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ORIGINAL ARTICLE

Di-PEGylated insulin: A long-acting insulin conjugate with superior safety in reducing hypoglycemic events

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Abstract Although the discovery of insulin 100 years ago revolutionized the treatment of diabetes, its therapeutic potential is compromised by its short half-life and narrow therapeutic index. Current long-acting insulin analogs, such as insulin-polymer conjugates, are mainly used to improve pharmacokinetics by reducing renal clearance. However, these conjugates are synthesized without sacrificing the bioactivity of insulin, thus retaining the narrow therapeutic index of native insulin, and exceeding the efficacious dose still leads to hypoglycemia. Here, we report a kind of di-PEGylated insulin that can simultaneously reduce renal clearance and receptor-mediated clearance. By impairing the binding affinity to the receptor and the activation of the receptor, di-PEGylated insulin not only further prolongs the half-life of insulin compared to classical mono-PEGylated insulin but most importantly, increases its maximum tolerated dose 10-fold. The target of long-term glycemic management *in vivo* has been achieved through improved pharmacokinetics and a high dose. This work represents an essential step towards long-acting insulin medication with superior safety in reducing hypoglycemic events.

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1. Introduction

As the most classical drug for glycemic management, insulin drugs are taken regularly by over 9 million type I diabetics and 60 million type II diabetics worldwide. However, two inherent liabilities (*i.e.*, short half-life and narrow therapeutic index) limit its full therapeutic potential[1]. Similar to many other protein drugs, insulin has a very short blood half-life, *ca.* 9 min[2]. To achieve blood glucose targets, diabetics usually need to inject insulin several times a day, which seriously affects their quality of life. What's worse, the narrow therapeutic index of insulin implies that overdosing with insulin may lead to serious side effects, namely hypoglycemia in diabetes. Symptoms of hypoglycemia include dizziness, shakiness, and confusion, which in the most extreme cases can cause life-threatening hypoglycemic coma[3]. Repeated hypoglycemic episodes are known to increase the risk of cerebrovascular disease and ultimately impair cognitive function[4]. Needle phobia and fear of hypoglycemia often lead to inadequate insulin administration and consequently poor management of blood glucose, which ends up with serious complications, such as cardiovascular disease, diabetic foot, and diabetic nephropathy[5].

With advances in protein engineering technology, increasingly long-acting insulin analogs have been appeared on the market or in clinical trials, in which conjugation with polymer has been proven to be an efficient pathway to extend the half-life of insulin by decreasing renal clearance[6,7]. In this respect, polyethylene glycol (PEG) remains the most used polymer for protein conjugation due to its hydrophilicity, biocompatibility, and demonstrated synthetic process[8]. To avoid sacrificing the bioactivity of insulin, PEG is usually mono-modified at the Phe B1 or Lys B29 position[9]. Thus, PEGylated insulin binds normally to the insulin receptor as native insulin. Though PEGylated insulin has demonstrated moderately improved pharmacokinetics, it has no benefit on the therapeutic index of insulin and overdose can still cause hypoglycemia. Diabetics still need to frequently self-monitor their blood glucose and promptly adjust the dose of insulin to avoid the incidence of hypoglycemia. Hence, long-acting insulin drugs with an improved therapeutic index are highly desirable for diabetics.

