Electronic Supplementary Information (ESI)

Trehalose-functional glycopeptide enhances glycerol-free cryopreservation of red blood cells

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Experimental Methods

Materials

ɛ-Polylysine (ɛ-PL, average molecular weight: ~4000) was purchased from Nanjing Xuankai Biotechnology, China. N{subscript}α-({tert-}butyloxycarbonyl)-N{subscript}ω-(4-toluenesulfonyl)-L-arginine (Boc-Arg(Tos)-OH, R) was provided by Shanghai Bide Pharmatech, China. Fluorescein isothiocyanate (FITC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI), N-hydroxysuccinimide (NHS), triethylamine and succinic anhydride were provided by Tianjin Heowns Biochemical Technology, China. Sterile defibrinated sheep red blood cells (RBCs) were provided by Guangzhou Future Biotechnology, China. Anhydrous trehalose (Tre) and trifluoroacetic acid (TFA) were obtained from J&K Scientific Ltd., China. Sodium hydroxide, 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), anhydrous dimethyl sulfoxide (DMSO), N,N’-dimethylformamide (DMF) and other solvents were supplied by Kermel Chemical Technology, China. 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), n-octyl-β-D-glucopyranoside, deuterium dimethyl sulfoxide (DMSO-d<sub>6</sub>) and deuterium oxide (D<sub>2</sub>O) were purchased from Sigma Aldrich (Energy Chemical, China). Dulbecco’s modified eagle medium (DMEM) and fetal bovine serum certified (FBS) were purchased from Gibco. Alamar blue cell viability reagent was provided by Shanghai BestBio Biotechnology, China. All chemicals were used directly as received without further purification unless mentioned.

Synthesis of carboxylated trehalose

As illustrated in Scheme S1, carboxylated trehalose (Tre-COOH) was prepared by Tre reacting with succinic anhydride adopted from the reference. 1 Tre (3.5 g, 10.23 mmol) was dissolved in dry dimethylformamide (DMF, 100 mL), and succinic anhydride (960 mg, 9.6 mmol) was dissolved in dry DMF (20 mL). Then, Tre solution was mixed with succinic anhydride solution, and 7% triethylamine DMF solution was added to the above mixtures.
The reaction was continued for 12 h at 80 °C under a nitrogen atmosphere. The product was precipitated using a diethyl ether/acetone mixture (70/30, v/v) for three times and dried in a vacuum oven at 50 °C for 12 h.

4 Synthesis of (glyco)peptides

As shown in Fig. 1A, trehalose-functional (glyco)peptides were prepared by sequent reactions of $\varepsilon$-PL with Boc-Arg(Tos)-OH (R) and carboxylated trehalose (Tre-COOH), adopted from the reference. Typically, Boc-Arg(Tos)-OH (3.32g, 7.75 mmol), EDCI (3.56 g, 18.57 mmol) and NHS (2.14 g, 18.59 mmol) were dissolved in DMSO (40 mL) and stirred at room temperature for 30 min. $\varepsilon$-PL (1.0 g, 0.25 mmol) dissolved in deionized water (10 mL) was added into the mixture solution and maintained at room temperature for 3 days. The mixture solution was dialyzed against deionized water for 3 days (cut off 2000 Dalton) and freeze-dried. The obtained white solid product was then dissolved in TFA and reacted at room temperature for 4 h. The reaction mixture was precipitated with diethyl ether and centrifugated. The crude product $\varepsilon$-PL-g-Arg(Tos) ($\text{PLR}_8$) was purified via dialysis in deionized water for 3 days (cut off 2000 Dalton) and freeze-dried. Then, $\text{PLR}_8$ was further reacted with carboxylated trehalose to synthesize $\varepsilon$-PL-g-Arg(Tos)-g-Tre ($\text{PLR}_8\text{T}_{13}$) in the same way. The product was purified via dialysis (cut off 2000 Dalton) against deionized water for 3 days and lyophilized.

