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Aqueous biphasic systems composed of cholinium chloride and polymers as effective platforms for the purification of recombinant green fluorescent protein

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Abstract

Green Fluorescent Protein (GFP) has excellent properties as biosensor and biomarker, however, its widespread use is limited by its purification costs. Alternative low-cost purification techniques can overcome this issue. The aim of this work was to evaluate aqueous biphasic systems (ABS) composed of cholinium chloride ([Ch]Cl) and different polymers as effective platforms to recover GFP from cell lysate of recombinant *Escherichia coli* BL21. All systems completely extracted GFP from cell lysate (>99%) into the polymeric- or [Ch]Cl-rich phases. In general, [Ch]Cl-based ABS allowed a good purification capacity (GFP 80-100% pure), with best results (approximately 100% pure GFP) achieved with polypropylene glycol (PPG)-400/[Ch]Cl ABS in a single-step extraction or in a two-step extraction (back-extraction) by the integration of polyethylene glycol (PEG)/sodium polyacrylate+[Ch]Cl ABS with a following stage using PEG/[Ch]Cl-based ABS. Additionally, to demonstrate the potential of PPG-400/[Ch]Cl ABS in downstream processing, solvent recyclability and GFP polishing was carried out using ultrafiltration. Finally, the capacity of PPG-400/[Ch]Cl ABS to extract other fluorescent proteins was also confirmed. The results clearly demonstrated that PPG-400/[Ch]Cl ABS can be applied in the downstream processing for the purification of proteins, enhancing not only purification yields but providing simpler, quicker, cost-effective and biocompatible processes.

Keywords: aqueous biphasic systems; cholinium chloride; green fluorescent protein; purification; integrated downstream process.

Introduction

Recent advances in biotechnology have pushed the discovery of many bio-products for diagnostic and therapeutic use, allowing medical care for previously untreatable diseases. However, medical applications require high levels of purity and demand complex processes of production and purification, resulting in expensive end-products, inaccessible to the general public.[1-2] The optimization and development of sustainable industrial purification techniques is essential to reduce the production costs and allow the use of these novel bioproducts at large scale. Among the biomolecules discovered in recent decades, the green fluorescent protein (GFP) stands as one with a high biotechnological potential, mainly due to its potential applications as biosensor and biomarker,[3] limited by the aforementioned purification and production issues.

GFP is a globular protein originally isolated from jellyfish *Aequorea victoria* species that has an intense and natural fluorescence,[4-5] exhibits a large pH and temperature stability range, and through the proper manipulation of its structure, its fluorescence intensity and spectra can be altered for different applications,[6] including in biological systems as a biomarker and biosensor.[3] Through the isolation and cloning of the gene responsible for the production of GFP, the protein was successfully produced by other recombinant organisms such as *Escherichia coli* (*E. coli*) and *Caenorhabditis elegans*.[3] However, even with the advances in GFP production through innovative molecular biology approaches, the development of downstream processes remains incomplete, and thus, GFP purification costs are still very high, restricting its application to research and small scale procedures.

For its application as a biomarker and biosensor, commercial GFP needs high purity levels, requiring a series of laborious and expensive chromatography steps,[7-14] with no efficient purification methodology that combines selective GFP recovery and affordable costs.[15] Even promising purification techniques for GFP like elastin-like polypeptide tag[16] are still not used commercially,[17] and regarding the pharmaceutical application of GFP, additional tests are required to guarantee that the tag or its residues are not immunogenic or detrimental for medical use.[17-18] The high number of costly purification stages required for the acquisition of pure GFP impacts the price of

the final product, e.g. BioVision® commercial GFP that costs approximately US$ 2,000.00 per mg.[19] Regarding the economical concern, the search for alternative purification techniques (such as liquid-liquid extraction) to reduce the production costs is under study. GFP recovery from cell lysate using organic solvent extraction has already been accomplished, and a complete purification of GFP was achieved by combining the liquid-liquid extraction stages with a further chromatography step.[15, 20] However, the process involved multiple extraction steps and the use of organic solvents, which can be toxic, volatile and/or flammable, and the current industrial trend is to replace these compounds with safer and more environmentally friendly alternatives,[21] like polymers[22-23] and ionic liquids (ILs).[24-25]

Polymers such as polypropylene glycol (PEG) and polyethylene glycol (PPG) and salts like cholinium chloride [(2-hydroxyethyl)trimethylammonium] chloride, [Ch]Cl, exhibit excellent properties for industrial use, having low toxicity[26-28] and enabling milder work conditions than organic solvents.[29-31] Recently, combinations of these biocompatible polymers and cholinium-based salts or ILs have been used on the formation of aqueous biphasic systems (ABS),[26, 32] which have been proposed as biocompatible, efficient, cheaper and easy to scaled-up platforms for the purification of several biomolecules.[26, 32-44] ABS consist of two aqueous-rich immiscible phases that are formed by the mixture (at certain concentrations) of, at least, two structurally different compounds, such as polymers, salts and/or ILs in an aqueous media.[38, 45] There are already some successful studies showing the use of ABS for the extraction of GFP,[46-47] but higher levels of purification were still not demonstrated and are essential for the commercial success and economic viability of the process.

Taking into consideration the excellent capabilities of cholinium-based ABS for the purification of pharmaceuticals[32, 48] and proteins,[26, 49] this work aimed to evaluate the systems potential for the recovery and purification of GFP from cell lysate of recombinant *E. coli* BL 21. Thus, a comprehensive study to evaluate the GFP extraction and purification aptitude of different ABS composed of [Ch]Cl and biocompatible polymers like PEG, PPG and/or sodium polyacrylate (NaPA) was carried. The GFP extraction efficiencies of each ABS were determined and their purification aptitudes evaluated by 3D fluorescence and SDS-polyacrylamide gel (SDS-PAGE) electrophoresis, and compared with more traditional polymer/buffer-based ABS. To improve the GFP

purification yields, a back-extraction procedure was performed. Envisaging the industrial application of the methodologies developed in this work, an integrated process of purification of GFP was assembled by using ABS (with the highest purification performance) with ultrafiltration, thus demonstrating the recycling of the phase-forming agents and allowing the GFP polishing.

Results and discussion

GFP Extraction

In the present work, the extraction efficiencies and purity of enhanced GFP (eGFP, which will be referred as GFP in all the manuscript) extracted from the supernatant of the cell lysate of recombinant *E. coli* BL21 with plasmids pLysS and pET28(a) using various ABS were determined to identify the system allowing the complete recovery and purification of GFP without losses on its fluorescence structural integrity. The details of GFP production and preparation of cell lysate are described in **section "Bacterial strain and growth conditions for production of GFP"** from the **Electronic Supporting Information**† (**ESI**†). The success of the use of ABS as purification platforms is dependent on the properties of coexisting aqueous phases and their ability to separate the target protein from other contaminants. Therefore, the physicochemical characterization of the target bio-product and the ABS coexisting phases are essential for the understanding and design of the best fractionation system.

