. Characterization of the CHO cell culture supernatant: (A) SE-HPLC chromatograms of the CHO cell culture supernatant (---), pure IgG solution 100 mg L⁻¹ (---), and pure BSA solution 200 mg L⁻¹ (---); (B) SDS-PAGE gel: lane 1 – molecular weight marker; lane 2 – CHO cell culture supernatant; lane 3 – pure IgG solution 1 g L⁻¹; lane 4 – pure BSA solution 1 g L⁻¹. BSA (red) and IgG (green) heavy (H.C.) and light (L.C.) chains are identified.

The cell culture was maintained for several months, resulting in a natural variation of the proteins content between each feedstock. The anti-IL-8 mAbs concentration varied between 40.5 and 99.4 mg L⁻¹, with an average content of 67.7 ± 23.7 mg L⁻¹ and an average purity of 16.9 ± 3.8 %. However, in all cases where ABS and TPP systems were applied, the original feedstock was always analyzed to infer the improvements achieved in respect to the original feedstock.

After the characterization of the phase diagrams and of the mAbs-containing feedstock, the potential of IL-based ABS as purification routes for anti-IL-8 directly from CHO cell culture supernatants was addressed for three mixture compositions (15 wt% K₂HPO₄/K₃HPO₄ pH 7.0 + 22.5 wt% H₂O + 37.5 wt% CHO cell culture supernatant + IL ranging from 25 wt% to 40 wt%). The performance of all ABS was investigated in terms of recovery yield (%Yield) and purification factor (PF), whose results are shown in Figure 4. The use of the purification factor allows to reduce discrepancies in the results derived from the variability of the biological matrix, and is valuable to carry out a first screening of the several AGB-IL-based systems performance. The composition of each phase for the mixture points used in the separation studies, i.e. the respective TLs, and detailed data on the recovery yields and purification factors are given in the ESIF.

For the mixture composition with a lower IL content (25 wt%), a preferential partition of all proteins to the top (IL-rich) phase was observed with all ILs (Figure 4 (A)). This phenomenon may be explained by the salting-out effect induced by the phosphate-based salt, promoting the exclusion of proteins into the opposite phase. Nevertheless, biomolecules affinity and interactions with ILs play a significant role since this behavior does not commonly occur in polymer-salt ABS, where the salt also acts as a salting-out agent. In IL-based ABS, a multitude of specific interactions (van der Waals, hydrogen-bonding and electrostatic interactions) occurring between the amino acids residues at the proteins surface and ILs seem to govern their partitioning to the IL-rich phase, as previously reported with other proteins. The isoelectric point (pI) of anti-IL-8 is ca. 9.3, and as the pH of the investigated IL-based systems is 7.0, antibodies are positively charged and electrostatic interactions may play a role. Nevertheless, if they were the most relevant type of interactions, IgG should preferentially partition to the salt-rich phase, as typically observed in polymer-salt ABS. In this work, the partition of IgG is triggered to the top phase of ABS using ILs instead of polymers, which may be due to preferential hydrophobic-bonding and dispersive forces between the target protein and ILs.

When using 25 wt% of IL in the ABS composition, recovery yields of mAbs ranging from 73.1 % up to 100.0 % (for [Bu₄N]Br) are obtained in a single step, with purification factors ranging from 1.0 to 1.5. Moreover, all AGB-IL-based ABS composed of 25 wt% of IL allow a high recovery of mAbs (>92%), performing better than the commercial ILs that lead to a maximum recovery yield of 73.1 %. mAbs losses with these ILs are probably associated to precipitation since some turbidity was detected in these systems. Overall, the best results were obtained using the ABS composed of [Bu₄N]Br, allowing the complete recovery of the antibodies in a single step, with a purification factor of 1.5 ± 0.1. According to the recovery yields achieved, it is clear that the IL cation core and the respective alkyl side chain length have a
significant influence on the mAbs partitioning between the phases and in the maintenance of their structure. The separation of biomolecules from a complex mixture in ABS can be manipulated by various factors, e.g., by the nature of the phase-forming components and their concentrations. This last possibility was taken into account, and conducted by increasing the concentration of the IL from 25 wt% to 30 wt% and 40 wt% while maintaining the composition of the salt at 15 wt%. The goal was to optimize the purification process with the four ILs that led to the most promising results in the first screening ([Pr[N(C3)]2]Br, [Bu[N(C3)]2]Br, [Mepy[N(C3)]2]Br and [C4 mim]Br). The results obtained are depicted in Figure 4 (B) and (C).

