Supporting Information

Membrane Stabilization of PEG-*b*-Polypeptide-*g*-Trehalose Assists Cryopreservation of RBCs

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EXPERIMENTAL SECTION

Materials. Amino terminated polyethylene glycol (PEG-NH_2, average molecular weight 2000), N_ε-benzyloxycarbonyl-$_L$-lysine (Cbz-Lys, **K**), *N*-benzyloxycarbonyl aspartic acid (Asp, **D**), L-phenylalanine (Phe, **F**), triphosgene, anhydrous trehalose (Tre), trifluoroacetic acid (TFA) and hydrobromide (HBr) in acetic acid (HAc) (33% w/w) were purchased from J&K Scientific Ltd., China. Fluorescein isothiocyanate (FITC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI), *N*-hydroxysuccinimide (NHS), triethylamine, succinic anhydride, and 3-(4,5-dimethlthiazol-2-yl)-2,5,-diphenyltetrazoliumbromid (MTT) were provided by Tianjin Heowns Biochemical Technology Co., Ltd., China. Sterile defibrinated sheep red blood cells (RBCs) were provided by Guangzhou Future Biotechnology Co., Ltd., China. Sodium hydroxide, anhydrous dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), *N, N'*-dimethylformamide (DMF, extra dry) and other solvents were supplied by Kermel Chemical Technology Co., Ltd., China. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum certified (FBS) were purchased from Gibco. All chemicals were used directly as received without further purification unless mentioned.

Synthesis of Amino Acid *N*-Carboxyanhydrides (NCA). Synthesis of *N*-carboxyanhydride (NCA), i.e., N_ε-benzyloxycarbonyl-L-lysine (L-Lys(Z)-NCA), asparate acid (L-Asp(Bn)-NCA) and phenylalanine (L-Phe-NCA), was depicted in Figure S1 taking a literature procedure with slightly modification.[3, 19] Briefly, in synthesis of L-Lys(Z)-NCA, N_ε-benzyloxycarbonyl-L-lysine (8.0 g, 28.5 mmol) was suspended in anhydrous THF (100 mL) in an oven-dried three-necked round bottomed flask under dry argon, and the suspension was heated to 50 °C. Triphosgene (3.4 g, 11.4 mmol) was dissolved in anhydrous THF and slowly added into the suspension. The

reaction was continuous for 3 h until the solution was clear. After cooling to room temperature, the clear solution was added into *n*-hexane to precipitate the L-Lys(Z)-NCA. The crude product was recrystallized from hexane and ethylacetate (1:1, *v*/*v*). ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra were obtained on a Bruker Avance 400 MHz at room temperature with the deuterated solvent as the reference and analyzed with MestReNova software. L-Asp(Bn)-NCA and L-Phe-NCA were obtained *via* similar method.

Synthesis of PEG-*b*-Polypeptides. Polypeptides were synthesized as similar approach in the reference.[3] For all polymerizations, freshly prepared batch of NCA monomers were adopted. The synthetic polypeptides investigated in this study were obtained through NCA ring-opening polymerization (NCA-ROP) using PEG-NH_2 as the initiator, respectively, which was illustrated in Figure S2 and Table S1. Typically, L-Lys(Z)-NCA (3.06 g), L-Asp(Bn)-NCA) (2.49 g) and L-Phe-NCA (0.38 g) were dissolved thoroughly in anhydrous DMF (50 mL) under argon atmosphere. Then, PEG-NH_2 (0.44 g) was added into the solution and the solution was stirred at 25°C for 72 h. The reaction mixture was precipitated in diethyl ether and the products were collected by centrifugation. The crude PK(Z)D(Bn)F1 was obtained.

PK(Z)**D**(Bn)**F1** was dissolved in TFA (10%), and 33% HBr in acetic acid was added. After overnight reaction at room temperature, the mixture was precipitated into diethyl ether. The precipitate was purified via dialysis (M_w, 2000) in deionized water for 3 days. The deprotected polypeptide **PKDF1** was obtained after lyophilization. The other polypeptides, **PKD** and **PKDF2**, could be prepared by similar method as shown in Table S1.

Synthesis of PEG-*b*-Polypeptide-*g*-Trehalose. As shown in Figure S2, PEG-*b*-poly(Lys-*co*-Asp-*co*-Phe)-*g*-trehalose was prepared by the sequent reaction of PEG-*b*-poly(Lys-*co*-Asp-*co*-Phe) with carboxylated trehalose (**Tre-COOH**, **T**), adopted from our previous report.[1] Typically,

Tre-COOH (3.32g, 7.51 mmol), EDCI (3.56 g, 18.57 mmol) and NHS (2.14 g, 18.59 mmol) were dissolved in DMSO (40 mL) and stirred for 30 min at room temperature. Then, PEG-*b*-poly(Lys-*co*-Asp-*co*-Phe) (1.0 g, 0.25 mmol) was dissolved in deionized water (10 mL) and added into the mixture solution and maintained at room temperature for 72 h. The product was purified *via* dialysis (cut off 2000 Dalton) against deionized water for 3 days and lyophilized. PEG-*b*-(Lys_{26}-*co*-Asp_{41}), PEG-*b*-(Lys_{23}-*co*-Asp_{45})-*co*-Phe_6, and PEG-*b*-(Lys_{24}-*co*-Asp_{43})-*b*-Phe_9, were synthesized and designated as **PKD**, **PKDF1** and **PKDF2**, respectively. For fluorescent labeling, the glycopeptide solutions of **PKDT**, **PKDF1T** and **PKDF2T** were reacted with fluoresceinisothiocyanate (FITC) at 0.01 molar ratio to polymer for 3 days at room temperature.[1] FITC-polymer was purified by dialysis (Cut off 2000 Dalton) and freeze-dried.

