Electronic Supplementary Material (ESI) for Green Chemistry.

Supporting Information

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

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Table S1. Recovery towards the top and bottom phases (in percentage) of the unreacted cytochrome c (Cyt-c) and the site-specific PEGylated forms (Cyt-c-PEG-4 and Cyt-c-PEG-8) in the ABS composed of 15 wt% of PEG + 20 wt% of potassium phosphate buffer (pH = 7).

	Cyt-c		Cyt-c-PEG-4		Cyt-c-PEG-8	
PEG MW ($g.mol^{-1}$)	**Rec Top (%)**	**Rec Bot (%)**	**Rec Top (%)**	**Rec Bot (%)**	**Rec Top (%)**	**Rec Bot (%)**
300	20. ± 3	80 ± 3	99.98 ± 0.01	0.02 ± 0.01	99.94 ± 0.01	0.06 ± 0.01
600	24.8 ± 0.2	75.2 ± 0.2	99.93 ± 0.01	0.07 ± 0.01	99.86 ± 0.01	0.14 ± 0.01
1000	0.3 ± 0.1	99.6 ± 0.1	99.5 ± 0.2	0.4 ± 0.2	99.9 ± 0.01	0.10 ± 0.01
1500	0.12 ± 0.05	99.88 ± 0.05	99.3 ± 0.6	0.7 ± 0.6	99.87 ± 0.01	0.13 ± 0.01
2000	0.11 ± 0.04	99.89 ± 0.04	92 ± 3	8 ± 3	98.5 ± 0.4	1.5 ± 0.4
4000	0.02 ± 0.01	99.98 ± 0.01	0.02 ± 0.01	99.98 ± 0.01	22 ± 2	78 ± 2
6000	0.02 ± 0.01	99.98 ± 0.01	0.02 ± 0.02	99.98 ± 0.02	45 ± 1	55 ± 1
8000	0.02 ± 0.01	99.98 ± 0.01	0.05 ± 0.05	99.95 ± 0.05	46 ± 1	54 ± 1

Table S2. Recovery towards the top and bottom phases (in percentage) of the unreacted cytochrome c (Cyt-c) and site-specific PEGylated forms (Cyt-c-PEG-4 and Cyt-c-PEG-8) in the ABS composed of PEG 1500 + of potassium phosphate buffer (pH = 7) considering different mixture points.

	Cyt-c		Cyt-c-PEG-4		Cyt-c-PEG-8	
Mixture point	**Rec Top (%)**	**Rec Bot (%)**	**Rec Top (%)**	**Rec Bot (%)**	**Rec Top (%)**	**Rec Bot (%)**
(15; 15 wt%)	0.07 ± 0.01	99.93 ± 0.01	99.94 ± 0.01	0.06 ± 0.01	99.92 ± 0.03	0.08 ± 0.03
(17.5; 15 wt%)	0.10 ± 0.01	99.90 ± 0.01	99.90 ± 0.01	0.10 ± 0.01	99.89 ± 0.01	0.11 ± 0.01
(20; 15 wt%)	0.12 ± 0.01	99.88 ± 0.01	99.3 ± 0.6	0.7 ± 0.6	99.87 ± 0.01	0.13 ± 0.01
(22.5; 15 wt%)	0.12 ± 0.01	99.88 ± 0.01	97.1 ± 0.4	2.9 ± 0.4	99.83 ± 0.03	0.17 ± 0.03
(25; 15 wt%)	0.12 ± 0.02	99.88 ± 0.02	92.4 ± 0.1	7.6 ± 0.1	99.81 ± 0.01	0.19 ± 0.01

Table S3. Weight fraction compositions for TLs and respective TLLs at the Top and Bottom (Bot) phases of the phase formers, namely the potassium phosphate buffer (PB), PEG (1500 and 8000) and water (W).

PEG 1500								
[PB] (wt%)	**[PEG] (wt%)**	**$[PB]_{Top}$ (wt%)**	**$[PEG]_{Top}$ (wt%)**	**$[W]_{Top}$ (wt%)**	**$[PB]_{Bot}$ (wt%)**	**$[PEG]_{Bot}$ (wt%)**	**$[W]_{Bot}$ (wt%)**	**TLL**
15	15	2.55	40.56	56.89	21.79	1.06	77.15	43.94
17.5	15	1.79	46.42	51.78	24.83	0.33	74.83	51.53
20	15	1.29	51.55	47.16	27.63	9.13×10^{-2}	72.28	57.81
22.5	15	0.89	56.97	42.14	30.21	2.21×10^{-2}	69.76	64.06
25	15	0.69	60.33	38.98	33.04	3.50×10^{-3}	66.95	68.45
PEG 8000								
[PB] (wt%)	**[PEG] (wt%)**	**$[PB]_{Top}$ (wt%)**	**$[PEG]_{Top}$ (wt%)**	**$[W]_{Top}$ (wt%)**	**$[PB]_{Bot}$ (wt%)**	**$[PEG]_{Bot}$ (wt%)**	**$[W]_{Bot}$ (wt%)**	**TLL**
20	15	1.12	57.49	41.39	26.67	4.95×10^{-6}	73.33	62.91

Table S4. Composition of the streams of the single-step and multistep processes using polymer-based ABS regarding all the phase formers, namely potassium phosphate buffer (PB), polyethylene glycol (PEG MW = 1500 and 8000) and water (W).