Apart from Phe B1 and Lys B29, there is another residual amino group on insulin, namely, Gly A1. Upon binding to the insulin receptor, the direct interaction of insulin with the first repeat domain (L1) of the insulin receptor is relatively sparse and the contact part of insulin is restricted to insulin B-chain residues. However, insulin engages the insulin receptor carboxy-terminal α-chain (αCT) segment, resulting in its remodeling on the face of L1 and the conformational switch of the insulin receptor. Specifically, the two most pivotal residues involving the bind interaction in αCT are (1) His 710, which plugs into a pocket formed by the residues Val A3, Gly B8, Ser B9 and Val B12 of insulin; and (2) Phe 714, which occupies a hydrophobic crevice formed by the residues Gly A1, Ile A2, Tyr A19, Leu B11, Val B12 and Leu B15 of insulin[10]. Thus, modification of Gly A1 has been proven to significantly compromise the blood glucose-lowering activity *in vivo* of insulin due to its reducing binding affinity for insulin receptor[11,12]. However, Gly A1 insulin-polymer (*e.g.*, PEG and trehalose glycopolymer) conjugates were not regarded as a good candidate for diabetes treatment in the minds of chemists, who usually insisted that modification should not have a deleterious effect on the activity of drug[13–15]. Except for renal clearance, insulin is also eliminated by the receptor-mediated clearance pathway[16] (Scheme 1A). Thus, we hypothesized that di-PEGylation of insulin (Scheme 1B) at both Gly A1 and Lys B29 could further prolong its half-life by simultaneously decreasing renal clearance and receptor-mediated clearance compared to mono-PEGylation (Scheme 1C). Furthermore, the therapeutic index of insulin may be greatly enlarged due to the reduced binding affinity between di-PEGylated insulin and insulin receptor, and its *in vivo* glycemia-lowering efficacy could be compensated by long circulation time and high dose. To test this hypothesis, two PEG chains were grafted to both Gly A1 and Lys B29 positions of insulin by precisely manipulating reaction conditions. *In vitro* activity results showed that di-PEGylated insulin exhibited significantly lower receptor binding affinity and activation efficacy compared to native insulin and Lys B29 mono-PEGylated insulin. *In vivo* studies demonstrated that di-PEGylated insulin completely prevented the incidence of hypoglycemia in healthy mice at a high dose of 10 U/kg, while a majority of mice treated with native insulin or mono-PEGylated insulin at this dose died from hypoglycemia. Di-PEGylated insulin exhibited more sustained glycemia management performance with negligible hypoglycemia in diabetic mice compared to native insulin and mono-PEGylated insulin, while long-term administration could alleviate a common diabetes complication—diabetic nephropathy. Overall, these results demonstrate the potential of di-PEGylated insulin as a long-acting insulin drug for treating diabetes with improved safety in reducing the risk of hypoglycemia.