Characterization for Structure and Composition. Chemical structures of PEG-*b*-polypeptide and PEG-*b*-polypeptide-*g*-trehalose were verified by analyses of ^{1}H nuclear magnetic resonance (^{1}H NMR, Brucker AV 400 MHz, Germany). The ^{1}H NMR measurement was performed *via* dissolving 10 mg of the specimen in DMSO-d^6 or D_2O. The 2D ^{1}H-^{1}H nuclear Overhauser effect spectroscopy (NOESY) NMR measurement was performed *via* dissolving 50 mg of the specimen in D_2O. The molecular weight and polydispersity of PEG-*b*-polypeptide and PEG-*b*-polypeptide-*g*-trehalose were obtained using an aqueous gel permeation chromatography (GPC) system (Viscotek, UK) with poly(ethylene glycol) as the standard. The eluent was sodium acetate buffer solution (0.5 M of NaAc and 0.5 M of HAc, pH= 4.5) at a flow rate of 1.0 mL·min^{-1} at 30 °C. The Circular dichroism (CD) spectra of (glyco)peptide were obtained using a CD spectrometer (J-810, Jasco, Japan) at room temperature under a constant flow of N_2. The polymer aqueous solutions (0.1mg·mL^{-1}, filtered by 0.22 μm filter) were

introduced into a quartz cuvette with a path length of 1 mm. The wavelengths were set at 185~250 nm.

Hemolysis Assay. Sterile defibrinated sheep RBCs were used as cell model for evaluation hemolysis of synthesized glycopeptide.[2] RBCs washed with single strength PBS solution (306 mOsm, pH 7.4) for three times (centrifugation, 2000 rpm, 10 min, 4 ºC). Then, RBCs were resuspended in glycopeptide (**Tre)** solutions. After being incubated at 37 ˚C for 2 h, the optical density (OD) values of released hemoglobin were measured at 541 nm using a microplate reader (TECAN, Switzerland). The sample of RBCs in PBS buffer was taken as the negative control, and the sample of RBCs lysed with deionized water was used as the positive control. The relative hemolysis of each sample was calculated according to the following equation (1).

$$\text{Relative hemolysis (\%)} = \frac{OD_{Sample} - OD_{Negative\ control}}{OD_{Positive\ control} - OD_{Negative\ control}} \times 100 \quad (1)$$

Cytotoxicity. The cytotoxicity of the glycopeptide was examined by using L929 fibroblasts *via* MTT assay.[3, 4] Briefly, L929 cells were seeded into a 96-well plate (Corning, USA) containing medium (0.2 mL per well) at a density of 1×10^4 cells per well for 24 h, respectively. The medium was replaced with 0.2 mL of sterilized serum-free Dulbecco's Modified Eagle Medium (DMEM) containing **PKDT**, **PKDF1T**, or **PKDF2T** at specific concentrations. The cultural medium of 96-well plate containing L929 cells was replaced with 90 μL of fresh culture and 10 μL of MTT solution (5 $mg\cdot mL^{-1}$ in PBS) after being incubated for 24 h and cultured another 4 h. Subsequently, the liquid was removed, and 200 μL of DMSO were added and shocked at 100 rpm for 30 min to dissolve the blue crystal per well. The OD_{490} value was measured at 490 nm using the microplate reader.The relative cell viability was calculated as following equation (2). During the measurement, the medium without polymer was as the positive control, and each sample was repeated in triplicate.

$$\text{Relative cell viability (\%)} = \frac{OD_{490,\ Sample}}{OD_{490,\ Positive\ control}} \times 100 \quad (2)$$

Cryopreservation Protocol. Cryopreservation and evaluation of RBCs after post-thaw recovery were performed according to the references.[2, 5] A 100 μL aliquot of RBCs was added to 1 mL glycopeptides (**Tre)** solutions. RBCs were incubated at approximately 15% haematocrit (measurement by BM 830, Beijing Baolingman Sunshine Technology Co. Ltd, China) in either 0.36 M **Tre** or glycopeptides (**Tre**) for 7 h at 37 ºC and pH 6.0, 7.4. Each sample was then directly plunged into liquid nitrogen immediately. For post-thaw recovery, cells were immediately thawed in a 37 ºC water bath. Post-thaw RBCs samples and control samples were centrifugated at 2000 rpm for 10 min, and the optical density (OD) values of supernatant in each sample were measured at 541 nm using the microplate reader. The RBC cryosurvival was calculated in quadruplet samples by subtracting the hemolysis with the following equation (3).

$$\text{RBC cryosurvival (\%)} = \left(1 - \frac{OD_{Sample} - OD_{Negative\ control}}{OD_{Positive\ control} - OD_{Negative\ control}}\right) \times 100 \quad (3)$$

Confocal Laser Scanning Microscope Observation. RBCs (10^7 mL^{-1}) were resuspended and cultured at 37 ºC for 2 h in the buffer solution (pH = 6.0 and 7.4). Then, the RBCs mixtures were centrifuged at 3500 rpm for 3 minutes and the supernatant was removed and the RBCs were resuspended in a 200 μL PBS solution. After RBCs being washed three times, the images of cells were recorded using an inverted laser scanning confocal microscope (CLSM, Olympus FV1000s-IX81, Japan) at the excitation wavelengths of 488 nm for FTIC-polymers.