19 Characterization

The molecular weight of carboxylated trehalose was measured by high resolution mass spectrometry (HRMS, miorOTOF-QII, Bruker). Chemical structures of $\text{PLR}$ and $\text{PLRT}_s$ were verified by analyses of $^1$H nuclear magnetic resonance (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$_2$O. The 2D $^1$H-$^1$H nuclear Overhauser effect spectroscopy (NOESY) NMR measurement was performed via dissolving 50 mg of the specimen in D$_2$O. The molecular
weights of PLT, PLRs and PLRTs were calculated by the integral of T and R related signals.
The molecular weight and polydispersity of (glyco)peptides 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 Fourier transform infrared (FTIR)
spectra were obtained in the range from 1800 to 1400 cm\(^{-1}\) in PerkinElmer (Spectrum 100,
USA) using the KBr pellet technique. RBCs before and after treatment with the
(glyco)peptide were also observed with a scanning electron microscope (SEM, Hitachi
su1510, Japan). The washed RBCs in 50 µL were added to 1 mL (glyco)peptide solution (0.1
mg·mL\(^{-1}\)). After being incubated at 37 °C for 2 h, the RBCs suspensions were dropped onto
the slide, followed by fixation with 2.5% glutaraldehyde in PBS overnight. The fixed RBCs
were dehydrated with a series of graded ethanol solutions (30, 50, 60, 70, 80 90, 95 and 100%,
15 min each), and then dried in air. The samples were gold-coated prior to being viewed by
SEM. The circular dichroism (CD) spectra of (glyco)peptides were obtained using a CD
spectrometer (J-810, Jasco, Japan) at room temperature under a constant flow of N\(_2\). The
polymer aqueous solutions (0.1 mg·mL\(^{-1}\)) were introduced into quartz cuvettes with a path
length of 1 mm. The wavelengths were set at 185~250 nm with a scan speed of 100 nm·min\(^{-1}\).
The integration time was set at 1 second and the bandwidth was set to 0.5 nm. The water was
used as the reference. All (glyco)peptide solutions were previously filtered using 0.22 μm
filter and each sample was measured in triplicate.

**Hemolysis assay**
Sterile defibrinated sheep RBCs were used as cell model for evaluation hemolysis of
synthesized (glyco)peptide.\(^3\) RBCs washed with single strength PBS solution (306 mOsm, pH
7.4) for three times (centrifugation, 2000 rpm, 10 min, 4 °C). Then, RBCs were resuspended
in (glyco)peptide or (glyco)peptide/Tre buffer 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 (S1).

\[
\text{Relative hemolysis (\%)} = \frac{\text{OD}_{\text{Sample}} - \text{OD}_{\text{Negative control}}}{\text{OD}_{\text{Positive control}} - \text{OD}_{\text{Negative control}}} \times 100
\]

(S1)

7 Cytotoxicity

The cytotoxicity of the (glyco)peptide was examined by using L929 fibroblasts via Alamar Blue assay and MTT assay.\(^{4-6}\) Firstly, cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin in a humidified incubator with 5% CO\(_2\) at 37 °C for 24 h. L929 cells were seeded into a 96-well plate (Corning) 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 DMEM containing 10% FBS and PL, PLT, PLR or PLRTs at specific concentrations, and the cells were cultured for another 24 h. The cytotoxicity was determined by MTT assay. For Alamar Blue assay, the cultural medium of 96-well plate containing L929 cells was replaced with the fresh culture composed of 10% Alamar Blue reagent after 24 h of incubation. Further incubated for 4 h was conducted according to the manufacturer’s instruction, and the fluorescence of each well was measured using a microplate reader at excitation wavelength of 525 nm and emission wavelength of 590 nm. The relative cell viability was calculated as following equation (S2). For MTT assay, 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·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 value was measured at 490 nm using the microplate reader.
The relative cell viability was calculated as following equation (S3). During the measurement, the medium without polymer was as the positive control, and each sample was repeated in triplicate.

