

Supporting Information

Functional Nanochaperones for PEGylated Insulin Delivery in Long-Term Glycemic Control

Xiaohui Wu,^{†a,b} Yanli Zhang,^{†a,b} Shuoshuo Song,^{a,b} Sainan Liu,^{a,b} Feihe Ma,^{*a} Rujiang Ma,^{*a} and Linqi Shi^{*a,b}

^a Key Laboratory of Functional Polymer Materials of Ministry of Education, Institute of Polymer Chemistry, State Key Laboratory of Medicinal Chemical Biology, Frontiers Science Center for New Organic Matter, College of Chemistry, Nankai University, Tianjin 300071, PR China. E-mail: feihema@nankai.edu.cn; marujiang@nankai.edu.cn; shilinqi@nankai.edu.cn

^b Haihe Laboratory of Sustainable Chemical Transformations, Tianjin 300192, PR China. E-mail: shilinqi@nankai.edu.cn

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1. Materials

All chemicals were obtained from commercial suppliers unless otherwise specified and were used as received. All aqueous solutions were prepared using Milli-Q water. ϵ -caprolactone (ϵ -CL), nitrilotriacetic acid (NTA), 3-aminophenylboronic acid (APBA), zinc acetate, α -D (+)-Glucose, thioflavin T (ThT) were purchased from Sigma-Aldrich and TCI. Fluorescein isothiocyanate (FITC), TRITC phalloidin and proteinase K were purchased from MCE. PEGylated Insulin (PEG5K-Insulin) was purchased from Xi'an ruixi Biological Technology. Insulin was purchased from Genview.

2. Preparation of Nanochaperones

Nanochaperones were prepared according to our previous reports.¹ Briefly, PCL-*b*-PEG, PCL-*b*-P(Asp-*co*-AspPBA) and PCL-*b*-P(Asp-*co*-AspNTA) were dissolved in DMSO overnight. Then, alkaline water (pH 11) was quickly added to the polymer solution under ultrasound. After being dialyzed in PBS buffer (PBS 7.4, 10 mM), micelles were obtained with a concentration of 0.6 g L⁻¹. Subsequently, zinc acetate solution was added and stirred for 1 h, and the excess Zn²⁺ was removed by dialysis. Finally, nanochaperones chelated with Zn²⁺ were successfully synthesized. According to the mass ratios of different block copolymers, we successfully obtained different nanochaperones, named PN-nChaps and CRT-nChaps (**Table S1**).

3. The Förster resonance energy transfer (FRET) measurements

Sulfo-Cyanine5 (Cy5) were labeled at the PEG terminus of SMs (Cy5-SMs) and at the glucose-responsive segment P(Asp-*co*-AspPBA) of PN-nChaps (Cy5-PN-nChaps). Cy5-CRT-nChaps and PN-nChaps were mixed with equal amount of FITC labeled insulin (FITC-insulin) or FITC fluorescently labeled PEG-insulin (FITC-PEGylated insulin), respectively. The mixture was then incubated at 37°C for 6 h. The fluorescence emission spectrum was measured using fluorescence spectrophotometer at 550-750 nm (excitation at 515 nm).

4. Proteolytic degradation

Proteinase K (PK) was used to verify the proteolytic stability of free insulin (Ins/PBS), free PEGylated insulin (PEG-Ins/PBS), insulin loaded on PN-nChaps (Ins/PN-nChaps) and

PEGylated insulin loaded on PN-nChaps (PEG-Ins/PN-nChaps). Briefly, each sample (1 mL) was mixed with the freshly prepared proteinase K solution (0.1 mL, 0.1 g L⁻¹) and incubation for 8 h at 37°C. Aliquots of the samples (50 µL) were withdrawn at different time for concentration determination. In order to simulate the high glucose environment in diabetic patients, glucose solution (4 g L⁻¹) was added to the mixture and incubated at 37°C for another 48 h. The residual amounts of samples were determined at 0 h and 48 h after adding glucose. The residual amount of insulin and PEGylated insulin were measured using ELISA kits. In addition, CD spectras of Ins/PBS, PEG-Ins/PBS, Ins/PN-nChaps and PEG-Ins/PN-nChaps after incubation with PK were determined, and the secondary structure content of each sample was analyzed by CDpro.