Ice Recrystallization Inhibition (IRI) Assay. The IRI measurement was performed by the splat-cooling method as previously reported.[1, 6, 7] A 20 μl droplet of sample solution was dropped onto the cover glass precooled to -60 °C from a height of 1.5 m forming a piece of polycrystalline ice. The temperature was increased to -6 °C at a rate of 25 °C·min^{-1}, and then the samples were annealed at -6 °C for 45 min on the Linkman cooling stage (C194). The polarized

optical microscopy (POM) images were obtained randomly using a Nikon polarized optical microscope (LV 100ND, Japan) equipped with a digital camera (Nikon Y-TV55, Japan). For every sample, three experimental runs were performed, and 5 images were obtained for each experimental run. The size of the largest 10 grains of each image was measured using Image J. Among these 150 data for each sample, 100 corresponding to the largest grains were chosen to calculate the mean largest grain size (MLGS). This average value was then compared to that of a PBS buffer negative control.

Molecular Dynamic Simulation. The GROMACS 4.67 package[8-11] and the GROMOS96 force field were applied for the Molecular dynamics simulations. The DMPC, DMPE and trehalose molecular model and force field employed in this study were chosen form Mark's work.[12, 13] And the three glycopeptides (**PKDT**, **PKDF1T** and **PKDF2T**) models, which captured the key feather of the three glycopeptides were generated from the small-molecule topology generator PRODRG.[14] The partial charge and force field parameters for the three glycopeptide models were taken from GROMOS96 force field mentioned above (as shown in Figure 1B). The bilayer was comprised by 64 DMPC (outer layer) and 64 DMPE molecules (inner layer). To mimic the cell behaviors, two bilayers were applied to bullied the cell system, with 4412 water molecules and 12 NaCl ions inside. To study the anti-freezing protection process of glycopeptides on the cell membrane system, one and four three kinds of glycopeptides, with 65 trehalose molecules was placed on the top of DMPC/DMPE bilayers, respectively. And then, 10195 water molecules with 28 NaCl ions were insert into simulation box. The ratios of NaCl/water and trehalose/water were set as experimental conditions. Finally, the dimensions of simulation box were close to $5.71\times5.71\times23.15$ nm^3. All the model's bond lengths were constrained using the LINCS algorithm. Simulations were performed using the

semiisotropic NPT ensemble with XY dimensions and Z dimension at 1 bar, the temperature was maintained at -196 °C using the Berendsen temperature coupling method,[15] respectively. The cutoff distance of the short-range non-bonded interactions was set to be 12 Å and long-range electrostatic forces were performed using the PME method.[16, 17] The snapshots of simulation results were captured by VMD.[18]

ATP and 2,3-DPG Assay. Herein, RBC after cryopreservation was used to evaluated its functions including ATP and 2,3-DPG. ATP content was measured by enhanced ATP assay kit (Beyotime) according to manufacturer instructions and by using the microplate assay procedure. 2,3-DPG was tested using sheep 2,3-DPG ELISA assay kit (Nanjing Herbaceous Source Biotechnology Co. Ltd) according to manufacturer instructions.

Statistical Analysis. All data points were repeated in triplicate. Reported results and graphical data are mean values with standard deviation encompassing a 95% confidence interval. Statical analyses were performed using Student's *t*-test. A *p*-value less than 0.05 indicated statistically significant.

SUPPLEMENTARY FIGURES

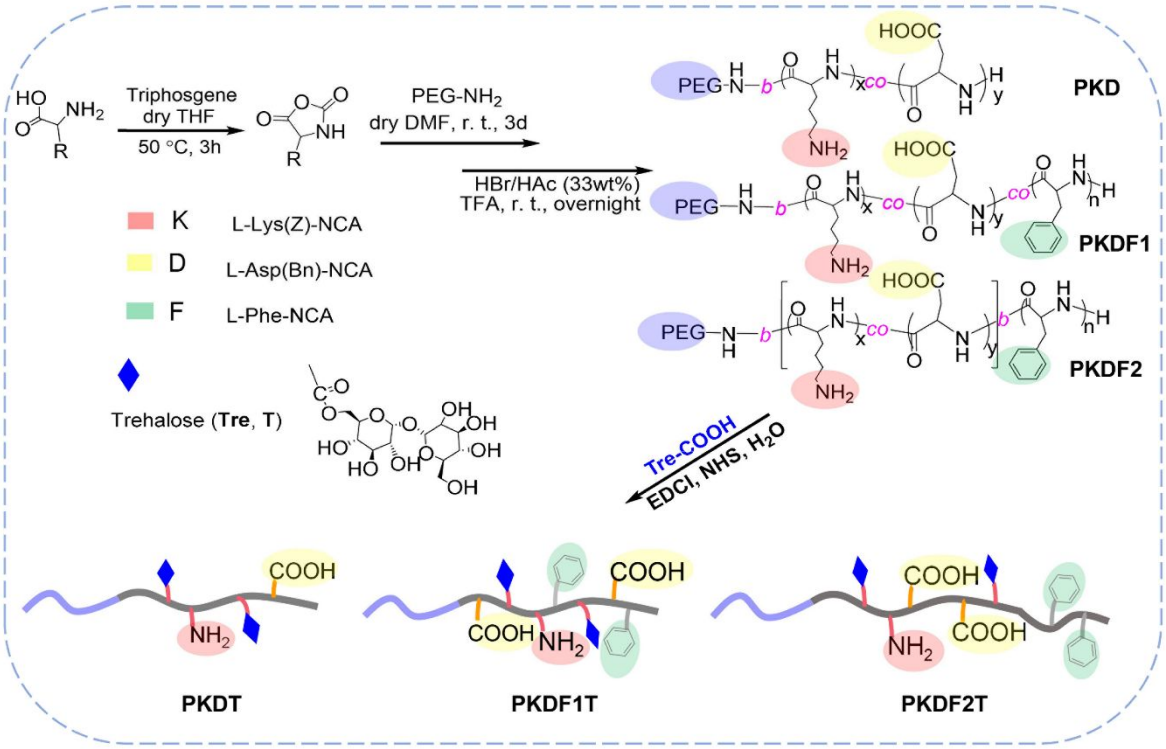

Figure S1. Synthesis pathway of PEG-*b*-polypeptide and PEG-*b*-polypeptide-*g*-trehalose.