\[
\text{Relative cell viability (\%) } = \frac{I_{590, \text{Sample}}}{I_{590, \text{Positive control}}} \times 100
\]

(S2)

\[
\text{Relative cell viability (\%) } = \frac{\text{OD}_{490, \text{Sample}}}{\text{OD}_{490, \text{Positive control}}} \times 100
\]

(S3)

**7 LF \textsuperscript{1}H NMR spectroscopy**

The LF NMR spectra were obtained through a Bruker Minispec mq20 spectrometer operating at a proton resonance frequency of 20 MHz at ambient temperature according reference.\textsuperscript{7-9} Samples were placed in the NMR tube with a 10 mm outer diameter. The free induction decay signals were recorded with Carr-Purcell-Meiboom-Gill (CPMG) pulse sequences, where the transverse spin-spin relaxation time ($T_2$) distribution curve was obtained through an inverse Laplace transform-based CONTIN analysis on the CPMG decay curves. Each sample was run with 4 scans and a 2 s recycle delay.

**15 DOPC liposome preparation**

The preparation of DOPC liposomes was adopted from the reference\textsuperscript{10,11} DOPC was suspended in the HBS-N running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, Nanjing SenBeiJia Biological Technology Co., Ltd., China) and then treated through froze-thaw-vortex for 5 times to completely agitate it. The obtained DOPC suspension (10 mM) was stirred ultrasonically for 20 min using an ultrasonic cell disruptor (Scientz-IID, JY92-IIN) and the nanoscale liposomes were formed.

**22 Surface plasmon resonance (SPR) spectroscopy**

SPR spectroscopy analysis was used for characterizing of the interaction of glycopeptide or
The L1 sensor chip was placed in the Biacore 3000 system (GE Healthcare) and washed with 40 mM \( n \)-octyl-\( \beta \)-D-glucopyranoside at the flow rate of 10 \( \mu \text{L}\cdot\text{min}^{-1} \) for 10 min. The 0.5 mM DOPC liposome, which was diluted in the HBS-N running buffer, was added to the sensor chip at a flow rate of 2 \( \mu \text{L}\cdot\text{min}^{-1} \) to generate a lipid bilayer on the chip until achieving the resonance unit (RU) value at 6400. Then, the surface of the sensor chip was washed with 50 mM NaOH at 2 \( \mu \text{L}\cdot\text{min}^{-1} \) for 5 min, and 100 \( \mu \text{g}\cdot\text{mL}^{-1} \) BSA flowed over the sensor chip at 2 \( \mu \text{L}\cdot\text{min}^{-1} \). It was regarded that the stabilization of the liposome onto the sensor chip was achieved when the increased extent by the addition of BSA (RU values) was <100. The HBS-N buffer solutions were prepared containing (glyco)peptide or Tre. Sample solution was injected into the Biacore 3000 system at the flow rate of 10 \( \mu \text{L}\cdot\text{min}^{-1} \) for 10 min, and then the running buffer was eluted. The SPR data were obtained using BIA evaluation software.

**Differential scanning calorimetry (DSC) analysis**

The energetics of the interactions between (glyco)peptide and DOPC were measured using a differential scanning calorimeter (PerkinElmer Diamond).\(^{14-16}\) DSC assessment of ice formation was performed.\(^{14,17}\) Samples were added to 40 \( \mu \text{L} \) aluminum pans and transferred to a Freon IntraCooler 2P cooling accessory (Heat flow, \( \text{W}\cdot\text{g}^{-1} \)). The measurement was recorded against an empty pre-weighted 40 \( \mu \text{L} \) aluminum reference pan from 25 \( ^\circ\text{C} \) to -40 \( ^\circ\text{C} \) at 10 \( ^\circ\text{C}\cdot\text{min}^{-1} \), followed by -40 \( ^\circ\text{C} \) to 25 \( ^\circ\text{C} \) at 2 \( ^\circ\text{C}\cdot\text{min}^{-1} \), with the presence of two large endothermic peaks which demonstrated water freezing and ice melting.

