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Purification of green fluorescent protein using fast centrifugal partition chromatography

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ABSTRACT

The green fluorescent protein (GFP) is a biomolecule used in many biological applications such as biomarkers and biosensors, which require high purity levels. It is usually obtained from recombinant *Escherichia coli* strains, which also produces other endogenous proteins, demanding multiple purification steps, and consequently, increasing the overall costs to obtain pure GFP. Simpler and cheaper purification methods like Aqueous Biphasic Systems (ABS) were already successfully applied to purify GFP at lab scale. Therefore, the development of automatized industrially compatible purification platforms, such as countercurrent chromatography using ABS, can potentially improve the GFP production. This work studied the continuous purification of the variant enhanced GFP (EGFP) by applying ABS composed of polyethylene glycol (PEG 8000), sodium polyacrylate (NaPA 8000) and sodium sulfate (Na_2SO_4) as electrolyte. An initial screening was carried by changing the electrolyte content in the ABS. The increase of this condition has demonstrated an increase on the EGFP partition for the PEG-rich phase. The most efficient ABS and, at the same time, with the most appropriate conditions, namely the system composed of 15 wt% PEG 8000 + 4.5 wt% NaPA 8000 + 2.5 wt% Na_2SO_4 was chosen and applied on the fast centrifugal partition chromatography (FCPC). After optimization, the best operational conditions were identified, *i.e.* a flow rate of 2.5 mL.min^{-1} and rotation speed of 2000 rpm at ascending mode, and the best results obtained, meaning a purification of 89.93% and a recovery yield of 82.3%, confirming the potential of FCPC to the continuous purification of EGFP.

1. Introduction

The green fluorescent protein (GFP) emits a natural and intense visible fluorescence without needing cofactors. It can be genetically encoded by a series of organisms, an advantage that enables its application as biomarker and biosensor [1,2]. It is a relatively small protein (27 kDa) consisting of 238 amino acid residues [3], firstly isolated by Shimomura *et al.* [4] from the jellyfish *Aequorea victoria* in 1960s. Almost 30 years later, some studies revealed GFP as an important tool for mapping cells by connecting its gene with those encoding for other proteins, thus functioning as a tracking lamp in living organisms, forming a fused protein [5–8]. Through the isolation and cloning of the gene coding sequence to obtain GFP, the protein was successfully produced by recombinant organisms such as *Escherichia coli (E. coli)* and *Caenorhabditis elegans* (*C. elegans*) [5]. Moreover, it has been successfully expressed in many other organisms, such as bacteria, fungi, plant, and animal cells [9]. In addition to that, the development of modified GFP with improved fluorescence properties and folding for biological applications, such as its most popular variant, the Enhanced Green Fluorescent Protein (EGFP) [10], broadened its use as a fluorescent genetic marker and tracking agent of analyzes within living cells or organelles [11].

Endowed by its remarkable fluorescence, recombinant variants, such as EGFP, exhibit high thermal stabilities and high resistances to alkaline pH, detergents, organic salts and proteases, being thus capable to retain fluorescence up to a pressure of 600 MPa [9,12]. With these properties, recombinant fluorescent proteins became a powerful tool for applications such as fusion tags, reporter gene, data storage, diagnosis,

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stability of proteins and many photochemical applications such as Fluorescence Resonance Energy Transfer (FRET) experiments and photobleaching [3,13,14]. For biomarker and biosensor applications, GFP and its variants need to interfere as little as possible with the reactions to be visualized, must not disturb the physiological conditions of the cells, and must be efficiently detectable [2]. All these characteristics strongly depend on high purity levels, requiring multi-stage conventional chromatographic and non-chromatographic techniques (*i.e.* ultrafiltration and precipitation). Examples of chromatographic technique are size exclusion and ion exchange chromatography (SEC and IEX), hydrophobic interaction chromatography (HIC), immobilized metal ion affinity chromatography (IMAC) and affinity. The downstream processing of recombinant GFP generally exhibits a cost of 80% of the total production, due to the need of several unit operations that contribute to the process cost [15–17]. Furthermore, owing to the multiple unit operations applied during the purification, mostly chromatography, a significant decrease in the recovery is observed at the end of the downstream processing, with considerable losses of recombinant GFP being reported [18–21].

Aqueous biphasic systems (ABS) have been widely studied for the purification of proteins and other biomolecules [22,23], including GFP variants [24,25]. They consist in two aqueous-rich phases formed upon mixing two structurally different components, such as two polymers, two salts or one polymer and one salt in aqueous media [26]. Polymer-based ABS composed by the combination of polyethylene glycol (PEG) and sodium polyacrylate (NaPA) have been successfully applied to recombinant GFP purification [24,27]. From the plethora of available ABS, various polymers (PEG-600, PPG-400 and PEG-600/NaPA-8000) [28] combined with cholinium chloride, different sizes of PEG combined with inorganic salts (e.g. PEG-1500/potassium phosphate and PEG-2000/potassium phosphate) [28] and alcohol combined with various salts (e.g. EtOH + $Na_3C_6H_5O_7$, EtOH + $K_3C_6H_5O_7$ and 1-PrOH + $K_3C_6H_5O_7$) [29] have been investigated to purify recombinant GFP in batch regime. These purification platforms exhibited high recoveries and purification levels (*Rec*% > 90% and purity between 70 and 100%), however none of these studies was performed in a continuous flow or in larger scale.

The ABS studied in this work are based on PEG and NaPA polymers, that by the addition of an electrolyte (e.g. inorganic/organic salt), allow the separation of two polymer-rich phases, a PEG-rich top-phase and a NaPA-rich bottom-phase. A small amount of electrolyte (circa 5 wt% or less) is needed to induce the NaPA compartmentalization and promote phase separation, leading to the formation of a biphasic system with low polymers' concentration. For this reason, PEG/NaPA-based ABS are highly biocompatible (> 70 wt% of water), generally exhibiting low viscosity. Moreover, both NaPA and PEG are easily recovered [30], allowing thus to overcome the high costs of the phase-forming polymers [31], one of the major drawbacks associated to ABS. It was recently described that, in certain conditions, recombinant GFP [5] partitions to the PEG-rich phase, being then recovered by back-extraction by the addition of salts (sodium sulfate (Na_2SO_4) or sodium chloride (NaCl)) [24]. The low viscosity and high water content of the phases are advantageous conditions making these ABS appropriate to be applied in counter-current liquid–liquid chromatography. Centrifugal partition chromatography (CPC), by its turn, is capable to enhance the purification performance in a continuous flow. It is considered a versatile, scalable and mechanistically simple approach, which permits the separation of structurally similar molecules in a continuous regime [32]. For that, this strategy has been considered of easy scale-up allowing the processing of small of some mL to several liters [33,34].