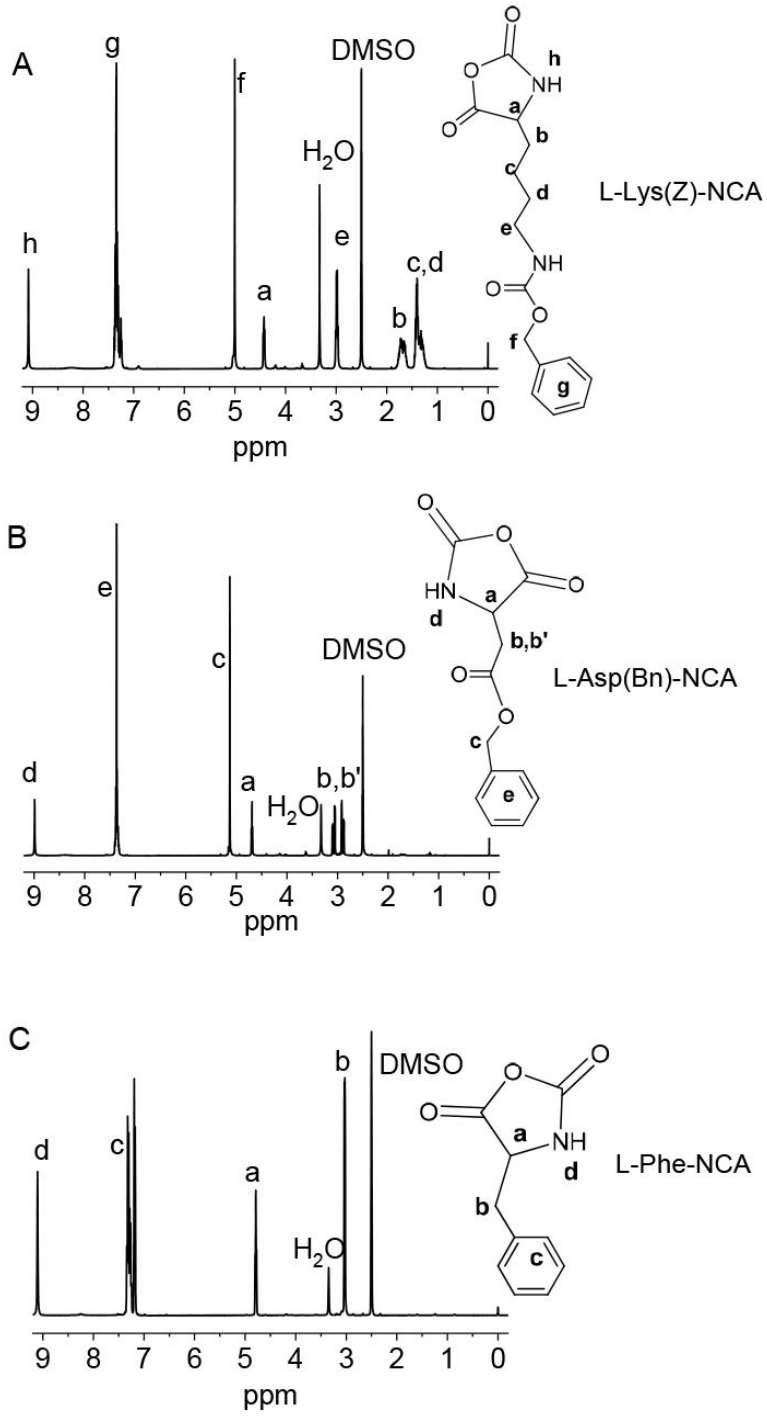

Figure S2. ^{1}H NMR spectra of (A) L-Lys(Z)-NCA, (B) L-Asp(Bn)-NCA and (C) L-Phe-NCA.

L-Lys(Z)-NCA: ^{1}H NMR (400 MHz, DMSO-d^{6}) δ 9.08 ppm (s, 1H), 7.41-7.26 ppm (m, 5H), 7.26 ppm (q, J = 5.8 Hz, 1H), 5.00 ppm (s, 2H), 4.43 ppm (dd, J = 7.3, 5.1 Hz, 1H), 2.99 ppm (q, J = 6.3 Hz, 2H), 1.80-1.59 ppm (m, 1H), 1.48-1.21 ppm (m, 4H).

L-Asp(Bn)-NCA: ^{1}H NMR (400 MHz, DMSO-d^{6}) δ 8.99 ppm (s, 1H), 7.44-7.28 ppm (m, 6H), 5.13 ppm (s, 2H), 4.69 (ddd, J = 5.1, 4.4, 1.1 Hz, 1H), 3.08 ppm (dd, J = 17.8, 4.9 Hz, 1H), 2.90 ppm (dd, J = 17.8, 4.3 Hz, 1H).

L-Phe-NCA: ^{1}H NMR (400 MHz, DMSO-d^{6}) δ 9.11 ppm (s, 1H), 7.31-7.18 ppm (d, J = 7.4 Hz, 5H), 4.79 ppm (s, 2H), 3.03 ppm (d, J = 5.2 Hz, 2H).

Table S1. Compositions and molecular weights of PEG-*b*-polypepetide.