![Image](image_url)

**Figure 4.** Recovery yields (% Yield<sub>igg</sub> – ■) and purification factors (P<sub>ig</sub> – □) of anti-IL-8 mAbs in the IL-rich phase using ABS composed of IL + 25 wt% KH2PO4/K2HPO4 pH 7 + 40 wt% H2O + 37.5 wt% CHO cell culture supernatant: (A) 25 wt% IL; (B) 30 wt% IL; and (C) 40 wt% IL.

The recovery yields for IgG of the systems composed of 30 wt% of IL range from 19.8% to 100%, however, when 40 wt% of IL is used (Figure 4 [C]), recovery yields ranging from 49.8% to 100% are obtained at the IL-rich phase. In general, ABS formed by [Pr[N(C3)]2]Br present the lowest performance regarding the IgG recovery in the top phase. Similarly, ABS composed of 30 wt% of [Mepy[N(C3)]2]Br lead to a decrease in the anti-IL-8 recovery and purification factor (61.3 ± 6.1 % and 0.8 ± 0.1, respectively), being these two ILs discarded for the evaluation with 40 wt% of IL. For comparison purposes, the systems composed of the commercial IL [C4 mim]Br were studied. These systems allow the complete recovery of anti-IL-8 mAbs with the increase of the IL concentration up to 40 wt%, with a purification factor of 1.30 in both cases. Regarding AGB-ILs, ABS composed of [Pr[N(C3)]2]Br and [Mepy[N(C3)]2]Br are not suitable to purify the target biomolecule from the complex medium under any concentration of IL (from 25 wt% to 40 wt%). The most promising ABS is constituted by [Bu[N(C3)]2]Br at 30 wt%, where a recovery yield of 100% and purification factors of 1.6 are achieved in one-step. Overall, the most hydrophilic ILs, which present smaller biphasic regions in their phase diagrams and shorter TLs, are the ones that lead to the less promising results (e.g. [Pr[N(C3)]2]Br and [Mepy[N(C3)]2]Br). On the other hand, more hydrophobic ILs (e.g. [Bu[N(C3)]2]Br, but still miscible in water), with larger biphasic regions and longer TLs, are more efficient options to recover and purify mAbs.

In addition to the IgG recovered at the IL-rich phase, the best identified ABS composed of [Bu[N(C3)]2]Br also leads to the creation of a protein-rich interface precipitate (cf. the ES†), particularly visible at the higher IL concentrations (40 wt%) and responsible for the lower recovery yields shown in Figure 4 (C). This phenomenon is related with the water content in the top phase, which decreases as the IL concentration increases (data given in the ES†), thus not allowing the complete solubilization of proteins and resulting in the formation of ILTPP systems. Mixture compositions comprising higher concentrations of IL, corresponding to longer tie-lines, imply higher concentrations of salt in the bottom phase. Due to the salt ions preferential solvation, less water will be available for the proteins solvation, resulting in the exposure of hydrophobic patches which promotes protein-protein interactions that ultimately lead to the proteins precipitation. This salting-out phenomenon has been reported for the precipitation of food proteins at the interface of IL-salt ABS, defined as ILTPP, although no complex mixtures have been addressed.25, 26

The antibodies precipitation in ABS has been systematically considered as a “bad feature” in the literature and, consequently, ABS promoting IgG precipitation are usually discarded due to their “low performance”.44-49 Nevertheless, if precipitation is selective and does not compromise the antibodies stability and activity, this approach should be considered as an alternative and effective IgG downstream strategy. Accordingly, the presence of a significant amount of precipitate in ABS composed of higher amounts (40 wt%) of [Bu[N(C3)]2]Br encouraged the investigation of the ILTPP approach as a potential strategy to purify and recover mAbs.

