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Multistep purification of cytochrome c PEGylated forms using polymer-based aqueous biphasic systems†

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Chemical PEGylation of proteins has been used to improve their physicochemical properties and kinetics. However, the PEGylation reactions lead to a heterogeneous mixture of PEGylated conjugates and unreacted protein, which is a challenge for the design of an efficient downstream process. The purification of PEGylated proteins should address the two main issues: the separation of PEGylated conjugates from the unreacted protein and the fractionation of the PEGylated conjugates on the basis of their degree of PEGylation. The present study aims at the development of liquid–liquid extraction processes for the purification of PEGylated conjugates. An initial study of the partition behavior of cytochrome c and their PEGylated conjugates (Cyt-c-PEG-4 and Cyt-c-PEG-8) on polyethylene-glycol (PEG) + potassium phosphate buffer (pH = 7) aqueous biphasic systems (ABS) shows that PEGs with intermediate molecular weights (PEG MW = 1000–2000) allow the separation of the PEGylated conjugates from the unreacted protein in a single step. It is further shown that the PEGylated conjugates can be efficiently separated using ABS based on PEGs with high molecular weight (PEG MW = 6000–8000) and a study of the protein stability after purification was carried using circular dichroism. A downstream process to separate Cyt-c, Cyt-c-PEG-4 and Cyt-c-PEG-8 with high purities (96.5% Cyt-c, 85.8% Cyt-c-PEG-4, and 99.0% Cyt-c-PEG-8) was developed. The process proposed addresses not only the efficient separation of each of the protein forms but also the recycling of the unreacted protein purified and the ABS phases, which was successfully used in a new step of PEGylation.

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Introduction

The use of PEGylation as a drug delivery technology was firstly proposed by Abuchowski *et al.*[1] It consists of the covalent attachment of polyethylene glycol (PEG) chains to proteins. This approach can be used for the drug delivery of biopharmaceuticals; its success depends on the characteristics of the PEGylated conjugates obtained, such as its enhanced pharmacokinetic properties, increased blood residence time and reduced immunological response and proteolytic degradation.[2–4] This protein modification technique can enhance the "green" character of the final product, by preserving the efficacy of the function, while decreasing its toxicity,[5] and thus their impact to the user and the environment. Through PEGylation, novel biobetters[6,7] have been developed and commercialized. Pegadamase (Adagen), a PEGylated form of the enzyme adenosine deaminase[8] used in the treatment of severe combined immunodeficiency disease (SCID),[9] and Pegaspargase (Oncaspar), a PEGylated form of the enzyme asparaginase[10] used for the treatment of childhood acute lymphoblastic leukemia (ALL)[11] are just two examples. In the field of biosensors, the PEGylation also showed to be a promising approach to enhance the thermal and long-term stability of the protein-based bionsensors.[12–14]

Given the interest of the applications of protein PEGylation, the downstream processing of the PEGylated conjugates remains a bottleneck to its widespread use.[15] The PEGylation reaction normally results in a heterogeneous mixture of unreacted protein with a variety of PEGylated conjugates with different degrees of PEGylation.[16] The purification of the PEGylated conjugates implies thus three main challenges: (i) the isolation and recycling of the unreacted protein from

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the PEGylated conjugates, (ii) the fractionation of PEGylated conjugates according to their degrees of PEGylation, and (iii) the isolation of each protein form from the reaction products (*e.g.* PEG derivate, hydroxylamine and buffer).[17] Nowadays, the downstream processes most used to fractionate the PEGylated conjugates are based on size exclusion chromatography (SEC)[18,19] and ion exchange chromatography (IEX).[20–22] Recently, non-chromatographic techniques such as capillary electrophoresis,[23,24] ultrafiltration[25–28] and aqueous biphasic systems (ABS)[29,30] have been proposed to selectively purify the PEGylated conjugates.

Aqueous biphasic systems (ABS) were originally proposed by Albertsson[31] as a cleaner and more biocompatible alternative to conventional liquid–liquid extraction processes that often use hazardous volatile organic solvents while ABS present a high water content, providing milder and more biocompatible conditions for bioseparations.[32] ABS are designer processes, with a wide range of compounds that can be combined as phase formers, *e.g.* polymer + polymer,[33–35] polymer + salt,[36,37] salt + salt,[38] among others.[39–41] This tailoring ability allows the design of specific downstream processes for a wide range of biomolecules.[42–45] Furthermore, the scale-up of these systems is rather straightforward.

