Electronic Supporting Information (ESI)

Continuous separation of Cytochrome-c PEGylated conjugates by fast Centrifugal Partition Chromatography

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1. Materials and Methods

1.1. Materials

Cyt-c from equine heart (purity ≥ 95%) was acquired from Merck. The PEG derivative used in the PEGylation reaction was the methoxyl polyethylene glycol succinimidyl NHS ester (mPEG-NHS), obtained from Nanocs (purity > 95%) with a molecular weight of 20 kDa. The salts used on the protein PEGylation step were the potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4), and hydroxylammonium chloride all acquired from Merck, with purities of 95%.

For the FCPC separation process, the polyethylene glycol series (PEGs) used included PEG 600, 1000, 1500, 2000, and 4000, all bought from Merck. The potassium phosphate buffer was prepared using the salts KH_2PO_4 and K_2HPO_4.

The mobile phase applied in the HPLC analysis was composed of acetonitrile (purity ≥ 99.9 wt%), trichloroacetic acid (purity ≥ 99.5 wt%) from Acros organics, both HPLC grade, and ultra-pure water, double distilled and passed through a Milli-Q plus 185 water purification apparatus. Syringe filters (0.45 μm) acquired from GE Healthcare, Whatman, were used.

1.2. PEGylation reaction of Cyt-c

The PEGylation reactions were conducted following standard protocols described in literature.[1,2] Briefly, 2 mL of a Cyt-c solution (0.5 $mg.mL^{-1}$) in 100 mM of potassium phosphate buffer (pH = 7) was added to a flask containing 20.8 mg of mPEG-NHS with 20 kDa (protein:PEG molar ratio = 1:25). The mixtures were stirred at 400 rpm, for 30 min at room temperature with a magnetic stirrer. To stop the PEGylation reaction, 10% (v/v) of hydroxylammonium chloride (1 M) was added. After, the samples were stored at -20^{o}C for further use in FCPC. The PEGylation yield obtained is calculated by the ratio between the weight of PEGylated protein and unreacted protein.

1.3. Fast Centrifugal Partition Chromatography Purification

1.3.1. FCPC equipment

A FCPC system, model FCPC-C, from Kromaton Rousselet-Robatel (Annonay, France), was used to investigate the continuous separation of PEGylated and non-PEGylated Cyt-c forms. The equipment used in this work is the same presented in our previous study,

where phenolic compounds were purified in a continuous regime.[3] In order to verify the PEG + potassium phosphate buffer ABS behaviour in the equipment, to increase the stationary phase retention ratio and to decrease the purification time and operating conditions, such as flow-rate and rotation speed were investigated (**Table S1**). The best flow rate and rotation speed found were of 2.5 mL.min^{-1} and 200 rpm with a S_f of 41.18%. The stationary phase retention, S_f, was calculated by the ratio of the stationary phase volume (V_S) and the column volume (V_C): $S_f = V_S/V_C$.

Table S1. FCPC assays with PEG 1000 + phosphate buffer ABS, mixture points adopted, stationary phase retention (S_f) achieved and operating conditions.

PEG (g.mol^{-1})	Potassium phosphate buffer	Mixture point (PEG; buffer wt%)	CPC	S_f	Flow rate (mL.min^{-1})	Rotation speed (rpm)
1000	pH 7	15; 20	✓	11.76	3.0	2000
		15; 20	✓	9.31	3.0	2500
		15; 20	✓	16.67	2.5	2500
		15; 20	✓	41.18	2.5	2000

1.3.2. FCPC purification of PEGylated and non-PEGylated Cyt-c

Ternary phase diagrams of PEG + potassium phosphate buffer were measured using the cloud point titration method[4] to further 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 buffer. The mixture points composed of 15 wt% of PEG + 20 wt% of potassium phosphate buffer (pH = 7) using different PEG molecular weights (**Table S2**) were selected. This system was set to work in the descending mode. The rotor was entirely filled with the PEG-(top)-rich phase at 5 mL.min^{-1} and 600 rpm to achieve the homogeneous solvent re-equilibration on the rotor. Then, the rotation was set up at the highest speed (2000 rpm), needed for the appropriate stationary phase retention. After the set-up of the working rotational speed, the potassium phosphate buffer-rich-(bottom) phase was pumped (2.5 mL.min^{-1}) through the stationary phase to reach the equilibrium, *i.e.* when only the mobile phase

came out of the column and the signal baseline is stabilized. The stationary phase retention parameter for all the ABS applied was calculated, ranging from 38% to 46%, depending on the PEG molecular weight used (**Table S2**). The sample loop was filled with 2 mL of the PEGylation Cyt-c samples (obtained by the method 1.2.). An elution-extrusion CCC method was applied,[5,6] this is composed of two steps: first an elution with potassium phosphate buffer-rich phase and then an extrusion with water. The first 20 min of elution were performed using the bottom(salt)-rich phase as mobile phase to extract the unreacted Cyt-c, then the PEGylated conjugates of Cyt-c were eluted with water by extrusion. By applying an elution-extrusion process, full sample recovery can be achieved with high resolution of the peaks.