Abbreviation	Polypeptide	Ratio of [PEG]:[**K**]:[**D**]:[**F**] (mol:mol:mol:mol)		$M_{n,\ NMR}$ [b] (×10⁴)	$M_{n,\ GPC}$ [c] (×10⁴)	$Đ$ [c]
		Feeding ratio [a]	Actual ratio [b]			
PKD	PEG-*b*-(Lys_{26}-*co*-Asp_{41})	1:45:45:0	1:26:41:0	1.01	1.03	1.04
PKDF1	PEG-*b*-(Lys_{23}-*co*-Asp_{45})-*co*-Phe_6	1:45:45:9	1:23:45:6	1.13	1.17	1.03
PKDF2	PEG-*b*-(Lys_{24}-*co*-Asp_{43})-*b*-Phe_9	1:45:45:9	1:24:43:9	1.24	1.34	1.06

[a] The feed ratio is the stoichiometric molar ratio of **K**, **D** or **F** to the amino group of PEG2000, where "**K**", "**D**" and "**F**" are referred to lysine, aspartic acid and phenylalanine, respectively.

[b] The actual mole ratio and molecular weight of the synthesized PEG-*b*-polypeptides were calculated from ^{1}H NMR spectra by the integral ratio of **PEG**, **K**, **D** or **F**.

[c] The molecular weight $M_{n,\ GPC}$ and $Đ$ of the synthesized PEG-*b*-polypeptides were measured by GPC with poly(ethylene glycol) as the standard.

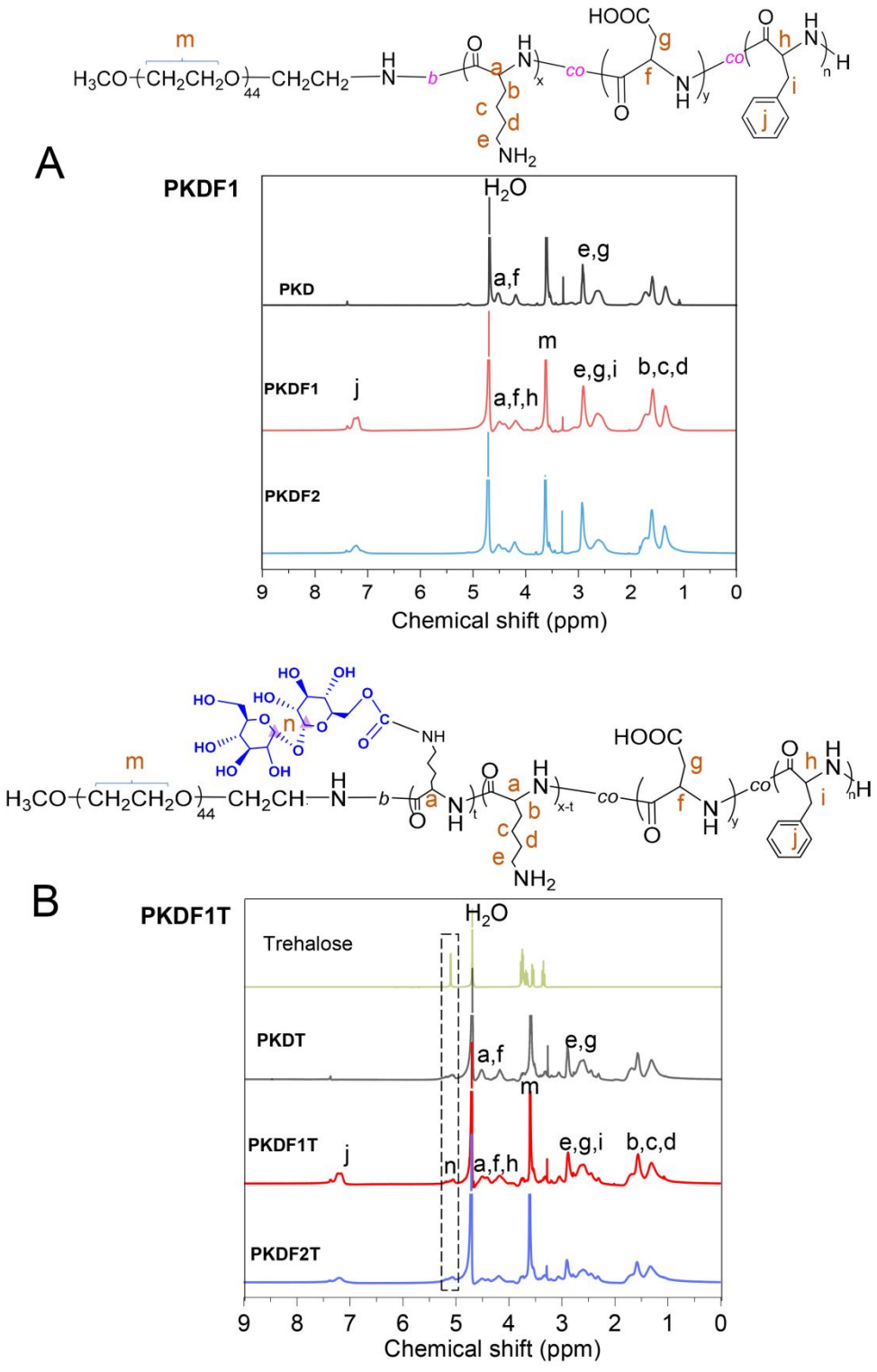

Figure S3. ^{1}H NMR spectra of **PKDT** (A) and **PKDF1T** (B).

