Supplementary Materials

Lysine-PEGylated Cytochrome c with Enhanced Shelf-Life Stability

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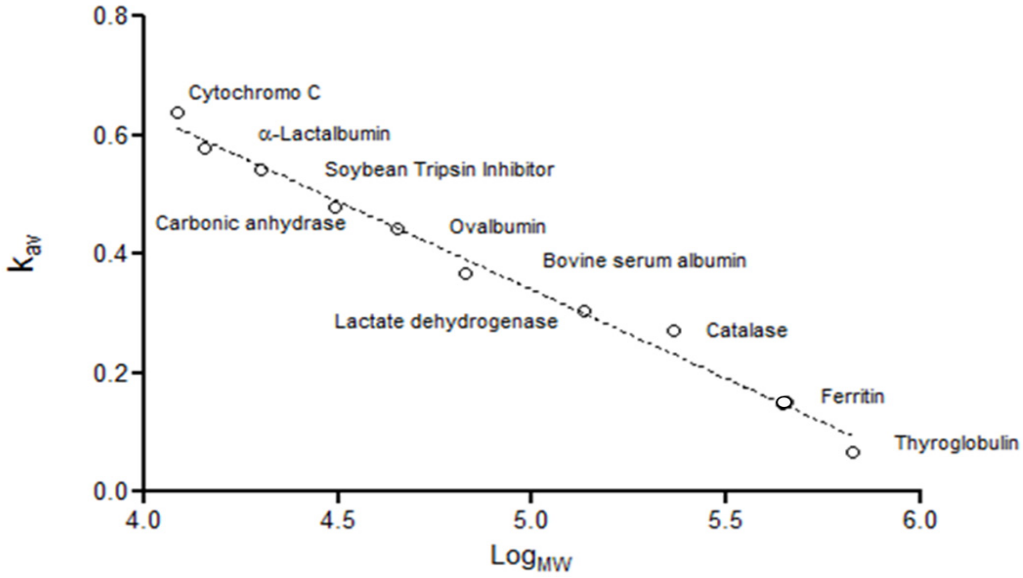

Figure S1. Calibration curve of size exclusion chromatography (SEC) using a Superdex 200 Increase 10/300 GL column (crosslinked agarose-dextran resin, Cytiva) in an AKTA™ purifier system (Cytiva). Data obtained by plotting the Kav value of each protein marker and the logarithms of their molecular weights (MW), namely: thyroglobulin 669 kDa, ferritin 450 kDa, catalase 232 kDa, lactate dehydrogenase 136.7 kDa, bovine serum albumin 67 kDa, ovalbumin 45 kDa, carbonic anhydrase 31 kDa, soybean trypsin inhibitor 20.1 kDa, α-lactalbumin 14.4 kDa and cytochrome c 12.2 kDa.