The proteins precipitate formed between the two coexisting phases in the ABS was recovered, resuspended in an aqueous solution of PBS (pH 7.0, 100 mM-L<sup>-1</sup>) and further analyzed. Through this approach and at the conditions under discussion, there is the selective (one-step) precipitation of IgG directly from the CHO cell culture supernatant, with a recovery yield of 41.0 ± 2.6% and a purification factor of 2.7 ± 0.1 (%Purity<sub>ig</sub> = 60.9 ± 2.0 %). Overall, with the ABS formed by 40 wt % of...
[Bu₄N]ClBr, 60.4 ± 5.2% of IgG with a purification factor of 0.8 ± 0.1 is recovered in the IL-rich phase, whereas the remaining antibody is recovered at the ABS interface with a recovery yield of 41.0 ± 2.6% and a purification factor of 2.7 ± 0.1. In Figure 5, the SDS-PAGE gel of all the recovered fractions is provided, allowing not only to infer the mAbs integrity, but also to corroborate the discussed purification results. It should be remarked that although promising results were obtained with higher concentrations of IL, higher concentrations of IL (> 40 wt%) are not experimentally feasible given the amount of feedstock that needs to be loaded to create ABS.

![SDS-PAGE](image)

**Figure 5.** SDS-PAGE of the recovered fractions using the system composed of 40 wt% [Bu₄N]ClBr + 15 wt% KH₂PO₄/K₂HPO₄ pH 7.0 ± 22.5 wt% H₂O + 37.5 wt% CHO cell culture supernatant (ABS fractions + UF fractions). Lane 1 — molecular weight marker (KDa); lane 2 — CHO cell culture supernatant; lane 3 — ABS top phase; lane 4 — ABS precipitate; lane 5 — ABS bottom phase; lane 6 — UF retentate of top phase; lane 7 — UF filtrate of top phase; lane 8 — UF retentate of precipitate; lane 9 — UF filtrate of precipitate. The bands corresponding to BSA, IgG heavy chain (H.C.) and IgG light chain (L.C.) are also labeled.

To fully characterize the potential of ABS and TPP as IgG one-step purification platforms, we evaluated the biological activity of the anti-IL-8 mAbs in the IL-rich phase and in the precipitate by competitive ELISA, which was found to be 90.5 ± 2.0 % and 74.7 ± 3.2, respectively. The detailed mAbs activity results are given in the ESI†. The mAbs activity in the IL-rich phase is similar to that presented by the antibodies in the CHO cell culture supernatant before the purification process (90.6 ± 1.3 %), meaning that the extraction using the IL-based approach does not affect or decrease the anti-IL-8 biological activity. Despite the remarkable results obtained with ILTPP, the anti-IL-8 activity slightly decreases in the precipitate fraction, probably due to structural changes induced by precipitation, but still in the order of that reported for anti-IL-8 purification using ProA affinity chromatography (79 ± 9 %).32 Since the biological activity of proteins is closely related with their 3D structure, these observations allowed us to infer that minimal conformational changes occur with IL-based ABS and TPP systems.

Albeit large efforts have been carried out with more traditional polymer-based ABS to purify antibodies, as reviewed by Capela et al.,1 the IgG recovery and purification results gathered in this work outstand those reported up to date using IL-based ABS.37,50 For instance, Ferreira et al.57 using commercial ILSs as adjuvants in polymer-salt ABS achieved 26% of IgG purity, whereas Mondal et al.59 recovered 85% of IgG from rabbit serum with a purity level of 25%. Ramalho et al.51 reached the best results, being able to recover all the IgG from rabbit serum with a purity level up to 49%. Considering the best results found in the literature with ABS comprising ILSs, it can be concluded that the systems herein developed allow higher purification levels. Furthermore, the previous works were carried out with mammal’s serum samples, where in this work the use of IL-based ABS for mAbs recovery/purification from CHO cell culture supernatants was considered for the first time. In the same line, for the first time ILTPP approaches were considered to recover IgG.