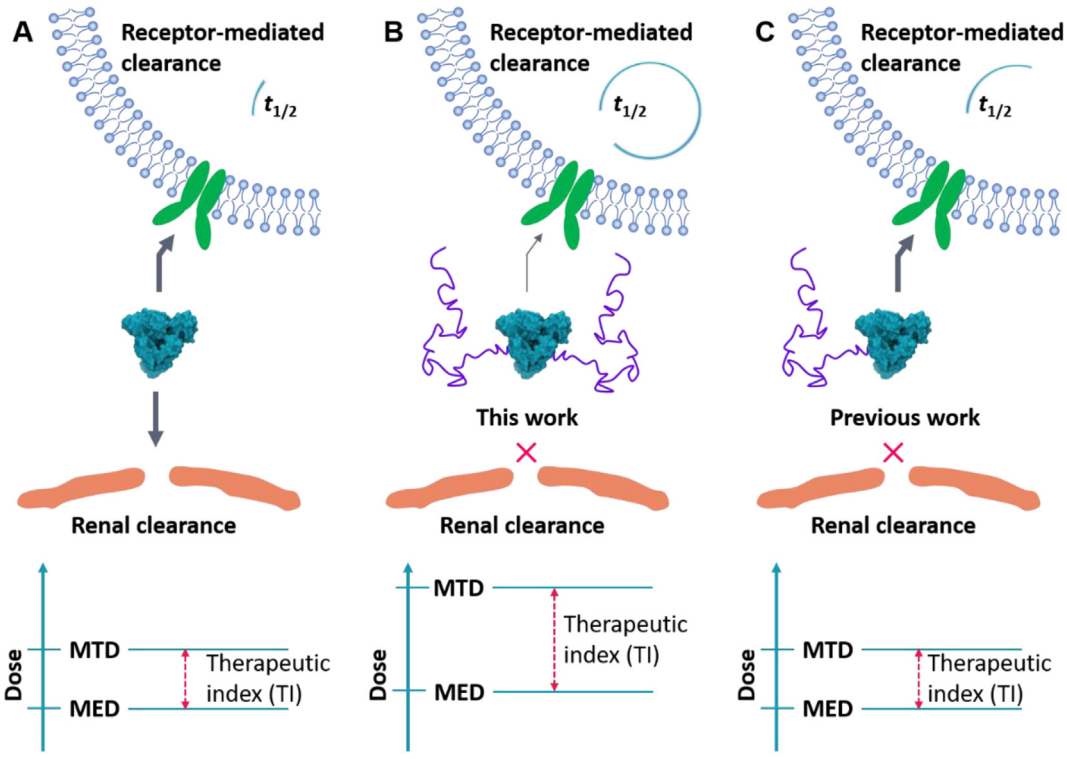
2. Materials and methods

2.1. Materials

Recombinant human insulin (26 U/mg) was obtained from Tonghua Dongbao Pharmaceutical Co., Ltd. (Tonghua, China). Methoxy PEG succinimidyl carboxymethyl ester (PEG-NHS, MW = 5000) was obtained from JenKem Technology (Beijing, China). Sulfo-cyanine7 NHS ester (Cy7-NHS) was obtained from Lumiprobe Corporation (Maryland, USA). Dithiothreitol (DTT) and streptozotocin were obtained from Sigma−Aldrich (St. Louis, MO, USA). BCA assay kit was obtained from Thermal Fisher Scientific (Waltham, USA). Phospho-AKT (Ser473) cellular HTRF kit was obtained from Cisbio (Bedford, USA). C2C12 cell was obtained from Procell Life Science & Technology Co., Ltd. (Wuhan, China). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) with high glucose, penicillin, and streptomycin were obtained from Gibco (New York, USA). The insulin ELISA kit and Iso-insulin ELISA kit were obtained from Mercodia (Uppsala, Sweden).

2.2. Synthesis of mono-PEGylated insulin and di-PEGylated insulin

PEGylated insulin was synthesized using a stepwise-feeding approach (Supporting Information Fig. S1). Briefly, insulin (58 mg) was dissolved in a mixed solvent of DMSO (0.5 mL) and carbonate-bicarbonate buffer (0.1 mol/L, pH = 10, 3.5 mL). Then one equivalent of PEG-NHS (pre-dissolved in DMSO) was added to the insulin solution every 15 min, 6 times in total. At 90 min, this reaction was terminated by the addition of glycine (10 mg/mL, 0.35 mL). After dialysis and freeze-drying, the crude product was purified using an AKTA protein purification system equipped with a HiTrap SP HP cation ion exchange column. Specifically, crude PEGylated insulin product was



Scheme 1 Schematic illustration of di-PEGylated insulin improving therapeutic index and pharmacokinetics. (A) Two clearance pathways of native insulin. (B) Di -PEGylated insulin reduces both renal clearance and receptor-mediated clearance, resulting in improved half-life and therapeutic index of insulin. (C) Classical mono-PEGylated insulin reduces renal clearance with only improving half-life of insulin. MTD: maximum tolerated dose, MED: minimum effective dose.

dissolved in acetate buffer (10 mmol/L, pH 4.0) and loaded on the column. Then the column was eluted with acetate buffer with a NaCl gradient (0−0.25 mol/L) over 100 min. The target products were collected when appropriate *via* continuous monitoring of the eluate with a UV detector (280 nm). After dialysis, the purified product was freeze-dried and stored at −80 °C. The yield of di-PEGylated insulin was about 30% based on the weight of freeze-drying powder.

2.3. *Native polyacrylamide gel electrophoresis (PAGE)*

After mixing with native-PGAE loading buffer, different insulin samples (native insulin, unpurified PEGylated insulin mixture, mono-PEGylated insulin, and di-PEGylated insulin) were loaded onto a polyacrylamide gel (5% acrylamide for stacking gel and 15% acrylamide for resolving gel). Then samples were running at 90 V for 15 min and 150 V for 90 min, respectively. The running buffer was the mixture solution of 25 mmol/L Tris and 0.2 mol/L glycine. Coomassie blue R-250 was used as the stain.

