Electronic Supplementary Information (ESI)

Facilitating trehalose entry into hRBCs at 4 ºC by alkylated ε-poly(L-lysine) for glycerol-free cryopreservation

Xingwen Liu,[a] Shuhui Gao,[a] Qingjing Niu,[a] Kongying Zhu,[b] Lixia Ren,[a] and Xiaoyan Yuan*[a]

[a] *School of Materials Science and Engineering, and Tianjin Key Laboratory of Composite and Functional Materials, Tianjin University, Tianjin 300350, China*

[b] *Analysis and Measurement Center, Tianjin University, Tianjin 300072, China*

* E-mail: yuanxy@tju.edu.cn

Materials

ε-Poly(L-lysine) (ε-PL, average molecular weight ~4000) was purchased from Nanjing Shineking Biotech Co., Ltd., China. Anhydrous trehalose was obtained from J&K Scientific, Beijing, China. Octanoic acid, capric acid, dodecanoic acid, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), dimethyl sulfoxide (DMSO) and pyrene were supplied by Heowns Biochem, Tianjin, China. Poly(ethylene glycol) (PEG) was purchased from Solarbio Science & Technology, Beijing, China. Poly(vinyl pyrrolidone) (PVP) was bought from Macklin Biochemical (Shanghai, China). 16-Doxyl stearic acid (16-DSA) was purchased from Sigma Aldrich (USA). All chemicals were used as received without further purification. Enhanced adenosine triphosphate (ATP) Assay Kit and Detergent Compatible Bradford Protein Assay Kit were supplied by Beyotime Biotechnology, China. Human 2,3-diphosphoglycerate (2,3-DPG) ELISA Kit was purchased from Jingmei Biotechnology, China. Methemoglobin (MetHb) Assay Kit was supplied by

Nanjing Jiancheng Bioengineering Institute, China. FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA) was used for determination of phosphatidylserine (PS) exposure by flow cytometer (FCM, BD, FACSAria III, USA).

Synthesis

Alkylated ε-PL was synthesized *via* coupling reaction of -COOH in octanoic acid, capric acid and dodecanoic acid with $–NH_2$ of ε-PL as shown in Fig. 1A.[1] ε-PL was dissolved in deionized water and followed by adding a given amount of EDC and NHS that were dissolved in DMSO before. When EDC and NHS were completely dissolved, it was mixed with the ε-PL solution after 30 min of activation under stirring conditions. A certain amount of octanoic acid, capric acid and dodecanoic acid were also added to the flask, respectively. Detailed amount of them are shown in Table 1. The reaction was carried out at room temperature for 24 h. Then the reactant was dialyzed against deionized water for 3 days using a cellulose membrane dialysis tube (MWCO 2 kDa) and freeze-dried to yield the product, named as PL-C_n-*x*, where, n referred to the carbon number in the alkyl chain and *x* was the grafting ratio.

Characterization

Chemical structures of the synthesized PL-C_n-*x* were verified by a ^1H nuclear magnetic resonance (^1H NMR) spectrometer (AVANCE III TM HD 400 MHz NanoBAY, Bruker, Germany) using D_2O as solvent. And, Fourier transform infrared spectroscopy (FTIR) spectra was also measured on a FTIR spectrometer (IRtracer100, SHIMADZU, Japan) using KBr pellet technique ranging from 4000 cm^{-1} to 400 cm^{-1}. The critical aggregation concentration

(CAC) of the samples was determined by fluorescence spectrophotometer (F-4600, HITACHI, Japan). Pyrene was dissolved in methanol in a 25 mL volumetric flask. When the solvent was evaporated, 25 mL of PL-C_n-*x* solution was added, and the final pyrene concentration was 0.12 μg/mL. After heated at 45°C for 1.5 h, the sample solution was cooled to room temperature for tests. Emission spectra of the solutions was measured with excitation wavelength of 335 nm and emission wavelengths of 350~550 nm. The CAC was determined from the change of the I_{373}/I_{384} value of pyrene since the emissions of pyrene at 373 and 384 nm wavelengths are sensitive to the surrounding environment.[2]

The self-assembled nanoparticles of PL-C_n-*x* were prepared in PBS (pH 7.4, 100 mOsm/L) with a concentration of 40 μg/mL. After stabilization at room temperature for 48 h, the hydrodynamic diameters and Zeta potential were determined by a dynamic light scatterer (DLS, Zetasizer Nano ZS90, Malvern, UK). PL-C_n-*x* was dissolved in PBS (pH 7.4, 100 mOsm/L) with a concentration of 40 μg/mL and the self-assembled nanoparticles were fixed on the copper mesh with carbon film for observation under a transmission electron microscope (TEM, JEOL Jem-2100f, Japan).