**Hybrid processes and IgG recovery**

Aiming the IgG recovery from the IL-rich phase and the IL removal from the antibodies’ fractions, ultrafiltration (UF) was finally applied, allowing to propose the use of hybrid processes to purify IgG by combining ABS + UF and TPP + UF steps. UF also allows to recover antibodies in a biological buffer, viable for the envisioned IgG therapeutic applications, and to recover the IL for further use. As a proof of concept, an ultrafiltration step was applied to the IL-rich phase and precipitate containing IgG of the most promising identified ABS (composed of 40 wt% of [Bu₄N]ClBr + 15 wt% of KH₂PO₄/K₂HPO₄ pH 7.0 ± 7.5 wt% of H₂O + 37.5 wt% of CHO cell culture supernatant). The performance of this additional step was investigated in terms of IgG concentration ([IgG]), recovery yield (%YieldIgG), purity level (%PurityIgG) and purification factor (PF). These parameters were determined for the different strategies that could be combined: (i) TOP phase + UF retentate; (ii) TOP phase + UF filtrate; (iii) Precipitate + UF retentate, and (iv) Precipitate + UF filtrate. Detailed data are given in the ESI†. A summary of the results, while highlighting the developed integrated processes in which the purification, recovery and final formulation of mAbs in phosphate-based solutions can be performed, is given in Figure 6.

The UF step of the top IL-rich phase allows to recover 47.3 ± 13.5% of IgG in the retentate, with an increase of 88.5 % in the purity level, up to 32.8 ± 1.5% vs. 17.4 ± 2.0 % before UF, and with an overall IgG recovery yield of 28.6 ± 18.7% in route (i). The IgG recovery yield in the filtrate is 45.1 ± 16.1%, presenting an IgG purity of 29.8 ± 5.7%, and with an overall yield of the two-step process (route (ii)) of 27.2 ± 21.3 %, with the anti-IL-8 mAbs biological activity of 66.4 ± 2.4%. Nevertheless, it should be remarked that the filtrate is the fraction where the IL is also present, and therefore the choice of using this fraction for IgG formulation or for IL recovery for further reuse should be made based on the final desired application.

Despite the good results accomplished using the ABS IL-rich phase, an even better performance was achieved with the ILTPP
approach combined with ultrafiltration. The IgG recovery yield in the retentate is 52.7 ± 2.2%, with a purity level of 63.5 ± 1.8%, whereas the overall recovery yield of the two steps is 21.6 ± 4.8% (overall process/route (iii)). The UF step in the filtrate results in a low IgG recovery yield of 18.1 ± 4.0% (that only results in an overall yield of the two-step process of 7.4 ± 6.6%); however, this approach leads to an increase in the purity level up to 67.2 ± 1.8% (route (iv)).

![Diagram showing the process of IgG purification](image)

**Figure 6.** Schematic representation of the integrated and hybrid processes developed for mAbs downstream processing: routes to recover IgG with different purity levels and allowing the IL recovery and reuse are identified.