**Raman spectroscopy**

Raman spectra were collected using a DXR Raman microscope with a 532 nm laser wavelength (Thermo Fisher Scientific Co. Ltd., USA).\(^{8,15,18}\) The samples for the Raman spectra measurement were dropped into aluminum pans and placed the test bench to measure at a specified temperature. All samples were loaded on a temperature-controlled Linkam stage.
(BCS196, UK) mounted on a Raman sample stage. The sample was cooled at a cooling rate of 2 °C·min⁻¹ from 20 ºC to -25 ºC and 10 °C·min⁻¹ from -25 ºC to -50 ºC. The samples were measured after being maintained at 20 ºC, 0 ºC, -25 ºC and -50 ºC for 5 min, respectively.

4 Cryopreservation protocol

Cryopreservation and evaluation of RBCs after post-thaw recovery were performed according to the references.³,¹⁹ A 100 µL aliquot of RBCs was added to 1 mL (glyco)peptides or (glyco)peptides/Tre solutions in cryovials (Corning, 1.8 mL). RBCs were incubated at approximately 15% haematocrit (measured by BM 830, Beijing Baolingman Sunshine Technology Co., Ltd, China) in either 0.36 M Tre or 0.36 M Tre/1 mg·mL⁻¹ (glyco)peptides for 7 h at 37 ºC and pH 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-thawed RBCs and control samples were centrifugated at 2000 rpm for 10 min, and the 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 (4).

\[
RBC\ cryosurvival\ (\%) = \left(1 - \frac{OD_{Sample} - OD_{Negative\ control}}{OD_{Positive\ control} - OD_{Negative\ control}}\right) \times 100
\]

17 Confocal laser scanning microscopy

ε-PL, PLR₈, PLT₅, PLR₈T₁₃ were reacted with fluoresceinisothiocyanate (FITC) at 0.01 molar ratio to polymer for 3 days at room temperature. FITC-polymer was purified by dialysis (Cut off 2000 Dalton) and freeze-dried. RBCs (10⁷ mL⁻¹) were resuspended and cultured at 37 ºC for 2 h in the buffer solution (pH = 7.4) in the presence of FTIC-polymers. Then, the RBCs mixtures were centrifuged at 3500 rpm for 3 minutes. When the supernatant was removed, 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, Leica SP8, Germany) at the excitation wavelengths of 488 nm for FTIC-polymers.

3 **Statistical analysis**

4 All data points were repeated in triplicate. Results and graphical data are mean values with standard deviation encompassing a 95% confidence interval. Statistical analyses were performed using Student’s $t$-test. A $p$-value less than 0.05 indicated statistically significant.
Scheme S1  Synthesis of carboxylated trehalose.

Fig. S1  $^1$H NMR spectra of carboxylated trehalose.
Fig. S2  HRMS spectrum of carboxylated trehalose.
Table S1  Percentage of trehalose, mono-carboxylated trehalose (Tre-(COOH)$_1$), di-carboxylated trehalose (Tre-(COOH)$_2$) and tri-carboxylated trehalose (Tre-(COOH)$_3$) calculated by HRMS spectrum.

<table>
<thead>
<tr>
<th>Monomer</th>
<th>Formula</th>
<th>Calculated molecular weight $^a$</th>
<th>Detected molecular weight $^b$</th>
<th>m/z</th>
<th>Percentage (%)</th>
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</thead>
<tbody>
<tr>
<td>Trehalose</td>
<td>C$<em>{12}$H$</em>{30}$O$_{11}$</td>
<td>350.30</td>
<td>350.02</td>
<td>350</td>
<td>20.7</td>
</tr>
<tr>
<td>Tre-(COOH)$_1$</td>
<td>C$<em>{16}$H$</em>{26}$NaO$_{14}$</td>
<td>465.36</td>
<td>465.13</td>
<td>465</td>
<td>45.1</td>
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<tr>
<td>Tre-(COOH)$_2$</td>
<td>C$<em>{20}$H$</em>{30}$NaO$_{17}$</td>
<td>565.43</td>
<td>565.15</td>
<td>565</td>
<td>26.8</td>
</tr>
<tr>
<td>Tre-(COOH)$_3$</td>
<td>C$<em>{24}$H$</em>{34}$NaO$_{20}$</td>
<td>665.51</td>
<td>665.16</td>
<td>665</td>
<td>7.4</td>
</tr>
</tbody>
</table>