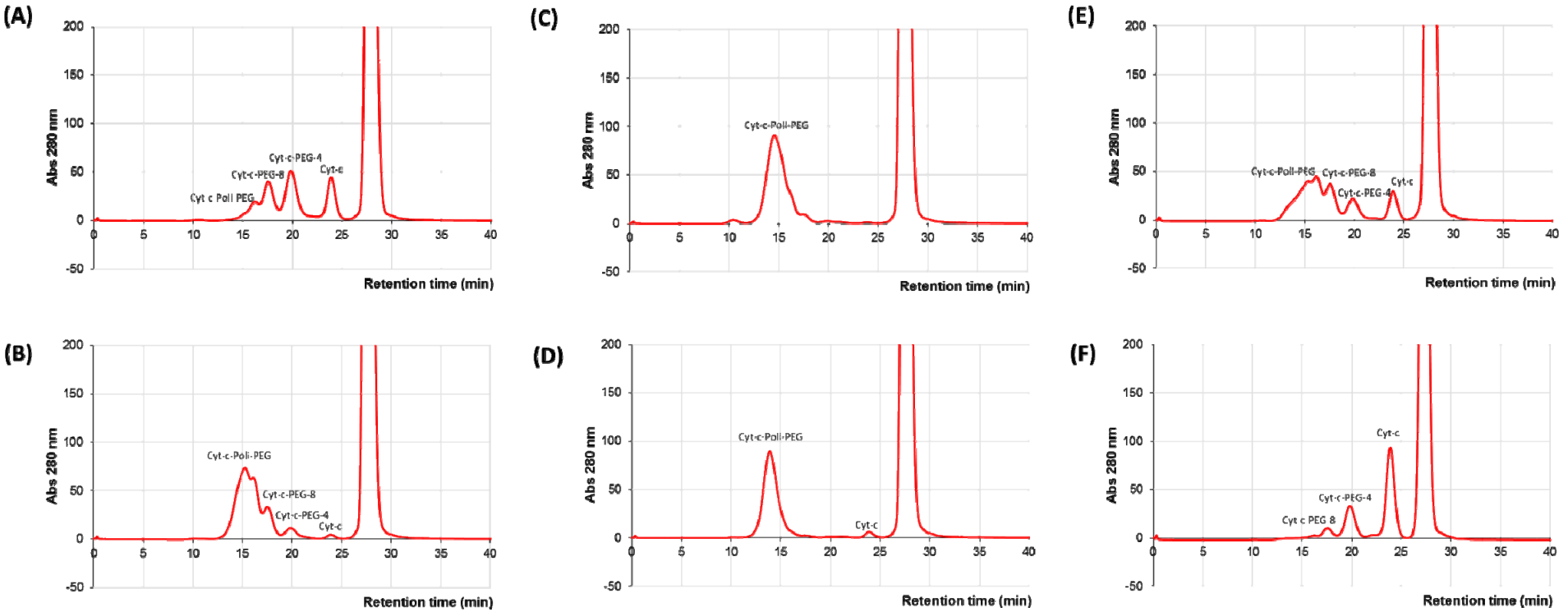

Figure S2. Effect of pH on cytochrome c (Cyt-c) PEGylation yield. Chromatogram of the PEGylation reaction mixture for the Cyt-c PEGylation in 0.1 M of potassium phosphate buffer at different pH values: 7 **(A)**, 8 **(B)**, 9 **(C)**, 10 **(D)**, 11 **(E)**, and 12 **(F)**. Reaction conditions: molar ratio 1:25 (protein:mPEG-NHS, 5 kDa), room temperature, 30 min. The peaks correspond to both Cyt-c PEGylated forms and unreacted protein.

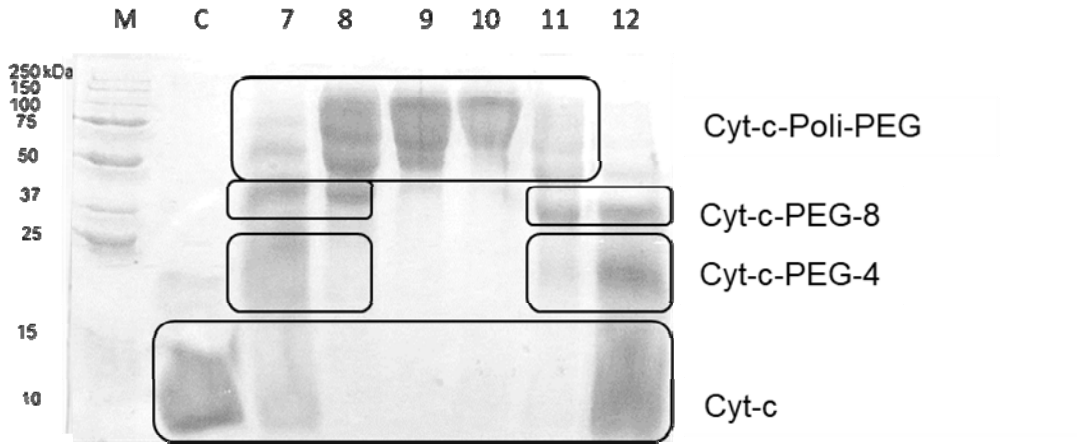

Figure S3. SDS-PAGE for the conjugation of mPEG-NHS to cytochrome c (Cyt-c) at different pHs. Lane 1: molecular weight marker, Lane 2: cytochrome c, Lane 3: pH 7, Lane 4: pH 8, Lane 5: pH 9; Lane 6: pH 10, Lane 7: pH 11, Lane 8: pH 12. The PEGylation reaction was performed in 0.1 M potassium phosphate buffer, 1:25 molar proportion (Cyt-c:mPEG-NHS, 5 kDa), at room temperature, for 30 minutes, and with a constant stirring of 400 rpm.