As shown in Fig. S1, signals at δ 0.77 (-NHCO$(CH_2)_m\underline{CH_3}$, 3H), δ 1.23 (-NHCO$(C\underline{H_2})_mCH_3$, 2H; -COCHR$CH_2C\underline{H_2}CH_2CH_2$NH-, 2H) , δ 2.90 (-COC$\underline{H}RCH_2CH_2CH_2CH_2$NH-, 1H), δ 1.68 (-COCHR$C\underline{H_2}CH_2CH_2CH_2$NH-, 2H), δ 1.47 (-COCHR$CH_2CH_2C\underline{H_2}CH_2$NH-, 2H), and δ 3.13 (-COCHR$CH_2CH_2CH_2C\underline{H_2}$NH-, 2H) were found.[3] The ratio of the integral 0.66~0.80 ppm to the integral 2.92~3.21 ppm was used to calculate the grafting ratio of the synthesized PL-C_n-*x*, as shown in Table 1, which was

further used to calculate the molecular weights of the PL-C_n-*x*. Alkyl grafting to ε-PL backbone was further analyzed by FTIR technique (Fig. S2). ε-PL and PL-C_n-*x* showed the characteristic peaks at 1643 cm^{-1}, 3140 cm^{-1} and 3490 cm^{-1}, which corresponds to stretching of C=O, stretching of NH, stretching of NH_2, respectively.[4] As the grafting ratio, the integral of the peaks in C=O and NH gradually enhanced, while the integral of the peak of NH_2 gradually reduced. FTIR results corroborated ^{1}H NMR conclusions by the presence of PL-C_n-*x* with various grafting ratio.