$^a$) Calculated results according the chemical formula of trehalose and its derivatives.

$^b$) Detected results according to the HRMS spectrum.
**Fig. S3** $^1$H NMR spectra of $\varepsilon$-PL-\(g\)-Tre (PLT).

$^1$H NMR spectra of $\varepsilon$-PL and Tre-functional (glyco)peptides, PLR$_8$ and PLR$_8$T$_{13}$. $\varepsilon$-PL, PLR$_8$ and PLR$_8$T$_{13}$ exhibited similar signals at $\delta$ 1~2 ppm, 3.0~3.5 ppm and 3.8~4.0 ppm in the $^1$H NMR spectra, attributed to -C\(\text{H}_2\)- and -C\(\text{H}\)- of the polymer backbone. The signal at approximately $\delta$ 7~8 ppm in the spectra of PLR$_8$ was assigned to -C\(\text{H}_2\)- of phenyl. PLR$_8$T$_{13}$ exhibited similar signals at $\delta$ 7~8 ppm in the spectra, assigned to the phenyl groups. In addition, the signal at $\delta$ 4.8 ppm in the spectra of PLR$_8$T$_{13}$ was attributed to -C\(\text{H}_2\)- of Tre residues.
Fig. S5  CD spectra of \( \text{PLR}_3 \text{T}_5 \) and \( \text{PLR}_7 \text{T}_{12} \) in aqueous solution at pH 7.4.

In Fig. S5, the CD spectrum of \( \text{PLR}_3 \text{T}_5 \) showed one negative band at 195~210 nm and one weak positive band at 215~220 nm. Similarly, the spectrum of \( \text{PLR}_7 \text{T}_{12} \) showed one negative band at 195~205 nm and one weak positive band at 210~220 nm. The results indicated that the coexistence of the similar amphipathic polyproline II helix structure (negative signal) and random coils (weak positive signal), and the content of a similar amphipathic polyproline II helix structure for \( \text{PLR}_7 \text{T}_{12} \) was higher than that of \( \text{PLR}_3 \text{T}_5 \).
Fig. S6 2D \(^1\)H-\(^1\)H NOESY spectra of (glyco)peptides in D\(_2\)O solutions. A) \textbf{PLR}_8\textbf{T}_{13}; B) \(\varepsilon\textbf{-PL}; C) \textbf{PLT}_5. D) FTIR spectra for selected fractions of (glyco)peptides in the amide I and amide II regions.

As shown in previous studies, NOESY spectra provided information about protons that was 5 Å or less apart in space, and the presence of a NOE peak (cross-peak) was the direct evidence that two protons are within 5 Å from each other in space.\(^{20,21}\) As shown in Fig. S3 and Fig. S4, the chemical shifts of the hydrogen protons for \textbf{PLR}_8\textbf{T}_{13}, \(\varepsilon\textbf{-PL}\) and \textbf{PLT}_5 have been discussed. Herein, Fig. S6A showed that hydrogen atoms bonded to carbon atoms (j, k, Arg(Tos)-CH, Fig. S4) produced NOE signals with hydrogen atoms in the backbone of \(\varepsilon\textbf{-PL}\) (a, b, c, d, e,
Lys-CH$_2$, Fig. S4). In addition, compared with NOESY spectrum of ε-PL (Fig. S6B), the intensities of cross-peaks were stronger among hydrogen atoms bond to carbon atoms with each other. The hydrogen atoms in the trehalose residue (m, Tre-CH, Fig. S4) also produced NOE signals with hydrogen atoms in the backbone of ε-PL. The results indicated that PLR$_8$$T_{13}$ might exist in the folded state in aqueous solution. In contrast, no obvious signal NOE cross-peaks were found in PLT$_5$ (Fig. S3 and Fig. S6C) between hydrogen atoms and other hydrogen atoms located farther away, indicating that the PLT$_5$ might be extended in conformation.