To evaluate the selective extraction of GFP from the raw extract (after lysis) the following ABS were studied: PEG-1500/potassium phosphate buffer; PEG-2000/ potassium phosphate buffer; PEG-600/NaPA-8000 + [Ch]Cl as adjuvant; PEG-600/[Ch]Cl; PPG-400/[Ch]Cl. These systems were chosen according to the main mechanisms that control the partitioning of biomolecules, namely, salting out effect (PEG/phosphate ABS), hydrophobicity/hydrophilicity balance (polymer/[Ch]Cl ABS) and electrostatic repulsion (PEG/NaPA ABS). Different mixtures at the biphasic region were chosen according to previously published phase diagrams for each type of ABS, as listed in **Table S1** (**ESI**†). The phase diagram of the PEG-2000 + K_2HPO_4/KH_2PO_4-based ABS is presented in **Fig. S4** (**ESI**†), as well as the corresponding ternary weight fraction composition (**Table S3**, **ESI**†) and detailed description of phase diagrams determination

methodologies (**section "Phase diagram determination"**, **ESI†**). The methods for preparation and characterization of ABS are described in **section "Extraction and purification of GFP"** from **ESI†**, and methods for determination of GFP 3D fluorescence spectrum and quantification of GFP and Total Protein (TP) are detailed in **section "Determination of GFP 3D fluorescence spectrum and quantification of GFP and Total Protein"** (**ESI†**).

The extraction and purification of GFP in each series of ABS was assessed in terms of GFP extraction efficiency (EE_{GFP}), GFP mass balance (MB_{GFP}) and relative concentration of GFP by total protein ($[GFP]_{rel}$), according to **Equations S1** to **S3** from **ESI†**. The EE_{GFP} was used to evaluate the capacity of extraction of GFP in the GFP-rich phase of each system, the MB_{GFP} was assessed to verify GFP fluorescence losses in the extraction process and the $[GFP]_{rel}$ (%) was calculated to effectively validate the purification capabilities of each system, and thus define which ABS can be used in the purification of GFP.

Each phase of the ABS studied was also characterized for their physicochemical properties considering the variables pH, conductivity ($mS.cm^{-1}$ at 25°C), viscosity (mPa.S), density ($g.cm^{-3}$) and water content (wt%), as presented in **Table S2** from **ESI†** and methods described in **section "Physicochemical characterization of ABS coexisting phases"** (**ESI†**). This characterization was important to determine the overall composition of each phase in association with the FT-IR analysis presented in **Fig. S5** to **S8** from **ESI†**. In addition to the determination of the ABS phases' composition, the physicochemical characterization of the phases allows the understanding of the partitioning mechanisms that will be addressed in the next paragraphs.

The ABS presented pH values between 5.3 and 8.2, an environment in which GFP is overall stable[4] and in general negatively charged (considering its isoelectric point (pI) of 5.52).[50] The water content in the PEG/NaPA + [Ch]Cl-based ABS was almost identical in the top and bottom phases (with an average difference of only 2.72 ± 1.56 wt%), both rich in water (around 60 wt%). As for the PEG/potassium phosphate buffer ABS, the buffered phase was slightly more hydrophilic (around 5 wt% of difference), but both phases were rich in water (64.58 to 78.85 wt% of water). However, in the polymer/[Ch]Cl systems, there was a greater difference in the water content among the phases, ranging from 29.14–33.08 and 56.44–63.15 wt% for the

[Ch]Cl phase in the PEG-600/[Ch]Cl and PPG-400/[Ch]Cl-based ABS, respectively, and 12.31–14.81 and 13.06–24.70 wt% for the polymer phase of the cited ABS. The average difference of top and bottom phase for the PEG-600 and PPG-400 ABS were 18.11 ± 1.21 wt% and 42.48 ± 4.34 wt%, respectively. As for the conductivity, in the PEG/NaPA + [Ch]Cl ABS, it ranged from 4.61 to 18.25 $mS.cm^{-1}$ in the PEG rich-phase and from 42.05 to 49.21 $mS.cm^{-1}$ in the NaPA rich-phase (average difference of 37.3 ± 3.4 $mS.cm^{-1}$); in the PEG/potassium phosphate buffer ABS, the PEG phase presented a conductivity of 5.35 to 6.74 $mS.cm^{-1}$ and the buffer phase of 122.30 to 124.40 $mS.cm^{-1}$; and finally, the polymer/[Ch]Cl ABS had less than 1 $mS.cm^{-1}$ of conductivity for the polymer phase and the [Ch]Cl phase for the PEG-600 system presented readings from 31.32 to 37.31 $mS.cm^{-1}$ and the PEG-600 system from 26.70 to 64.21 $mS.cm^{-1}$, with respective average differences between top and bottom phases of 33.1 ± 3.4 and 45.2 ± 19.0 $mS.cm^{-1}$.

The ABS described in **Table S1** were initially studied for GFP extraction, where the influence of the type and length of the polymers, type of salts and the concentration of the phase-forming agents were the parameters evaluated. The comparison of the average EE_{GFP} (%) of three independent assays (and respective standard deviations) for all systems are compiled in **Fig. 1**, and the detailed weight fraction compositions (wt%), MB_{GFP} (%), EE_{GFP} (%), $[GFP]_{rel}$ (%) and physicochemical characteristics of coexisting phases are fully provided in **Table S2** of the **ESI**†.