The use of PEG + salt, PEG + polymer and PEG + polymer + salt (adjuvant) ABS for the separation of the PEGylated conjugates has been described for several proteins, *e.g.* bovine serum albumin,[46] immunoglobulin G,[46] α-lactalbumin,[29] RNase A,[29,47] lysozyme[48] and glycoproteins granulocyte-macrophage colony-stimulating factor (GM-CSF).[30,46] Delgado *et al.*[30,46] reported a correlation between $\log(K)$ and the PEGylation degree of the conjugates that allowed the purification of protein conjugates from the unreacted protein. González-Valdez *et al.*[29] proved that PEG + phosphate buffer ABS were able to efficiently fractionate the PEGylated conjugates from their unreacted precursors for two proteins: RNase A and α-lactalbumin. Nevertheless, the most remarkable application of ABS considering the downstream PEGylation process was attributed to Sookumnerd and Hsu,[48] which used PEG + phosphate buffer countercurrent distribution in aqueous biphasic systems (CCD-ABS) to selectively fractionate the unmodified lysozyme, and its mono- and di-PEGylated forms. Galindo-López & Rito-Palomares[47] also attempted the separation of mono-PEGylated and di-PEGylated RNase A, using CCD-ABS followed by ultrafiltration. However, low selectivities (*i.e.* co-elution of mono-PEGylated RNase A and di-PEGylated RNase A in the purified fractions) were obtained and significant product loss (%Rec mono-PEGylated RNase A = 34% and %Rec di-PEGylated RNase A = 45% of 50 kDa of PEG) occurred for both PEGylated conjugates. Despite the promising results of ABS in the fractionation of different protein PEGylated forms, their use is still compromised by the poor results obtained. While Cyt-c has been purified with ABS[49,50] and stabilized with ionic liquids,[51,52] the results for the purification of PEGylated cytochrome c (Cyt-c) with ABS are, to the best of our knowledge, previously inexistent. Cyt-c is a biocatalyst[53–56] used as a biosensor,[12,57] and enzyme-based biosensors suffer from the fact that proteins have a fragile conformation, being affected by several exogenous conditions, namely pH, salt concentration, the presence of proteases, and temperature, among others.[12,58] In order to overcome these drawbacks, the chemical modification of proteins and enzymes is frequently employed.[13,59] Actually, the chemical modification of protein-based biosensors through PEGylation improves their properties, like their biocompatibility, long-term stability, enhanced thermal stability, and solubility in organic solvents.[2] In this context, some authors have already shown the potential of PEGylation in nanostructured Cyt-c biosensors, by improving its long-term stability even under accelerated conditions.[12] However, there is a need to purify the PEGylated conjugates of Cyt-c in order to have a control batch-to-batch and guarantee that all purified conjugates have a similar biosensing activity.

In this work, polymer-based ABS were used to develop an integrated process to fractionate different cytochrome c PEGylated forms and separate them from the unreacted protein. In the optimization of this integrated process, a multi-step strategy was developed to separate firstly the unreacted protein from the conjugates and, secondly, the conjugates from each other. Aiming at the industrial application of the process, the recycling of the unreacted protein to a novel PEGylation cycle was also investigated, as well as the recycling and reuse of each phase former used in the ABS preparation.

Experimental

Materials

Horse heart cytochrome c (Cyt-c) was acquired in Sigma-Aldrich with a purity of ≥95%.

The PEG derivative used in the PEGylation reaction was the methoxyl polyethylene glycol succinimidyl NHS ester (mPEG-NHS or just mPEG), obtained from Nanocs (purity >95%).

Hydroxylammonium chloride acquired from Sigma-Aldrich was used to stop the PEGylation reaction and to avoid the formation of undesirable and unstable products.

The aqueous buffer used in the PEGylation reaction was 100 mM potassium phosphate buffer, being the pH adjusted using a solution of NaOH 2 M. The inorganic salts, K_2HPO_4 and K_2HPO_4, were purchased from Sigma-Aldrich (a purity of 95%).

The phase formers used to prepare the different ABS were polyethylene glycol (PEG) with different MW (300, 600, 1000, 1500, 2000, 4000, 6000, and 8000), obtained from Sigma-Aldrich with high purity and the potassium phosphate buffer was prepared using K_2HPO_4 and K_2HPO_4.

PEGylation reaction of Cyt-c

The PEGylation reactions were conducted according to literature.[60] Briefly, 1 mL of a Cyt-c solution (0.5 mg mL^{-1}) in potassium phosphate buffer (100 mM, pH = 7) was added to a

flask containing 5.2 mg mPEG with 5 kDa. The mixtures were stirred at 400 rpm, for 15 min at room temperature with a magnetic stirrer, and then stored at −20 °C for further use.

Purification studies of unreacted Cyt-c and PEGylated forms using ABS

Ternary mixtures of PEG + potassium phosphate buffer were used to study the fractionation of the unreacted Cyt-c and PEGylated forms. In these systems, the top phase corresponds to the PEG-rich phase while the bottom phase is mainly composed by potassium phosphate buffered salt. The mixture points used in the purification studies were chosen based on phase diagrams described in literature.[61,62]

For the first screening, the effect of the PEG molecular weight was evaluated (PEG 300, 600, 1000, 1500, 2000, 4000, 6000 and 8000 g mol^{-1}) considering a fixed mixture with 15 wt% of PEG + 20 wt% of potassium phosphate buffer (pH 7). Regarding the second set of experiments carried, in which the water content of the top phase was studied, the PEG 1500 + potassium phosphate buffer (pH = 7) system was studied considering the mixture points: (15; 15 wt%), (17.5; 15 wt%), (20; 15 wt%), (22.5; 15 wt%), (25; 15 wt%), (potassium phosphate buffer; PEG 1500). The tie-lines (TLs) were calculated for all mixture points aforementioned as previously reported.[63,64]