CPC is a form of counter-current liquid–liquid chromatography with a separation mechanism based on the concept of the differential partition of the components between two immiscible aqueous phases. The centrifugal force field is created through rotation of a disc formed by a series of chambers containing the stationary phase, which are connected by ducts to a chamber-cascade, while the mobile phase is pumped through. Due to the liquid nature of the phases, CPC can operate in ascending or descending modes, and by selecting the top and bottom phases of the ABS as the mobile or stationary phase. CPC does not require a solid packing material to maintain the stationary phase, contrarily to what happens with the traditional chromatography techniques. In this sense, the sample losses are reduced, which could occur by irreversible adsorption and by avoiding protein degradation or precipitation [35,36]. CPC and ABS have been studied by several research groups as a strategy to up-scale and work the flow in continuous regime to purify various biomolecules including proteins [36–40]. Our group has a series of works reporting the advantages and good results of using fast centrifugal partition chromatography (FCPC) and PEG/NaPA-based ABS on the separation of different phenolic acids ($Rec_{PHENOLICS}$% = between 65 and 87% and purity up to 95%) and later for cytochrome-c PEGylated conjugates (Rec_{PROT}% = 99% and purity around 100%) [32,39]. FCPC operates similarly to CPC, however it has a fast performance due to its excellent retention of the stationary phase, thus allowing high flow rates and better mixing of the phases in each channel [41].

In this work, PEG 8000 + NaPA 8000-based ABS, using Na_2SO_4 as electrolyte in concentrations ranging from 1.25 to 7.5 wt%, were studied in the purification of GFP, variant EGFP, produced intracellularly by *E. coli* BL21 cells. The best electrolyte concentration for the ABS formation, considering the partition coefficient (K_P), the volume ratio (V_R) and the selectivity (S) was performed, followed the application of the most performant ABS in FCPC. The optimization of FCPC' operational conditions was also performed for the flow rate, mode (*i.e.* ascending or descending) and rotation speed.

2. Experimental

2.1. Materials

The microorganism *E. coli* BL21(DE3) pLysS carrying the plasmid pET-28(a) encoding the gene for expression of recombinant EGFP was kindly provided by the Molecular and Cellular Biology Laboratory of the School of Pharmaceutical Sciences from São Paulo State University (UNESP), Araraquara, Brasil.

The components of Luria-Bertani (LB) culture media, namely tryptone and yeast extract, were purchased from Oxoid while NaCl was acquired from Panreac. The antibiotics used in cell cultivation, kanamycin disulfate salt from *Streptomyces kanamyceticus* and chloramphenicol (≥98 wt%), were supplied by Sigma-Aldrich as well as the isopropyl β-$_D$-1-thiogalactopyranoside (IPTG, ≥ 99 wt%). The components of the Tris–HCl buffer, namely tris(hydroxymethyl)aminomethane (PA) and chloridric acid (37 wt%), were acquired from Pronalab and Sigma-Aldrich, respectively. Bovine serum albumin (BSA) used was purchased at Thermo Scientific.

The PEG 8000 g.mol^{-1} (purum) and NaPA 8000 g.mol^{-1} (45 wt% in water), were both purchased from Sigma-Aldrich. Na_2SO_4 was acquired at Sigma-Aldrich, with a purity ≥ 99 wt%.

2.2. Cell cultivation for production of EGFP

The cell cultivation was carried out following the protocol described elsewhere [42,43]. Briefly, after 6 h of protein incubation and 17 h of protein induction, the fermented broth was centrifuged at 4700 *g* for 30 min at 4 °C, the supernatant discharged, and the weight of wet cells determined. The cell pellets containing EGFP were resuspended in 50 mmol.L^{-1} of Tris–HCl buffer (pH 8) at approximately 0.05 of wet cells (wt%).

2.3. EGFP release: Cell disruption

The wet cells at 0.05 wt% were disrupted using a digital ultrasonic cell disrupter (Branson 450 Digital Sonifier) at an amplitude of 40%

Table 1
FCPC assays with 15 wt% of PEG 8000 + 4.5 wt% of NaPA 8000 + 2.5 wt% of Na_2SO_4. The data of the operating conditions and stationary phase retention (S_f) achieved is reported.

Flow rate (mL.min^{-1})	Rotation speed (rpm)	Mode	Mobile phase	S_f (%)
2.0	2000	Ascending	PEG-rich phase	19.12
2.0	2500	Ascending	PEG-rich phase	19.12
2.5	2000	Ascending	PEG-rich phase	19.12

(corresponding to 112 W). During the ultrasonication, to avoid temperatures higher than 40 °C and to prevent protein denaturation, the cell suspension was maintained in an ice water bath. The number of cycles of the ultrasonication process previously studied was adopted [42], in a total of 30 cycles, each cycle with 5 s ON/10 s OFF (corresponding to a total of 7.5 min). The cell suspensions were centrifuged at 4700 *g* for 30 min at 4 °C and the supernatant was removed from the pellet containing the cellular debris. Then the supernatant, the EGFP raw extract, was collected for further used.

2.4. Preparation of polymer-based ABS to screen the electrolyte concentration

The ABS used for the partition studies of EGFP extract were prepared using graduated centrifuge tubes by weighing the appropriate amount of each phase component and the EGFP extract. The extraction point adopted to study the partition of EGFP extract was 15 wt% of PEG 8000 + 4.5 wt% of NaPA 8000 + X wt% of Na_2SO_4 as electrolyte (with X ranging from 1.25; 2.5; 3.75; 5; to 7.5 wt%). For the extraction mixture point, the EGFP aqueous raw extract obtained after cell disruption was added in the ABS to totalize 100% of the system (15 wt% + 4.5 wt% + X wt% of electrolyte + Y wt% of EGFP raw extract). To prepare the blank controls the EGFP raw extract was replace by water. For each electrolyte concentration, triplicates containing aqueous EGFP extract and blanks were analyzed. Possible interferences of the phase-forming compounds, including the electrolytes, were subtracted using the blank controls.