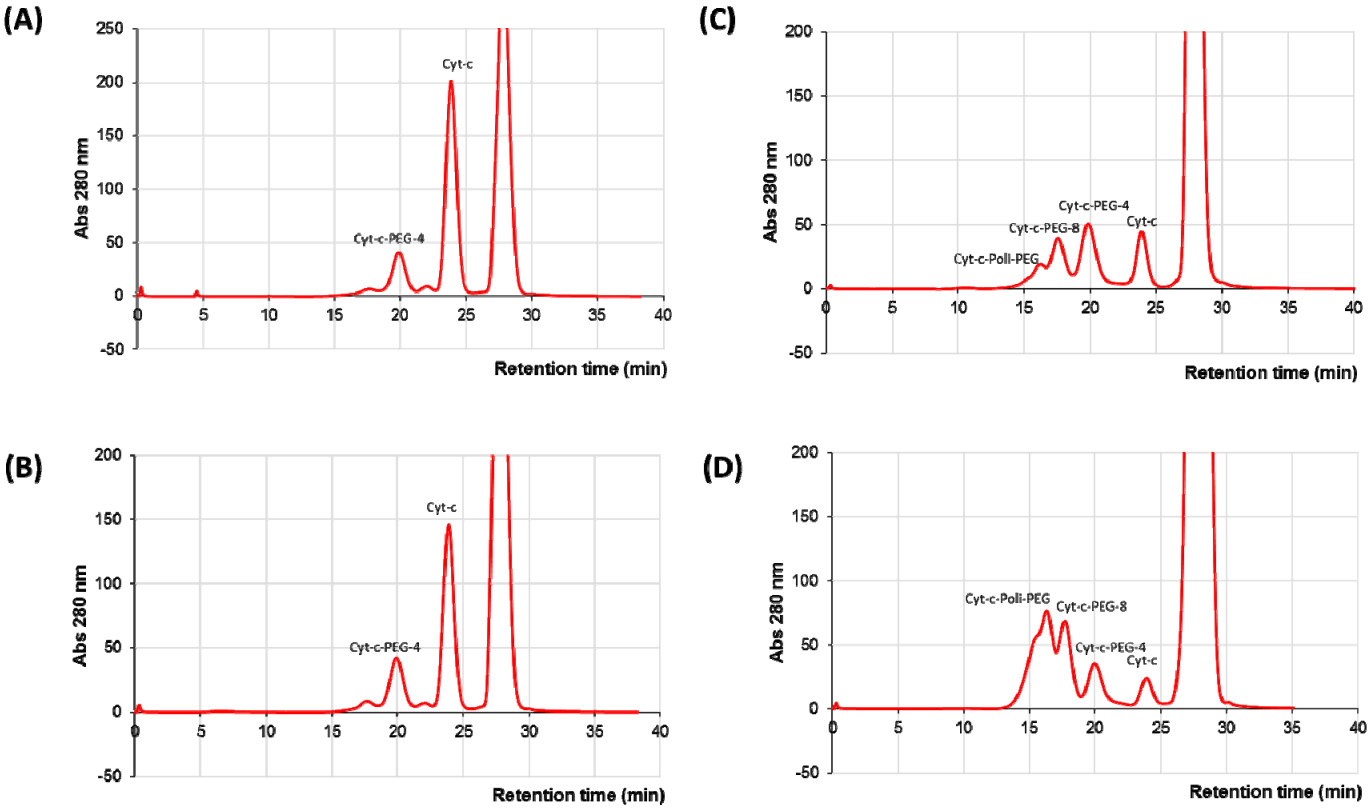

Figure S4. Effect of molar ratio (protein:mPEG-NHS) on cytochrome c (Cyt-c) PEGylation yield. Chromatogram of the PEGylation reaction mixture from a PEGylation reaction in 0.1 M phosphate buffer (pH =7) at different molar ratios (protein:mPEG-NHS, 5 kDa): 1:5 **(A)**, 1:10 **(B)**, 1:25 **(C)**, and 1:35 **(D)**, at room temperature for 30 minutes. The peaks correspond to both Cyt-c PEGylated forms and unreacted protein.