5. ThT Assay

Thioflavin T (ThT) is a fluorescent dye commonly employed to monitor protein aggregation, especially amyloid fibril formation. When ThT binds to amyloid fibrils, it exhibits enhanced fluorescence, allowing for the quantification and observation of aggregation kinetics. In this experiment, ThT was dissolved in 1x PBS (10 mM, pH 7.4) to prepare a working solution with a final concentration of 10 µM. Insulin and PEGylated insulin, both with and without PN-nChap, were incubated at 37°C under constant stirring at 700 rpm. At predetermined intervals, 50 µL of the incubated solution was sampled and mixed with 450 µL of the ThT working solution. The fluorescence intensity of ThT was then measured using a spectrophotometer, with an emission wavelength at 485 nm and an excitation wavelength at 440 nm. Both the excitation and emission slit widths were adjusted to 5 nm to ensure optimal signal detection. This method enables real-time monitoring of insulin aggregation under different conditions.

6. Animals

BALB/c male mice (19-22 g) were purchased from YiShengYuan Biotechnology Co., Ltd. (Tianjin, China). Streptozotocin (STZ)-induced diabetic rat (Sprague Dawley, male, 290–330 g) and streptozotocin (STZ)-induced diabetic mice (BALB/c, male, 15–19 g) were obtained

from Tianjin Key Laboratory of Radiation Medicine and Molecular Nuclear Medicine. All animal experiments were performed in the context of the animal protocol approved by the Animal Ethics Committee of Nankai University (Tianjin, China) and under the Guidelines for Care and Use of Laboratory Animals of Nankai University.

7. Biological Activity of PEGylated Insulin

BALB/c male mice (19-22 g) were randomly divided into 2 groups ($n = 5$ in each group). Then, they were treated with insulin (Ins/PBS) and PEGylated insulin (PEG-Ins/PBS) (a single dose of 2 nmol kg^{-1}) by tail vein injection, respectively. Blood samples were obtained at the scheduled time (0 h, 0.25 h, 0.5 h, 0.75 h, 1h, 1.5 h, 2 h and 3 h), and glucose levels were measured using a commercially available glucose meter. The blood glucose level (BGLs), the area under curve (AUC), the nadir values (C_{nadir}) and the time to reach the nadir values (T_{nadir}) were calculated by the average experimental data. The relative biological activity of PEGylated insulin (PEG-Ins/PBS) was determined by comparing its AUC Value to that of the same dose of unmodified insulin.

8. Pharmacokinetic Study

Streptozotocin (STZ)-induced diabetic rat (Sprague Dawley, male, 290–330 g) were used as Type 1 Diabetic animal model and randomly divided into four groups. Five rats for each group were selected for subcutaneously injected with free insulin (Ins/PBS), PEGylated insulin (PEG-Ins/PBS), insulin-loaded nanochaperones (Ins/PN-nChaps), and PEGylated insulin-loaded nanochaperones (PEG-Ins/PN-nChaps), with a single dose of 133 nmol kg^{-1} for each rat. Blood samples collected from the orbital venous plexus were placed into 1.5mL centrifuge tubes containing heparin sodium at predetermined time points and centrifuged (5000 rpm, 4°C , 10 min). The concentration of insulin and PEG insulin in plasma were determine by insulin ELISA kit.

9. In Vivo Studies Using STZ-Induced Type 1 Diabetic Mice

For diabetes treatment, streptozotocin (STZ)-induced diabetic mice (BALB/c, male, 15-19 g) were randomly divided into five groups (n = 5 in each group) and subcutaneously injected with PBS, Ins/PBS, PEG-Ins/PBS, Ins/PN-nChaps, and PEG-Ins/PN-nChaps. The blood glucose level (BGLs) was measured from tail vein blood samples using a glucose meter at different time points. The intraperitoneal glucose tolerance test (IPGTT) was performed to confirm the in vivo glucose responsiveness of PEG-Ins/PN-nChaps. Four groups of diabetic mice (n = 5 in each group) were subcutaneously injected with Ins/PBS, PEG-Ins/PBS, Ins/PN-nChaps, and PEG-Ins/PN-nChaps (Dosage: 133 nmol of drug/kg body mass). One hour later, glucose solution (Dosage: 1.5g of glucose/kg body mass.) was required for intraperitoneal injection, and BGLs of tail vein blood samples were monitored using a glucose meter. Glucose solution was injected again when the blood sugar level returned to normal. For hypoglycemia risk assessment, normal mice were subcutaneously injected with PBS, PEG-Ins/PBS and PEG-Ins/PN-nChaps, respectively. Blood samples from tail vein was collected and BGLs were monitored at predetermined time points. Healthy mice (BALB/c, male, 15-19 g) were used as Normal Control group without treatment.