After the complete dissolution of all components by stirring, all mixtures were left to equilibrate for 2 h in an air oven, at 25 °C and centrifuged at 1610 *g* for 10 min at 25 °C to achieve the complete partition of the EGFP raw extract constituents between the two aqueous phases. After the careful separation of the phases, the EGFP content was measured by fluorescence around the highest peak of fluorescence intensity (excitation at 485 nm and emission at 530 nm) in a microplate reader (Synergy HT microplate reader – BioTek). The total protein concentration was determined with the Pierce BCA Protein Assay and Micro BCA Protein Assay (Thermo Scientific, Schwerte, Germany), according to the product recommendations and using BSA as standard protein.

Different parameters were determined to evaluate the partition performance of EGFP and contaminant proteins, namely their partition coefficients K_P (K_{EGFP} and K_{TP}, respectively), their recovery in the top phase (Rec_{Top}EGFP%), which corresponds to the PEG-rich phase, and the selectivity data (S), as represented by Eqs. (1)–(3), respectively:

$$K_P = \frac{[P]_{Top}}{[P]_{Bot}} \quad (1)$$

$$Rec_{Top}EGFP(\%) = \frac{100}{1 + \left(\frac{1}{K_{GFP} x R_V}\right)} \quad (2)$$

$$S = \frac{K_{EGFP}}{K_{TP}} \quad (3)$$

where $[P]_{Top}$ and $[P]_{Bot}$ represent the protein concentration (EGFP or total protein (TP)) at the top and bottom phases, respectively. V_R represents the volume ratio between the top and bottom phase volumes. The detailed recovery data are presented in Table S1 in ESI. The experimental procedure and conditions used in the size-exclusion high-performance liquid chromatography (SEC-HPLC) analysis and the equation for purification yield and mass balance of total purification yield are given in ESI.

2.5. Purification using FCPC

A FCPC system, model FCPC-C, from Kromaton Rousselet-Robatel (Annonay, France), was used to study the purification of EGFP in continuous flow. The FCPC separations were carried using the system composed of 15 wt% of PEG 8000 + 4.5 wt% of NaPA 8000 + 2.5 wt% of Na_2SO_4. This system was set to work in the ascending mode. The rotor was entirely filled with the NaPA-(bottom)-rich phase at 600 rpm to achieve homogeneous solvent re-equilibration on the rotor. Then, the rotation was set up at 2000 or 2500 rpm for an appropriate stationary phase retention. After, the working rotational speed was set up, the PEG 8000-rich-(top) phase was pumped through the stationary phase to reach the equilibrium, *i.e.* when only the mobile phase came out of the column and the signal baseline was stabilized. The mobile phase flow rate was studied to increase the stationary phase retention ratio and to decrease the purification time, being applied the flow rates of 2.0 and 2.5 mL.min^{-1}. The processual conditions, namely rotation speed and flow rate, were selected according to the ABS applied and the sample injected (EGFP extract), allowing good liquid–liquid partition conditions and a reasonable operation time. The stationary phase retention, S_f, was calculated by the ratio of the stationary phase volume (V_S) and the column volume (V_C): $S_f = (V_S/V_C) \times 100$. Although several operational conditions were investigated, a value of 19.2% of S_f was constantly achieved (Table 1). For the purification of EGFP extract, the sample loop was filled with 3 mL of the extract. Fractions of 2 mL were collected during the process.

The quantification of EGFP after FCPC was carried by SEC-HPLC following standard protocols. The experimental details of the procedure and respective conditions used are detailed in ESI.

3. Results and discussion

3.1. Effect of the electrolyte concentration in PEG/NaPA-based ABS for the partition of EGFP

The phase diagrams of the systems investigated in this work were reported in a previous study [44]. The EGFP raw extract was obtained by ultrasonic *E. coli* cell disruption. The partition tests were performed using systems composed of 15 wt% of PEG 8000 + 4.5 wt% of NaPA 8000 + Na_2SO_4 as electrolyte. From the set of salts previously investigated [44], Na_2SO_4 was selected as electrolyte due to the low concentrations required to form the two phases, as well as the adequate values of K and S it represents. The range of Na_2SO_4 concentrations, *i.e.*, 1.25, 2.5, 3.75, 5 and 7.5 wt%, was selected considering the binodal curves [44], ensuring that the system would remain in the biphasic region. In these PEG/NaPA systems, the manipulation of the electrolyte concentration affects not only the EGFP partition but also the volume ratio between the phases and, consequently, the systems selectivity, which is a result of specific interactions occurring between the phase-forming components, the electrolyte, and the target protein or the

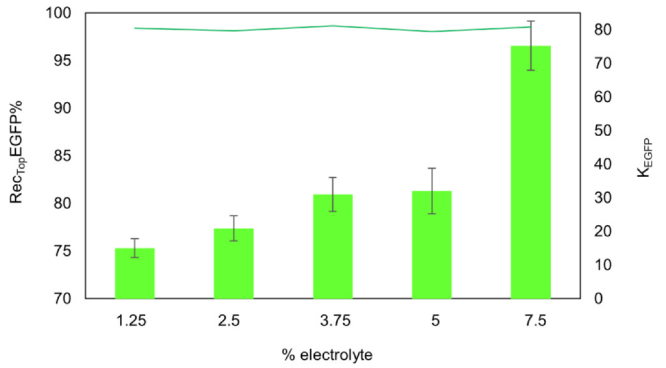

Fig. 1. The recovery of EGFP in the top phase (Rec_{Top}EGFP%, orange line) and the partition coefficients of EGFP (K_{EGFP}, green bars) in the ABS containing 15 wt% of PEG 8000 + 4.5 wt% of NaPA 8000 + water and different concentrations of Na_2SO_4 as electrolyte. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

contaminants.

The effect of the salt concentration on the EGFP partition was initially studied. According to Fig. 1, by increasing the salt concentration, the partition coefficients of the EGFP increase. The presence of Na^+ and SO_4^{2-} disturb the equilibrium, modifying de binodal curve by moving it closer to the axis. By increasing the electrolyte concentration, the volume of the PEG-rich phase becomes smaller, and more concentrated the EGFP is in that phase [27]. This happens because the increase of the salt concentration moves the binodal curve for the PEG rich phase, which "powers" the salting out effect, increasing the repulsion of GFP to this phase [24].

Electrolyte concentrations above 7.5 wt% were tested, however, the phases became increasingly viscous, which difficulted the phase separation and made the use of CPC impracticable. Particularly, at 10 wt % of Na_2SO_4, the high viscosity of the ABS coexisting phases was noticed, while for a concentration around 12.5 wt% of electrolyte, a salt precipitation occurred. As previously reported for other negatively charged proteins [45] such as EGFP [46], by increasing the salt concentration, the partition of proteins is favored to the top PEG-rich phase. This is coherent with our results, where a Rec_{Top}EGFP% higher than 98% is reported, independently of the electrolyte concentration, as shown in Fig. 1 (orange line).