2.4. *MALDI−TOF mass spectrometry*

Matrix-assisted laser desorption ionization−time of flight mass spectrometry (MALDI−TOF-MS, Bruker UltrafleXtreme) was used to analyze the molecular weight of insulin, mono-PEGylated insulin, and di-PEGylated insulin. Specifically, insulin, mono-PEGylated insulin, and di-PEGylated insulin with/without DTT treatment were mixed 1:1 with the matrix (cyano-4-hydroxycinnamic acid) in Eppendorf tubes and applied to the MALDI−TOF sample plate. The acceleration voltage was held at 19 kV and the mass spectra were averaged from approximately 300 laser shots.

2.5. *Size exclusion chromatography (SEC)*

The molecular weight of insulin, mono-PEGylated insulin, and di-PEGylated insulin were analyzed on an Agilent 1260 Infinity II HPLC/GPC system equipped with a PL aquagel-OH column and a refractive index detector. The mixture solution (pH 2.3) of acetic acid (200 mL), acetonitrile (300 mL), and water (500 mL) were used as the mobile phase. The column temperature and the flow rate were set as 30 °C and 0.5 mL/min, respectively.

2.6. *N-terminal Edman sequencing*

Insulin, mono-PEGylated insulin, and di-PEGylated insulin solutions were dropped onto a PVDF membrane and the PVDF was then placed in the reactor of a protein sequencer (PPSQ-33A, Shimadzu). Data was processed using the PPSQ-30 DataProcessing software.

2.7. *Dynamic light scattering (DLS)*

The hydrodynamic size of insulin, mono-PEGylated insulin, and di-PEGylated insulin was measured using ZetaSizer Nano-ZS (Malvern Instruments, UK). The results are presented as the average of three repeated measurements.

2.8. Circular dichroism (CD)

Insulin, mono-PEGylated insulin, and di-PEGylated insulin were dissolved in PBS. J-810 spectropolarimeter (JASCO, France) was used to obtain their CD spectra. The scanned wavelength range was from 180 to 260 nm and the light-path length was 1 mm. Three measurements were performed on each sample and the results were presented as average. CDNN software was used to analyze the secondary structure (*e.g.*, α-helix, β-sheet) proportions.

2.9. In vitro activity test

A cell-based insulin receptor activation assay was used to detect the effect of mono/di-PEGylation on insulin activity. In brief, C2C12 cells were cultured in DMEM with 10% FBS, 1% penicillin and streptomycin (5% CO_2, 37 °C). After seeding in a 96-well plate with a density of 5000 cells/well and culturing overnight, followed by a starvation process with serum-free medium for 4 h. Starved cells were treated with a series of concentrations of insulin, mono-PEGylated insulin, and di-PEGylated insulin for 30 min. Then phosphorylated AKT at ser473 was detected by a phospho-AKT (Ser473) cellular HTRF kit after cell lysis according to the manufacturer's protocol. The fluorescence emission at 665 and 620 nm (excitation: 330 nm) were recorded using a Microplate reader (Synergy H1, BioTek). EC50 values of insulin, mono-PEGylated insulin, and di-PEGylated insulin were determined by GraphPad Prism software 7.0 after curve fitting with dose-responsive stimulation (four parameters) mode.

2.10. Hypoglycemia effect

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-sen University. Male C57BL/6 mice (8 weeks) were purchased from the Guangdong Medical Laboratory Animal Center. Fasted healthy C57BL/6 mice received a series of doses of insulin, mono-PEGylated insulin, and di-PEGylated insulin injection. Mouse blood glucose was measured using a ONETOUCH UltraVue glucose meter (Johnson Medical Ltd., Shanghai, China) from the tail vein at different time points.