10. Antibody Titer Test

Diabetic mice were given long-term treatment with Ins/PBS, PEG-Ins/PBS, Ins/PN-nChaps and PEG-Ins/PN-nChaps for three weeks with subcutaneous injection every three days. Blood samples collected from the orbital venous plexus was centrifuged after long-term treatment. Supernatants were taken for antibody titer test. Briefly, antigen coating of insulin and PEGylated insulin were performed on 96-well plates, respectively. Subsequently, 50 μ L of plasma sample was added to each well and incubated at 37°C for 2 h. After washing with PBST, HRP-labeled Goat Anti-Mouse IgG was added and incubated for 1 h at room temperature, then a mixture of 100 μ L TMB I and TMB II (volume ratio 1:1) was added and incubated for 20 min. The absorbance at 450 nm was measured using the spark multimode microplate reader.

Healthy mice and diabetic mice without treatment were treated as Normal Control and Model Control.

11. Statistical Analysis

All data were presented as mean \pm s.d. Statistical analysis was performed by using the one-way ANOVA when more than two groups were compared, multiple comparisons were performed using Tukey's test. All statistical analyses were performed with GraphPad Prism. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Table S1. Composition, size and zeta potential of the nanochaperones.

Sample code	Components (wt%)			D_h (nm)/ PDI	Zeta potential (mV)
	PCL- <i>b</i> -PEG	PCL- <i>b</i> -P(Asp- <i>co</i> -AspPBA)	PCL- <i>b</i> -P(Asp- <i>co</i> -AspNTA)		
CRT-nChap	100	0	0	91.5 / 0.203	-1.7 ± 0.6
PN-nChap	25	50	25	123 / 0.187	-4.8 ± 1.7

Table S2. In vivo bioactivity of insulin and PEGylated insulin. And the blood glucose level (BGLs), the area under curve (AUC), the nadir values (C_{nadir}) and the time to reach the nadir values (T_{nadir}) were calculated by the average experimental data, n=5.

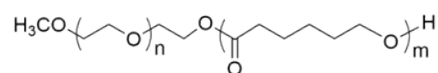
Sample code	T_{nadir} (h)	$C_{nadir} \pm SD$ (g L ⁻¹)	AUC (g h L ⁻¹)	Bioactivity ^a
Insulin	0.5	0.52 ± 0.03	1.02	100
PEGylated insulin	0.5	0.66 ± 0.03	1.15	88

^a Calculated relative to insulin**Table S3.** Pharmacokinetic parameters of insulin and PEGylated insulin in SD rats after subcutaneous injection of Ins/PBS, PEG-Ins/PBS, Ins/PN-nChaps, and PEG-Ins/PN-nChaps.

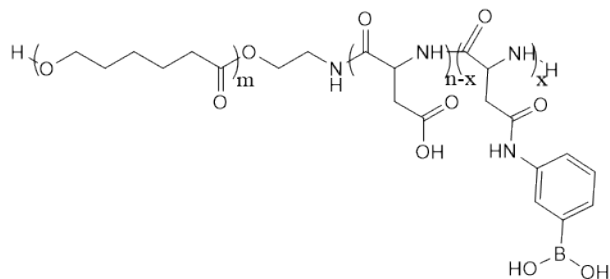
Sample code	$t^{1/2}$ (h) ^a	AUC _(0-inf) (ng/mL*h) ^b	CL[(nmol/kg)/(ng/mL)/h] ^c	MRT (h) ^d
Ins/PBS	2.14±0.45	331.56±49.61	0.41±0.066	2.71±0.33
PEG-Ins/PBS	8.05±0.21	838.47±37.62	0.16±0.0071	8.94±0.22
Ins/PN-nChaps	9.17±1.73	1699.18±362.53	0.081±0.019	14.10±2.01
PEG-Ins/PN-nChaps	18.66±1.13	3810.56±337.42	0.035±0.0029	27.44±2.49

^a $t^{1/2}$: Half-life of insulin and PEGylated insulin^bAUC: area under the plasma concentration–time curve^cCL: Clearance^dMRT: Mean residence time.