1.4. Integrated process applying continuous purification by FCPC

An integrated process was created to reuse the unreacted Cyt-c in a novel PEGylation reaction. This integrated process combines the PEGylation reaction, followed by FCPC purification and then the isolation of the purified fractions of Cyt-c by applying an ultrafiltration step. The purified Cyt-c fractions were filtrated through Amicon Ultra centrifugal filters (Merck) with a MWCO of 3 kDa to remove the mobile phase (composed of potassium phosphate buffer with small quantities of PEG). The ultrafiltration step consisted in three washes of 100 mM of potassium phosphate buffer (pH = 7) to change the solvent of the unreacted Cyt-c to the appropriate PEGylation medium. The polished Cyt-c fraction was then PEGylated with a molar ratio of 1:25 (protein:PEG) and applying the same conditions of reaction time, temperature, and agitation speed, described in section 2.2. The recovery yield of proteins (*Rec*%) is defined as the ratio between the weight of PEGylated protein found in the purified fraction and the initial weight of PEGylated protein after bioconjugation reaction. It was calculated for both the integrated and simple processes, meaning the process with and without recycling of Cyt-c and main solvents.

1.5. Polishing of purified Cyt-c products (Cyt-c and Cyt-c-PEG) and recycling of phase components

Both purified fractions containing the PEGylated form and the unreacted protein were ultrafiltrated through Amicon Ultra centrifugal filters (Merck) with a MWCO of 3 kDa for

the removal of phase components of the mobile phase. The recycling of the phase components from the fractions without protein was achieved in the integrated process. This additional step aims to decrease the environmental impact and to seek for the higher sustainability of the purification process proposed.

1.6. Analytical procedures: HPLC quantification of Cyt-c and Cyt-c-PEG

The quantification of purified fractions of PEGylated Cyt-c and unreacted protein from the FCPC was carried by HPLC-DAD (Shimadzu, model PROMINENCE). 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 trifluoracetic acid (TFA)-ultra-pure water (phase A) and 0.1% TFA-acetonitrile (phase B), previously degassed by ultrasonication. The separation was conducted using the following gradient mode, 0 min 25% of B, 42 min 42% of B, 45 min of B, and then returning to initial conditions during 20 min to ensure the column stabilization. The flow rate used was 0.8 $mL.min^{-1}$ with an injection volume of 60 µL. DAD was set at 409 nm. Each sample was analysed at least in duplicate. The column oven and the autosampler operated at a controlled temperature of 25°C. Cyt-c and Cyt-c-PEG presented retention times of 16.5 and 19.3 minutes, respectively.

The purification performance of FCPC process was evaluated based on the recovery of protein purified (*Rec*%) and purity (%) determined for both Cyt-c and Cyt-c-PEG. The recovery was calculated by dividing the protein weight in the purified fraction (either Cyt-c or Cyt-c-PEG) by the initial protein weight (before purification). The purity (%) was calculated by the weight percentage of the desirable protein (either Cyt-c or Cyt-c-PEG) in the purified fraction.