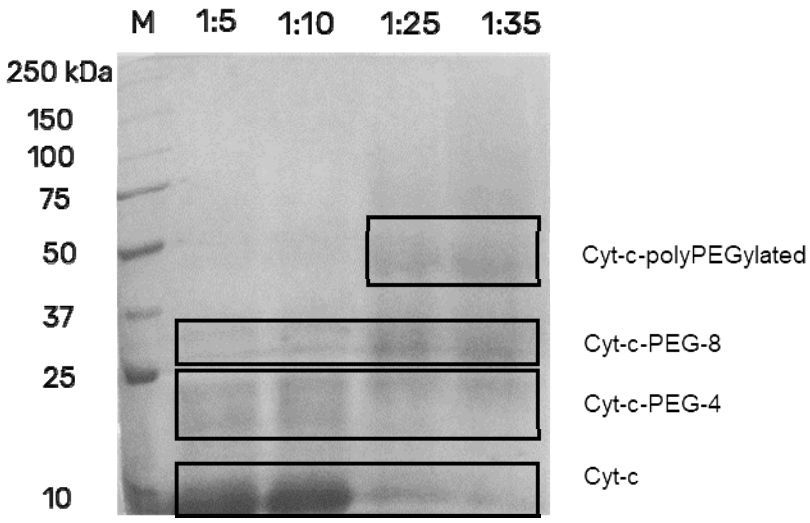

Figure S5. SDS-PAGE for the conjugation of mPEG-NHS with cytochrome c (Cyt-c) at different molar ratios (protein:mPEG-NHS). Lane 1: molecular weight marker, Lane 2: 1:5, Lane 3: 1:10, Lane 4: 1:25, Lane 5: 1:35. The PEGylation reaction was performed in 0.1 M potassium phosphate buffer (pH 7), at room temperature, for 30 minutes, and with a constant stirring of 400 rpm.

