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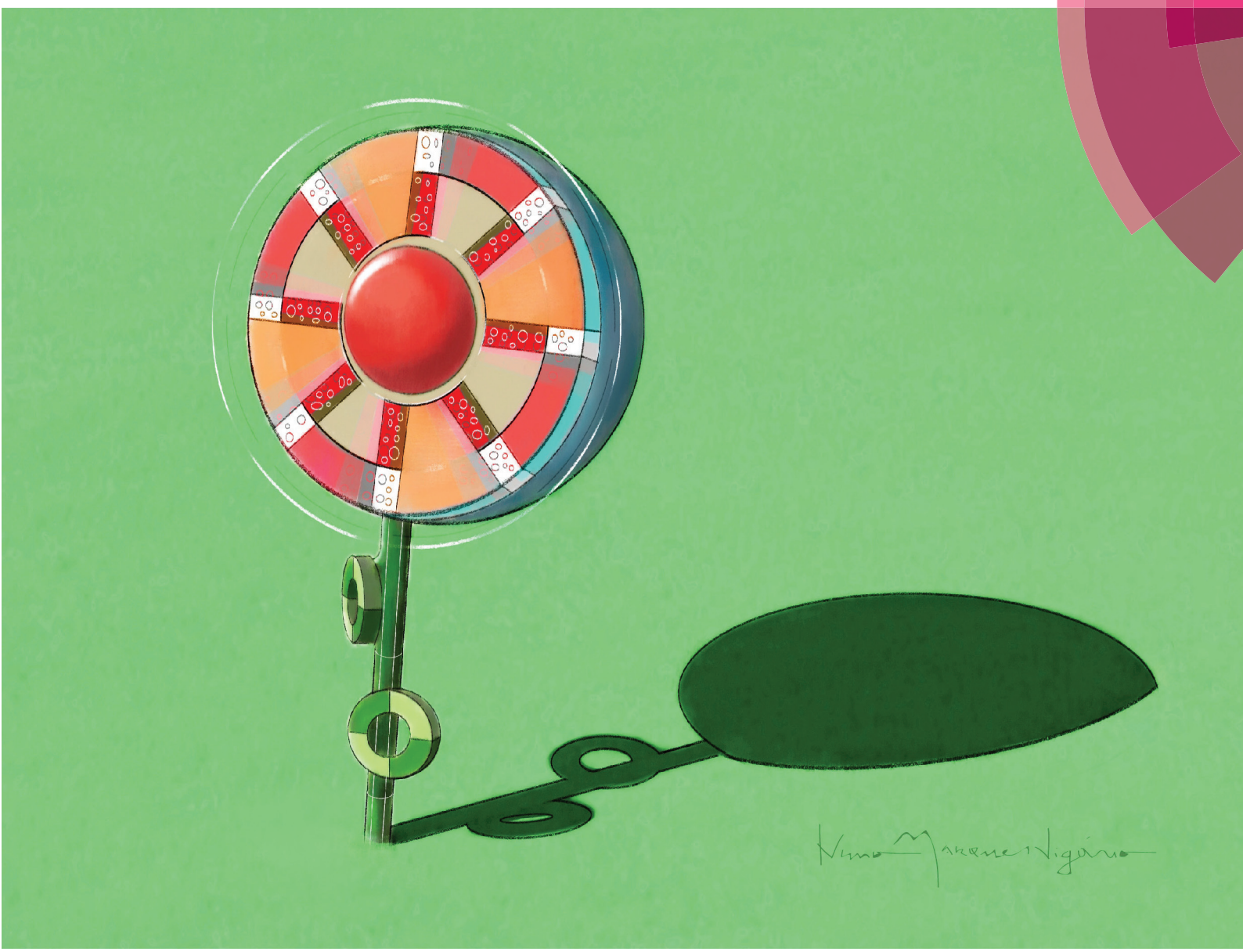

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Continuous separation of cytochrome-c PEGylated conjugates by fast centrifugal partition chromatography†

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Herein, the effective use of aqueous biphasic systems (ABS) in Fast Centrifugal Partition Chromatography (FCPC) for the purification of PEGylated cytochrome c conjugates is shown. High recoveries (between 88% and 100%) and purities (~100%) were obtained. Both the unreacted cytochrome c and solvents may be recovered and reused, thus allowing the design of a sustainable process in a continuous regime for the isolation of bioconjugates. This process allowed the reduction of the complete E-factor and carbon footprint at *circa* 100% and 67%, respectively, reinforcing the important environmental contribution of recycling units.