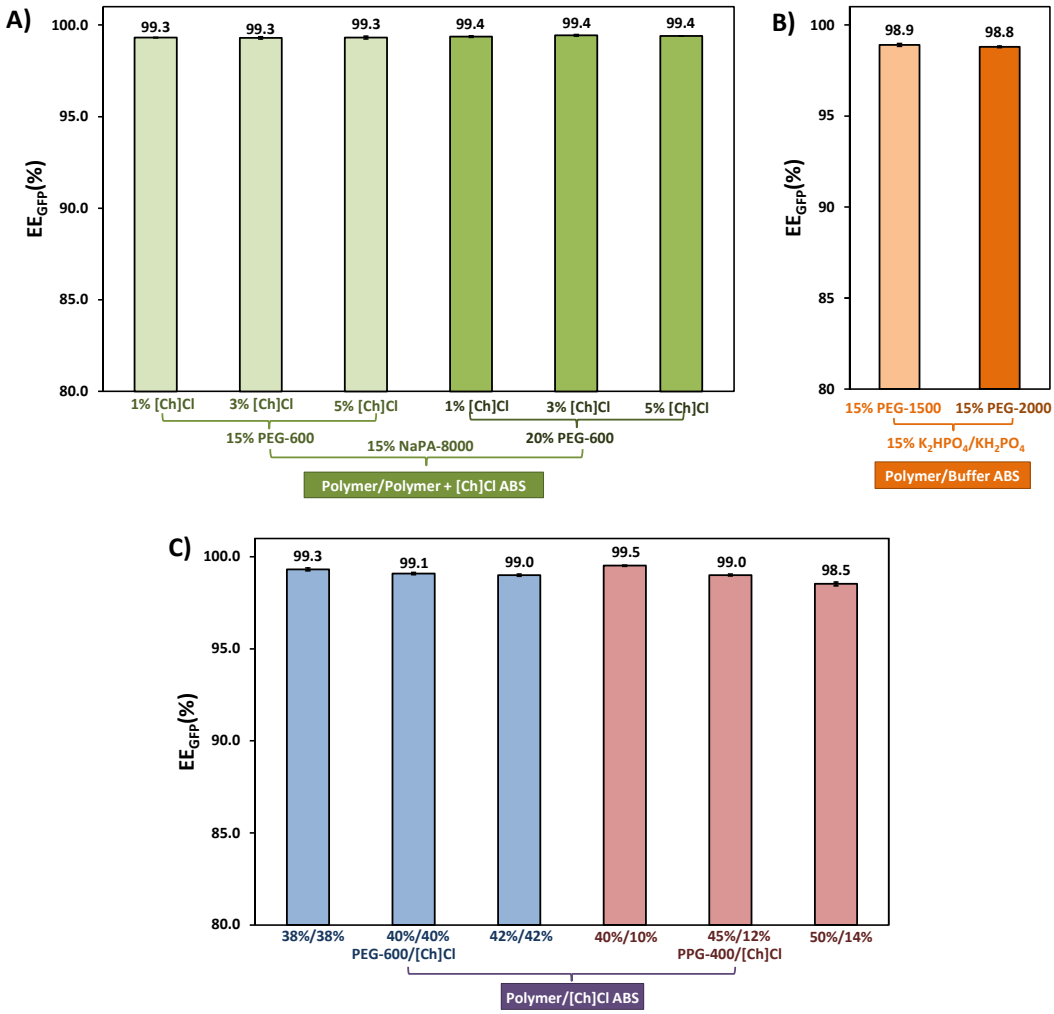

Fig. 1. GFP extraction efficiency [EE_{GFP} (%)] for the: **A)** PEG-rich phase using ABS composed of 15 wt% PEG-600 + 15 wt% NaPA-8000 + [Ch]Cl (1, 3 or 5 wt%) ( ); and 20 wt% PEG-600 + 15 wt% NaPA-8000 + [Ch]Cl (1, 3 or 5 wt%) ( ); **B)** PEG-rich phase using ABS composed of 15 wt% PEG-1500 + 15 wt% K_2HPO_4/KH_2PO_4 ( ); 15 wt% PEG-2000 + 15 wt% K_2HPO_4/KH_2PO_4 ( ). **C)** [Ch]Cl-rich phase using ABS composed of PEG-600 (38, 40 or 42 wt%) + [Ch]Cl (38, 40 or 42 wt%) ( ) and PPG-400 (40, 45 or 50 wt%) + [Ch]Cl (10, 12 or 14 wt%) ( ) (concentration: polymer%/[Ch]Cl%). The results represent the average of three independent experiments ± standard deviation errors.

The results depicted in **Fig. 1 A)** and **B)** show that, independently of the composition of PEG-600 and [Ch]Cl in the PEG-600/NaPA + [Ch]Cl-based ABS and the MW of the PEG used in the PEG/potassium phosphate buffer systems, GFP was fully extracted into the PEG-rich phase (EE_{GFP}(%) > 99%). Also, the MB_{GFP} (%) for these systems were higher than 90% (**Table S2** from **ESI**†), demonstrating that these ABS were quite compatible with GFP and caused no significant losses on its fluorophore structural integrity and conformational structure. It is important to note that even with 20 wt% of PEG-600

and 15 wt% of NaPA-8000, the GFP maintained its fluorescence capability on the PEG-rich phase, as demonstrated by MB_{GFP} around 100%. The maintenance of GFP structure and fluorescence integrity using these ABS is expected, since the concentrations of each polymer, [Ch]Cl and buffer are low and adequate for protein stability,[47, 51] avoiding the precipitation of proteins or undesirable structural changes. GFP present many variants and stability ranges, but overall, GFP is a robust protein,[3-4] showing a pH stability between 5.5 to 12[52] and, although in its native form it undergoes denaturation above 37°C,[53-54] many recombinant GFP (as the one in this study) retain its fluorescence at 65°C. Regarding the stability of GFP in the presence of solvents, it can withstand low concentrations of surfactans like 1% m/v SDS solutions and even some concentrations of chaotropic agents.[55-57] Also, there are GFP extraction studies showing that GFP is stable in the presence of phosphate salts,[58] PEG and NaPA-8000,[47] results that are aligned with the outcomes of this study.

In the PEG-600/NaPA-8000 + [Ch]Cl-based ABS, GFP was completely recovered in the PEG-rich phase at all compositions studied. These results agree with previous works of Johansson *et al.*,[47] in which more than 90% of GFP was recovered in the PEG-rich phase using systems composed of PEG-400 + NaPA-8000 + Na_2SO_4 as adjuvant. The authors explained the protein preferential partition to the PEG-rich phase as a combination of hydrophobic and entropic effects, suggesting that due to the small size of PEGs (from 400 to 1500 $g.mol^{-1}$), the proteins are more easily accommodated in the PEG-rich than in the NaPA-rich phase.[47] This preferential partition to the PEG-rich phase (considered by the authors as the hydrophobic phase) was suggested to be a result of the reduction of entropic (due to the low PEG size) and the increase of enthalpic driving forces (due the high PEG-concentration).[47] Herein, it was observed that the GFP fully partitioned to the PEG-600-rich phase, and considering **Table S2** from **ESI†**, there was no significant difference in the phases water content (with an average difference of only 2.72 ± 1.56 wt%, the PEG-rich phase cannot be considered as the hydrophobic one), so further understanding of the GFP partition mechanism is needed. As observed in previous studies using PEG/NaPA-based ABS, the partition of proteins,[47, 59] irrespective of its hydrophilic/hydrophobic nature, was always for the PEG-rich phase. Considering that the GFP isoelectric point (pI) is 5.52,[50] the protein is negatively charged at pH 7 (pH of the system). Thus, since at neutral pH the NaPA-8000

is negatively charged due to the presence of carboxylic acid groups in the principal polymeric chain,[59] electro-repulsive forces of NaPA-8000 over charged solutes govern the protein partitioning towards the PEG-rich phase. Therefore, more than the entropic effects with volume exclusion or hydrophobic effects, the complete extraction for the PEG-600-rich phase seem to be a result of the electro-repulsiveness nature of NaPA-8000 over the negatively charged GFP.