The effect of Na_2SO_4 as electrolyte in ABS composed of PEG/NaPA is in agreement with the reports of Johansson *et al.* [24], which evaluated the partition of pGFP and the study of dos Santos *et al.* [28] where EGFP was partitioned in PEG/NaPA systems using [Ch]Cl as electrolyte. From these works it was possible to observe that, at circa the neutral pH of the polymer coexisting phases, the EGFP is negatively charged (isoelectric point of 5.8) [47] thus justifying its preference for the PEG-rich phase. This was justified, mainly by the electro-repulsiveness nature of NaPA 8000 (negatively charged due to the presence of carboxylic acid groups in the main polymeric chain) over the negatively charged protein [28].

In Table 2, the volume ratio of each system at different Na_2SO_4 concentrations is presented in order to understand the changes in the selective partition of EGFP upon the increase of the electrolyte concentration. It can be noted that the volume ratio decreases while the concentration of electrolyte is increased. Indeed, by varying the salt concentration we are changing the composition of the phases composing the ABS and their ionic strength. The presence of Na^+ and SO_4^{2-} disturb the equilibrium, modifying de binodal curve by moving it closer to the axis [30,44]. By changing the binodal curve, the V_R also changes. Since the V_R is the ratio between the top and the bottom phases, by increasing the electrolyte concentration, the PEG-rich phase becomes more concentrated and the smaller is the volume of water in the phase. As a consequence, the total volume of the phase decreases and more concentrated the EGFP is in the PEG-rich phase.

Table 2
Volume ratio (V_R) and selectivity (S) for the partition of EGFP for the different concentrations of electrolyte tested.

[Na_2SO_4] (wt%)	Volume ratio (V_R)	Selectivity (S)
1.25	4.08 ± 0.35	10.49 ± 0.92
2.50	2.53 ± 0.11	12.23 ± 1.82
3.75	2.42 ± 0.08	17.92 ± 4.54
5.00	1.58 ± 0.06	17.64 ± 3.48
7.50	0.91 ± 0.02	30.44 ± 0.47

Despite the properties and volumes of the coexisting phases, the data regarding the target-protein and total protein partition coefficients, *i.e.* selectivity, are also a key parameter to define adequate systems to be used in CPC mode. As shown in Table 2, the highest selectivity (S = 30.4 ± 0.5) was obtained for the highest electrolyte concentration (7.5 wt%), which means that the EGFP was mainly concentrated in PEG-rich phase, while the contaminant proteins were concentrated in the NaPA-rich phase (K_{TP} between 1.4 ± 0.1 and 2.5 ± 0.2). It is important to note that all the concentrations of salt used are defined as low enough to prevent the formation of a PEG/salt-based ABS [48].

The results of this screening indicate that the most promising ABS to be used in FCPC is the one containing 7.5 wt% of electrolyte, in which the highest K_{EGFP} and S values were obtained, as well as a V_R close to the unit (ideal value for FCPC mode). However, because of the increase of ABS phases' viscosities observed by increasing the electrolyte, it was not possible to apply the system with 7.5 wt% of Na_2SO_4 in FCPC equipment. Therefore, we selected the system with 2.5 wt% of electrolyte to be used in FCPC, since it showed also a good selectivity for the EGFP, guarantying good separation parameters for FCPC performance, *i.e.* low viscosity of the phases and the appropriate volume ratio (2.5 ± 0.1).

Despite the concentrations of 3.75, 5 or even 7.5 wt% have demonstrated to be better for the partition of EGFP using PEG/NaPA + electrolyte, it should be noted that when applying ABS in CPC, further hydrodynamic overpressure issues occur, mostly related with the viscosity of the mobile and stationary phases. Following the previous findings of Oelmeier et al. 2012 [49], which stated that *"a system of higher density difference and viscosity (…) generated a higher backpressure and retained more stationary phase"*. Besides that, according to Armstrong, 1988 [50], the total pressure drop in CPC during the experiment is originated from hydrostatic and hydrodynamic sources following a model equation (4), in which the pressure drop (P) is directly proportional to the balance between viscosities and densities, given by:

$$P = n(\Delta\rho\omega^2 Rh + \eta\gamma F) \quad (4)$$

where n is the total number of channels, Δρ is the difference in liquid densities ($kg.m^{-3}$), h is the height of the stationary phase in one channel, ω is the spin rate for the rotor with radius R, η is the liquid mobile phase viscosity ($kg.m^{-1}.s^{-1}$), γ is a coefficient correlated to the geometrical characteristics of the tube (m^{-3}) and F is the flow rate ($cm^3.s^{-1}$).

The minor differences between partition coefficients for the different ABS (*i.e.*, using 2.5, 3.75 or 7.5 wt% of Na_2SO_4), and to avoid technical overpressure issues in the equipment, we decided to follow the studies with the system composed of 2.5 wt% (which will exhibit the lowest viscosities from all the systems under study).

3.2. Purification of EGFP using FCPC

To select the most adequate processual FCPC conditions (mobile flow rate and rotation speed), the system containing 15 wt% of PEG