Protein PEGylation is widely used by the pharmaceutical industry,[1] and also to obtain protein-based biosensors.[2–5] PEGylated drugs are commonly classified as biobetters,[6–8] since the polyethylene glycol (PEG) attachment on proteins normally results in pharmacokinetic and pharmacodynamic improvements.[9,10] PEGylation enhances drug solubility/bioavailability and decreases immunogenicity,[1] while increasing the plasma half-life by reducing drug proteolysis and clearance, thereby contributing to a reduced dosing frequency by the patient.[8,11] Taking into consideration these benefits, a wide variety of therapeutic proteins,[12–14] peptides,[15] and antibody fragments,[10] as well as small molecule drugs,[16] are usually PEGylated. Regarding the development of protein-based biosensors, this approach allows overcoming the limitations related to the long term stability of the protein. PEGylated conjugates are, in general, more stable over extended periods of time while maintaining their biosensing performance.[2]

The conjugation reaction between PEG and proteins is performed by adding activated PEG (also known as a PEG derivative) to the target proteins under appropriate conditions (*i.e.* pH, PEG : protein molar ratio, time, temperature and agitation speed).[17,18] The chemistry behind protein PEGylation is well-known.[6,18,19] Protein PEGylation results in a complex heterogeneous colloidal solution, consisting of a residual polymer, a native protein, reaction by-products and, even more problematic, different forms of the modified protein conjugates differing in the number of PEG molecules attached to different residues.[18] Accordingly, two main problems persist when considering the design of efficient, continuous and scaled-up bioprocesses, namely the low yield of most PEGylation reactions and the need for efficient fractionation processes.

Currently, purification processes of PEGylated proteins are based on chromatographic techniques, namely size exclusion chromatography (SEC)[20,21] and ion exchange chromatography (IEX).[22,23] However, the separation of PEGylated proteins through conventional chromatographic techniques results in a large number of cases, in the co-elution of PEGylated products and native proteins (*i.e.* poorer resolution and separation).[24,25] Separations by size (SEC) and charge (IEX) may be of low efficiency when the differences of molecular weight and charge between PEGylated conjugates and the native protein are small. Besides the conventional chromatographic techniques, other approaches have been recently considered to fractionate PEGylated conjugates, namely membrane separations, electrophoresis, capillary electrophoresis, and aqueous biphasic systems (ABS).[26] ABS are liquid–liquid systems composed of water and two phase-forming components (*e.g.* two polymers or a polymer and a salt) that phase separate under given conditions of composition, pH, pressure and temperature. Due to their water-rich nature, which is advantageous when dealing with biologics, and the possibility of scale-up,

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†Electronic supplementary information (ESI) available: Experimental details explaining the methodologies followed also including the environmental analysis. In terms of figures in the ESI there are the binodal curves used to prepare the ABS, HPLC chromatograms, and schematic representations of the simple process evaluated in this work. Regarding tables, there are the partition coefficient data for Cyt-c and Cyt-c-PEG and data of the mixture points tested in FCPC, stationary phase retentions and operational conditions. The data used in the environmental analysis is also detailed. See DOI: 10.1039/c9gc01063g

ABS have been largely investigated in separation processes of a plethora of therapeutic compounds.[27,28]