Similarly, in the PEG/potassium phosphate buffer-based ABS, the results from **Fig. 1 B** also shown that GFP complete partitioned to the PEG rich-phase. Thus, in spite of the GFP pI of 5.52[50] and an overall negative surface charge at pH 7, GFP still had a higher affinity to the low ionic strength (PEG-rich) phase, where the conductivities ($mS.cm^{-1}$) are 5.35 or 6.75 for both PEG/potassium phosphate buffer studied (for comparison conductivities of the phosphate buffer phases are 122.3 or 124.4 $mS.cm^{-1}$). In addition, both phases are very rich in water (ranging from 64.58 to 78.85 %), being the difference in water content (wt%) between the two coexisting phases lower than 5%, **Table S2** from **ESI†**. Therefore, this rules out the preferential partition of the hydrophilic GFP due the hydrophobic-hydrophilic effects (the PEG-rich phase being the most hydrophobic). Thus, since phosphate salts have a strong salting-out ability,[60] it seems that the salting-out phenomenon is the mechanism that controls the partition of GFP on these ABS. A similar behavior was reported by Pereira *et al.*[61] for the purification of ovalbumin from egg white using PEG-salt ABS, also dominated by the salting-out effect.

In opposition, on both PEG-600/[Ch]Cl and PPG-400/[Ch]Cl-based ABS, the GFP was completely extracted for the [Ch]Cl-rich phase (EE_{GFP} (%) greater than 99%), as shown in **Fig. 1 C)**. These systems are also highly biocompatible with GFP, exhibiting MB_{GFP} (%) values closer to or higher than 90% (**Table S2** of **ESI†**). It is important to notice that for both systems there was no apparent difference between the three concentrations of polymer/[Ch]Cl evaluated. Regarding the mechanisms that control the GFP partition in the PEG-600/[Ch]Cl and PPG-400/[Ch]Cl-based ABS, these are also completely different from the previous ABS studied. In this system, GFP was extracted into [Ch]Cl-rich phase (the most hydrophilic phase), considering the water content (wt%) of the phases. In addition, the water content (wt%) was considerably different for each phase, being around 29-33 and 56-62 wt% for the [Ch]Cl phase in the PEG-

600/[Ch]Cl and PPG-400/[Ch]Cl-based ABS, respectively, and 12-15 and 13-25 wt% for the polymer phase of the aforementioned ABS, respectively. Thus, due to the hydrophilic character of GFP, the hydrophobic-hydrophilic interactions had a major role in the partition mechanism for both ABS, where the GFP partitioned always for the phase with the highest water content. Previously, Salabat *et al*.[62] performed some studies on the proteins extraction using polymer/salt ABS (by combining PEG-600 and PPG-425 polymers and $MgSO_4$, Na_2SO_4, $(NH_4)_2SO_4$ inorganic salts), demonstrating a selective extraction of hydrophobic protein for the polymeric phase and the partition of the hydrophilic proteins towards the salt-rich phase. The partition of GFP using other type of polymer/salt ABS was previously reported (PEG with salts $MgSO_4$, $Na_3C_6H_5O_7$, $(NH_4)_2SO_4$, Na_2SO_4 or buffer NaH_2PO_4 + Na_2HPO_4),[47, 58] but usually exhibiting quite low purification capacity (as will be further discussed in the next sections), thus requiring the association of other techniques like chromatography to significantly increase the purification levels.

Back-extraction of GFP

Although the PEG/NaPA + [Ch]Cl-based ABS were effective to completely extract the GFP from the supernatant of *E. coli* lysate, a purification using these systems was not completely satisfactory, as further detailed in **section "Purification of GFP"**. The low levels of purification using PEG/NaPA and adjuvants was previously reported by Johansson *et al.*,[47] being highlighted that these yields could not be increased due the impossibility of to perform a back-extraction of GFP with small size PEG polymers, which had the highest partition coefficients. However, as demonstrated in this study, GFP could be completely extracted to opposite phases: **(i)** for the PEG-600-rich phase for the systems composed of PEG-600/NaPA-8000 + [Ch]Cl and **(ii)** for the [Ch]Cl-rich phase considering the PEG-600/[Ch]Cl-based ABS. Thus, the combination of these PEG/[Ch]Cl-based ABS with the PEG/NaPA + [Ch]Cl system can enable the back-extraction of GFP, consequently increasing the GFP purification levels. Considering this, a back-extraction procedure combining two extraction stages using these two ABS platforms was designed, with methods detailed in **section "Back-Extraction of GFP"** from **ESI†**. The back-extraction was performed with a first purification step using the PEG-600/NaPA+[Ch]Cl, followed by a second fractionation

using an ABS composed of [Ch]Cl and PEG-600, as sketched in **Fig. S9** (**ESI†**). Moreover, the EE_{GFP} (%), MB_{GFP} (%) for the first and second purification steps are also provided.

The overall EE_{GFP} (%) and MB_{GFP} (%) of the back-extraction procedure was 99.60 ± 0.01% and 88.6 ± 1.2%, respectively, demonstrating that the integration of two purification units was effective on the GFP recovery, with no significant losses (lower than 12 wt%). The results here obtained are quite interesting, since they demonstrated the possibility of to create a back-extraction using PEG with low molecular weight that completely extracted GFP while also enhancing the purification yields, as shown in the next section.

Purification of GFP

Considering the excellent extraction efficiencies obtained with all the ABS under study, as well as with the back-extraction procedure, the purification ability of each system was then assessed. To compare the fraction of GFP on the total protein, and thus, to obtain an initial evaluation of the GFP purity, a method using 3D fluorescence spectra was developed (as detailed **section "Determination of GFP 3D fluorescence spectrum and quantification of GFP and Total Protein"**, **ESI†**). For this new method, purified samples (phases where GFP was extracted) and non-purified (GFP raw extract obtained after lysis) were diluted to present the same concentration of GFP (12.7 ± 0.2 $mg.L^{-1}$). Each solution was evaluated through the analysis of complete 3D fluorescence spectra, allowing the inspection of the GFP fluorescence region (maximum fluorescence at excitation wavelength (λ_{ex}) at 488 nm and emission wavelength (λ_{em}) at 510 nm, λ_{ex488}, λ_{em510}) and TP fluorescence region (residues of phenylalanine, tryptophan and tyrosine that exhibit a maximum fluorescence at λ_{ex276}, λ_{em336}) in the same figure. The ratio between the fluorescence intensity of GFP (UF, at λ_{ex488}, λ_{em510}) and the fluorescence intensity of TP (UF, at λ_{ex276}, λ_{em336}), GFP/PT (directly proportional to the GFP purity) was then determined. The complete 3D spectra, and respective GFP/TP ratios for the GFP raw extract and the ABS' phases where the GFP was extracted are shown in **Fig. S10** (**ESI†**).

