Electronic Supplementary Information (ESI)

Membrane stabilization versus perturbation by aromatic monoamine-modified γ-PGA for cryopreservation of human RBCs with high intracellular trehalose

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Materials

γ-Poly(glutamic acid) (γ-PGA, 100 kDa) was purchased from Nanjing Bioshineking Biotech, China. Anhydrous trehalose was obtained from J&K Scientific, Beijing, China. Benzylamine (BA), phenethylamine (PEA), 4-phenylbutylamine (PBA), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), hydroxyethyl starch (HES), polyvinyl pyrrolidone (PVP, 8 kDa), and Calcein were obtained from Heowns Biochem, Tianjin, China. Human serum albumin (HSA) was purchased from Tianjin Haoshengjie Biotech, China. Dextran (6 kDa) was obtained from Shanghai Energy Chemical, China. 3-Phenyl-1-propylamine (PPA) was purchased from Shanghai Aladdin Biotech, China.

5-Amino fluorescein (5-AF) was obtained from Shanghai D&B Chemical Technology, China. Glutaraldehyde (2.5%, *v*/*v*) was obtained from Shanghai Yuanye Biotechnology, China.

Enhanced adenosine triphosphate (ATP) Assay Kit and Detergent Compatible Bradford Protein Assay Kit were obtained from Beyotime Biotechnology, China. Human 2,3-diphosphoglycerate (2,3-DPG) ELISA Kit was purchased from Nanjing Herb-Source Bio-Technology, China. Methemoglobin (MetHb) Assay Kit was supplied by Nanjing Jiancheng Bioengineering Institute, China. FITC Annexin V Apoptosis Detection Kit I was purchased from BD Biosciences, USA.

The isotonic phosphate buffer solution (PBS, pH 7.4, ~306 $mOsm \cdot L^{-1}$) was prepared containing 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 138 mM NaCl, and 2.7 mM KCl. Hypertonic PBS (pH7.4, ~900 $mOsm \cdot L^{-1}$) was prepared containing 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO4, 435 mM NaCl, and 2.7 mM KCl.

Synthesis

As shown in Fig. S1, the amphiphilic γ-PGA (PGA-*g*-C_nP) polymers were prepared via grafting γ-PGA with aromatic monoamines with 1~4 methylene, namely BA, PEA, PPA, and PBA, respectively. The reaction was carried out with EDC/NHS as the coupling agent at room temperature for 24 h, according to the references.[1] γ-PGA (1.00 g, 7.75 unit mM) was dissolved in 50 mL deionized water, and then EDC (2.78 g, 15.5 mM) and NHS (1.78 g, 15.5 mM) were added. The solution was stirred and activated in an ice bath for 20 min. Aromatic monoamines (1.55 mM, including BA, PEA, PPA, and PBA) in 50 mL Dimethyl sulfoxide solution (DMSO) were added to the solution and then reacted at room temperature for 24 h. After the reaction,

the reaction solution was dialyzed (molecular weight cut-off 20 kDa) against deionized water for 3 days, and then lyophilized for 24 h. The modified γ-PGA products by PMA, PEA, PPA, and PBA were designated as PGA-*g*-C_1P, PGA-*g*-C_2P, PGA-*g*-C_3P, and PGA-*g*-C_4P, respectively.

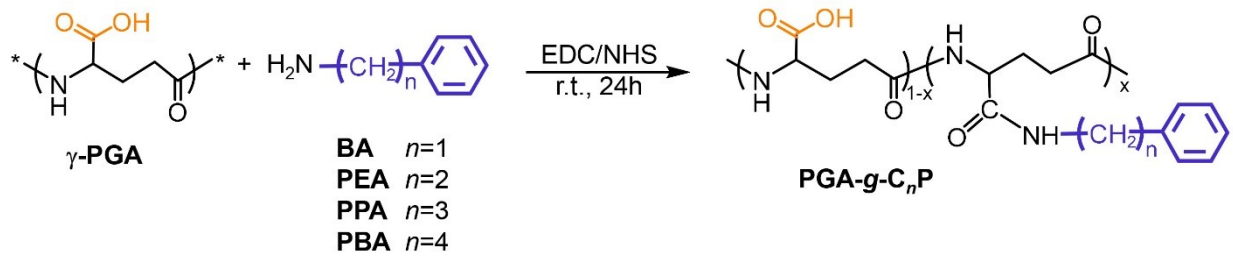

Fig. S1 Synthesis of PGA-*g*-C_nP by grafting γ-PGA with aromatic monoamines with 1~4 methylene spacer, i.e., BA, PEA, PPA, and PBA, respectively.