Regarding the scale-up of liquid–liquid systems and their use in a continuous regime, countercurrent chromatography (CCC) is commonly investigated and applied by both academia and industry.[29,30] It is a liquid–liquid chromatographic technique where a support-free liquid stationary phase is maintained by centrifugal force.[31] The application of liquid–liquid systems in CCC has been shown to be highly efficient and selective for the separation of structurally similar proteins; however very distinct partitioning trends between the molecules being separated are required for its successful application.[29,31] When the partition behaviour is quite similar between the biomolecules being fractionated and, when ABS are intended to be used, hydrostatic CCC columns, known as fast centrifugal partition chromatography (FCPC), should be applied.[29,32,33] FCPC, in contrast to CCC, uses single axial rotation through a constant centrifugal field, enclosing geometrical volumes, tubes, channels, or locules, repeated through connecting tubes, thus allowing a continuous separation process.[31] FCPC can operate in the ascending (mobile phase rises through the retained denser stationary phase) and descending (mobile denser phases descend through the lighter phase) modes. It is considered a versatile, scalable and mechanistically simple approach, which permits the separation of structurally similar molecules in a continuous regime.[34–36]

This work aims to investigate the potential of ABS applied in FCPC for the continuous separation of PEGylated and non-PEGylated species, using cytochrome-c as the model protein. PEGylated Cyt-c forms (Cyt-c-PEG) are produced through an acylation reaction using NHS-PEG ester, in which site-specific Cyt-c PEGylated conjugates are produced.[33] Details on the experimental procedure and materials to perform the PEGylation reaction, the optimization of the ABS conditions and the application of the most relevant system on FCPC are given in the ESI (sections 1.2 to 1.6).† Moreover, and while foreseeing the development of an efficient, sustainable and continuous separation process that could meet some of the requirements of the Green Chemistry principles, two distinct scenarios were considered: the separation process was studied with and without the recycling of the unreacted protein and main solvents (see section 1.5 of the ESI† for experimental details) as a way to increase sustainability. In the end and, still considering both scenarios, the environmental impact of the final process was investigated, by considering both the complete E-factor and carbon footprint metrics, as final indicators. These metrics are in line with the high demand for the design of more sustainable chromatographic-based processes, including CPC-based ones.

ABS formed by PEG 600 g mol^{-1}, PEG 1000 g mol^{-1}, PEG 1500 g mol^{-1}, and PEG 2000 g mol^{-1} (respectively, PEG 600, PEG 1000, PEG 1500 and PEG 2000) and potassium phosphate buffer aqueous solutions (pH = 7) were used. The respective phase diagrams were taken from the literature,[38] being summarized in Fig. S1 in the ESI.† These phase diagrams give an indication on the phase-forming component aptitude to phase separate and on the amounts required to form two-phase systems.[39,40] The mixture composition adopted in the ABS biphasic region to test the continuous purification using FCPC is as follows: 15 wt% of PEG + 20 wt% of potassium phosphate buffer (pH = 7) + 65 wt% of water (*cf.* Fig. S1 in the ESI†). To address the separation of both proteins (unreacted and PEGylated conjugate) and to design the conditions to apply in FCPC, their partition coefficient (K) was initially determined in batch experiments using the two pure proteins; the respective partition coefficients at room temperature (*ca.* 25 ± 1 °C) are given in Table S1 in the ESI.† Aiming at improving the separation efficiency of both proteins, the molecular weight of the polymer (PEG 600, PEG 1000, PEG 1500, PEG 2000 and PEG 4000) was additionally investigated, at the common mixture composition, to tailor the hydrophilic–hydrophobic nature of the phases. The partition coefficient corresponds to the concentration of each protein in the PEG-rich phase with respect to that in the salt-rich phase. Overall, the results obtained indicate the preferential partition of Cyt-c-PEG towards the PEG-rich phase and of the unreacted protein towards the opposite phase (*i.e.* the salt-rich phase). This behaviour may be justified by the preferential PEG–PEG interactions and by hydrophobic effects.[37,41–44] Partition coefficients between 5.24 and 1548 for Cyt-c-PEG, and between 0.001 and 0.50 for the unreacted Cyt-c protein were obtained. These values allow the definition of the operating conditions to apply in FCPC. The five ABS composed of PEGs with different molecular weights and potassium phosphate buffer (pH = 7) were then investigated in multi-step separations and a continuous regime, where the FCPC flow rate and rotation speed were kept constant and the stationary phase retention and the capability to isolate the different protein forms were addressed (detailed data given in Table S2 in the ESI†).