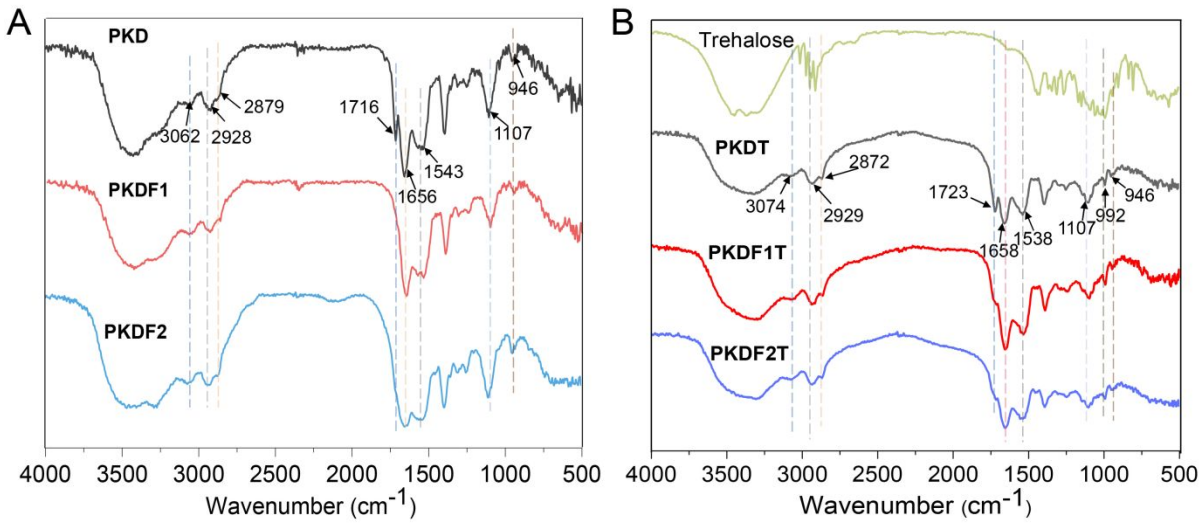

Figure S4. FTIR spectra of PEG-*b*-polypeptides (A) and PEG-*b*-polypeptides-*g*-trehalose(B). Stretching vibration absorptions of amide I and amide II bands were observed at 1656 cm^{-1} (C=O stretching) and 1543 cm^{-1} (C-N and N-H bending), respectively, and the absorption peak at 992 cm^{-1} was attributed to C-H band of trehalose.

The molecular weights and compositions of PEG-*b*-polypeptides were confirmed by ^{1}H NMR, FTIR and GPC (Figure S3, Table S1). As shown in Figure S3A, for **PKD**, the signals at δ 3.57 ppm, 2.5-3.0 ppm were assigned to the protons of CH_2-CH_2-O- (m) groups of PEG units, -CH_2-CH_2-NH_2 (e) groups of Lys units, and -CH_2-C(O)- (g) groups of Asp units, respectively. For **PKDF1** and **PKDF2**, the signal at δ 7.23 ppm was attributed to the protons of benzyl groups (j) of Phe units. As shown in **Figure** S**3B**, for **PKDT**, the signals at δ 3.57 ppm, 2.5-3.0 ppm were assigned to the protons of CH_2-CH_2-O- (m) groups of PEG units, -CH_2-CH_2-NH_2 (e) groups of Lys units, -CH_2-C(O)- (g) groups of Asp units, respectively. The results of FTIR spectra further confirmed that the successful PEG-*b*-polypeptides were successfully prepared (Figure S4A). It could be seen that amide I and amide II bands at stretching vibration absorption were observed at 1656 cm^{-1}(C=O stretching) and 1543 cm^{-1} (C-N and N-H bending), respectively. Figure S4B showed that the absorption peak at 992 cm^{-1} attributed to C-H band of trehalose. The molecular weights of **PKD**, **PKDF1** and **PKDF2** calculated from ^{1}H NMR were 1.01×10^4, 1.13×10^4 and 1.24×10^4, respectively (Table S1). In addition, the molecular weights of **PKD**, **PKDF1** and **PKDF2** estimated by GPC were 1.03×10^4, 1.17×10^4 and 1.34×10^4, respectively.

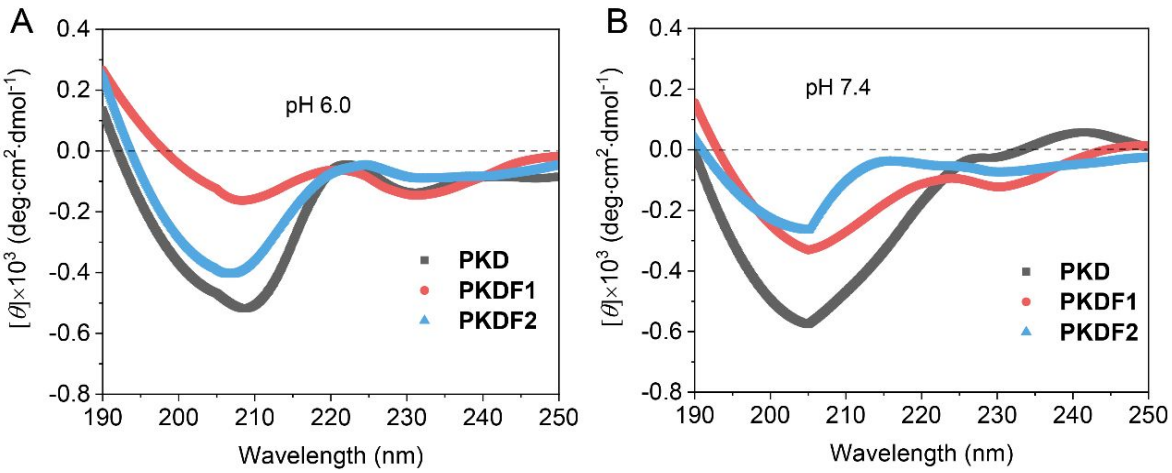

Figure S5. CD spectra of PEG-*b*-polypeptides. (A) pH 6.0, (B) pH 7.4, showing two negative bands at 200-205 nm and 230 nm, which indicated glycopeptides adopted random coil conformation in aqueous solution both at pH 6.0 and 7.4.