For the study of the multistep ABS strategy, three consecutive ABS were implemented: first step – **ABS I** using PEG 8000 + potassium phosphate buffer (pH = 7); second step – **ABS II** using PEG 8000 + potassium phosphate buffer (pH = 7); and third step – **ABS III** using PEG 1500 + potassium phosphate buffer (pH = 7). In all units (**ABS I**, **ABS II** and **ABS III**) the top-phase was recovered and used in the next step of purification.

For the partition studies the preparation of ABS was carried in eppendorfs, with a total volume of 2 mL, being the aqueous mixture vigorously homogenised in vortex. After the homogenisation, the systems were centrifuged for 15 min at 3500 rpm to induce the phase separation and both phases were carefully separated and weighted. After separation, both top and bottom phases were injected into an AKTA™ purifier system (GE Healthcare) size exclusion chromatographer equipped with a Superdex 200 Increase 10/300 GL chromatographic column prepacked with crosslinked agarose-dextran high resolution resin (GE Healthcare) in order to separate and quantify each PEGylated conjugate and the unreacted protein. The column was equilibrated with 0.01 M of a sodium phosphate buffer solution (0.14 M NaCl, pH = 7.4) and eluted with the same buffer with a flow of 0.75 mL min^{-1}. The quantification of the unreacted Cyt-c and each conjugate was carried at 280 nm by the FPLC/UV size-exclusion method.

All experiments were performed in triplicate, where the final absorbance was reported as the average of three independent assays with the respective standard deviations calculated.

To assess the performance of the purification parameters for the different ABS, the partition coefficients in the log scale $\log(K)$ of the unreacted Cyt-c and the PEGylated conjugates (Cyt-c-PEG-4 and Cyt-c-PEG-8), their recovery in the top (Rec Top – %) and bottom (Rec Bot – %) phases were determined (eqn (1)–(3), respectively):

$$\log(K) = \log\left(\frac{[\text{Prot}]\text{top}}{[\text{Prot}]\text{bot}}\right) \quad (1)$$

$$\text{Rec Top}(\%) = \frac{100}{1 + \left(\frac{1}{K \times R_v}\right)} \quad (2)$$

$$\text{Rec Bot}(\%) = \frac{100}{1 + R_v \times K} \quad (3)$$

where $[\text{Prot}]_{top}$ and $[\text{Prot}]_{bot}$ represent the protein concentration in the top and bottom phases, respectively. R_v represents the volume ratio between the top and bottom phase volumes. The purity of the three Cyt-c proteins was calculated considering the ratio between the (SEC-FPLC) peak area representing the target protein and the peak area defined for the sum of all proteins present in the phase.[65] The purification parameters were calculated for the unreacted Cyt-c and the PEGylated conjugates, respectively, Cyt-c-PEG-4 and Cyt-c-PEG-8. The mass balance (MB) was calculated for the three Cyt-c based products, considering each separation unit, and the overall process (OMB).

Recycling of unreacted Cyt-c and its application in a new cycle of PEGylation

In order to prove the reuse of the unreacted protein in a subsequent cycle of PEGylation, after its recovery from the reactional mixture using the ABS, two approaches were tested. The first strategy consists of adding directly to the reaction media the salt-rich phase with the unreacted Cyt-c. The second approach tested was the use of the unreacted Cyt-c free of the salt-rich phase, which was removed by precipitation using cold acetone. In this last approach, the unreacted protein was re-suspended in the same buffer used for the PEGylation reaction. For both cases, the quantification of the PEGylation conjugates and unreacted protein was performed by FPLC/UV SEC methodology. A SDS-PAGE of the samples was carried to evaluate the PEGylation yield.

Circular dichroism (CD) experiments

CD spectra of Cyt-c before and after ABS purification were obtained on a Jasco J-720 Spectropolarimeter (Jasco, Tokyo, Japan). The final spectra were the average of 6 scans, following subtraction of the spectrum representing 0.01 M of sodium phosphate buffer (0.14 M NaCl, pH = 7.4) obtained under the same conditions. CD spectra were obtained in the far-UV range (190–260 nm). Samples were placed in quartz cells (1.00 mm of optical length) with the concentration ranging from 13 to 15 μM. Spectral intensities (θ, mdeg) were converted to residual molar ellipticity ($[\theta]$, deg cm^2 $dmol^{-1}$) by the following expression:

$$[\theta] = \frac{\theta}{10 \cdot C \cdot l \cdot n} \quad (4)$$

where C is the protein concentration in mol L^{-1}, l is the optical length in cm and n is the number of residues in the protein, that for this specific case is 104 residues.