2.11. Animal mode of type 1 diabetes

Type 1 diabetic animal model was induced by streptozotocin (STZ) according to the published method[17]. Briefly, C57BL/6 mice received STZ solution (0.1 mol/L citrate buffer, pH 4.5) intraperitoneal injection at a dose of 50 mg/kg for 5 consecutive days. Two weeks after the first injection, mouse body weight and fasted blood glucose were monitored. Only those mice with blood glucose higher than 16.7 mmol/L were used in the following experiments.

2.12. Glycemic control

Fasted diabetic mice were randomly divided into 4 groups with 6 mice in each group. Then 5 group of mice received an s.c. injection of PBS, insulin (5 U/kg), mono-PEGylated insulin (5 U/kg), and di-PEGylated insulin (5 and 15 U/kg), respectively. Blood glucose variation was monitored using a glucose meter from the tail vein at predetermined time points. At 4.5, 7.5, and 10.5 h, mice were intraperitoneally injected with glucose at a dose of 2 g/kg.

2.13. Pharmacokinetics

Blood samples were collected from the retro-orbital plexus under anesthesia at different time points after insulin or PEGylated insulin injection. After coagulation, blood samples were centrifuged at 3500 rpm (Eppendorf Centrifuge 5425) for 10 min to collect serum. Then the concentrations of mono-PEGylated insulin and di-PEGylated insulin for diabetic mice and insulin for healthy mice were determined using an Iso-insulin ELISA Kit and an insulin ELISA Kit, respectively.

2.14. Absorption and urine excretion

To study the absorption and urine excretion, insulin, mono-PEGylated insulin, and di-PEGylated insulin were labeled with Cy7 following the previously reported method[18]. After purification with PD-10 desalting column, Cy7-labeled insulin, mono-PEGylated insulin, and di-PEGylated insulin were s.c. injected into mice at a Cy7 dose of 0.1 mg/kg. The concentration for Cy7-labeled insulin, mono-PEGylated insulin, and di-PEGylated insulin solution before injection was 19, 19.2, and 19.6 μg/mL, respectively. Mice were imaged using Small Animal In Vivo Imaging System (IVIS Lumina X5, PerkinElmer) at needed time points. Meanwhile, urine samples were collected at needed time points and imaged by Small Animal In Vivo Imaging System.

2.15. In vitro stability

Insulin, mono-PEGylated insulin, and di-PEGylated insulin were dissolved in PBS at an insulin concentration of 1 mg/mL, wherein insulin was solubilized with dilute HCl and adjusted back to neutral with NaOH solution. Insulin, mono-PEGylated insulin, and di-PEGylated insulin solution were shaken at a speed of 100 rpm (ZHTY-505, Zhichu shaking incubator) at 37 °C. Absorbance at 540 nm was measured at needed time points and the whole procedure lasted 80 h. Insulin aggregation induced light scattering, resulting in a transmittance decrease of solution.

2.16. Long-term efficacy

To assess the long-term therapeutic efficacy of di-PEGylated insulin, diabetic mice were s.c. administrated with PBS, insulin, and di-PEGylated insulin every day. The dose of insulin and di-PEGylated insulin was 1 and 3 mg/kg, respectively. During the whole 2-week treatment, water consumption was recorded daily. On Days 7 and 14, blood samples were withdrawn and separated serum was used to analyze the level of glycated serum protein (A037-1), CREA (C011-2-1), and BUN (C013-2-1). All the assay kits were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). On Day 14, mouse kidneys of all three groups were harvested for H&E staining, PAS, and Masson staining.

2.17. Biosafety

Healthy C57 mice were randomly assigned to three groups and received an injection of PBS, insulin (1 mg/kg), and di-PEGylated insulin (3 mg/kg), respectively. The frequency of injection was once a day. Mouse body weight was recorded every 2 days. Moreover, blood samples were collected after two weeks, and blood cell counts were analyzed using an auto hematology analyzer (BC-2800 Vet). Serum biochemical indicators (*e.g.*, ALT,

AST, ALP, and ALB) were analyzed using an automated clinical chemistry analyzer (Chemray 800). The major organ and skin surrounding the injection site (5 mm in diameter) were collected for tissue sections.