Characterizations

The chemical structure of PGA-*g*-C_nP was characterized by ^{1}H nuclear magnetic resonance (^{1}H NMR, Bruker AV 400 MHz, Germany) and Fourier Transform infrared spectroscopy (FTIR, Perkin-Elmer Spectrum100, USA). Samples were dissolved in D_2O for the ^{1}H NMR measurement. The FTIR samples were prepared by the KBr pellet method and measured in the wavenumber range of 400~4000 cm^{-1}.

The structure of modified γ-PGA by aromatic monoamines was characterized and analyzed by FTIR and ^{1}H NMR. The graft ratios of modified γ-PGA were calculated by ^{1}H NMR. As shown in Fig. S2, hydrogen NH-C*H*(C=O)-CH_3 on the main chain was located at δ ~4 ppm, and -C*H* in side-chain phenyl was located at δ ~7 ppm, which indicated successful grafting of aromatic monoamines. The grafting ratios were calculated by the peak area of the above two peaks. The grafting ratios of PGA-*g*-C_nP were 23.2, 18.3, 19.8, and 16.8%, respectively.

Original -CH_2CH_2- and newly grafted -(CH_2)$_n$- belong to δ 1.71~2.38 ppm and δ 2.88~3.42 ppm, respectively.

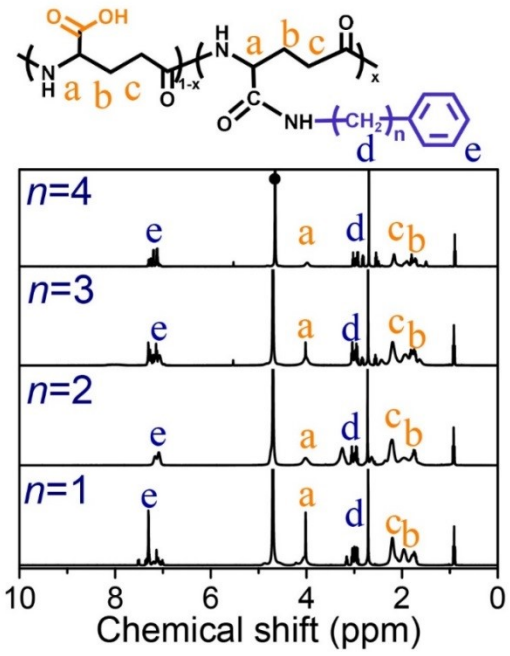

Fig. S2 ^{1}H NMR spectra of PGA-*g*-C$_n$P.

As shown in Fig. S3, both PGA-*g*-C_nP and γ-PGA had C=O telescopic vibration peaks at 1650 cm^{-1}, C-N and N-H bending vibration peaks at 1532 cm^{-1}, and C-O bond absorption peaks at 1394 cm^{-1}. Furthermore, the absorption peak at 3058 cm^{-1} was the C-H extended vibration peak of the phenyl ring in the pendant group. Two small peaks at 694, 732 cm^{-1} of the phenyl ring proved that the aromatic monoamines were successfully grafted.

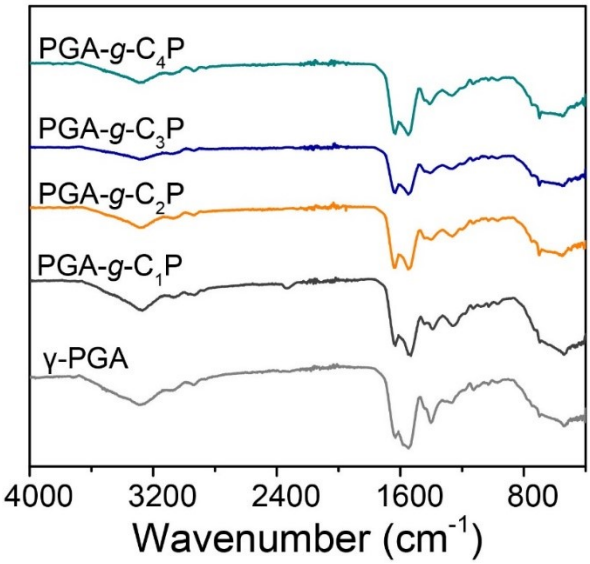

Fig. S3 FTIR spectra of γ-PGA and PGA-*g*-C_nP.

Ice recrystallization inhibition (IRI) activity

Generally, 10 μL of the above solution was dropped onto a quartz crucible cooled by liquid nitrogen vapor from a height of 1.5 meters, then transferred to a microscope cold stage at -80 °C, and then heated up to -8 °C at a warming rate of 25 °C·min^{-1}.[2] After incubation at -8 °C for 30 min, five photos of samples were taken, and ten of the largest ice crystals for each photo was measured to calculate the mean largest ice crystal size. IRI activity was calculated as the mean largest grain size (MLGS) of the samples compared to that of the PBS solution. As shown in Fig. S4, compared with pure PBS solution, it can be seen that the MLGS of PGA-*g*-C$_n$P decreased significantly, and the IRI activity of these four polymers was about 70% size of PBS, increasing indistinctively with hydrophobicity.