The polymer-based ABS composed of the PEG of higher molecular weight, *i.e.* PEG 4000, resulted in a highly viscous top-phase, preventing the application of FCPC due to overpressure. On the other hand, the ABS formed by the PEG with the lower molecular weight (PEG 600) exhibits the lowest value of liquid stationary phase retention factor (Sf), and thus presents a weak capacity in what concerns the retention of the stationary phase. Taking into account all the processual restrictions identified, the work with FCPC was further performed using PEGs with intermediate molecular weights, namely PEG 1000, PEG 1500, and PEG 2000.

After selecting the most appropriate ABS to use combined with FCPC, they were then applied to the real matrix resulting from the PEGylation reaction of Cyt-c (12 kDa). In this step, the reaction medium was directly used in the ABS formulation. A PEG derivative (mPEG-NHS) of 20 kDa was used to carry out the PEGylation reaction, resulting in a complex medium containing a heterogeneous mixture of proteins (Cyt-c and Cyt-c-PEG) and salts (hydroxylamine and potassium phosphate buffer). Further experimental details are given in the ESI.† The yield of PEGylation observed was *circa* 43.5%, with only one PEGylated conjugate formed (Cyt-c-PEG). This yield is quite reasonable when compared to other PEGylation yields obtained for Cyt-c.[2,3,45] Fig. S2 in the ESI† presents the chro-

matogram resulting from the batch injection of 2 mL of reaction medium, performed in the descending mode and with a mobile phase flow rate of 2.5 mL min^{-1}. The ascending mode was also tested resulting in a weaker fractionation performance; for this reason it is discarded.

The potassium phosphate buffer-rich phase was used as the mobile phase during the initial stage of elution (from 0 to 20 min), whereas the PEG-rich phase was used as the stationary phase. Afterwards and for the elution of unreacted Cyt-c, the mobile phase was changed to water and the extrusion method was applied[46,47] (from 20 to 40 min). This change in the mobile phase decreases the elution time of Cyt-c-PEG since the PEGylated form has a higher affinity to the stationary phase, *i.e.* the PEG-rich phase. Previous studies[37] and the partition batch tests performed here for both proteins (Table S1 in the ESI†) demonstrated that Cyt-c exhibits $K < 1$, resulting in its favourable partition to the most hydrophilic phase. In contrast, the PEGylated form is mostly concentrated in the PEG-rich phase ($K > 1$). The FCPC results are in good agreement with these batch assays, where the unreacted protein preferentially partitions to the mobile phase, being firstly separated, followed by the further elution of the PEGylated conjugate by extrusion. No losses of the stationary phase were observed during the separation run and the complete separation of the two compounds was achieved. Cyt-c elutes in fractions 7–15, whereas Cyt-c-PEG elutes in fractions 35–39, being dependent on the PEG molecular weight used in the ABS formulation. The removal of the phase-forming components (PEG and potassium phosphate buffer) was assessed by ultrafiltration, using molecular weight cut-off, MWCO = 3 kDa Amicon centrifugal filters, and the proteins were identified and quantified by HPLC, whose results are shown in Fig. 1.

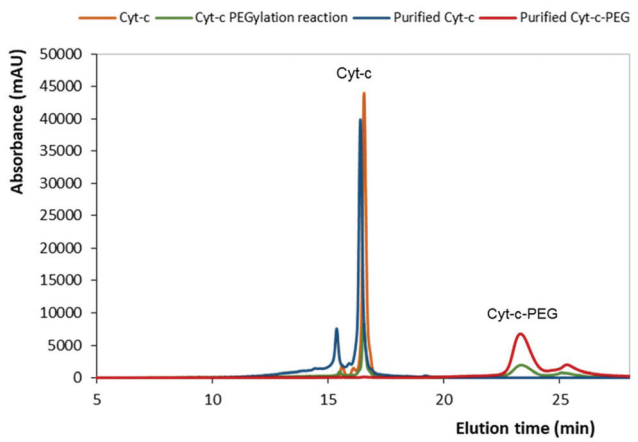

Fig. 1 High performance chromatography (HPLC) chromatogram of native Cyt-c, Cyt-c PEGylation reaction media, purified Cyt-c and purified Cyt-c-PEG after the process without recycling of solvents and proteins. HPLC analyses were performed with an analytical C18 reversed-phase column (250 × 4.60 mm), kinetex 5 μm C18 100 A, from Phenomenex. The mobile phase used was a gradient system of 0.1% of trifluoroacetic acid (TFA)-ultra-pure water (phase A) and 0.1% TFA-acetonitrile (phase B).