2.18. *Statistical analysis*

All data are presented as average ± standard deviation (SD). Data statistical significance was analyzed by one-way ANOVA with Turkey/Dunnett's multiple comparisons test (GraphPad Prism software 7.0). Differences were regarded as significant when the *P* value was less than 0.05.

3. Results and discussion

3.1. *Synthesis and characterization of di-PEGylated insulin*

In known methods, PEG was usually only modified at the Phe B1 or Lys B29 position to avoid sacrificing the activity of insulin[19]. For example, in the reaction of synthesis of mono-PEGylated insulin (modified at Lys B29) reported by Hinds et al.[19], only equimolar or slightly excess activated PEG (*e.g.*, PEG-NHS) with insulin was used to ensure the specificity of the modification. However, in this work, di-PEGylated insulin was synthesized using a stepwise-feeding method (Fig. S1), in which the molecular weight of the PEG-NHS used was 5000. As shown in the native PAGE image (Fig. 1A), two separated bands with high molecular weight appeared after the reaction. Meanwhile, the band corresponding to native insulin disappeared, implying that the reaction efficiency of insulin was nearly 100%. The PEGylated insulin mixture was separated into two pure substances using ion exchange chromatography (Supporting Information Fig. S2). MALDI−TOF mass spectroscopy (Fig. 1B) was further used to demonstrate their molecular weight. The peak distribution centered at 10,860 and 15,786 (*m*/*z*) confirmed that insulin was conjugated with one PEG chain and two PEG chains, respectively. The peak interval (44 Da) matched well with the molecular weight of ethylene glycol repeated units (Supporting Information Fig. S3). Moreover, we used dithiothreitol (DTT) to reduce the disulfide bridges of insulin. As shown in Supporting Information Fig. S4, two peaks corresponding to A chain (2,384, *m*/*z*) and B Chain (3,431, *m*/*z*) were observed in the spectra of native insulin after DTT treatment. For mono-PEGylated insulin, the peak corresponding to A chain was retained. However, a new Gaussian distributed peak centered at 8525 (*m*/*z*) matched well with the molecular weight sum of insulin B chain and one PEG. For di-PEGylated insulin, the peak corresponding to A chain also disappeared. Meanwhile, another Gaussian distributed peak centered at 7379 (*m*/*z*) close to the peak 8525 (*m*/*z*) was released, implying that PEG was also conjugated to insulin A chain. Size exclusion chromatography (SEC) analysis (Fig. 1C) showed that di-PEGylated insulin (11.7 min) exhibited a retention time shorter