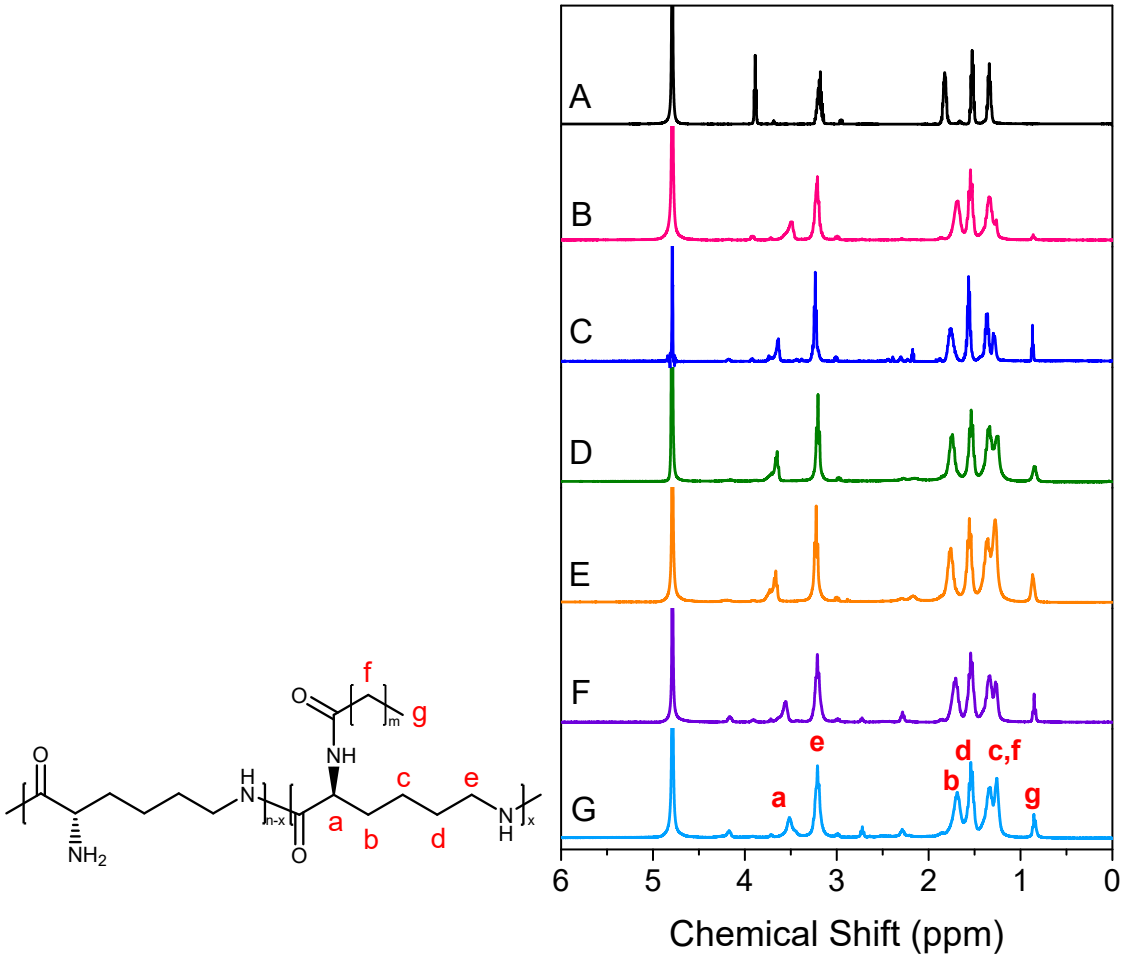

Fig. S1 ^{1}H NMR spectra of ε-PL and alkylated ε-PL. (A) ε-PL, (B) PL-C_{12}-5, (C) PL-C_{12}-13, (D) PL-C_{12}-18, (E) PL-C_{12}-22, (F) PL-C_8-13, (G) PL-C_{10}-13.

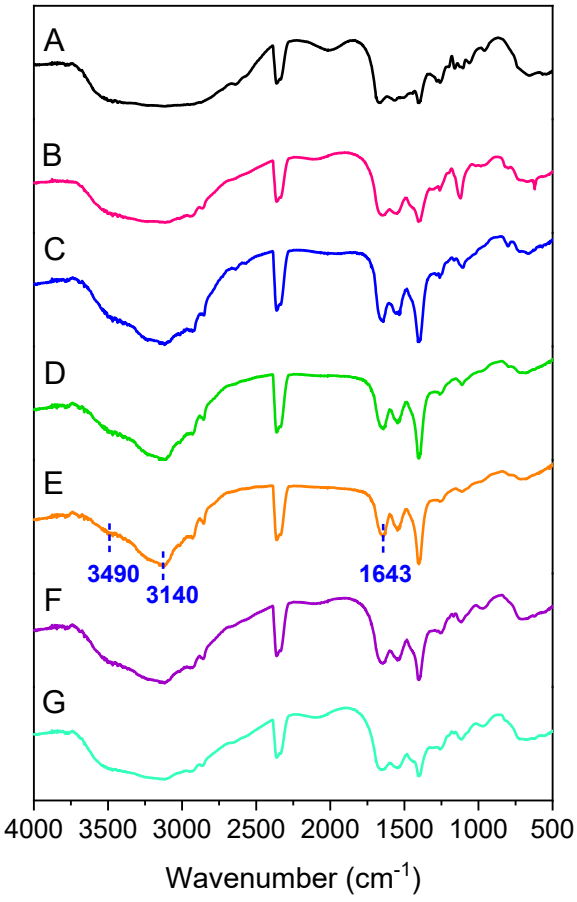

Fig. S2 FTIR spectra of ε-PL and alkylated ε-PL. (A) ε-PL, (B) PL-C_{12}-5, (C) PL-C_{12}-13, (D) PL-C_{12}-18, (E) PL-C_{12}-22, (F) PL-C_{8}-13, (G) PL-C_{10}-13.