Fig. S10 has two distinct regions for all the spectra (the first around λ_{ex276} and λ_{em336} and the second around λ_{ex488} and λ_{em510}), corresponding to the fluorescence of the proteins containing the three-fluorescent aromatic amino acids (phenylalanine,

tyrosine, and tryptophan) and GFP, respectively. Since the two regions are clearly separated, it was possible to obtain a rough evaluation of the GFP purity through the analysis of the GFP/TP fluorescence ratio. Comparing the GFP/TP ratio from the GFP raw extract and those from the PEG-1500 + K_2HPO_4/KH_2PO_4, PEG-2000 + K_2HPO_4/KH_2PO_4 and PEG-600/NaPA-8000 + [Ch]Cl ABS, only a slight increase was observed, from 4.3 to 4.7, 5.0, and 4.5 respectively. On the other hand, the polymer/[Ch]Cl-based ABS exhibited a more significant increase in GFP/TP ratio, namely, from 4.3 to 6.5, in the PEG-600/[Ch]Cl ABS, and from 4.3 to 8.6 in the PPG-400/[Ch]Cl ABS. Interestingly, the back-extraction procedure also enhanced the GFP/TP ratio to 7.0. Although this comparison is based on the total intensity of fluorescence units, without the determination of GFP and TP concentration through calibration curves, it suggests that the PEG/potassium phosphate buffer and PEG-600/NaPA-8000 + [Ch]Cl-based ABS have a limited purification ability, while the PEG-600/[Ch]Cl, PPG-400/[Ch]Cl ABS and back extraction systems are quite effective in removing the contaminant proteins, consequently improving the purification of GFP. To quantify the purification capabilities of each system, the $[GFP]_{rel}$, was calculated and the comparison of the average of $[GFP]_{rel}$ (%) of three independent assays (and respective standard deviations) for all systems and GFP raw extract are shown in **Fig. 2**.

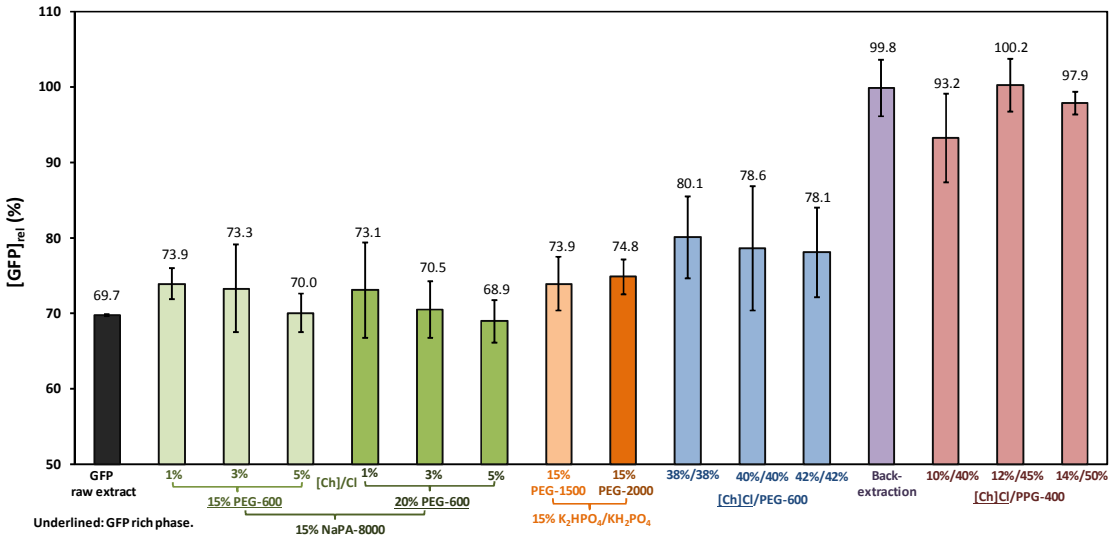

Fig. 2. Relative concentration of GFP by TP ($[GFP]_{rel}$ (%)) for the GFP raw extract ( ) and the GFP rich-phases for the ABS composed of: (light green bars) 15% PEG-600, 15% NaPA-8000 + [Ch]Cl (1, 3 or 5%) ( ); 20% PEG-600, 15% NaPA-8000 + [Ch]Cl (1, 3 or 5%) ( );15 wt% PEG-1500 + 15 wt% K_2HPO_4/KH_2PO_4 ( ); 15 wt% PEG-1200 + 15 wt% K_2HPO_4/KH_2PO_4 ( );

[Ch]Cl/PEG-600 ( ) , back-extraction ( ) and [Ch]Cl/PPG-400 ( ). The results are the average of three independent experiments ± standard deviation.

From **Fig. 2**, it is observed that the GFP raw extract (black bar) after lysis has a $[GFP]_{rel}$ (%) of almost 70% of the total protein of the cell lysate, which is expected because GFP recovered is arising from recombinant *E. coli* BL21(DE3) and other proteins are also produced as result of microorganism metabolism. As observed in the 3D fluorescence (GFP/TP ratio) from **Fig. S10**, the determination of $[GFP]_{rel}$ (%) clearly demonstrates that the use of the systems PEG/potassium phosphate buffer (PEG-1500 and PEG-2000, light and dark orange bars respectively) or the PEG-600/NaPA-8000 + [Ch]Cl (light and dark green, respectively) did not promote an increase on the GFP purity, since there was no significant increase of $[GFP]_{rel}$ (%) for any of the concentrations of the aforementioned ABS. For the PEG-600/[Ch]Cl-based ABS, the $[GFP]_{rel}$ (%) showed only a slight increase (around 10%), with no apparent differences between the various concentrations of polymer and [Ch]Cl, which corresponds to a limited potential for the purification of the target protein. However, if integrating the prior PEG-600/NaPA-8000 + [Ch]Cl-based system with a further extraction step using a PEG-600/[Ch]Cl ABS (purple bar), the GFP purification yields are effectively enhanced, being attained $[GFP]_{rel}$ (%) close to 100%, revealing that the back-extraction procedure completely purifies the GFP, removing the other contaminant proteins. This is consistent with the GFP/TP ratio, increasing from 4.5 from the single step extraction using PEG-600/NaPA-8000 + [Ch]Cl ABS to 7.0 with the back-extraction associating the aforementioned ABS with a following extraction with the PEG-600/[Ch]Cl-based ABS. Interestingly, the total purification of the GFP was also achieved in a single purification step with the PPG-400/[Ch]Cl-based ABS, in which $[GFP]_{rel}$ (%) values from 93 to 100% were achieved. The high GFP purification is in close agreement with the GFP/TP ratio (increase from 4.3 to 8.6), proving that this PPG-400/[Ch]Cl ABS is the best option to perform a quick and simple GFP purification. To further confirm the purification, a SDS-PAGE analysis was used, method described in **section "Evaluation of GFP purification by SDS-PAGE"** (**ESI**†). To identify the protein present in the extracts after the use of each ABS, samples of each system and the raw extract containing 15 µg of GFP were added to the gel, and a SDS-PAGE run was performed, as shown in **Fig 3.**