Results and discussion

Partition studies

PEGylation is one of the most attractive strategies used to increase the half-life time of therapeutic proteins. Despite its high applicability, the efficiency of the PEGylation reaction is low, thus compromising the production of site-specific PEGylated proteins. In order to establish an efficient process to separate the PEGylated forms from the unreacted protein, polymer-based ABS composed of PEG and potassium phosphate buffer were investigated. The influence of the various process and system variables on the partition of these biomolecules was evaluated by using the heterogeneous mixture obtained after PEGylation. From the PEGylation reaction three forms were obtained, the unreacted Cyt-c, the Cyt-c PEGylated 4 times (Cyt-c-PEG-4) and the Cyt-c PEGylated 8 times (Cyt-c-PEG-8). The recovery results at the ABS top and bottom phases are reported in the ESI (Tables S1 and S2†) and presented in Fig. 1–3.

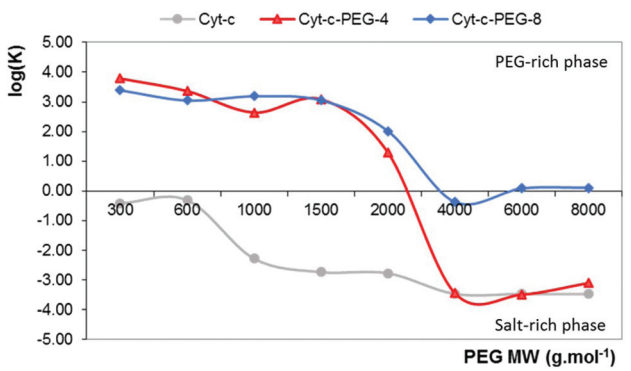

Fig. 1 Effect of PEG molecular weight (MW) on the partition coefficient represented by log (*K*) for (unreacted) Cyt-c and each PEGylated form (Cyt-c-PEG-4 and Cyt-c-PEG-8) for the ABS with 15.0 wt% of PEG + 20.0 wt% of potassium phosphate buffer (pH = 7). The polymers tested were PEG 300, 600, 1000, 1500, 2000, 4000, 6000 and 8000 g mol^{-1}.

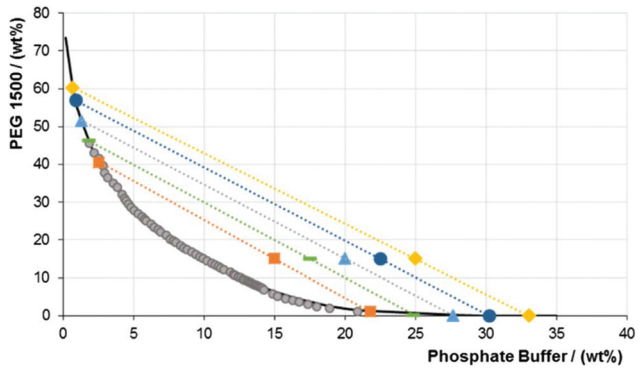

Fig. 2 Phase diagram (●) and respective tie-lines; TLs defined for the ABS composed of PEG 1500 + potassium phosphate buffer (pH = 7). The TLs were calculated considering the following mixture points (potassium phosphate buffer; PEG 1500 wt%): ■ (15; 15 wt%), — (17.5; 15 wt%), ◆ (20; 15 wt%), ● (22.5; 15 wt%), ▲ (25; 15 wt%).

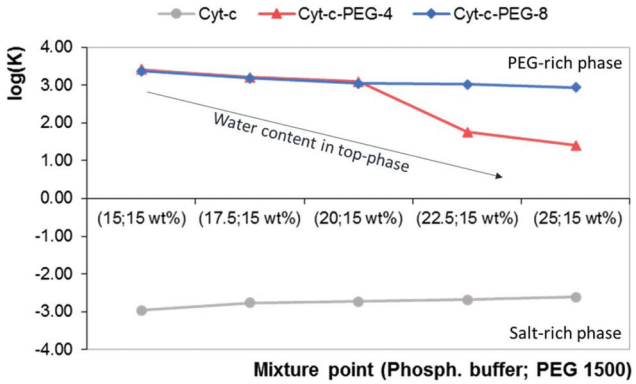

Fig. 3 Effect of the top-phase water content on the log (*K*) of Cyt-c and each PEGylated form (Cyt-c-PEG-4 and Cyt-c-PEG-8) for the system PEG 1500 + potassium phosphate buffer (pH = 7). The mixture points (potassium phosphate buffer; PEG 1500 wt%) studied were: (15; 15 wt%), (17.5; 15.0 wt%), (20; 15.0 wt%), (22.5; 15.0 wt%) (25; 15.0 wt%).