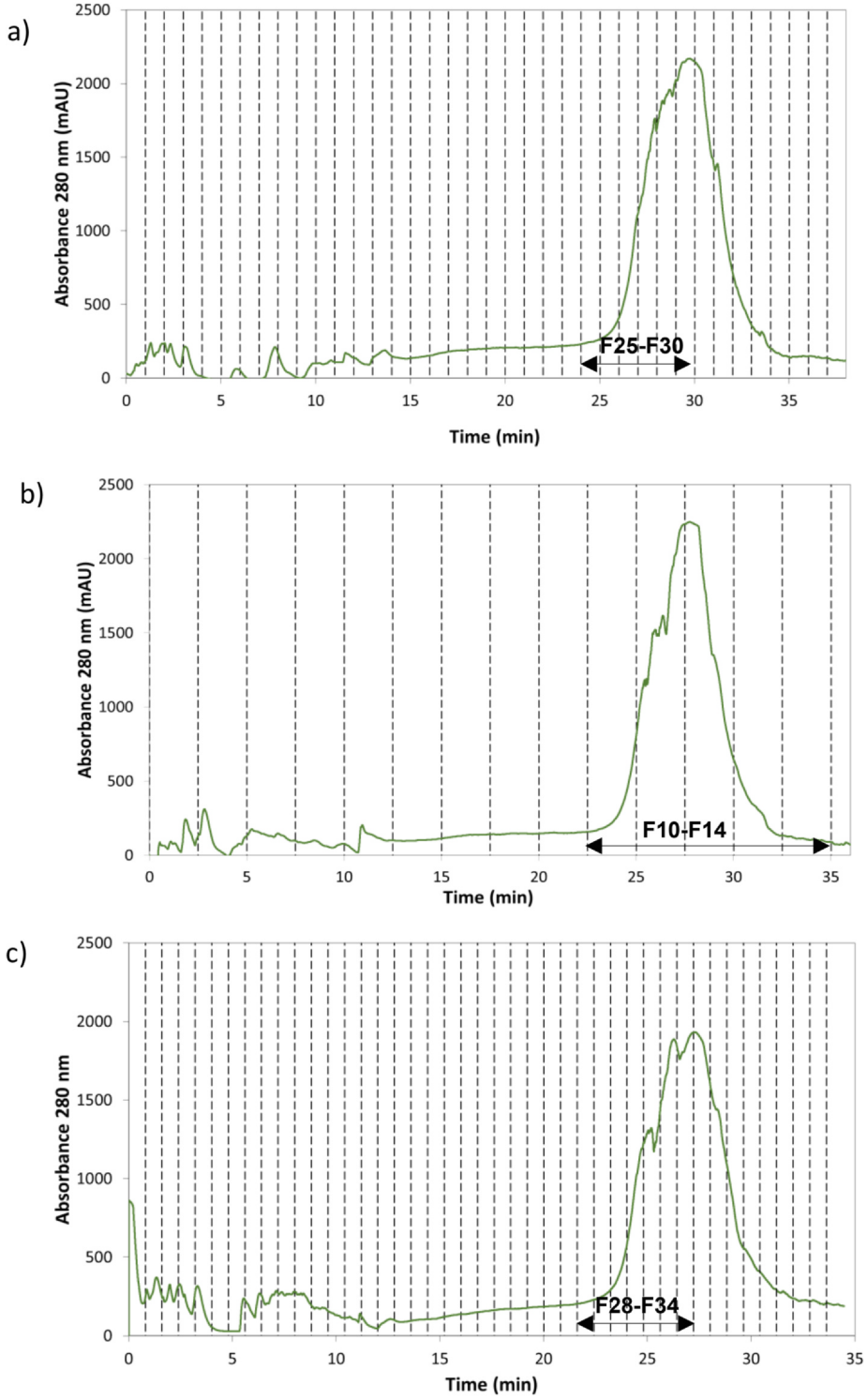

Fig. 2. Purification of EGFP by FCPC using the ABS composed of 15 wt% of PEG 8000 + 4.5 wt% of NaPA 8000 + 2.5 wt% of Na_2SO_4 + 78 wt% of water. The experimental conditions tested were *Sf* = 19.2%; P ≈ 4.5 MPa; detection wavelength of 280 nm, (a) rotation speed of 2000 rpm; flow-rate of 2.0 mL.min^{-1}, (b) rotation speed of 2500 rpm; flow-rate of 2.0 mL.min^{-1} and (c) rotation speed of 2000 rpm; flow-rate of 2.5 mL.min^{-1}.

8000 + 4.5 wt% of NaPA 8000 + 2.5 wt% of Na_2SO_4 + 78 wt% of water was prepared. Considering that the EGFP was preferentially partitioned in the PEG (top)-rich phase (less dense), the FCPC experimental procedure was carried in ascending mode. The column was firstly filled with the heavier NaPA-rich phase, being this retained as a stationary phase at two rotation speeds (2000 and 2500 rpm). After filling, the PEG-rich phase was flooded through the NaPA-rich phase at two moderate flow rates (2.0 and 2.5 mL.min^{-1}). It should be noted that the range of both FCPC processual conditions was selected to guarantee the protein integrity, avoiding protein denaturation/precipitation during the purification.

During the process, the mobile and stationary phases were collected and the chromatograms for the eluted fractions containing purified EGFP (F) determined as shown in Fig. 2.

The eluted fractions shown in chromatograms A, B and C in Fig. 2 are those for which the purity of EGFP is greater than the initial purity

Table 3
Purity, purity factor (PF) and yield of EGFP for each fraction obtained after FCPC experiments using the system composed of 15 wt% of PEG 8000 + 4.5 wt % of NaPA 8000 + 2.5 wt% of Na_2SO_4 + 78 wt% of water.

Condition A: Flow 2.0 mL.min^{-1} and rotation speed 2000 rpm			
	Purity (%)	***PF (fold)***	**Yield (%)**
Initial extract	15.10	1.00	100
Purified fractions			
F25	63.13	4.18	10.39
F26	58.19	3.85	11.40
F27	46.82	3.10	8.97
F28	20.78	1.38	14.68
F29	27.71	1.83	18.59
F30	17.21	1.14	13.48
Total purified fractions		**MB/total yield (%)**	77.52
Condition B: Flow 2.0 mL.min^{-1} and rotation speed 2500 rpm			
	Purity (%)	***PF (fold)***	**Yield (%)**
Initial extract	15.82	1.00	100
Purified fractions			
F10	47.18	2.98	25.33
F11	16.32	1.03	26.00
F12	16.88	1.07	30.04
F13	18.44	1.17	1.64
F14	19.64	1.24	4.48
Total purified fractions		**MB/total yield (%)**	87.49
Condition C:Flow rate 2.5 mL.min^{-1} and rotation speed 2000 rpm			
	Purity (%)	***PF (fold)***	**Yield (%)**
Initial extract	17.29	1.00	100
Purified fractions			
F28	89.83	5.19	6.76
F29	71.80	4.15	10.17
F30	38.89	2.25	16.06
F31	43.69	2.53	12.39
F32	21.41	1.24	13.99
F33	23.17	1.34	11.07
F34	17.46	1.01	11.89
Total purified fractions		**MB/total yield (%)**	82.34

of the protein, while the remaining phases mostly contained impurities from the extract. No losses of the stationary phase (NaPA-rich phase) were observed during all the separation runs. The correspondent purities, purity factor and yields of each fraction for the systems are shown in Table 3 and the correspondent equations for calculation are given in the ESI.