Single-step process				Multistep process				
Stream	**[PB] (wt%)**	**[PEG 1500] (wt%)**	**[W] (wt%)**	**Stream**	**[PB] (wt%)**	**[PEG 1500] (wt%)**	**[PEG 8000] (wt%)**	**[W] (wt%)**
I	20	15	65	**i**	20	0	15	65
ii	1.29	51.55	47.16	**ii, iii**	26.67	0	4.95E-06	73.33
iii	27.63	9.13E-02	72.28	**iv, v**	1.12	0	57.49	41.39
				vi	27.63	9.13E-02	< 4.95E-06	72.28
				vii	1.29	51.55	< 4.95E-06	47.16

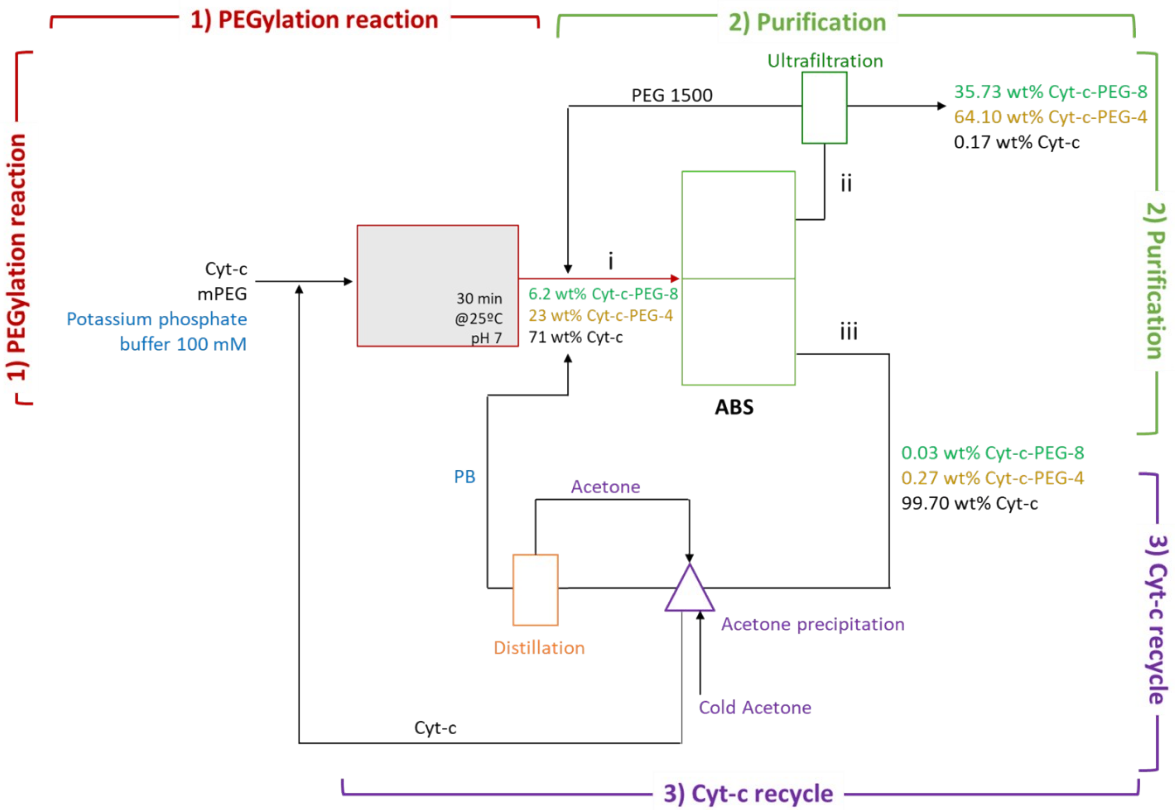

Fig S1. Diagram of a single-step process to fractionate selectively the unreacted cytochrome c from their PEGylated conjugates (Cyt-c-PEG-4 and Cyt-c-PEG-8) using ABS. The proposed strategy indicates that after the PEGylation reaction the ABS composed of PEG 1500 + potassium phosphate buffer (pH = 7) is applied and able to separate the PEGylated conjugates (Cyt-c-PEG-4 and Cyt-c-PEG-8) from Cyt-c with the presented recovery yields. The recycling of Cyt-c for a novel PEGylation reaction is herein projected through a cold acetone precipitation step followed by the resuspension of the protein pellet in the conventional PEGylation buffer (100 mM potassium phosphate buffer, pH =7).

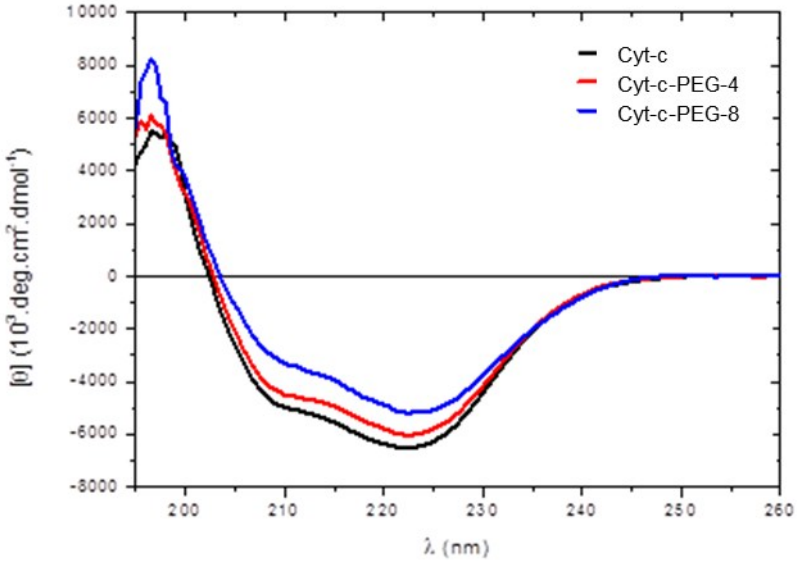

Fig S2. Far-UV CD spectra of Cyt-c control (—), Cyt-c-PEG-4 (—), and Cyt-c-PEG-8 (—) purified through aqueous biphasic system.