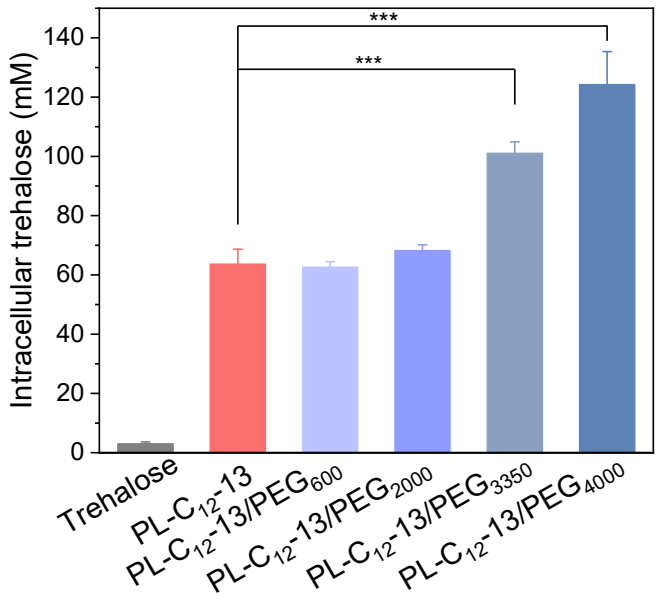

Fig. S3 Effect of the molecular weight of PEG on the intracellular trehalose loading content. All samples (haematocrit ~30%, *n*=3) were incubated at 4 °C for 30 h with 0.8 M trehalose, and the concentrations of PL-C_{12}-13 and PEG were 40 μg/mL and 30 mM, respectively.

Confocal laser scanning microscope images

5-Carboxyfluorescein (5-FAM) labeled PL-C_{12}-13 was first prepared.[4] PL-C_{12}-13 and 5-FAM were dissolved in DMSO in a molar ratio of 1:3 to enable PL-C_{12}-13 to be modified with 5-FAM *via* EDC and NHS. The reaction was performed under stirring at room temperature for 24 h. Then, it was dialyzed against deionized water for 3 days using a cellulose membrane dialysis tube (MWCO 2 kDa) and freezed-dried to yield 5-FAM-labeled PL-C_{12}-13. Incubation was carried out at 4 °C/300 rpm for 30 h in a vial containing 40 μg/mL 5-FAM-labeled PL-C_{12}-13, 0.8 M trehalose, and hRBCs (haematocrit ~30%) for a final volume of 1.5 mL. After washing once with PBS (pH 7.4, 900 mOsm/L) or without washing, hRBCs were observed under a confocal laser scanning microscope (CLSM, A1R+, Nikon, Japan) with an excitation wavelength of 5-FAM at 488 nm.

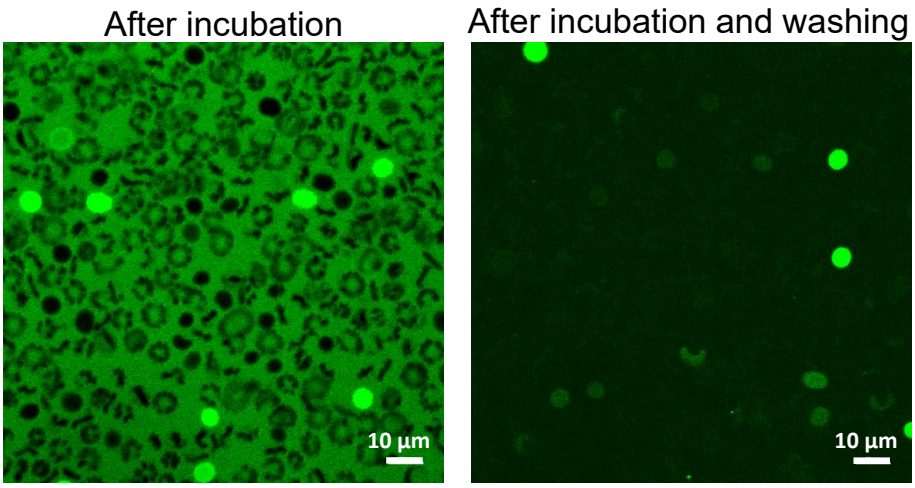

Fig. S4 CLSM images of hRBCs after incubation with 0.8 M trehalose and 40 μg/mL 5-carboxyfluorescein-modified PL-C_{12}-13 at 4°C for 30 h (A), and followed by washing (B).