The experimental procedure and conditions used in the HPLC analysis are given in the ESI.† This procedure allows obtaining the target proteins in proper buffered aqueous solutions while allowing the recovery and reuse of the phase-forming components as discussed below.

Overall, after FCPC and ultrafiltration, high recovery yields (Rec%) and purities for both proteins (*i.e.* Rec% > 96% and purity = 100% for Cyt-c; Rec% > 88% and purity = 100% for Cyt-c-PEG) were achieved with all systems (*cf.* Table 1). Among these, the system composed of PEG 2000 was identified as the most promising system due to the highest values of recovery (Rec% > 99%) for both proteins. This result may be justified by the fact that, for its high molecular weight, a higher stationary phase retention is obtained (*cf.* Table S2 in the ESI†), thus preventing significant losses of the stationary phase. These values support the high potential of suitable ABS for the separation of PEGylated conjugates using FCPC in a continuous regime, while allowing the technology scale-up. Currently, the biopharmaceutical industries are seeking the development of processes fitting within the principles of Green Chemistry and Sustainability.[48] Therefore, the recovery and reuse of both the phase-forming components and unreacted protein were appraised. In particular, this approach comprises two steps: (i) the reuse of purified unreacted Cyt-c for a new cycle of PEGylation reaction and purification and (ii) the reutilization of the phase-forming ABS components (PEG 2000 and potassium phosphate buffer) for a consequent FCPC purification run. A summary of the process proposed is depicted in Fig. 2; recovery yields and purification levels of both proteins are given in Table 2.

The recovered Cyt-c was used in a new PEGylation reaction, leading to a yield of reaction of 55 ± 2%. A subsequent FCPC purification step was performed using the PEG 2000 + potassium phosphate buffer system, applying the same methodology of elution–extrusion. Fig. 3 depicts the chromatogram of each protein after FCPC, where the complete separation of both proteins is achieved using the ABS formed by "recycled" PEG 2000, without losses of the stationary phase. Since the process was designed taking into account the recyling of the solvents, the respective tie-line was characterized for the mixture point adopted, as presented in Table S3.† By the tie-line determination, the composition of the top and bottom phases was defined and used for further preparation of the mixture point to test in the second cycle of PEGylation + separation of protein formation. The Cyt-c eluted is represented by fractions

Table 1 Purified fractions, protein recovery (Rec%) and purity of Cyt-c and Cyt-c-PEG obtained after fast centrifugal partition chromatography (FCPC) purification (without recycling)

	Cyt-c			Cyt-c-PEG		
	Fractions	Rec (%)	Purity	Fractions	Rec (%)	Purity
PEG 1000	14–15	96.1%	100	39	88.2%	100
PEG 1500	12–14	98.9%	100	38–39	90.6%	100
PEG 2000	7–12	100%	100	35	99.2%	100