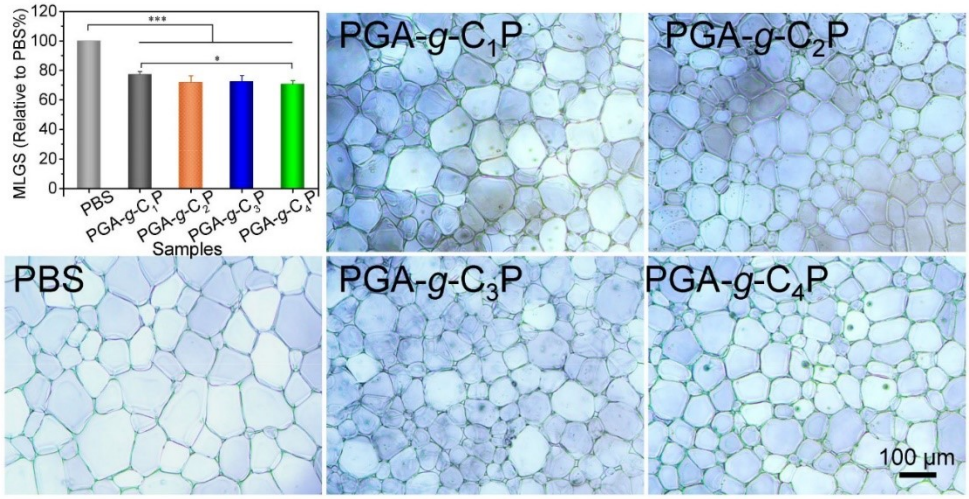

Fig. S4 IRI activity of 1 mg mL^{-1} PGA-*g*-C$_n$P in isotonic PBS and crystal pictures after frozen at -80 °C and held at -8 °C for 30 min (PBS as the control).

Confocal laser scanning microscopy

To determine the location of the polymers on the cell membrane, PGA-*g*-C_nP was labeled with a fluorescent 5-AF with EDC/NHS as coupling agents to obtain FITC-labeled PGA-*g*-C_nP.[3] Briefly, hRBCs were incubated with 1 mg·ml^{-1} PGA-*g*-C_nP-FITC at 10% HCT, at 4 °C for 24 h. After being washed thrice, resuspended hRBCs were observed with a confocal laser scanning microscope (CLSM, A1R+, Nikon, Japan) at an excitation wavelength of 488 nm. As can be seen from Fig. S5, after being washed thrice, there were certain residues of fluorescent on the membrane surface. In other words, PGA-*g*-C_nP adsorbed on the surface and interacted with the cell membrane.

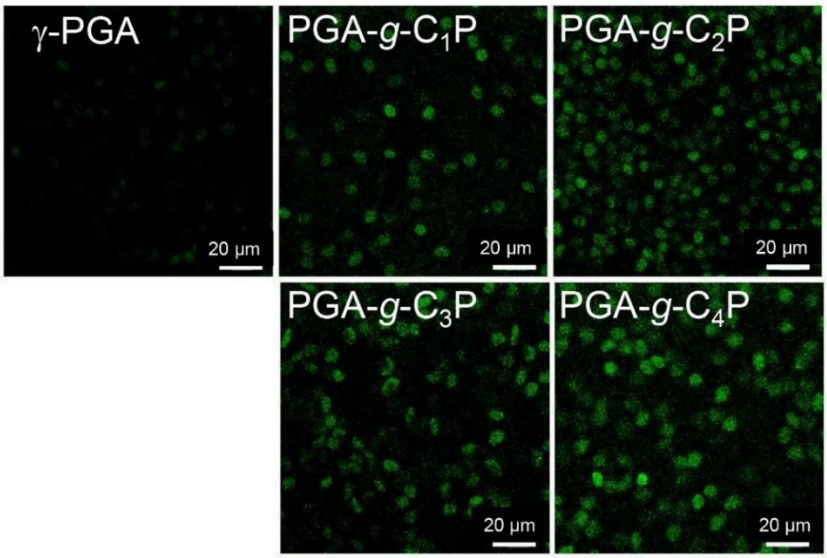

Fig. S5 CLSM images of hRBCs after incubation with 1.0 mg·mL^{-1} FITC labeled γ-PGA or FITC labeled PGA-*g*-C_nP at 4 °C, 24 h. All images were taken after being washed thrice with isotonic PBS solution.