Cytotoxicity

The cytotoxicity of alkylated ε-PL was examined by using cell counting kit-8 (CCK8) against smooth muscle cells (SMCs). SMCs were seeded into the 96-well plate containing DMEM containing 10% (*v*/*v*) fetal bovine serum (FBS) and 1% (*v*/*v*) penicillin–streptomycin (0.1 mL per well) at a density of 1×10^4 cells/well in a humidified incubator with 5% CO_2 at 37 °C for 24 h. Then, the culture medium was replaced by 0.1 mL of DMEM containing alkylated ε-PL with various concentrations and the medium without alkylated ε-PL was used as the control. After 24 h of incubation, 0.1 mL CCK8 reagent was added into wells to replace medium and the fluorescence of sample was obtained at the wavelength of 450 nm. The relative cell viability was calculated from the fluorescence intensity reading values with following equation (S1):

$$Relative\ cell\ viability\ (\%) = \frac{OD_{Sample}}{OD_{positive}} \times 100 \qquad \text{(S1)}$$

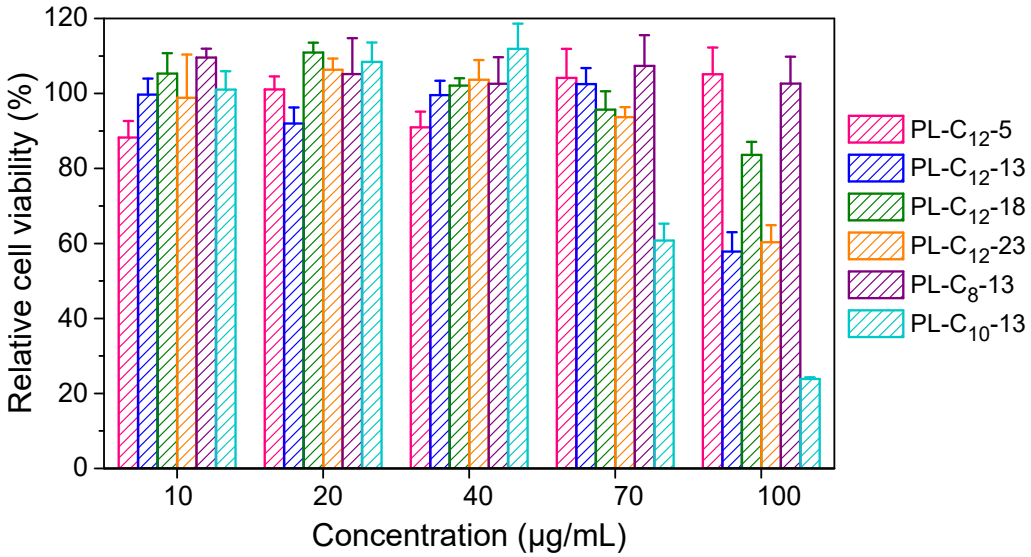

Fig. S5 Relative cell viability of alkylated ε-PL with different grafting ratios and alkyl chain lengths against smooth muscle cells (SMCs).

References

1 B. Liu, Q. Zhang, Y. Zhao, L. Ren and X. Yuan, *J. Mater. Chem. B*, 2019, **7**, 5695-5703.

2 H. Zhou, W. Yu, X. Guo, X. Liu, N. Li, Y. Zhang and X. Ma, *Biomacromolecules,* 2010, **11**, 3480-3486

3 B. Chen, Y. Huang, Y. Huang, T. Wen and J. Jan, *ACS Macro Lett.*, 2014, **3**, 220-223.

4 Q. Zhang, B. Liu, J. Chong, L. Ren, Y. Zhao and X. Yuan, *Sci. China-Technol. Sc.*, 2021, **64**, 806-816.