The effect of the PEG MW was the first variable studied. A fixed mixture point with 15 wt% of PEG + 20 wt% of potassium phosphate buffer (pH = 7) was adopted and the partition of Cyt-c, Cyt-c-PEG-4 and Cyt-c-PEG-8 is shown in Fig. 1. From the data it is possible to observe that the native/unreacted Cyt-c has a clear tendency to partition towards the salt rich (bottom) phase. This tendency is even more pronounced with the increase of the MW of PEG (*i.e.* PEG MW ≥ 1000), thus with the increased hydrophobicity of the PEG-rich phase. Since Cyt-c is a small (12 kDa) and highly hydrophilic heme protein,[53] its higher affinity towards the most hydrophilic phase is thus expected. The PEGylated conjugates, by their turn, exhibited a different behaviour, their partition being dependent on the PEG MW. For PEGs with lower MW (*i.e.* PEG MW ≤ 2000) both conjugates partition towards the PEG-rich (top) phase; however for PEGs with higher MW, the partition of the PEGylated forms towards the bottom phase becomes more significant. This is especially true for the form with a lower degree of PEGylation (Cyt-c-PEG-4) that exhibits a pronounced preference for the bottom phase in ABS with the heavier PEGs. The partition coefficients obtained for the different protein forms show that these ABS can be applied as purification platforms for (i) the separation of unreacted protein from the PEGylated conjugates using the ABS composed of PEG 1000/1500 + potassium phosphate buffer (Rec $Bot_{Cyt\text{-}c}$ > 99% and Rec $Top_{Cyt\text{-}c\text{-}PEG\text{-}4}$ > 99% and Rec $Top_{Cyt\text{-}c\text{-}PEG\text{-}8}$ > 99%) and (ii) the partial fractionation of the PEGylated conjugates according to their degree of PEGylation considering the use of ABS based in PEG 6000/8000 + potassium phosphate buffer (Rec $Bot_{Cyt\text{-}c}$ > 99% and Rec $Bot_{Cyt\text{-}c\text{-}PEG\text{-}4}$ > 99% and Rec $Top_{Cyt\text{-}c\text{-}PEG\text{-}8}$ ≈ 45%).

In order to fully explore the potential for the separation of the PEGylated forms, and aiming at increasing the selectivity of the systems previously studied towards the fractionation of these conjugates, different tie-lines were studied using mixture points with variable concentrations of water. The ABS were chosen aiming at promoting the total partition of the

PEGylated conjugates towards the top phase and the unreacted protein towards the opposite phase. The phase diagram and the respective tie-lines (TLs) of the selected ABS (PEG 1500 + potassium phosphate buffer, pH = 7) are depicted in Fig. 2. The TLs were studied for the following mixture points: (15; 15 wt%), (17.5; 15 wt%), (20; 15 wt%), (22.5; 15 wt%), (25; 15 wt%) potassium phosphate buffer; PEG 1500 wt%. The composition of the top phase was determined as described in Table S3.† Table S3† presents the mixture points studied, their compositions, respective TLs and tie-line lengths (TLLs) obtained. As can be noticed from the results of Table 1, by increasing the tie-line length (TLL), the water content in the top phase decreases (from 56.89 to 38.98 wt%). The partition behaviour of both unreacted Cyt-c and Cyt-c-PEG-8 showed no significant dependency with the TLL as shown in Fig. 3. On the other hand, the partition of Cyt-c-PEG-4 is shown to decrease with the increase of the TLL. This could be explained by the fact that, when the concentration of water in the top phase decreases, these less PEGylated proteins, with intermediate hydrophobicity, start to be excluded from the most hydrophobic phase towards the bottom phase (decrease of 3 fold on the log (K) from 3.42 to 1.40). The amount of water in the top phase has been described in the literature as one of the driving-forces controlling the partition of PEGylated proteins.[29] Analysing the results, it can be observed that the effect of volume exclusion affects only the PEGylated conjugate with a lower degree of PEGylation, thus "PEG–PEG" interactions between the PEG phase forming agent and the PEG covalently bound to the protein are most certainly occurring. Those specific "PEG–PEG" interactions were already observed by other authors.[29] Previous PEGylated protein partition studies in PEG-dextran ABS showed partition coefficients to increase exponentially with the amount of PEG bound to the protein and a linear relationship between log (K) values and the number of grafted PEG chains.[30] Other studies also assume that as the number of chains linked to the protein increase, "PEG–PEG" interactions become an important driving-force for the partition on this ABS, since conjugates with a higher degree of PEGylation will have stronger interactions with the PEG-rich phase. Overall, in the polymer-salt studied ABS, the partition of the protein conjugates seems to be driven by "PEG–PEG" interactions, while the partition of the unreacted protein is dominated by its hydrophilic character, promoting hydrophilic interactions with the most polar phase.

Process design

Considering the application of the various PEGylated forms, their use as both pure compounds or mixtures can be envisaged since PEGylated protein mixtures may result in a synergetic performance in terms of bioactivity, and for that reason in specific cases, *e.g.* biosensing applications,[17] the fractionation of PEGylated forms could not be necessary.