From the screening, the best condition to perform FCPC using PEG/NaPA-based ABS in ascending mode was represented by a flow rate of 2.5 mL.min^{-1} and rotation speed of 2000 rpm (condition C), reaching purities up to 89.8%. The results show that high purity levels were achieved in the first fractions although the extraction yield is still relatively low compared to less pure phases. In condition B, for example the phase F10 enhanced the purity of the extract in approximately 3-fold and reached higher yields (25.33%) than the ones obtained in phase F28 condition C (purity enhancement up to 5-fold), achieving yields 3.7 times lower than the condition previously described. Since the design of the purification process for EGFP and other proteins depends substantially on the final application of the purified protein, even in continuous systems such as FCPC, the highest purities were achieved for the lowest yields of extraction.

Although the three conditions tested have the same stationary phase retention value, the increase in flow rate allows greater purification factors than the systems at 2.0 mL.min^{-1}. This may be justified by the fact that by reducing the time, the protein remains less time in the rotor of FCPC equipment and thus the denaturation of EGFP potentially caused by the temperature increase is avoided. Moreover, also the concerted loss of visible fluorescence, thermal stability and alteration of absorption characteristics caused by the force field of the equipment are inhibited. Nevertheless, at higher temperatures, the protein is denatured by interacting with PEG [51]. This is consequently imposing the use of shorter residence times, e.g. rotation speed of 2000 rpm (conditions A and C in Table 3) instead of 2500 rpm (condition B). Furthermore, the increase of the different flow rates is associated to a better dispersion caused by the increased interfacial area between the phases when increasing the volume flow. Despite the limited range of equipment processual conditions under study, from the results it is evident that the EGFP purification performance in the FCPC is favored by the increase of the mobile phase flow rate, exhibiting the rotor speed a less significant effect on the purity yields.

Considering the excellent purification performance, a possible integrated platform for the purification of EGFP was proposed in Fig. 3, including the units of production and extraction and purification using ABS applied in FCPC. The values of purification support the high potential of ABS for the purification of EGFP using FCPC in a continuous regime from a complex matrix extracted from recombinant *E. coli*, while allowing the technology scale-up.

4. Conclusions

ABS composed of PEG 8000/NaPA 8000 + Na_2SO_4 were investigated and shown to be biocompatible and efficient to purify EGFP. The concentration of the electrolyte was firstly screened, and the best condition selected by taking into account the selectivity, partition coefficients, recovery, volume ratio and viscosity. By applying the best conditions, an integrated process using FCPC was developed to purify EGFP extracted from recombinant *E. coli* BL21 cells from the

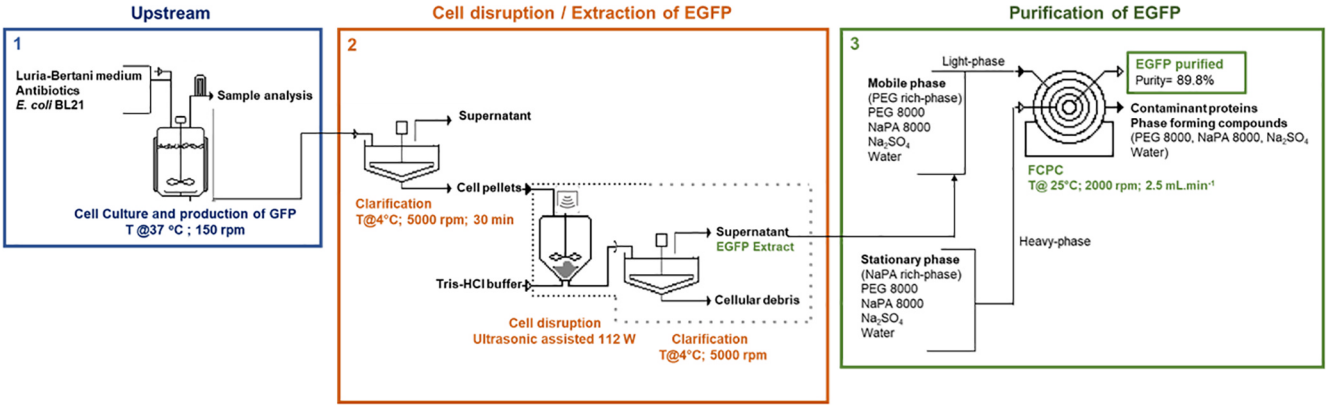

Fig. 3. Process diagram of the EGFP purification process using ABS in FCPC.

contaminant proteins. The operational parameters of FCPC have demonstrated a significant influence on the yield and purity of EGFP. Higher flow rates and lower rotation speeds act better to maintain the integrity of the protein, increasing the final purity of the product. In addition, for all conditions tested, the higher the purity the lower the purification yield. The high purities obtained in the fractions collected from the FCPC demonstrates how promising the integration of ABS is when conjugated with FCPC to purify biological samples.

Declaration of Competing Interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2020.117648.