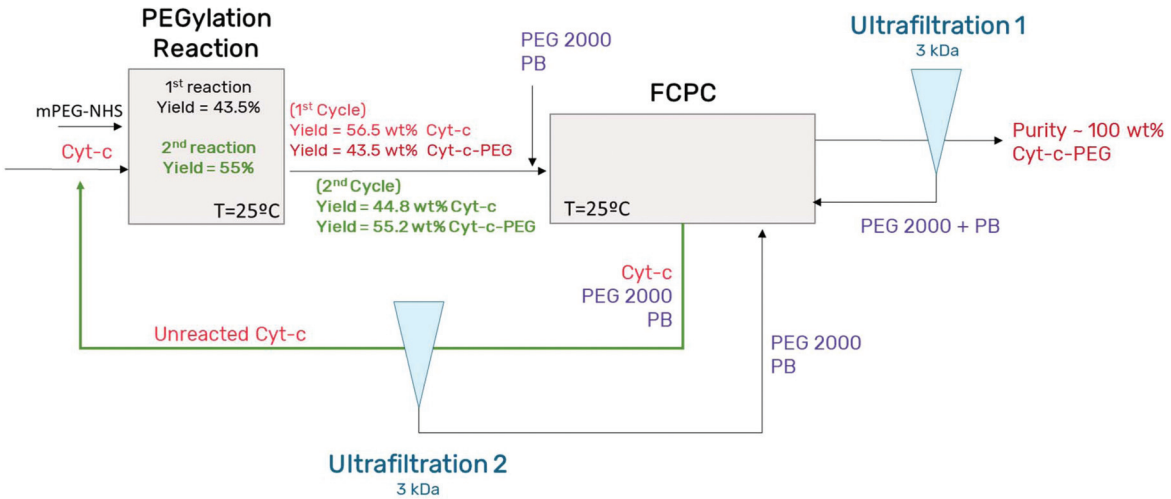

Fig. 2 Process diagram of the integrated process of purification in a continuous regime applying fast centrifugal partition chromatography (FCPC) with PEG 2000 and phosphate buffer (PB), including the recycle of the unreacted Cyt-c and main solvents used.

Table 2 Purified fractions, recoveries (Rec%) and purity (%) of Cyt-c and Cyt-c-PEG obtained after fast centrifugal partition chromatography (FCPC) purification in both processes designed (with and without recycling)

	Cyt-c			Cyt-c-PEG		
	Fractions	Rec (%)	Purity	Fractions	Rec (%)	Purity
PEG 2000	7–12	100%	100	35	99.2%	100
PEG 2000 recycled	10–12	96.0%	100	36–37	90.9%	100

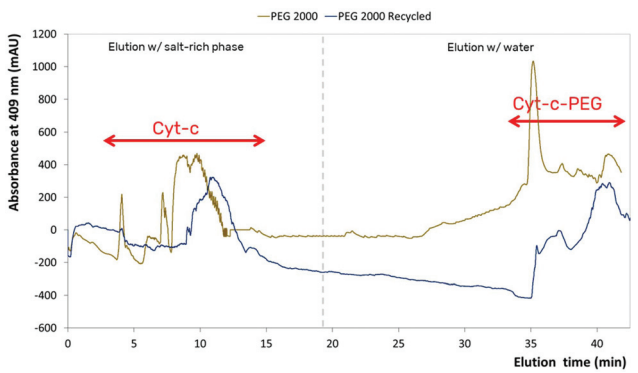

Fig. 3 Chromatogram of fast centrifugal partition chromatography (FCPC) representing the separation of unreacted Cyt-c and Cyt-c-PEG, obtained after two-steps of PEGylation + fractionation using FCPC and the ABS comprising 15 wt% of PEG 2000 + 20 wt% of potassium phosphate buffer at pH 7.0.

10–12, and the Cyt-c-PEG corresponds to fractions 36–37. In general, the yields of the PEGylation reaction increase from 43.5 ± 0.3% to 55 ± 2%, and the recovery yields (ratio between the weight of the PEGylated protein found in the purified fraction and the initial weight of the PEGylated protein after the bioconjugation reaction) and purity levels of both proteins are maintained with high values. Recovery values from 100% to 96.0% for Cyt-c and from 99.2% to 90.9% for Cyt-c-PEG, both with a purity of *ca.* 100%, were obtained. Overall, the proposed integrated process in which both the phase-forming components and unreacted protein are used leads to similar or even higher results of recovery and purity. Furthermore, in this work, remarkable purities and recoveries were obtained, even using the recycled components, when compared for instance with results obtained by applying countercurrent distribution (CCD) to purify PEGylated RNAse A[49] or PEGylated lysozyme.[50] In comparison with FCPC, CCD is a preparative CCC scale technique firstly developed; it uses gravity to maintain the liquid stationary phase, resulting in very long elution times, lower resolution, highly laborious work (no-automation)[49] and a worse purification scenario, as already discussed in the literature.[47,48]

Two of the major contributors to the sustainability of a manufacturing process are the water and solvent consumption, which can account for 80 to 90% of the total mass in a typical process, and the low reaction conversions.[51] With the integrated process developed and proposed in this work, the steps of the PEGylation reaction and separation of the PEGylated products from the unreacted proteins were integrated by including the recycling of the ABS phase-forming compounds and use of the unreacted Cyt-c in a new PEGylation reaction.