If only the extraction of the PEGylated forms from the reaction mixture is required, a simplified process can be designed, as sketched in Fig. S1† to achieve the separation of the unreacted protein from the PEGylated forms. Using this approach, the isolation and recycling of the unreacted protein from the PEGylated conjugates can be accomplished with recoveries higher than 99% for both the PEGylated forms, at the top, PEG 1500-rich phase, while more than 99% of the unreacted protein is recovered at the bottom, salt-rich phase. This means that, when the PEGylation reaction is not complete the reuse of the non-reacted proteins is possible, thus increasing the efficiency and sustainability of the overall process. To date, to the best of our knowledge, no studies have been reported on the possibility of purification and reuse of the unreacted protein.

The most important challenge in terms of purification is, however, the complete separation of each PEGylated conjugate obtained. The selective fractionation of each conjugate was achieved through a multistep strategy of purification sketched in Fig. 4. In this process, two liquid–liquid separation units were applied, namely those based in PEG 8000 + potassium phosphate buffer (pH = 7) (**ABS I** and **ABS II**), representing two stages of the same unit, and the second unit corresponding to **ABS III**, PEG 1500 + potassium phosphate buffer (pH = 7). The first unit was used in the separation of each PEGylated form, in which the conjugates were partially concentrated in opposite phases. The Cyt-c-PEG-4 was concentrated in the bottom phase, rich in salt, and Cyt-c-PEG-8 preferentially partitions towards the most hydrophobic phase, rich in polymer. With the application of the first process unit, the complete separation of both PEGylated forms was achieved, as demonstrated by the recoveries reported in Fig. 4.

Table 1 Recoveries towards top and bottom phases, log (K), purities and mass balances (MB) obtained for the unreacted Cyt-c and for the PEGylated conjugates, respectively, Cyt-c-PEG-4 and Cyt-c-PEG-8, in the integrated multistep process using polymer-based ABS as purification platforms, for the system using PEG (8000 and 1500) + potassium phosphate buffer (pH = 7). The overall mass balance (OMB) is also depicted in this table for unreacted and Cyt-c PEGylated forms

	Cyt-c					Cyt-c-PEG-4					Cyt-c-PEG-8				
	Rec Top (%)	Rec Bot (%)	Log (K)	Purity (%)	MB (%)	Rec Top (%)	Rec Bot (%)	Log (K)	Purity (%)	MB (%)	Rec Top (%)	Rec Bot (%)	Log (K)	Purity (%)	MB (%)
ABS I	0.02	99.98	−3.48	70.0	90.65	0.05	99.95	−3.10	29.7	93.74	45.78	54.22	0.11	87.4	88.23
ABS II	0.04	99.96	−3.13	85.2	92.59	0.13	99.87	−2.63	10.5	94.32	50.74	49.26	0.28	99.0	85.23
ABS III	0.09	99.91	−2.80	96.5	84.78	99.56	0.44	2.63	85.8	85.62	21.79	78.21	−0.28	99.0	96.52
OMB (%)			71.16					75.70					72.66		