References

[1] A.B. Cubitt, R. Heim, S.R. Adams, A.E. Boyd, L.A. Gross, R.Y. Tsien, Understanding, improving and using green fluorescent proteins, Trends Biochem. Sci. 20 (1995) 448–455, https://doi.org/10.1016/S0968-0004(00)89099-4.
[2] P.I.H. Bastiaens, R. Pepperkok, Observing proteins in their natural habitat: the living cell, Trends Biochem. Sci. 25 (2000) 631–637, https://doi.org/10.1016/S0968-0004(00)01714-X.
[3] D.E. Crone, Y.-M. Huang, D.J. Pitman, C. Schenkelberg, K. Fraser, S. Macari, C. Bystroff, GFP-based biosensors, in: State Art Biosens. - Gen. Asp., 2013: pp. 3–36. http://dx.doi.org/10.5772/52250.
[4] O. Shimomura, F.H. Johnson, Y. Saiga, Extraction, purification and properties of Aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*, J. Cell. Comp. Physiol. 59 (1962) 223–239, https://doi.org/10.1002/jcp.1030590302.
[5] M. Chalfie, Y. Tu, G. Euskirchen, W.W. Ward, D.C. Prasher, Green fluorescent protein as a marker for gene expression, Fed. Eur. Biochem. Lett. 263 (1994) 802–805, https://doi.org/10.1126/science.8303295.
[6] D.C. Prasher, V.K. Eckenrode, W.W. Ward, F.G. Prendergast, M.J. Cormier, Primary structure of the *Aequorea victoria* green-fluorescent protein, Gene 111 (1992) 229–233, https://doi.org/10.1016/0378-1119(92)90691-h.
[7] D.C. Prasher, Using GFP to see the light, Trends Genet. 11 (1995) 320–323, https://doi.org/10.1016/S0168-9525(00)89090-3.
[8] S. Inouye, F.I. Tsuji, *Aequorea* green fluorescent protein. Expression of the gene and fluorescence characteristics of the recombinant protein, FEBS Lett. 341 (1994) 277–280, https://doi.org/10.1016/0014-5793(94)80472-9.
[9] M. Zimmer, Green fluorescent protein (GFP): Applications, structure, and related photophysical behavior, Chem. Rev. 102 (2002) 759–781, https://doi.org/10.1021/cr010142r.
[10] N.V. dos Santos, C.F. Saponi, T.L. Greaves, J.F.B. Pereira, Revealing a new fluorescence peak of the enhanced green fluorescent protein using three-dimensional fluorescence spectroscopy, RSC Adv. 9 (2019) 22853–22858, https://doi.org/10.1039/c9ra02567g.
[11] M.M. Nalaskowski, P. Ehm, S. Giehler, G.W. Mayr, A toolkit for graded expression of green fluorescent protein fusion proteins in mammalian cells, Anal. Biochem. 428 (2012) 24–27, https://doi.org/10.1016/j.ab.2012.06.001.
[12] S.-H. Park, R.T. Raines, Green fluorescent protein as a signal for protein-protein interactions, Protein Sci. 6 (1997) 2344–2349, https://doi.org/10.1002/pro.5560061107.
[13] J.C. March, G. Rao, W.E. Bentley, Biotechnological applications of green fluorescent protein, Appl. Microbiol. Biotechnol. 62 (2003) 303–315, https://doi.org/10.1007/s00253-003-1339-y.
[14] J.M. Belardinelli, M. Jackson, Green fluorescent protein as a protein localization and topological reporter in mycobacteria, Tuberculosis 105 (2017) 13–17, https://doi.org/10.1016/j.tube.2017.04.001.
[15] M. Sabalza, P. Christou, T. Capell, Recombinant plant-derived pharmaceutical proteins: current technical and economic bottlenecks, Biotechnol. Lett. 36 (2014) 2367–2379, https://doi.org/10.1007/s10529-014-1621-3.
[16] M. Kafri, E. Metzl-Raz, G. Jona, N. Barkai, The cost of protein production, Cell Rep. 14 (2016) 22–31, https://doi.org/10.1007/s10529-014-1621-3.
[17] I. Shachrai, A. Zaslaver, U. Alon, E. Dekel, Cost of unneeded proteins in *E. coli* is reduced after several generations in exponential growth, Mol. Cell. 38 (2010) 758–767, https://doi.org/10.1016/j.molcel.2010.04.015.
[18] Y. Li, A. Agrawal, J. Sakon, R.R. Beitle, Characterization of metal affinity of green fluorescent protein and its purification through salt promoted, immobilized metal affinity chromatography, J. Chromatogr. A. 909 (2001) 183–190, https://doi.org/10.1016/S0021-9673(00)01100-6.
[19] A. Uretschläger, A. Einhauer, A. Jungbauer, Continuous separation of green fluorescent protein by annular chromatography, J. Chromatogr. A. 908 (2001) 243–250, https://doi.org/10.1016/S0021-9673(00)00833-5.
[20] S. Dalal, S. Raghava, M.N. Gupta, Single-step purification of recombinant green fluorescent protein on expanded beds of immobilized metal affinity chromatography media, Biochem. Eng. J. 42 (2008) 301–307, https://doi.org/10.1016/j.bej.2008.07.010.
[21] K. Sugo, T. Yoshitake, M. Tomita, S. Kobayashi, Y. Kurosawa, K. Kawamura, T. Okuyama, Simplified purification of soluble histidine-tagged green fluorescent protein from cocoon of transgenic silkworm in metal affinity hydroxyapatite chromatography, Sep. Purif. Technol. 76 (2011) 432–435, https://doi.org/10.1016/j.seppur.2010.11.015.
[22] M.V. Quental, M. Caban, M.M. Pereira, P. Stepnowski, J.A.P. Coutinho, M.G. Freire, Enhanced extraction of proteins using cholinium-based ionic liquids as phase-forming components of aqueous biphasic systems, Biotechnol. J. 10 (2015) 1457–1466, https://doi.org/10.1002/biot.201500003.
[23] J.H.P.M. Santos, E.V. Capela, I. Boal-Palheiros, J.A.P. Coutinho, M.G. Freire, S.P.M. Ventura, Aqueous biphasic systems in the separation of food colorants, Biochem. Mol. Biol. Educ. 46 (2018) 390–397, https://doi.org/10.1002/bmb.21125.
[24] H.O. Johansson, M. Ishii, M. Minaguti, E. Feitosa, T.C.V. Penna, A. Pessoa, Separation and partitioning of green fluorescent protein from *Escherichia coli* homogenate in poly(ethylene glycol)/sodium-poly(acrylate) aqueous two-phase systems, Sep. Purif. Technol. 62 (2008) 166–174, https://doi.org/10.1016/j.seppur.2008.01.017.
[25] Y. Li, R.R. Beitle, Protein purification via aqueous two-phase extraction (ATPE) and immobilized metal affinity chromatography. Effectiveness of salt addition to enhance selectivity and yield of GFPuv, Biotechnol. Prog. 18 (2002) 1054–1059, https://doi.org/10.1021/bp025526x.
[26] 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, https://doi.org/10.1039/c2cs35151j.
[27] A.M. Lopes, J.V.D. Molino, V.C. dos Santos-Ebinuma, A. Pessoa, S.R. Valentini, J.F.B. Pereira, Effect of electrolytes as adjuvants in GFP and LPS partitioning on aqueous two-phase systems: 1. Polymer-polymer systems, Sep. Purif. Technol. 206 (2018) 39–49, https://doi.org/10.1016/j.seppur.2018.04.090.
[28] N.V. Dos Santos, M. Martins, V.C. Santos-Ebinuma, S.P.M. Ventura, J.A.P. Coutinho, S.R. Valentini, J.F.B. Pereira, Aqueous biphasic systems composed of cholinium chloride and polymers as effective platforms for the purification of recombinant green fluorescent protein, ACS Sustain. Chem. Eng. 6 (2018) 9383–9393, https://doi.org/10.1021/acssuschemeng.8b01730.
[29] S.C. Lo, R.N. Ramanan, B.T. Tey, W.S. Tan, P.L. Show, T.C. Ling, C.W. Ooi, Purification of the recombinant enhanced green fluorescent protein from *Escherichia coli* using alcohol + salt aqueous two-phase systems, Sep. Purif. Technol. 192 (2018) 130–139, https://doi.org/10.1016/j.seppur.2017.09.072.
[30] H.-O. Johansson, E. Feitosa, A.P. Junior, Phase diagrams of the aqueous two-phase systems of poly(ethylene glycol)/sodium polyacrylate/salts, Polymers (Basel). 3 (2011) 587–601, https://doi.org/10.3390/polym3010587.
[31] S.M. Waziri, B.F. Abu-Sharkh, S.A. Ali, Protein partitioning in aqueous two-phase systems composed of a pH-responsive copolymer and poly(ethylene glycol), Biotechnol. Prog. 20 (2004) 526–532, https://doi.org/10.1021/bp0342349.
[32] J.H.P.M. Santos, A.M. Ferreira, M.R. Almeida, P.S.G.N. Quinteiro, A.C.R.V. Dias, J.A.P. Coutinho, M.G. Freire, C.O. Rangel-Yagui, S.P.M. Ventura, Continuous separation of cytochrome-c PEGylated conjugates by fast centrifugal partition chromatography, Green Chem. (2019), https://doi.org/10.1039/C9GC01063G.
[33] Y. Ito, Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography, J. Chromatogr. A. 1065 (2005) 145–168, https://doi.org/10.1016/j.chroma.2004.12.044.
[34] Kromaton, Fast Centrifugal Partition Chromatography ® products / FCPC® B & D, (n.d.). http://www.kromaton.com/en/products/fcpc-b-d (accessed January 28, 2020).
[35] J.B. Friesen, J.B. McAlpine, S.N. Chen, G.F. Pauli, Countercurrent separation of natural products: an update, J. Nat. Prod. 78 (2015) 1765–1796, https://doi.org/10.1021/np501065h.
[36] C. Schwienheer, A. Prinz, T. Zeiner, J. Merz, Separation of active laccases from