The proteins profile of each UF fraction is provided in Figure 5, in which is possible to identify that IgG is mainly retained in the retentate fractions. It is important to remark that IgG is present in all fractions evaluated, as shown in the SE-HPLC chromatograms (given in the ESIt†), even though in lower concentrations. After UF the peak corresponding to low molecular weight protein impurities is only present in the filtrate, meaning that with this step we completely remove one class of protein impurities, representing the basis for the increment of 88.5% in the purity level of IgG in the retentate fraction (comparing to the top IL-rich phase fraction). The precipitate fraction after UF does not contain protein impurities with lower molecular weights (that are only retained in the top phase of the system), supporting the high purity level attained with this fraction (up to 67.2%). Although a membrane with a 100 kDa cut-off was used, some 3D conformation of the antibodies allow, in some extension, their passage through the membrane, thus leading to some losses in the IgG recovery yield. This step is however of high relevance since the IL can be recovered and reused, and IgG can be recovered in a phosphate aqueous solutions viable to proceed to biotechnological and health related applications. Since the previously described processes allow the IL recovery, the IL recyclability was finally demonstrated. The respective experimental details are given in the Experimental Section. After the first separation step with “fresh” IL, the IL recycling and its use in the formation of a new system was investigated for two more times. The results obtained for the 3 cycles of separation in terms of mAbs recovery/purification are provided in Figure 7 (detailed values are given in the ESIt†). Overall, similar results in the recovery yield and purity level of IgG were obtained in the 3 separation cycles, both in the IL-rich phase and precipitate fractions, meaning that the proposed processes keep their separation performance even using recycled IL, while contributing to these processes sustainability. Through the tie-line data corresponding to the mixture compositions used in the separation of IgG (cf. Table S8 in the ESI†), the concentration of IL in the bottom salt-rich phase is significantly low (0.0003460 wt%). Accordingly, ILs are mainly lost during the recovery and recycling steps and transference between vials. The IL loss in each recycling step is below 3 wt%. However, it should be remarked that this loss corresponds to a lab-scale procedure,
being expected that it can be reduced by properly optimizing
the IL transference and by scaling-up the process.

![Figure 7. Recovery yields (%Yield$_{IGS}$ – ■) and purity levels (%Purity$_{IGS}$ – ◆) of anti-IL-8 mAbs in three separation cycles, where the last two correspond to the use of recycled IL.](image)

Based on the overall results obtained, an hybrid and integrated purification platform is proposed to purify mAbs from CHO cell culture supernatants, comprising the following steps: (1) IL-based ABD and TPP for mAbs recovery and purification; (2) UF for mAbs polishing and IL removal/salt exchange; and (3) a final product formulation through two routes, according to the IgG desired purity. Based on the combination of the fractions with similar purity levels summarized in Figure 6, such as routes (i) + (ii) and (iii) + (iv), two fractions of anti-IL-8 mAbs can be recovered with purity levels higher than 29% and 63%, respectively. Although further optimization studies can be carried out aiming at achieving the complete precipitation of mAbs at the ABS interface, as well as the testing of other membrane cut-offs, the developed platforms reveal a high potential and provide new directions in the design of more sustainable IL-based strategies for the purification and recovery of biopharmaceuticals such as mAbs.

**Conclusions**

The use of IL-based purification and recovery strategies for mAbs directly from CHO cell culture supernatants was here proposed and evaluated for the first time. To this end, novel ABS composed of glycine-betaine analogues ILs and $K_2HPO_4/KH_2PO_4$ at pH 7.0, the respective three-phase partitioning systems, and hybrid processes combined with ultrafiltration, were investigated. With the studied ABS, mAbs preferentially partition to the IL-rich phase, with recovery yields up to 100% and purification factors up to 1.6. With IL-based three-phase partitioning approaches a precipitate enriched in mAbs is obtained at the ABS interface, yielding 41.0% of IgG with a purification factor of 2.7 (purity of 60.9% achieved in a single-step). The biological activity of anti-IL-8 mAbs is maintained after the several purification and recovery steps, being superior and competitive with the activity of mAbs purified by ProA chromatography. Hybrid processes combining the two previous techniques and an ultrafiltration step were finally applied and evaluated, allowing the recovery of mAbs from the different fractions in an appropriate buffer solution for biopharmaceuticals formulation, while allowing the simultaneous IL removal and reuse. The IL recyclability was demonstrated, with no losses on the separation performance. The best results were obtained with the hybrid process combining IL-TPP and ultrafiltration, where IgG with a purity greater than 60% is obtained.

**Conflicts of interest**

There are no conflicts to declare.

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