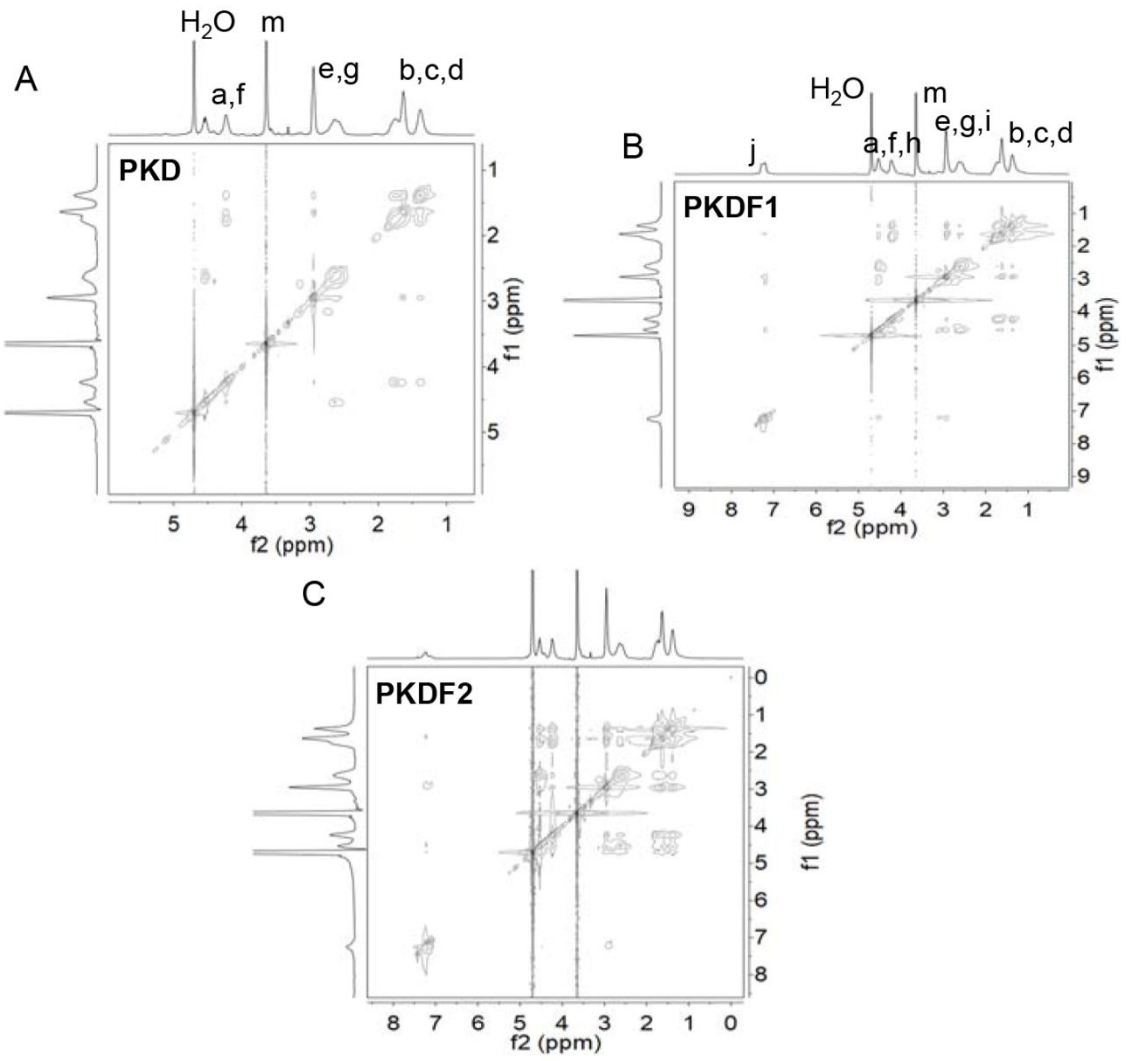

Figure S6. 2D ^{1}H-^{1}H NOESY spectra of PEG-*b*-polypeptides. (A) **PKD**, (B) **PKDF1**, (C) **PKDF2**. NOESY spectra provide information about protons that is 5 Å or less apart in space, and the presence of a NOE peak (cross-peak) is the direct evidence that two protons are within 5 Å from each other in space. Figure S6 showed that no obvious signals NOE cross-peaks were found in the NOESY spectra of PEG-*b*-polypeptides.