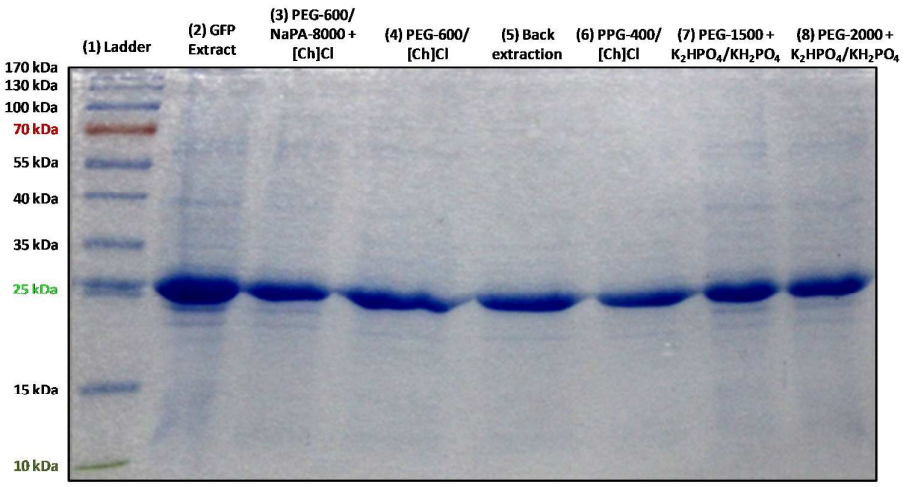

Fig. 3. SDS-PAGE electrophoresis of the GPF extract and the ABS containing the same mass of GFP in each well (15 µg). Ladder (lane 1), GFP raw extract (lane 2) and extracts after purification using the ABS: PEG-600/NaPA-8000 + [Ch]Cl (lane 3); PEG-600/[Ch]Cl (lane 4); back-extraction (lane 5); PPG-400/[Ch]Cl (lane 6); PEG-1500 + K_2HPO_4/KH_2PO_4 (lane 7); PEG-2000 + K_2HPO_4/KH_2PO_4 (lane 8). GFP has 27 kDa and is represented by the strongest bands between 25-35 kDa.

In the SDS-PAGE, a strong band around 27 kDa is indicative of GFP, while bands in other regions are indicative of the presence of contaminant proteins. All samples present a strong band between 25 and 35 kDa, corresponding to the 27 kDA of GFP. The SDS-PAGE results from **Fig. 3** are in close agreement with the GTP/TP ratio and $[GFP]_{rel}$ (%) data, suggesting that the 3D fluorescence spectra analysis can be used as a simple and quick method to evaluate the purification of proteins. Moreover, and as shown in the gel electrophoresis, the GFP raw extract (lane 2) exhibited, in addition to the GFP band, several bands outside the region between 25-35 kDa, these corresponding to contaminant proteins. While in the PEG-600/NaPA-8000 + [Ch]Cl (lane 3) and PEG/potassium phosphate buffer (lanes 7 and 8) the number and intensity of the impurities were not significantly reduced, these bands diminished in the PEG-600/[Ch]Cl ABS (lane 4), indicating a moderate purification potential. However, the best results were effectively obtained with the back-extraction system (lane 5) and PPG-400/[Ch]Cl (lane 6), in which the impurities bands are practically not visible, showing the purification potential of these ABS.

Considering the partition mechanisms discussed in the **section "GFP Extraction"**, it is possible to suggest that when the GFP migration is mainly controlled by electro-repulsive forces (for the PEG-600/NaPA-8000 + [Ch]Cl ABS) or by salting-out

mechanisms (for the PEG/phosphate buffer ABS), all the other contaminant proteins are concentrated in the same phase, leading to low GFP purification yields. On the other hand, when the partitioning mechanisms are based on the hydrophilicity-hydrophobicity balance, in both PEG-600/[Ch]Cl and PPG-400/[Ch]Cl, the GFP purification is considerably more efficient. It seems that the protein contaminants (other cellular proteins) are less hydrophilic than GFP, thus preferentially migrating into the most hydrophobic phase (PEG-rich phase). This is also clearly observed by the increase in the GFP purification yields when the PEG-600 was replaced by the PPG-400 polymer (more hydrophobic), resulting in a higher water content (wt%) difference among coexisting phases (as described in **Table S2**).

Previously, Ishihama *et al.* profiled more than 1000 proteins present in the cytosol of *E. coli*, which showed that the most abundant proteins in *E. coli* present a mean relative hydrophobicity in Kyte-Doolite scale of -0.25,[63] while GFP presents a mean value of -0.47, (lower values in Kyte-Doolite scale indicate that the proteins are more hydrophilic). The method for the calculations is described in **section "Determination of relative hydrophobicity of GFP"** (**ESI**[†]). Although the most abundant proteins in *E. coli* are still considered hydrophilic (negative values in hydrophobicity scale), GFP is even more hydrophilic, and thus, this can explain the difference in the migration pattern in the polymer/[Ch]Cl ABS. This effect was also observed by Salabat *et al.*[62] in a study with different polymer/salt-based ABS (polymers PPG-425 and PEG-600), where the PPG-425 systems had the best results for the selective separation of hydrophobic and hydrophilic proteins. Additionally, we have demonstrated that the integration of PEG/NaPA + [Ch]Cl and PEG/[Ch]Cl-based ABS, two purification platforms with different proteins' partitioning mechanisms, can be a feasible approach to increase the protein purification abilities.

To demonstrate the excellent GFP purification results achieved in this work, **Table 1** shows a comparison of the results obtained with the PPG-400/[Ch]Cl-based ABS and back-extraction system with the alternative GFP purification techniques already established in literature (in general, liquid-liquid extractions in a single step or associated with back-extraction or chromatography techniques). As can be seen in **Table 1**, most alternative techniques for GFP purification either involve a series of liquid-liquid extractions and their association with chromatography techniques, or

present low purification factors (PF) or partition coefficients (*K*). Thus, both approaches proposed in this study are effective alternatives, allowing high GFP recovery and purity yields through the use a simpler extraction platform using ABS. The results confirmed the ability of ABS composed of biocompatible and low toxicity solvents to purify GFP in a single or two-phase liquid-liquid extraction unit, however, to further assess their industrial potential, the recycling of the phase formers and GFP polishing were also investigated.