2. Figures and Tables

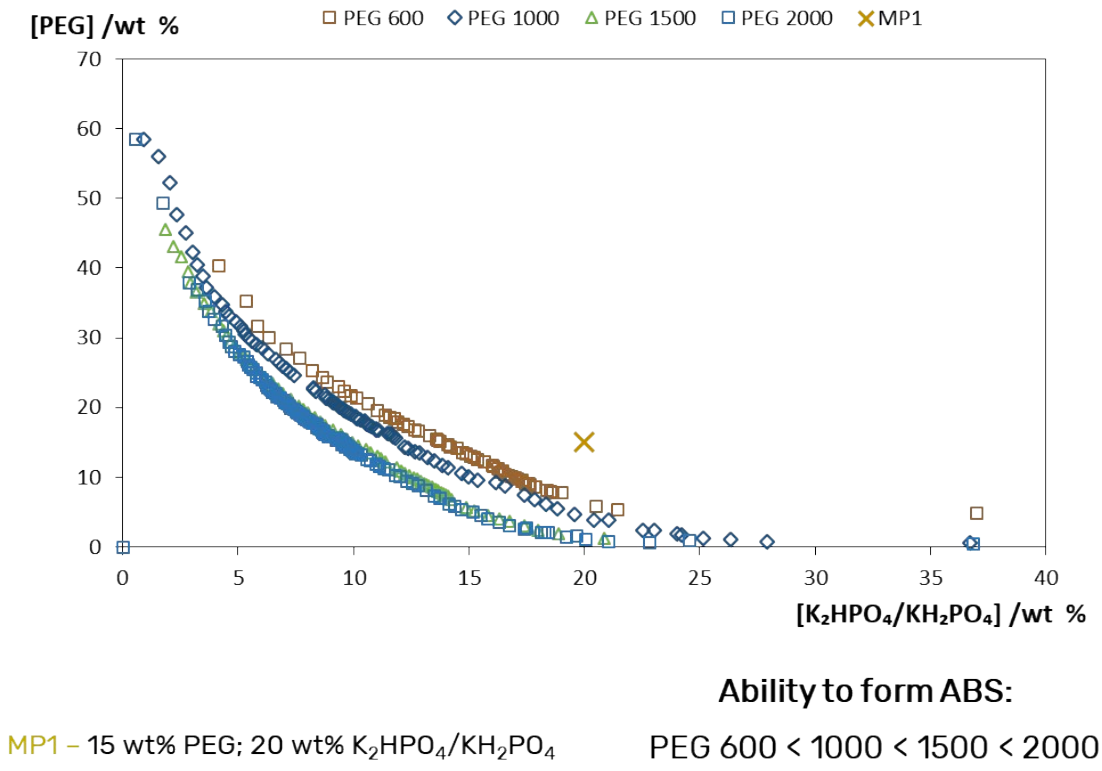

Figure S1. Phase diagrams of polyethylene glycol + potassium phosphate buffer and mixture point (MP) adopted to test the fast centrifugal partition chromatography (FCPC). The system PEG 1500 + K_2HPO_4/KH_2PO_4 were adopted from the literature.[7]

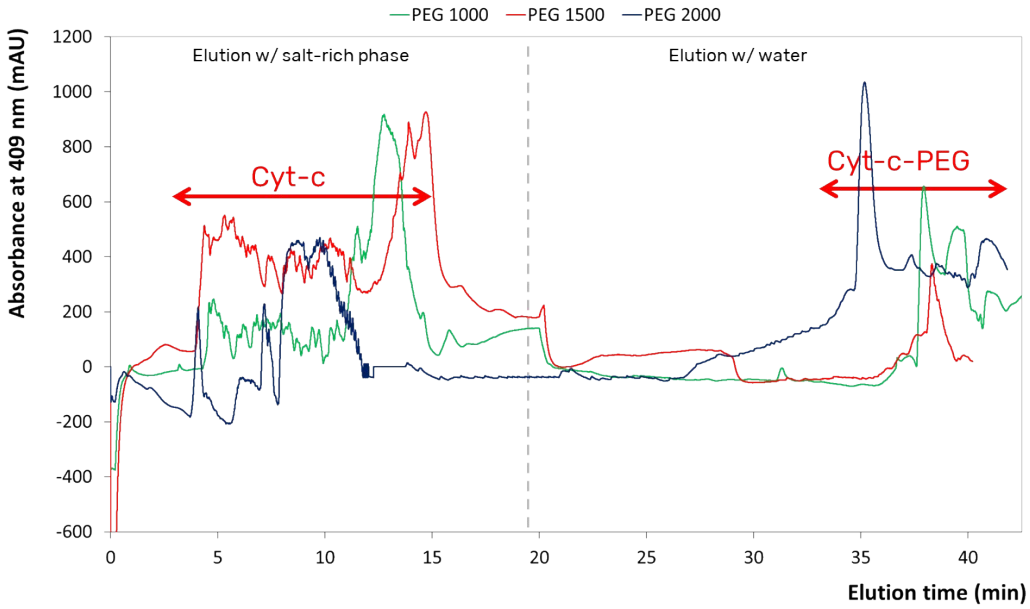

Figure S2. Chromatogram of fast centrifugal partition chromatography (FCPC) purification of Cyt-c and Cyt-c-PEG performed employing ABS comprising 15 wt% of PEG (1000, 1500 and 2000) + 20 wt% of potassium phosphate buffer, at pH 7.0.

Table S1. Partition coefficients (*K*) of Cyt-c and Cyt-c-PEG in PEG + potassium phosphate buffer in lab-scale ABS.