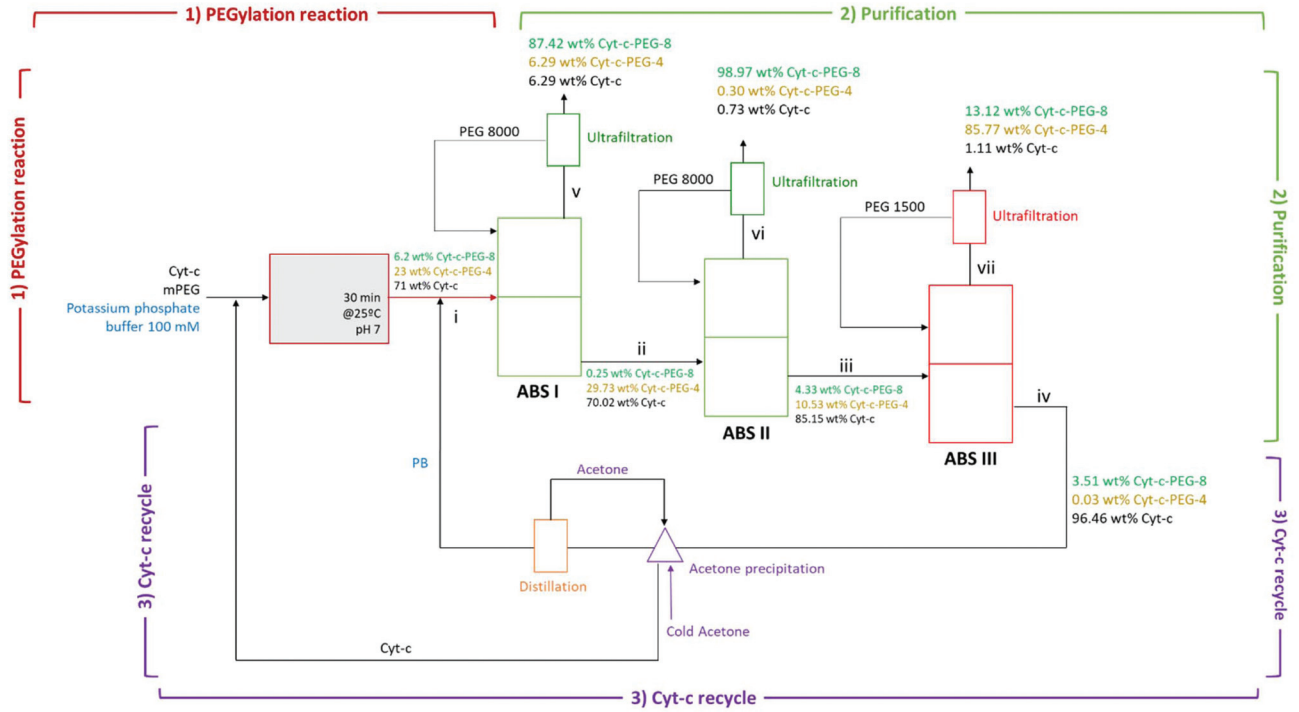

Fig. 4 Diagram of multistep process to fractionate selectively the unreacted cytochrome c and each one of the PEGylated conjugates (Cyt-c-PEG-4 and Cyt-c-PEG-8). The proposed strategy includes three steps of purification using ABS, namely considering the ABS I and ABS II composed of PEG 8000 + potassium phosphate buffer (pH = 7) to separate Cyt-c-PEG-8 and ABS III using PEG 1500 + potassium phosphate buffer (pH = 7) to fractionate the unreacted Cyt-c from the Cyt-c-PEG-4 conjugate. The recovery yield of each step is also provided in the present diagram. The recycle and reuse of the unreacted Cyt-c in a new PEGylation cycle after recovery in the last step is also demonstrated.

In the final of **ABS II**, the continuous removal of Cyt-c-PEG-8 was assessed and then, **ABS III** was applied to carry the final step of purification, in which the Cyt-c-PEG-4 was removed from the unreacted protein. From the results of log (K) for Cyt-c, Cyt-c-PEG-4 and Cyt-c-PEG-8 shown in Table 1, it is possible to reinforce that the application of the multistep process is effectively contributing to efficiently fractionate and purify each PEGylated form and the unreacted protein with high selectivity. High purities for each compound were obtained by applying this multistep approach as shown in Table 1, those being really appealing for the application of these biomolecules in the field of biosensors (purity of Cyt-c = 96.5%, purity of Cyt-c-PEG-4 = 85.8% and purity of Cyt-c-PEG-8 = 99.0%). Additionally, the analysis of the overall mass balance (OMB) of the protein conjugate shows that the protein losses during the multistep ABS are small. The detailed composition of the streams of the process are presented in Table S4.† It should be taken into consideration that the water content contemplates the Cyt-c based products, since their quantity in terms of weight is comparatively smaller than the phase forming agents (initial Cyt-c solution = 0.5 mg mL^{-1}). This is the first report of a multistep approach using ABS in cascade being efficiently applied in the development of an effective downstream process to purify PEGylated conjugates according to their degree of PEGylation.

The development of recycling strategies aiming at the reuse of the recovered unreacted Cyt-c to be used in new cycles of PEGylation is required. For that reason, two different approaches were tested: (i) the direct PEGylation of the salt-rich aqueous phase in which the purified Cyt-c is concentrated and (ii) the use of an acetone precipitation step to isolate the purified Cyt-c from the salt-rich layer as represented in Fig. 6. After the precipitation step, the unreacted protein is ressuspended in the same buffer used in the PEGylation. The results obtained, presented in Fig. 5, show the first approach to be inefficient due to the absence of any PEGylation of the recycled Cyt-c as can be gauged from the chromatogram presented in Fig. 5A, which could be due to the higher amounts of salt present. However, the approach based on the acetone precipitation proved to be efficient for the recovery of the Cyt-c for further PEGylation, as shown in the chromatogram presented in Fig. 5B and Lane 2 of the SDS-PAGE in Fig. 5C, thus increasing the viability of the overall process.

Finally, the stability and integrity of the unreacted Cyt-c purified was accessed using far-CD. Both the control and purified Cyt-c present a CD spectrum (Fig. 6) characteristic of α-helical secondary structures, displaying negative intensity bands centered at 208 and 222 nm and a positive peak in the 195–200 nm region of the spectrum, proving thus the structural integrity of the protein structure. Moreover, the PEGylated forms were also stable after the multistep downstream processing and confirmed by far-UV CD (Fig. S2†).

The results reported highlight the potential of the downstream process here proposed for an efficient fractionation of the Cyt-c PEGylated conjugates and recovery of unreacted Cyt-c.