PCL-*b*-PEG



PCL-*b*-P(Asp-co-AspPBA)



PCL-*b*-P(Asp-co-AspNTA)

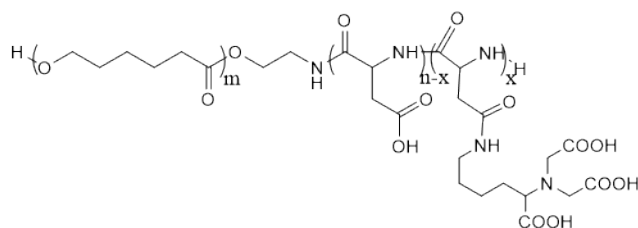


Figure S1. The chemical structures of PCL-*b*-PEG, PCL-*b*-P(Asp-*co*-AspPBA) and PCL-*b*-P(Asp-*co*-AspNTA).

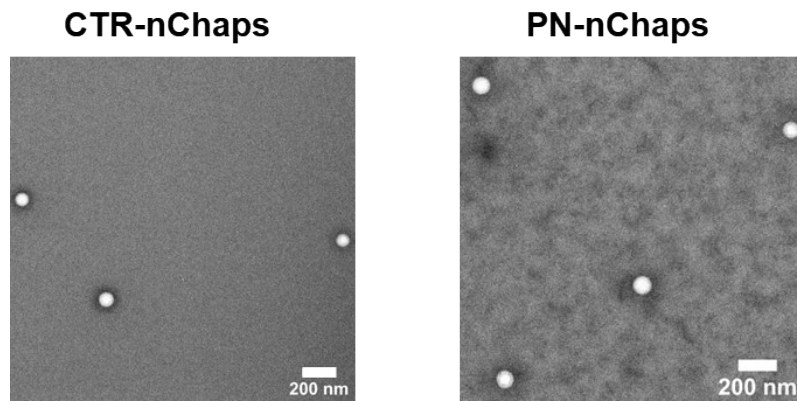


Figure S2. TEM images of CTR-nChaps and PN-nChaps.

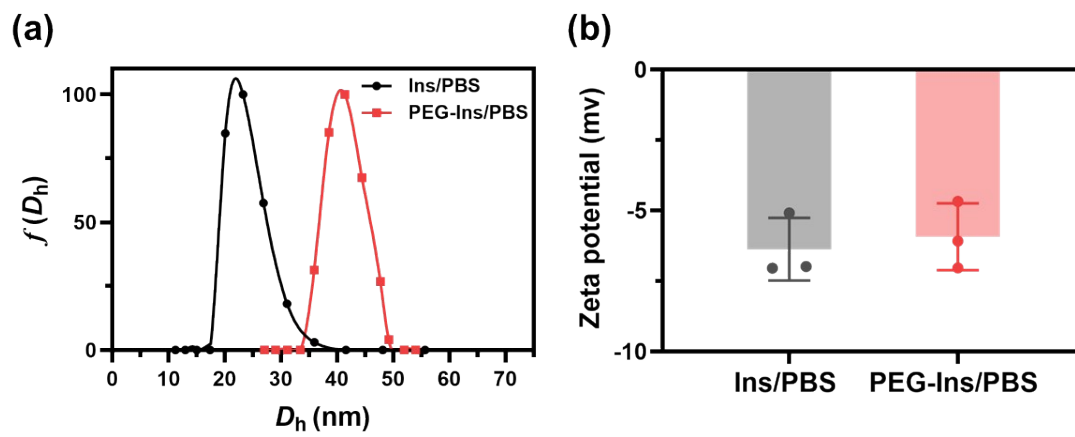


Figure S3. DLS (a) and zeta potential (b) of insulin and PEGylated insulin.