Pleurotus sapidus culture supernatant using aqueous two-phase systems in centrifugal partition chromatography, J. Chromatogr. B. 1002 (2015) 1–7, https://doi.org/10.1016/j.jchromb.2015.07.050.
[37] I.A. Sutherland, G. Audo, E. Bourton, F. Couillard, D. Fisher, I. Garrard, P. Hewitson, O. Intes, Rapid linear scale-up of a protein separation by centrifugal partition chromatography, J. Chromatogr. A. 1190 (2008) 57–62, https://doi.org/10.1016/j.chroma.2008.02.092.
[38] I. Sutherland, P. Hewitson, R. Siebers, R. van den Heuvel, L. Arbenz, J. Kinkel, D. Fisher, Scale-up of protein purifications using aqueous two-phase systems: Comparing multilayer toroidal coil chromatography with centrifugal partition chromatography, J. Chromatogr. A. 1218 (2011) 5527–5530, https://doi.org/10.1016/j.chroma.2011.04.013.
[39] J.H.P.M. Santos, M.R. Almeida, C.I.R. Martins, A.C.R.V. Dias, M.G. Freire, J.A.P. Coutinho, S.P.M. Ventura, Separation of phenolic compounds by centrifugal partition chromatography, Green Chem. 20 (2018) 1906–1916, https://doi.org/10.1039/c8gc00179k.
[40] F. Bezold, J. Goll, M. Minceva, Study of the applicability of non-conventional aqueous two-phase systems in counter-current and centrifugal partition chromatography, J. Chromatogr. A. 1388 (2015) 126–132, https://doi.org/10.1016/j.chroma.2015.02.021.
[41] A. Berthod, Countercurrent Chromatografy: The support-free liquid stationary phase, First ed., 2002.
[42] M. Martins, C.W. Ooi, M.C. Neves, J.F.B. Pereira, J.A.P. Coutinho, S.P.M. Ventura, Extraction of recombinant proteins from *Escherichia coli* by cell disruption with aqueous solutions of surface-active compounds, J. Chem. Technol. Biotechnol. 93 (2018) 1864–1870, https://doi.org/10.1002/jctb.5596.
[43] C. Lopes, N.V. dos Santos, J. Dupont, D.B. Pedrolli, S.R. Valentini, V. de Carvalho Santos-Ebinuma, J.F.B. Pereira, Improving the cost effectiveness of enhanced green fluorescent protein production using recombinant *Escherichia coli* BL21 (DE3): decreasing the expression inducer concentration, Biotechnol. Appl. Biochem. 21 (2019) 1–10, https://doi.org/10.1002/bab.1749.
[44] J.H.P.M. Santos, F.A.E. Silva, J.A.P. Coutinho, S.P.M. Ventura, A. Pessoa, Ionic liquids as a novel class of electrolytes in polymeric aqueous biphasic systems, Process Biochem. 50 (2015) 661–668, https://doi.org/10.1016/j.procbio.2015.02.001.
[45] H.O. Johansson, F.M. Magaldi, E. Feitosa, A. Pessoa, Protein partitioning in poly (ethylene glycol)/sodium polyacrylate aqueous two-phase systems, J. Chromatogr. A. 1178 (2008) 145–153, https://doi.org/10.1016/j.chroma.2007.11.071.
[46] D.B. Thompson, J.J. Cronican, D.R. Liu, Engineering and identifying supercharged proteins for macromolecule delivery into mammalian cells, Methods Enzym. 503 (2012) 293–319, https://doi.org/10.1038/jid.2014.371.
[47] A.J. Hughes, A.M. Tentori, A.E. Herr, Bistable isoelectric point photoswitching in green fluorescent proteins observed by dynamic immunoprobed isoelectric, J. Am. Chem. Soc. 42 (2012) 17582–17591, https://doi.org/10.1038/jid.2014.371.
[48] L.A. Ferreira, J.A. Teixeira, Salt effect on the aqueous two-phase system PEG 8000-sodium sulfate, J. Chem. Eng. Data. 56 (2011) 133–137, https://doi.org/10.1021/je1010467.
[49] S.A. Oelmeier, C. Ladd Effio, J. Hubbuch, High throughput screening based selection of phases for aqueous two-phase system-centrifugal partitioning chromatography of monoclonal antibodies, J. Chromatogr. A. 1252 (2012) 104–114, https://doi.org/10.1016/j.chroma.2012.06.075.
[50] D.W. Armstrong, Theory and use of centrifugal partition chromatography, J. Liq. Chromatogr. 11 (1988) 2433–2446, https://doi.org/10.1080/01483918808076738.
[51] L.C. de L. Novaes, P.G. Mazzola, A. Pessoa, T.C.V. Penna, Effect of polyethylene glycol on the thermal stability of green fluorescent protein, Biotechnol. Prog. 26 (2010) 252–256. http://doi.org/10.1002/btpr.296.