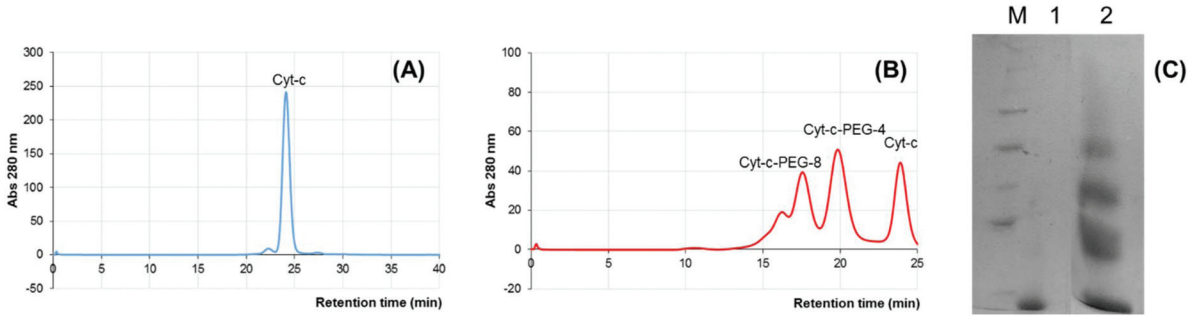

Fig. 5 SEC-FPLC chromatogram of (A) unreacted Cyt-c concentrated in the bottom-phase used directly in a new cycle of PEGylation; (B) unreacted Cyt-c used in a new cycle of PEGylation but after its isolation from the bottom phase through precipitation with cold acetone. (C) SDS-PAGE showing the successful use of the recycled Cyt-c in a new cycle of PEGylation: Lane M – Molecular Marker, Lane 1 – commercial equine heart Cyt-c, Lane 2 – PEGylation mixture obtained after a new cycle of PEGylation using the purified Cyt-c.

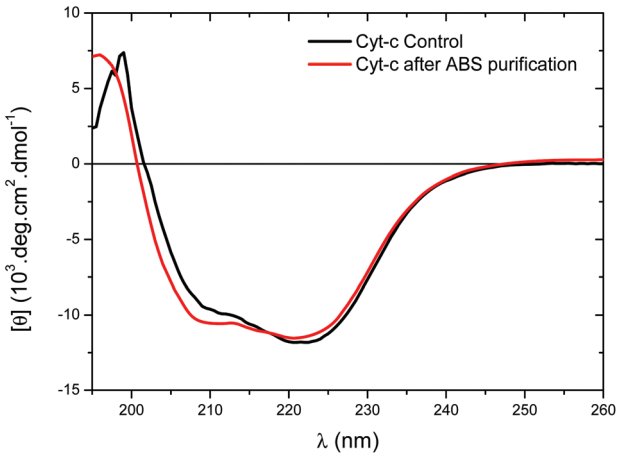

Fig. 6 Far-UV CD spectra of Cyt-c control (—) and Cyt-c purified through the aqueous biphasic system (—). Cyt-c concentrations used are 15 and 13 μM for the control and purified forms, respectively.

Conclusions

In order to overcome the difficulty in obtaining pure site-specific PEGylated conjugates of Cyt-c-based biosensors, an alternative process of downstream was here proposed. For that, the use of consecutive steps of purification using polymeric-based ABS were applied on the separation of (i) the unreacted protein from the PEGylated forms and (ii) each PEGylated conjugate. From the main results obtained in the partition studies, it was assessed that the impact of the molecular weight of PEG and water content composing the top phase enriched in PEG. These two conditions have a significant impact on the partition profile of the unreacted Cyt-c and the different PEGylated forms obtained in non-site-specific PEGylation reactions (in this case, specifically Cyt-c-PEG-4 and Cyt-c-PEG-8) towards opposite phases. The different partition trend obtained for each class of proteins is actually explained by the presence of two major driving-forces occurring, namely the exclusion volume of the polymer-rich phase and "PEG–PEG" interactions. Considering thus the main applications of each PEGylated form and the unreacted protein, different processes of purification were defined to achieve different purposes. Thus, a single step process using the ABS composed of PEG 1500 was successfully developed for the isolation of the unreacted protein from the PEGylated conjugates. Rec Bot of 99.88 ± 0.05% of Cyt-c towards the bottom phase were achieved while in the top phase, Rec Top of 99.31 ± 0.62% and 99.87 ± 0.01% were obtained for Cyt-c-PEG-4 and Cyt-c-PEG-8, respectively. For the case when each PEGylated form is required in its purer form, a multistep process was developed by the conjugation of three different steps, all using the polymer-based ABS studied. This multi-step approach strategy was efficiently developed for the fractionation of the three protein species, namely Cyt-c, Cyt-c-PEG-4 and Cyt-c-PEG-8, allowing purities higher than 95% of the unreacted Cyt-c and Cyt-c-PEG-8 and more than 85% of purity for Cyt-c-PEG-4 in the final of the overall process. Envisaging the industrialization of these integrated processes, the recycling of the unreacted Cyt-c for a novel PEGylation reaction was successfully achieved by applying a precipitation step carried out with cold acetone, in which the successful reuse of the unreacted Cyt-c after purification was proved, while maintaining the structural integrity of the protein.

In this work, an efficient process of PEGylation and purification of different site-specific PEGylated forms was developed with high efficiency.

Conflicts of interest

There are no conflicts of interest to declare.

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