PEG + potassium phosphate buffer ABS	$K_{Cyt\text{-}c}$	$K_{Cyt\text{-}c\text{-}PEG}$
PEG 600	0.496	1114
PEG 1000	0.005	1548
PEG 1500	0.002	1128
PEG 2000	0.002	154
PEG 4000	0.001	5.24
Preferential Partition	Potassium phosphate buffer-rich phase	PEG-rich phase

Table S2. PEG + potassium phosphate buffer ABS tested in FCPC, mixture points adopted, stationary phase retention and operating conditions.

PEG MW ($g.mol^{-1}$)	**Potassium phosphate buffer**	**Mixture point (PEG; buffer wt%)**	**CPC**	S_f	**Operating conditions**
600	pH 7	15; 20	✓	38.73	Flow: 2.5 $mL.min^{-1;}$ Rotation speed: 2000 rpm
1000		15; 20	✓	41.18	
1500		15; 20	✓	46.07	
2000		15; 20	✓	48.53	
4000		15; 20	×	--	Over-pressure Highly viscous top-phase

S_f - stationary phase retention; CPC – ability to perform the purification on centrifugal partition chromatography.

Table S3. Weight fraction percentage (wt%) composition of the initial mixture and of the coexisting phases of the PEG 2000 + potassium phosphate buffer –based ABS at pH = 7, used in the purification of Cyt c forms.

Weight fraction composition (wt%)					
$[PEG\ 2000]_M$	$[Phosphate\ buffer]_M$	$[PEG\ 2000]_T$	$[Phosphate\ buffer]_T$	$[PEG\ 2000]_B$	$[Phosphate\ buffer]_B$
15	20	35.1	3.3	1.76×10^{-4}	32.5

3. Environmental evaluation by determination of Complete E-factor and carbon footprint

The environmental performance of the integrated system proposed for the purification of the Cyt-c-PEG was assessed by calculating the complete E-factor and the carbon footprint. Two scenarios were evaluated: (i) without and (ii) with the reuse of Cyt-c, PEG 2000, KH_2PO_4, K_2HPO_4, and water. In the scenario without reuse, the purified Cyt-c-PEG was obtained by applying a PEGylation reaction and a fast centrifugal partition chromatography (FCPC) followed by an isolation step (designed as ultrafiltration 1) to separate the Pegylated protein from main solvents. In the scenario with reuse, besides these steps, an additional ultrafiltration unit (ultrafiltration 2) was considered to obtain the purified Cyt-c-PEG, by improving the solvents and water recovery yields.

The complete E-factor (cEF) assesses the efficiency of a process by measuring the total amount of chemical waste generated, including water, relative to each isolated product, and it is calculated according to Equation S1.

$$complete\ \mathrm{E}-factor\ =\ \frac{\sum \mathrm{w}_i}{\mathrm{p}} \qquad \text{S1}$$

where, the complete E-factor was obtained for each scenario studied ($kg_{waste}.kg^{-1}_{Cyt\text{-}c\text{-}PEG}$), w_i is the amount of each waste generated during the purification process presented in Table S4 (units in **Table S4**: kg) and p (in $kg_{Cyt\text{-}c\text{-}PEG}$) is the amount of purified Cyt-c-PEG obtained (1 of $kg_{Cyt\text{-}c\text{-}PEG}$ as shown in **Table S5**).

Table S4. Waste generated to produce the purified PEGylated conjugate formed (Cyt-c-PEG): (i) without and (ii) with the reuse of Cyt-c, PEG 2000, KH_2PO_4, K_2HPO_4, and water. The amounts refer to 1 kg of Cyt-c-PEG.

Waste generated	Unit	Without reuse	With reuse
mPEG-NHS	kg	4.64×10^{-2}	4.00×10^{-2}
Cyt-c	kg	1.31	1.14
PEG 2000	kg	3.48×10^{-2}	-
KH_2PO_4 (PB)	kg	2.32×10^{2}	-
K_2HPO_4 (PB)	kg	2.32×10^{2}	-
$[NH_3OH]Cl$	kg	3.23×10^{-2}	2.78×10^{-2}
Water	kg	1.51×10^{3}	-

The carbon footprint carbon footprint corresponds to the sum of greenhouse gas (GHG) emissions expressed as carbon dioxide equivalent (CO_2 eq) and calculated according to Equation S2.

$$carbon\ footprint = \frac{\sum A_j \times GHG_j}{p} \quad \text{S2}$$

where, the carbon footprint was obtained for each scenario studied (kg $CO_{2\ eq}.kg^{-1}_{\text{Cyt-c-PEG}}$), A_j is the amount of each input presented in **Table S5** (units in **Table S5**: kg or KWh), GHG_j is the GHG emission factor for each input *j* presented in Table S6 (units in **Table S6**: $kgCO_{2\ eq}.kg^{-1}_{\text{Cyt-c-PEG}}$ or $kgCO_{2\ eq}.kWh^{-1}$), and p is the amount of purified PEGylated conjugate formed (Cyt-c-PEG) obtained (1 of $kg_{\text{Cyt-c-PEG}}$ as shown in **Table S5**).