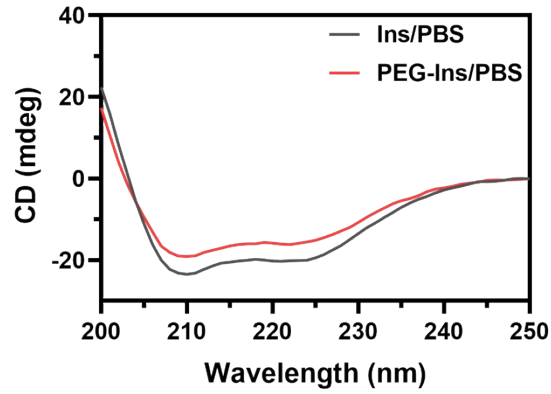


Figure S4. Circular dichroism (CD) spectra of insulin and PEGylated insulin in 1x PBS (pH=7.4) buffer solution.

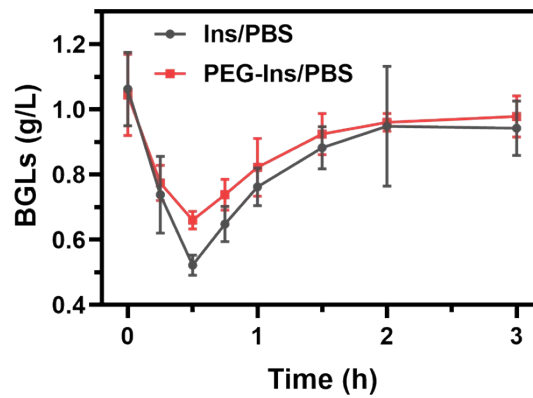


Figure S5. The glucose levels in normal mice (n = 5 in each group) after a single intravenous injection dose of insulin or PEGylated insulin.

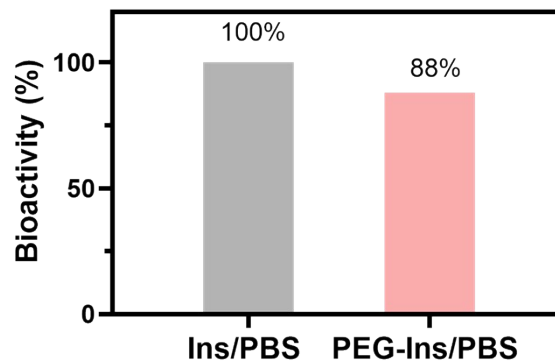


Figure S6. Bioactivity of PEGylated insulin relative to natural insulin.

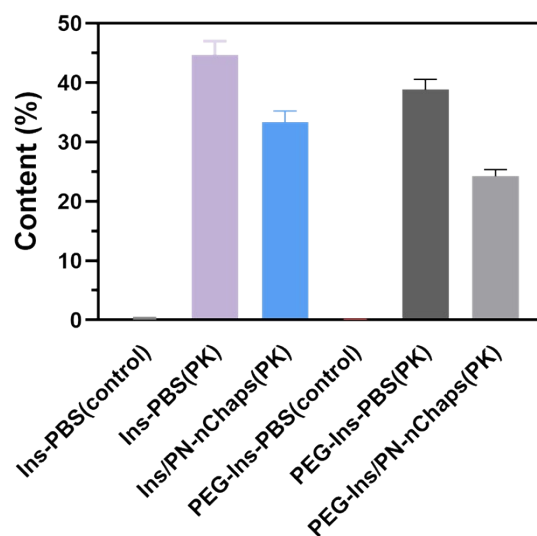


Figure S7. The random coil content of Ins/PBS, PEG-Ins/PBS, Ins/PN-nChaps, and PEG-Ins/PN-nChaps after incubation with PK in glucose solution for 48 h, free insulin and free PEGylation insulin without PK treatment were used as control.

References

1 Y. Zhang, C. Li, X. Wu, F. Deng, F. Huang, Y. Zhang, J. Liu, H. Gui, R. Ma and L. Shi, *Chem. Eng. J.*, 2022, 435,134866.