FTIR spectra of proteins and polypeptides exhibited amide absorptions which represented different vibrations of the peptide moiety, such as amide I and amide II.$^{22-24}$ As shown in Fig. S6D, ε-PL displayed an amide I absorption peak at 1671 cm$^{-1}$, and it shifted to 1656 cm$^{-1}$ (PLT$_5$) and 1648 cm$^{-1}$ (PLR$_8$$T_{13}$), respectively. Meanwhile, the amide II absorption peak at 1562 cm$^{-1}$ (ε-PL) shifted to 1557 cm$^{-1}$ (PLT$_5$) and 1542 cm$^{-1}$ (PLR$_8$$T_{13}$), respectively. It was proposed that a conformation might change during amide I and amide II shifting.
As shown in Fig. S7, the cryosurvival percentage of RBCs increased with concentration increase of Tre from 0 M to 0.36 M, and then the cryosurvival percentage of RBCs decreased when Tre concentration was 0.64 M. Although Tre could protect RBCs from cryoinjury during freezing and thawing, the higher concentration of Tre may cause significant increase of extracellular osmotic pressure from 300 mOsm to 600 mOsm (0.36 M Tre) or 1270 mOsm (0.64 M Tre), and then the RBCs could be damaged owing to severe dehydration. Thus, in this study, the optimum Tre concentration was 0.36 M, similar to the results in the reference.
Fig. S8  A,B) Hemolysis of RBCs after incubated with different concentrations of (glyco)peptides at 37 °C and pH 7.4 for 2 h. A) (glyco)peptides with 0.36M Tre. B) (glyco)peptides alone. The hemolysis of PLR₃ and PLR₇ was higher than that of PLR₃T₅ and PLR₇T₁₀ with or without 0.36 M Tre, but some agglutinations of RBCs appeared for both PLR₃ and PLR₇.  C,D) Concentration-dependent relative cell viability of different (glyco)peptides at 37 °C and pH 7.4 for 24 h against L929 cells. C) Alamar Blue assay. D) MTT assay. Values are means ± the standard deviation, *p < 0.05, **p < 0.01, n = 3, and all samples were compared with control sample without (glyco)peptides. The L929 cell viability was over 80% in the concentration ranges from 0.1 mg·mL⁻¹ to 2.0 mg·mL⁻¹ for PLR₃ and PLR₇, PLR₃T₅ and PLR₇T₁₂.
Fig. S9 Digital pictures of samples after incubation with 1 mg∙mL⁻¹ (glyco)peptides/0.36 M Tre and with 1 mg∙mL⁻¹ (glyco)peptides alone. It could be observed that the samples with PL₈T₁₃ exhibited negligible hemolysis, even similar to the negative control.
Dynamic light scattering (DLS) analysis

The zeta potentials were measured using a laser light scattering spectrometer (DLS, Zetasizer Nano Zs90, Malvern, UK). The (glyco)peptide and/or RBCs mixture solutions in the buffer at pH 7.4 were used for the DLS measurement which was performed at an angle of 90º in a 10 mm diameter cell at 25 ºC, and each sample was repeated in triplicate. Glycopeptide solution at 1.0 mg·mL⁻¹ was prepared in the buffer at pH 7.4. (Glyco)peptide (1mg·mL⁻¹) was mixed with RBCs (100/1, v/v).