Table 1. Comparison of different liquid-liquid extraction approaches (single step liquid-liquid extraction, or association with back-extraction or chromatography techniques) for the purification of GFP. GFP recovery (%), $[GFP]_{rel}$ (%) (indicating GFP purity), purification factor (PF) and partition coefficient (*K*) values are presented when available.

Purification techniques	GFP Recovery (%)	$[GFP]_{rel}$ (%)	PF	*K*	Reference
PEG-600/NaPA-8000/[Ch]Cl ABS + back extraction with PEG-600/[Ch]Cl ABS	89*	100			This study
PPG-400/[Ch]Cl ABS	92*	100			
Multiple liquid-liquid organic extractions + Phenyl-Sepharose Chromatography	36		14		15
PEG-6000/$C_6H_5Na_3O_7$ ABS	91		3.3		58
PEG-6000/$C_6H_5Na_3O_7$ ABS + IMAC[&]	81		89		
PEG-6000/$C_6H_5Na_3O_7$ ABS + back extraction with PEG-6000/Dextran T500 ABS	39		4.5		
PEG-6000/$C_6H_5Na_3O_7$ ABS + back extraction with PEG-6000/Dextran T500 ABS + IMAC*	32		126		
PEG-6000/$C_6H_5Na_3O_7$ ABS + back extraction with PEG-6000/NaH_2PO_4 + Na_2HPO_4 ABS	73		4		
PEG-6000/$C_6H_5Na_3O_7$ ABS + back extraction with PEG-6000/NaH_2PO_4 + Na_2HPO_4 ABS + IMAC[&]	71		94		
PEG-3000/NaPA-8000/Na_2SO_4 ABS	86			8.1	47
PEG-3000/NaPA-8000/Na_2SO_4 ABS + back extraction with PEG/Na_2SO_4 ABS	96[#]			0.2[#]	

*For this study, MB_{GFP} (%) represents GFP Recovery (%).
[&]IMAC - immobilized metal affinity chromatography.
[#]calculated in relation to the single extraction with PEG-3000/NaPA-8000/Na_2SO_4 ABS.

Considering the great potential of the PPG-400/[Ch]Cl ABS to extract and purify GFP from *E. coli* cell lysate, and to validate if this performance is maintained for other recombinant proteins produced by *E. coli*, the extraction of two other fluorescent proteins (FP) from *E. coli* cell lysate, Fezziwig Yellow Fluorescent Protein (YFP) and Red Fluorescent Protein (RFP), was also evaluated (as described in the **section "Extraction and purification of other recombinant fluorescent proteins produced by *E. coli* using**

PPG-400/[Ch]Cl ABS" from the **ESI†**). As can be seen in **Table S4** (**ESI†**), PPG-400/[Ch]Cl ABS was once more able to completely extract (Relative Extraction for the FP, RE_{FP} (%) >99%) both proteins in the [Ch]Cl rich-phase. Additionally, to evaluate the purification potential of the system, 3D Fluorescence spectra of YFP and RFP raw extracts (1 and 3, respectively) and YFP and RFP after the extraction step using PEG-600/[Ch]Cl ABS (2 and 4, respectively) were compared (**Fig. S11** from **ESI†**), and the FP/TP relation determined. As shown in **Fig. S11**, there was a small increase in the FP/TP relation for the YFP (from 4.4 to 4.8) and a larger increase for the FP/TP relation of RFP (from 4.0 to 5.5) after extraction, suggesting that the PPG-400/[Ch]Cl ABS can extract efficiently and purify, to a certain extent, other recombinant fluorescent proteins from *E. coli* cell lysate. These relative ratios do not allow to directly compare the purification efficiency of the three fluorescent proteins (GFP, YFP and RFP), but indicates qualitatively that all of them were purified to a certain extent using the PPG-400/[Ch]Cl ABS. Although, further studies are required to understand the degree of purification, these preliminary studies indicate that this system has a great potential to be used as a high-performance platform for the recovery and purification of different recombinant proteins produced by *E. coli*.

Recycling of the ABS phase former components and GFP polishing

To address one of the critical issues regarding the application of ABS for biomolecules' purification, namely the use of high concentrations of relatively expensive phase-forming agents,[43] a method for recycling the phase-forming components of the ABS with best GFP purification performance, PPG-400/[Ch]Cl-based ABS, was attempted (methods described in **"Recycling of the ABS phase former components and GFP polishing"** from **ESI†**). It is important to note that a successful recycling of the solvents decreases not only the costs, but also the environmental impacts of the downstream process. After some initial tests, the ultrafiltration was chosen for the recycling and polishing studies. Precipitation methods using cold acetone or ammonium sulfate were also attempted, but failed due to the precipitation of some phase-forming components with the GFP. Thus, an integrative approach for

the extraction/purification with PPG-400/[Ch]Cl ABS and the respective phases' recycling through ultrafiltration was proposed.

After a first purification step using PPG-400/[Ch]Cl ABS, both top (PPG-rich) and bottom ([Ch]Cl-rich phase, containing GFP) phases were recovered and submitted to ultrafiltration (with a 10 kDa membrane) processes. After filtration of the PPG-rich phase, the permeate containing the PPG-400 aqueous solution free of contaminant proteins, which were removed in the retentate, was then recycled in the liquid-liquid extraction unit. Similarly, the [Ch]Cl-rich phase was also filtered and then, the [Ch]Cl aqueous solution recovered in the permeate (without GFP) was also recycled in the liquid-liquid extraction unit. It is important to note that extra amounts of ABS phase-forming components can be added in the system, to fulfill some solvent losses occurring in the process. The pure GFP recovered in the retentate was collected and it can be either used in the liquid for the formulation of some bio-product of interest or go for further processing, as for example, freeze-dying or encapsulation procedures. The weight fraction (wt%), EE_{GFP} (%), MB_{GFP} (%) and $[GFP]_{rel}$ (%) for the PPG-400/[Ch]Cl-based ABS before and after solvent recycling using ultrafiltration were determined, as compiled in **Table 2**. Additionally, the purity of the polished GFP (recovered after the ultrafiltration step) was evaluated by means of the $[GFP]_{rel}$ (%). Additional information about the recycling process (mass of top and bottom phases recovered after ultrafiltration, total mass of the PPG-400/[Ch]Cl ABS before and after ultrafiltration and recovery percentage (wt%) of the recycled ABS) is presented in **Table S5** from the **ESI†**.

Table 2. Weight fraction (wt%), EE_{GFP} (%), MB_{GFP} (%) and $[GFP]_{rel}$ (%) for the PPG-400/[Ch]Cl ABS before and after solvent recycling using ultrafiltration. $[GFP]_{rel}$ (%) of the polished GFP, recovered after ultrafiltration step is also presented. Results correspond to the average of three independent assays and respective standard deviations.