Aiming to deeply explore the sustainability of the process developed, its environmental impact[52–54] was evaluated by determining the carbon footprint, as previously explored,[55,56]

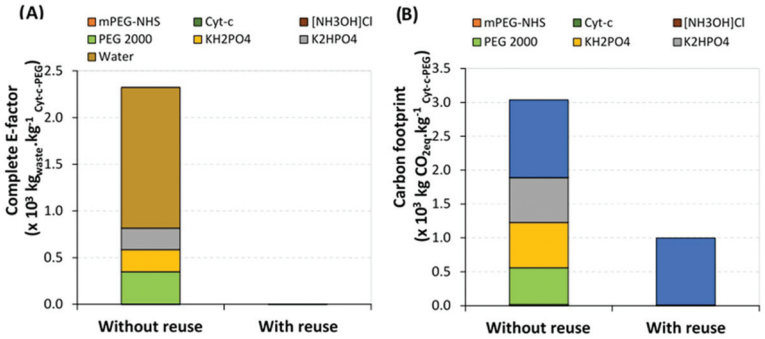

Fig. 4 Complete E-factor (A) and carbon footprint (B) of the integrated process to purify Cyt-c-PEG, for both scenarios explored, with and without the reuse of Cyt-c, PEG 2000, KH_2PO_4, K_2HPO_4, and water.

but also the complete E-factor (Fig. 4). Both scenarios proposed were evaluated, namely the purification of Cyt-c with and without the recycling and reuse of Cyt-c, PEG 2000, KH_2PO_4, K_2HPO_4 and water. Fig. 4A shows that without reuse the complete E-factor achieves a maximum of 2.3×10^3 kg_{waste} $kg^{-1}_{Cyt\text{-}c\text{-}PEG}$, the wastewater being generated during the purification of the Cyt-c-PEG contributing 65% of the complete E-factor, followed by a 15% contribution attributed to losses of PEG 2000. Meanwhile, for the scenario contemplating the reuse of Cyt-c, PEG 2000, KH_2PO_4, K_2HPO_4 and water, the complete E-factor diminishes by 3 orders of magnitude, which represents an almost 100% decrease to 1.20 kg_{waste} $kg^{-1}_{Cyt\text{-}c\text{-}PEG}$.

In addition to the complete E-factor, also the carbon footprint was determined (Fig. 4B). Again, both scenarios were contemplated, where for the approach without the reuse of proteins and solvents, the carbon footprint reached a value of 3.0×10^3 $kgCO_2eq$ $kg^{-1}_{Cyt\text{-}c\text{-}PEG}$, decreasing by around 67% to 1.0×10^3 $kgCO_2eq$ $kg^{-1}_{Cyt\text{-}c\text{-}PEG}$, when reuse was considered. In terms of the carbon footprint, the production of the electricity consumed, mainly in FCPC and the PEGylation reaction was the main contributor to the carbon footprint for both scenarios. Electricity consumed is indeed a contribution particularly relevant when the reuse of unreacted proteins and solvents is analysed, since it represents almost 100% of the total carbon footprint.

In addition to a decrease in the environmental metrics of the process, its overall cost is also significantly decreased when the reuse step is contemplated.

In a rough estimative and based on current prices of 50–60 \$ g^{-1} for Cyt-c (99% purity), and 1–2 \$ kg^{-1}, 1–3 \$ kg^{-1}, and 10 \$ kg^{-1} for PEG 2000 (99.9% purity), K_2HPO_4 (99.5% purity), and KH_2PO_4 (99% purity), respectively, the proposed system is economically advantageous.[57] Overall, these results open up a novel path for engineering and processing in the development of continuous purification strategies[58] for other biopharmaceuticals as well that can easily be scaled-up through the combination of ABS and FCPC.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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