Fig. S10 also showed that zeta potential values of PLR₃T₅, PLR₇T₁₂ and PLR₈T₁₃ were lower than those of ε-PL, PLT₅ or PLR₃, PLR₇ and PLR₈. Furthermore, the zeta potential values of all sample solutions decreased after mixing with RBCs possible due to negative charge existing on the surface of RBC membrane. Interestingly, the zeta potentials of samples with both Arg(Tos) and Tre mixing with RBCs were similar with that of control sample with RBCs alone, while the values of samples with Arg(Tos) and Tre mixing with RBCs increased. The results indicated that the samples incorporation with both Arg(Tos) and Tre has no zeta potential disruption on RBCs.

Fig. S10  Zeta potential of (glyco)peptides with RBCs or without RBCs at 1 mg·mL⁻¹.
Fig. S11  RBC cryosurvival. A) By using (glyco)peptides alone. B) By using PLR$_3$T$_5$/0.36 M Tre or PLR$_7$T$_{12}$/0.36 M Tre. Values are means ± the standard deviation, and all samples were compared with control sample without any (glyco)peptides.

As shown in Fig. S11, the cryopreservation of (glyco)peptides or (glyco)peptide/Tre on sheep RBCs was measured. Fig. S11A showed the cryosurvival of RBCs was below 10% when the polymer was used alone. Fig. S11B showed both PLR$_3$T$_5$ and PLR$_7$T$_{12}$ combing with 0.36 M Tre could improve RBC cryosurvival compared with 0.36 M Tre alone. The results suggested that the synthesized (glyco)peptides could not cryoprotect RBCs, but could synergistically improve the RBC cryosurvival with 0.36 M Tre.
Fig. S12  RBC cryosurvival with \( PLR_8T_{13}/0.36 \) M trehalose, 0.36 M trehalose, \( PLR_8T_{13}/0.36 \) M sucrose and 0.36 M sucrose. Values are means ± the standard deviation, ***\( p < 0.001, n = 3. \)

As shown in Fig. S12, under identical condition, the RBCs cryosurvival of \( PLR_8T_{13}/\)sucrose sample (51.7±1.0%) was significantly lower than that of \( PLR_8T_{13}/\)trehalose sample (67.3 ±2.8%). Meanwhile, the RBCs cryosurvival of sucrose was also significantly lower than that of trehalose. The results suggested that trehalose actually performed a special role in cryopreservation of RBCs, and \( PLR_8T_{13} \) could synergistically improve RBCs cryosurvival with 0.36 M trehalose.
Fig. S13  A) LF NMR relaxation time ($T_2$) inversion spectra of water in the different (glyco)peptides with 1mg·mL$^{-1}$. B) SPR sensorgrams for Tre.

In Fig. S13A, the spectra peaks of pure (glyco)peptide aqueous solutions showed similar transverse spin-spin relaxation time ($T_2$) values (Table S2) with that of pure water, and the peaks of PLR$_3$T$_5$ solution would get narrow and $T_2$ values would decrease among the samples of $\epsilon$-PL, PLT$_5$, PLR$_8$ and PLR$_3$T$_5$ solution. However, the peaks of samples of PLR$_3$T$_5$, PLR$_7$T$_{12}$ and PLR$_8$T$_{13}$ solution were similar with pure water. The results indicated that water molecules moved without being restricted due to weak interactions between (glyco)peptides and water molecules.

It could be found (Fig. S13B) that RU immediately decreased after the end of the addition of the Tre solution as a result of quick release of the interacted components, indicating that the interaction between Tre and DOPC liposome was weak due to stronger Tre-water interaction than Tre-DOPC interaction. In addition, the RU values of Tre increased with the concentration of sample solution increasing, suggesting that the interaction between Tre and DOPC could be enhanced under higher concentration.
Fig. S14  DSC heating thermograms. A) 1 mM DOPC or 1mg·mL⁻¹ (glyco)peptide mixtures.
B) 1 mM DOPC with PLR₈T₁₃ with different concentration. C) PLR₈T₁₃ with different concentration. Scans were obtained at a heating rate of 2 °C·min⁻¹.