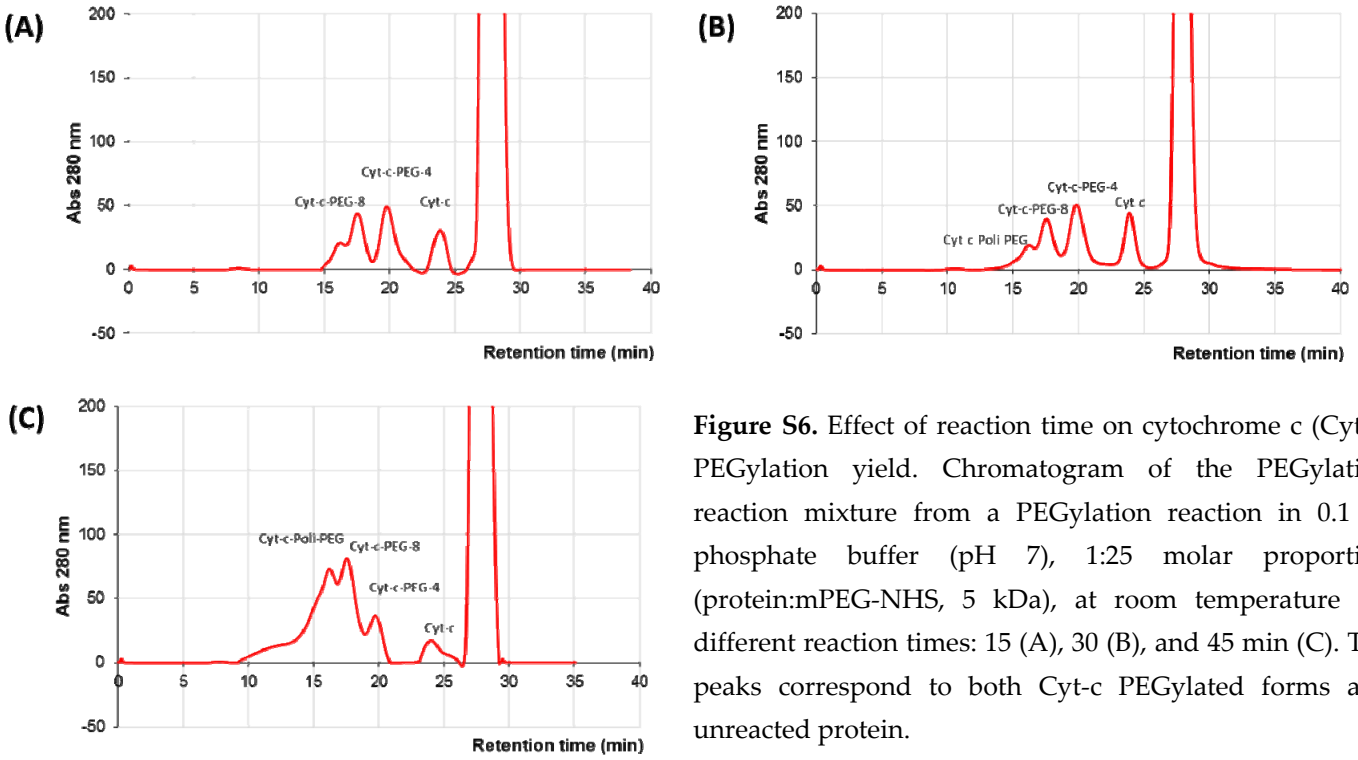

Figure S6. Effect of reaction time on cytochrome c (Cyt-c) PEGylation yield. Chromatogram of the PEGylation reaction mixture from a PEGylation reaction in 0.1 M phosphate buffer (pH 7), 1:25 molar proportion (protein:mPEG-NHS, 5 kDa), at room temperature for different reaction times: 15 (A), 30 (B), and 45 min (C). The peaks correspond to both Cyt-c PEGylated forms and unreacted protein.

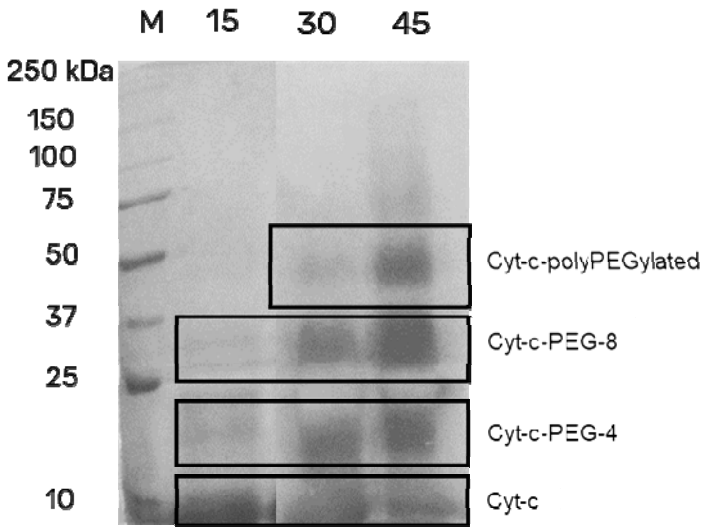

Figure S7. SDS-PAGE for the conjugation of mPEG-NHS with cytochrome c (Cyt-c) for different reaction times. Lane 1: molecular weight marker, Lane 2: 15 min, Lane 3: 30 min, Lane 4: 45 min. The PEGylation reaction was performed in 0.1 M potassium phosphate buffer (pH 7), 1:25 molar proportion (protein:mPEG-NHS, 5 kDa), at room temperature, and with a constant stirring of 400 rpm.

Table S1. Half-life ($t_{1/2}$) and long-term residual activity of native and PEGylated forms of cytochrome c (Cyt-c) stored in phosphate buffer at 4 ºC and 25 ºC.

Cyt-c form	**Half-life* (days)**		**Residual activity (%) on day 60**	
	4 ºC	**25 ºC**	**4 ºC**	**25 ºC**
Native	~44	~24	42.8	27.3
Cyt-c-PEG-4	>60	>60	62.2	53.4
Cyt-c-PEG-8	>60	>60	80.4	57.3

*Half-life (i.e. the time taken for the Cyt-c residual activity to be reduced by 50% of the initial) was estimated from data presented in Figure 6**.**