Molecular dynamics simulations

Molecular dynamics (MD) simulations were performed with GROMACS in the GROMOS96 force field to get an unambiguous and intuitive grasp of the interaction of PGA-*g*-C_nP and phospholipid bilayer.[4] The structure of PGA-*g*-C_nP was simplified to 10 repeating units of γ-PGA, with two carboxyl groups on the side chain grafted with aromatic monoamines to reduce the calculation time. The PGA-*g*-C_nP molecules (Fig. 1A) were constructed with the small molecule topology generator PRODRG.[5] Dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) were selected to simulate the outer and inner phospholipid components of hRBC membrane, respectively.[6] A simulation box with a volume of 8×8×8 nm^3 was established. The model phospholipid bilayer with 10×10×1 parallel-aligned DMPC and 10×10×1 parallel-aligned DMPE molecules was placed at the bottom of the simulation box. Then, 32 trehalose molecules and four minimized PGA-*g*-C_nP particles were placed above the phospholipid bilayer. The single point charge model was used to simulate the water in the system, and 60 chloride ions and 60 sodium ions were added to the aqueous solution to balance the system charge.[7] Subsequently, 300 steps of energy optimization were performed on the system using the steepest descent method. All simulations were performed in the isothermal-isobaric ensemble with temperature controlled to 300 K by Berendsen thermostat and pressure controlled to 1 bar by Berendsen barostat.[8] The molecular dynamics simulation was performed for 4 ns with a time step of 2 fs. The pictures were prepared using VMD.[9]

The number of hydrogen bonds between PGA-*g*-C_nP particles and the phospholipid bilayer,

PGA-*g*-C_nP particles, and trehalose, trehalose, and the phospholipid bilayer was examined to further understand the interaction between PGA-*g*-C_nP, trehalose, and membrane lipids.[4] The results are shown in Fig. S6.

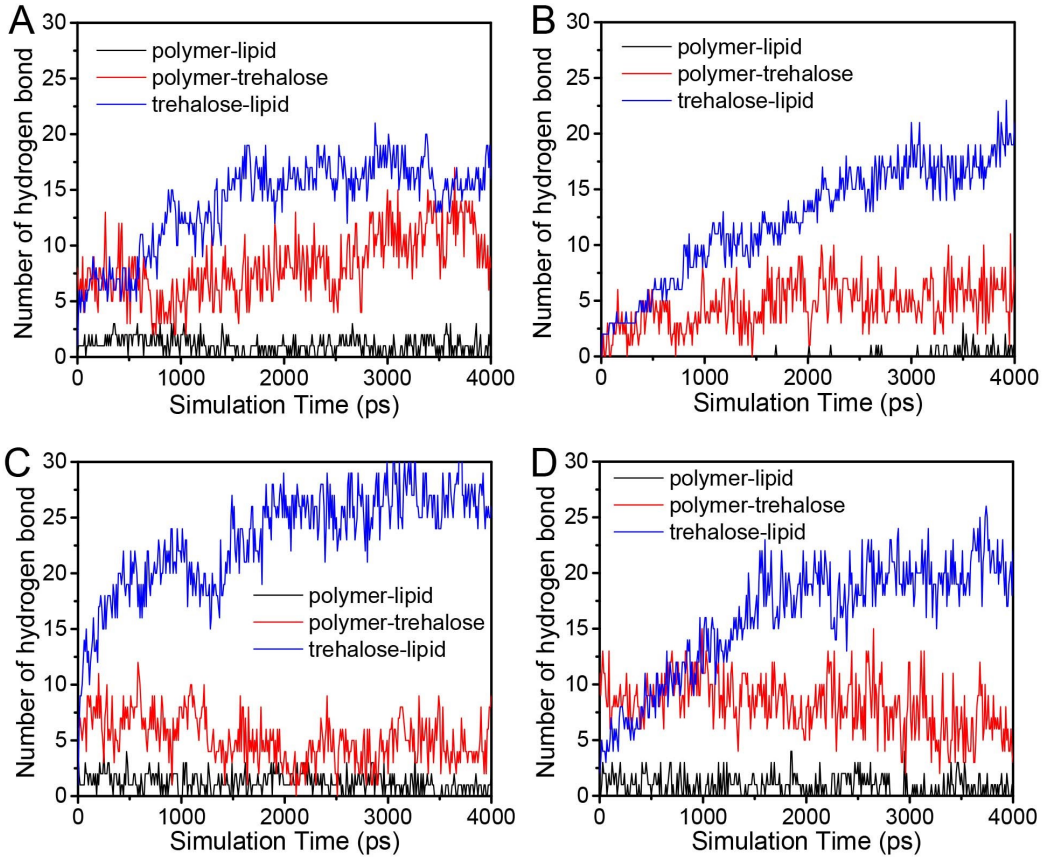

Fig. S6 The number of hydrogen bonds between PGA-*g*-C_nP and the phospholipid bilayer, PGA-*g*-C_nP and trehalose, trehalose, and the phospholipid bilayer in MD simulations. (A) PGA-*g*-C_1P; (B) PGA-*g*-C_2P; (C) PGA-*g*-C_3P; (D) PGA-*g*-C_4P.

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