Fig. S14A showed the onset temperature (T\textsubscript{onset}) of ice in ε-PL, PLT₅, PLR₈ and PLRTs solutions mixing with DOPC were slightly lower than that of pure water. Compared to pure water, Fig. S14B showed that the T\textsubscript{onset} of ice in DOPC/PLR₈T₁₃ solutions were slightly lower, but Fig. S14C showed the melt points of PLR₈T₁₃ solutions negligibly changed.
Table S2  Relaxation time ($T_2$) of water in the compound mixtures containing 1 mM DOPC, 1 mg·mL$^{-1}$ (glyco)peptide or 0.36 M Tre.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$T_2$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC/PLR$<em>8$T$</em>{13}$/Tre</td>
<td>1288</td>
</tr>
<tr>
<td>DOPC/PLR$<em>8$T$</em>{13}$</td>
<td>1755</td>
</tr>
<tr>
<td>DOPC/Tre</td>
<td>1390</td>
</tr>
<tr>
<td>DOPC</td>
<td>1635</td>
</tr>
<tr>
<td>PLR$<em>8$T$</em>{13}$/Tre</td>
<td>1080</td>
</tr>
<tr>
<td>Tre</td>
<td>950</td>
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1 Ice recrystallization inhibition (IRI) assay

2 The IRI measurement was performed by the splat-cooling method as previously reported.\textsuperscript{26-28}

3 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\textsuperscript{-1}, and then the samples were annealed at -6 °C for 45 min on the Linkman cooling stage (C194). Photographs 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 captured 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.

Fig. S15  IRI activity. The polarized optical microscopy images showing native ice crystal growth annealing at -6 °C for 45 min by addition of A) 1 mg·mL\textsuperscript{-1} PLR\textsubscript{8}T\textsubscript{13}/0.36 M trehalose, B) 0.36 M trehalose, C) 1 mg·mL\textsuperscript{-1} PLR\textsubscript{8}T\textsubscript{13} and D) PBS. E) The mean largest grain sizes (MLGS) obtained from buffer solutions of 1 mg·mL\textsuperscript{-1} PLR\textsubscript{8}T\textsubscript{13}/0.36 M trehalose, 1 mg·mL\textsuperscript{-1} PLR\textsubscript{8}T\textsubscript{13} and 0.36 trehalose relative to PBS solution alone. Error bars represent the STDEV of the size of 100 ice crystals.
Fig. S15 showed ice recrystallization inhibition activity of **PLR₈T₁₃** or **Tre**. In terms of **PLR₈T₁₃/Tre** and **Tre**, the MLGS of ice crystal was almost similar (about 47%), while that for **PLR₈T₁₃** addition alone was the largest (about 116%). The results indicated that **PLR₈T₁₃** has no IRI activity, but **PLR₈T₁₃** could protect cells from osmotic injury by membrane stabilization.

**Fig. S16** IRI activity. The polarized optical microscopy images showing native ice crystal growth annealing at -6 °C for 45 min by addition of A) 1 mg·mL⁻¹ **PLR₈T₁₃**/0.36 M sucrose, B) 0.36 M sucrose, C) 1.08 M trehalose and D) 1.08 M sucrose. E) The mean largest grain sizes (MLGS) obtained from buffer solutions of 1 mg·mL⁻¹ **PLR₈T₁₃**/0.36 M sucrose, 1.08 M trehalose and 1.08 M sucrose relative to PBS solution alone. Error bars represent the STDEV of the size of 100 ice crystals.

Fig. S16 showed the IRI activity of **PLR₈T₁₃** or sucrose. It could be observed that both **PLR₈T₁₃**/sucrose and sucrose showed IRI activity, and the size of ice crystal could be inhibited when the concentration was increased.
References