Table S5. Inputs considered to produce the purified PEGylated conjugate formed (Cyt-c-PEG): (i) without and (ii) with the reuse of Cyt-c, PEG 2000, KH_2PO_4, K_2HPO_4, and water. The amounts refer to 1 kg of Cyt-c-PEG.

	Unit	Without reuse	With reuse
Inputs			
PEGylation reaction			
mPEG-NHS	kg	4.64×10^{-2}	4.00×10^{-2}
Cyt-c	kg	2.32	1.14
[NH3OH]Cl	kg	3.23×10^{-2}	2.78×10^{-2}
Electricity	kWh	1.18×10^{3}	1.02×10^{3}
FCPC			
PEG 2000	kg	3.48×10^{2}	-
KH_2PO_4 (PB)	kg	2.32×10^{2}	-
K_2HPO_4 (PB)	kg	2.32×10^{2}	-
Water	kg	1.51×10^{3}	-
Electricity	kWh	1.74×10^{3}	1.50×10^{3}
Ultrafiltration 1			
Electricity	kWh	5.81×10^{-3}	1.71×10^{-2}
Ultrafiltration 2			
Electricity	kWh	-	2.70×10^{-2}
Output			
Cyt-c-PEG	kg	1	1

Table S6. Greenhouse gas (GHG) emission factors used to calculate the carbon footprint of the purified PEGylated conjugate formed (Cyt-c-PEG) and name of the processes taken from Ecoinvent version 3.5.[10]

Input	Reference unit	GHG emissions (kg CO_2 eq/reference unit)[a]	Name of the process in Ecoinvent
mPEG-NHS	kg	1.5677	Ethylene glycol production, Europe[b]
Cyt-c	kg	6.4359	Enzymes production, Europe[c]
[NH3OH]Cl	kg	14.96	Hydroxylamine production, Europe[d]
PEG 2000	kg	1.5677	Ethylene glycol production, Europe[b]
KH_2PO_4 (PB)	kg	2.8636	Sodium phosphate, Europe[e]
K_2HPO_4 (PB)	kg	2.8636	Sodium phosphate, Europe[e]
Water	kg	2.791×10^{-4}	Tap water production, conventional treatment, Europe without Switzerland
Electricity	kWh	0.39213	Market for electricity, low voltage, Portugal

[a]Global warming potentials for converting the mass of each GHG into mass of CO_2 eq are those recommended by the Intergovernmental Panel on Climate Change (IPCC)[8] for a time horizon of 100 years.
[b]In the absence of data for the production of methoxyl polyethylene glycol succinimidyl NHS ester (mPEG-NHS) and polyethylene glycol (PEG 2000), this process was selected as more similar.
[c]In the absence of data for the production of the protein (Cyt-c), this process was selected as more similar.
[d]In the absence of data for the production of the hydroxylammonium chloride [NH3OH]Cl, this process was selected as more similar.
[e]In the absence of data for the production of the potassium phosphate monobasic (KH_2PO_4) and potassium phosphate dibasic (K_2HPO_4), this process was selected as more similar.

To calculate the complete E-factor, data on the amounts of waste generated during the purification process from the use of mPEG-NHS, Cyt-c, PEG 2000, KH_2PO_4, K_2HPO_4, $[NH_3OH]Cl$ and water, were obtained during the experiment. To calculate the carbon footprint, data on the amounts of mPEG-NHS, Cyt-c, PEG 2000, KH_2PO_4, K_2HPO_4, $[NH_3OH]Cl$ and water were also obtained during the experiment. Data on electricity consumption in the PEGylation reaction and FCPC were collected during the experiment, and data on electricity consumption in both ultrafiltration units and reuse flows were collected from literature[9] and pump catalogue, respectively. Data on GHG emissions from the production of mPEG-NHS, Cyt-c, PEG 2000, KH_2PO_4, K_2HPO_4, $[NH_3O]Cl$ and water were taken from Ecoinvent database version 3.5.[10]

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