PPG-400/[Ch]Cl ABS	ABS Composition Weight fraction (wt%)			EE_{GFP} (%)	MB_{GFP} (%)	$[GFP]_{rel}$ (%)*
Before ultrafiltration	**PPG-400**	**[Ch]Cl**	**GFP raw extract**			
	44.96	12.04	10.05	99.14 ± 0.06	94.4 ± 3.0	98.2 ± 3.8
After Recycling#	**Recycled PPG-rich phase**	**Recycled [Ch]Cl-rich phase**	**GFP raw extract**			
	52.74	37.19	10.07	99.11 ± 0.09	93.0 ± 2.0	98.1 ± 2.9
GFP Polishing						**$[GFP]_{rel}$ (%)***

After ultrafiltration	98.5 ± 1.0

*$[GFP]_{rel}$ (%) of extract: 69.7 ± 0.1 %. #Solvent recovery: 60.7 ± 2.2 wt%.

As can be seen in **Table 2**, the values of EE_{GFP} (%), MB_{GFP} (%) and $[GFP]_{rel}$ (%) for the PPG-400/[Ch]Cl ABS were maintained even after solvent recycling (60.7 ± 2.2 wt% of the solvents were recovered). These results confirm that the reuse of the phase-forming agents after ultrafiltration does not compromise the purification capabilities of the ABS. However, it is important to note that it is also possible to make up the solvents lost in the process, which can potentially increase the solvent recycling capacities of this system. In addition, it was possible to obtain a concentrated aqueous solution of pure GFP, 98.5 ± 1.0 %. It is important to notice that after the filtration, GFP is not only free of contaminant proteins (removed in the purification process), but also from all the low molecular residual impurities, which were removed through the ultrafiltration step. Therefore, a highly pure GFP was obtained after ultrafiltration, with both PPG-400 and [Ch]Cl effectively recycled and reused to form a new PPG-400/[Ch]Cl-based ABS, allowing a closed and sustainable purification process. Summing up, an integrative process for the purification of GFP with PPG-400/[Ch]Cl ABS and the respective phases' recycling through ultrafiltration was herein proposed, as schematized in **Fig. 4**.

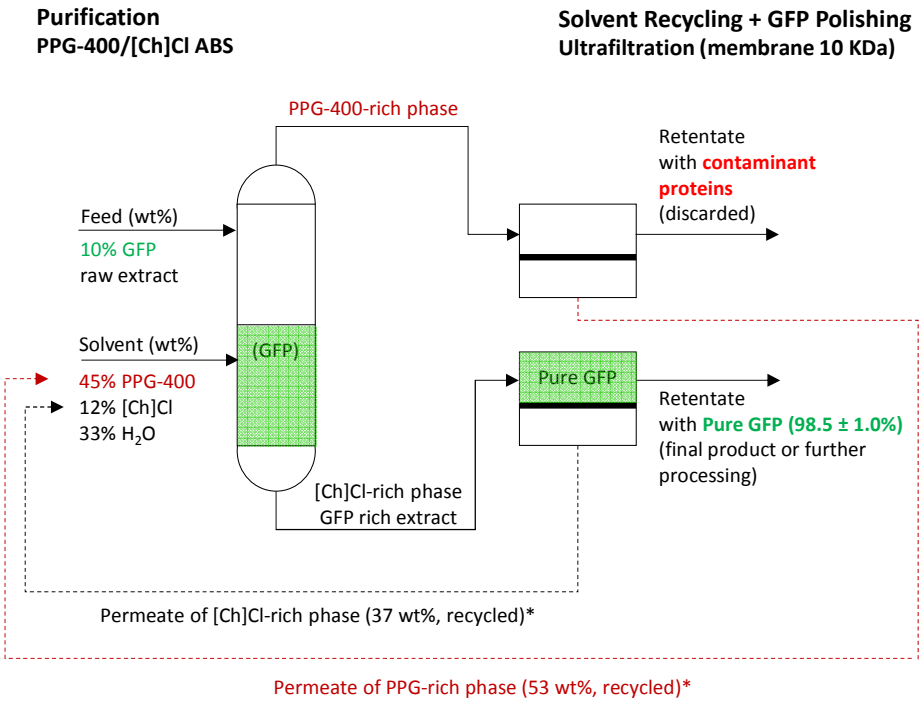

Fig 4. Diagram of the integrative process developed for the purification of GFP using PPG-400/[Ch]Cl ABS and the recycling of the phase-forming agents using ultrafiltration. *Composition (wt%) of the new ABS, after solvent recycling.

Conclusions

In this work, a biocompatible and effective process for the recovery and purification of GFP from cell lysate of a recombinant *E. coli* BL21 was established. In general, all ABS allowed the full partition of the target protein (above 99% of extraction efficiency) in the PEG-rich or [Ch]Cl-rich-phase, depending on ABS. The systems composed of PPG-400 and [Ch]Cl, in addition to the excellent extraction performance, also had great capacity to purify GFP from contaminant proteins in a single step process (GFP purity yields close to 100%). The ABS PEG-600/NaPA-8000 + [Ch]Cl and PEG-600/[Ch]Cl did not exhibit high GFP purification aptitudes when used as a single step, but when integrated in a two-purification step (as a back-extraction procedure), also allowed to obtain an almost 100% pure GFP. Additionally, the economic and environmental sustainability of the PPG-400/[Ch]Cl-based ABS was improved, being shown that approximately 60% of the phase-forming components can be effectively recycled through the integration of an ultrafiltration unit in the end of the process of purification to each phase. Therefore, considering that the PPG-

400/[Ch]Cl-based ABS can even recover and purify other fluorescent recombinant proteins (YFP and RFP) from *E. coli* cell lysate, this work demonstrates that the [Ch]Cl-based ABS can be a feasible and promising platform for the purification of proteins from complex media (such as fermented broth or cell lysates).

Electronic Supporting Information† (ESI†)

Experimental section; physical chemical characterization, extraction and purification parameters table; phase diagram determination for PEG/phosphate buffer ABS; ABS' phases FTIR-ATR analysis; back-extraction diagram; 3D Fluorescence spectra for the ABS; extraction and purification of other recombinant fluorescent proteins; table detailing the recycling of the ABS phase former components and GFP polishing.

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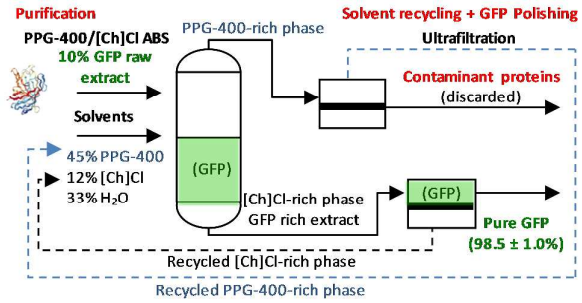
Abstract Graphic



Synopsis

A single-step purification technique which allow high yields and purity of green fluorescent protein produced by *Escherichia coli*.