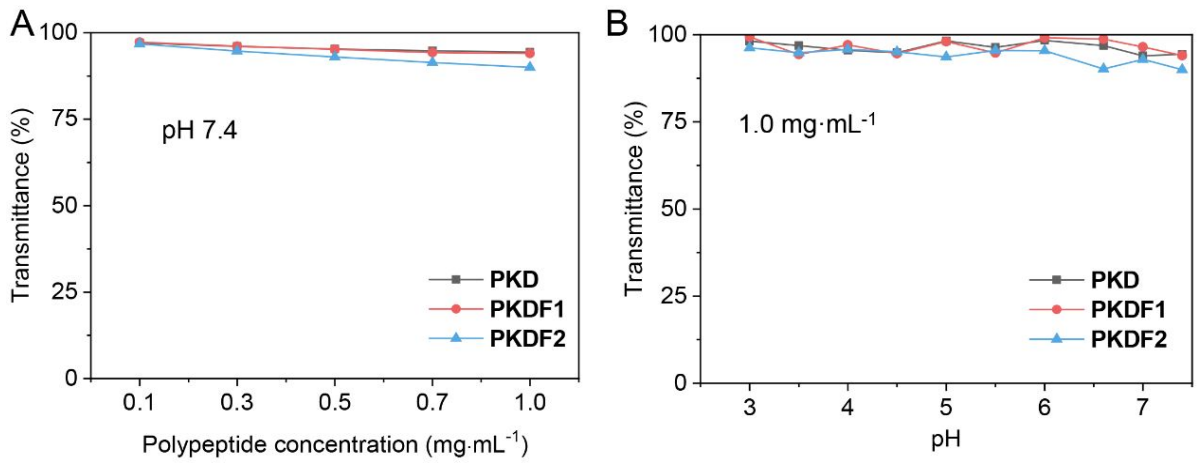

Figure S7. Concentration-dependent (A) and pH-dependent (B) transmittance of the PEG-*b*-polypeptides, indicating that they could easily dissolve in water in our experiment condition.

Table S2. Zeta-potential of glycopeptide solutions tested by DLS.

Sample	pH	Concentration ($mg \cdot mL^{-1}$)	Z-Average (nm)	PDI	Zeta potential (mV)
PKDT	6.0	1	—	—	-3.68±0.40
	7.4	1	779.7±57.1	0.85±0.08	-5.26±0.96
	7.4	10	582.2±26.8	0.59±0.11	-4.59±0.65
PKDF1T	6.0	1	3.2±0.3	0.22±0.30	-5.44±2.90
	7.4	1	658.8±58.8	0.77±0.08	-5.86±0.30
	7.4	10	560.8±29.6	0.61±0.05	-5.82±0.87
PKDF2T	6.0	1	33.7±1.0	0.25±0.00	-3.46±0.25
	7.4	1	85.6±13.3	0.37±0.06	-5.28±0.77
	7.4	10	61.7±1.8	0.72±0.07	-4.95±0.85

The zeta potentials and size distribution of the glycopeptide were measured by a dynamic light scattering (DLS) method using a laser light-scattering spectrometer (Zetasizer Nano Zs90, Malvern, UK). Table S2 showed that the zeta-potential values of PEG-*b*-polypeptides-*g*-trehalose solutions were from 0 to -6 mV (Table S2), resulting in particles of glycopeptides with unstable diameter.

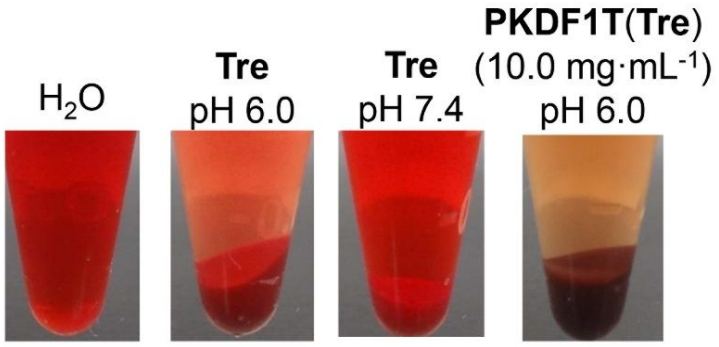

Figure S8. Digital pictures of post-thawing RBCs, showing that the content of post-thawing intact RBCs treated by **Tre** at pH 6.0 was higher than that at pH 7.4. However, darkening of post-thawing RBCs treated by **PKDF1T**(**Tre**) at glycopeptide concentration of 10.0 mg·mL^{-1} and pH 6.0 was observed, indicating that RBC function was damaged although the hemolysis value was not high.

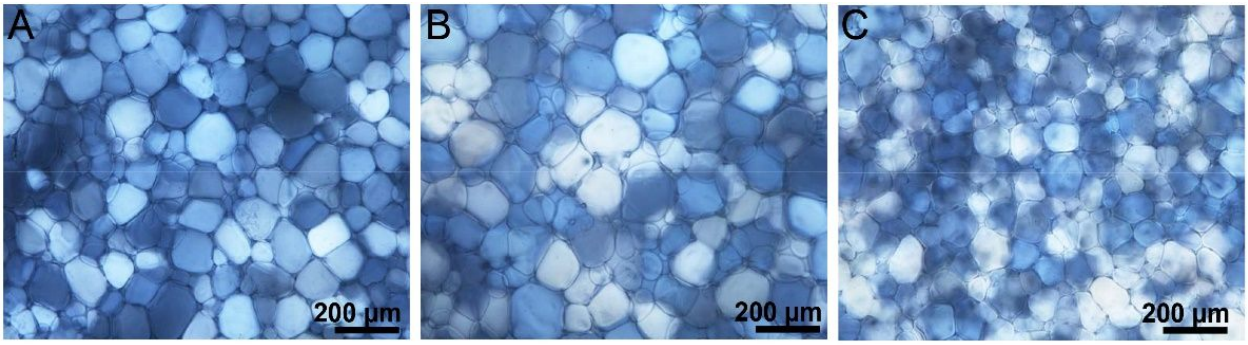

Figure S9. IRI activity. The polarized optical microscopy images showing native ice crystal growth annealing at -6 °C for 45 min by addition of (A) **PKDT**, (B) **PKDF1T**, (C) **PKDF2T** at 10.0 mg·mL^{-1}, showing a similar ice crystal size with PBS and indicating glycopeptides could not suppress the recrystallization of ice crystals.

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