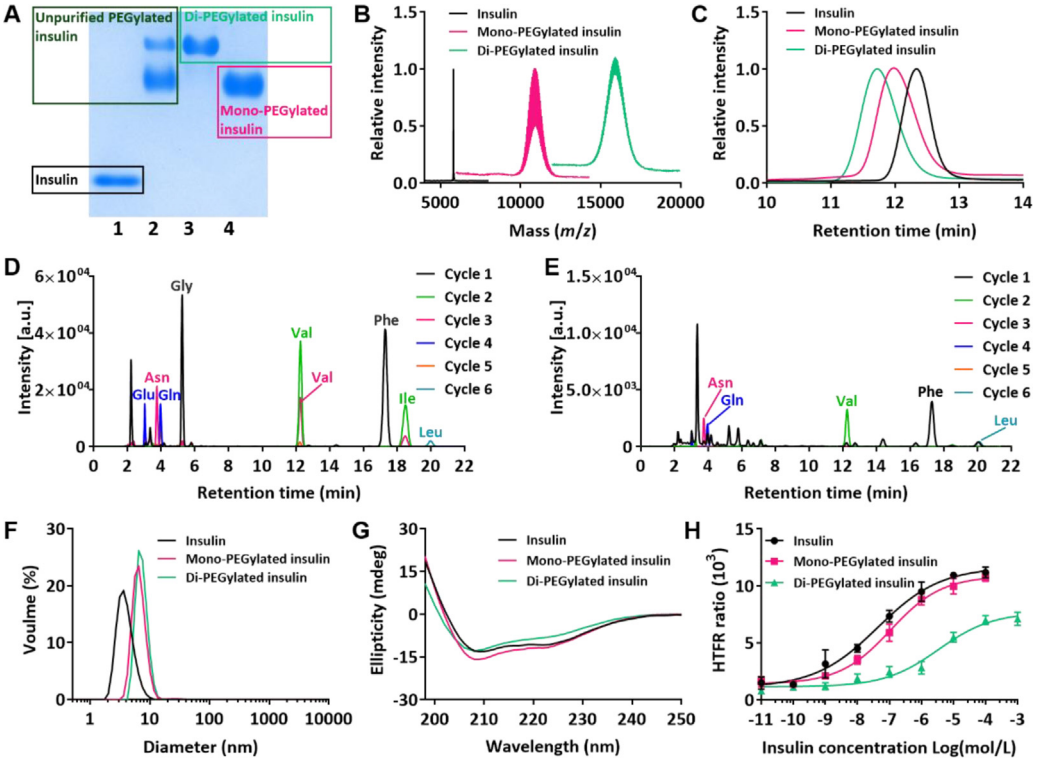


Figure 1 Characterization of di-PEGylated insulin. (A) Coomassie-stained native PAGE (from left to right, lane 1, native insulin; lane 2, crude PEGylated insulin reaction mixture; lane 3, purified di-PEGylated insulin; lane 4, purified mono-PEGylated insulin). (B) MALDI−TOF mass spectra of insulin, mono-PEGylated insulin, and di-PEGylated insulin. (C) SEC traces of insulin, mono-PEGylated insulin, and di-PEGylated insulin. *N*-terminal Edman sequencing chromatograms from the first six cycles of mono-PEGylated insulin (D) and di-PEGylated insulin (E). (F) Hydrodynamic sizes of insulin, mono-PEGylated insulin, and di-PEGylated insulin. (G) Circular dichroism (CD) spectra of insulin, mono-PEGylated insulin, and di-PEGylated insulin. (H) *In vitro* bioactivity of insulin, mono-PEGylated insulin, and di-PEGylated insulin, results are represented as average ± SD ($n = 3$), EC_{50}: 4.3×10^{-8} mol/L for insulin; 1.1×10^{-7} mol/L for mono-PEGylated insulin; 3.4×10^{-6} mol/L for di-PEGylated insulin. E_{max}: 100% for insulin; 95.7% for mono-PEGylated insulin; 63.5% for di-PEGylated insulin.

than mono-PEGylated insulin (12.0 min), which was shorter than native insulin (12.3 min). To precisely identify the PEGylated position, mono-PEGylated insulin and di-PEGylated insulin were characterized by *N*-terminal Edman sequencing. As shown in Fig. 1D and Supporting Information Fig. S6, *N*-terminal amino acids of the A chain and B chain of insulin were both observed for mono-PEGylated insulin. However, only the *N*-terminal amino acids of the B chain were observed for di-PEGylated insulin (Fig. 1E). For example, Gly (A1 amino acid) and Phe (B1 amino acid) were observed in the first cycle of mono-PEGylated insulin, whereas only Phe was observed for di-PEGylated insulin. The same rule applied to the remaining 5 cycles. From the above data, we could deduce that the PEGylated site of mono-PEGylated insulin was Lys B29 and the PEGylated sites of di-PEGylated insulin were Lys B29 and Gly A1. The hydrodynamic sizes of insulin, mono-PEGylated insulin, and di-PEGylated insulin in PBS were 4.1 ± 0.2, 7.5 ± 0.8, and 8.3 ± 1.1 nm, respectively (Fig. 1F). The close size of mono-PEGylated insulin and di-PEGylated insulin ensured that they had similar efficacy to reduce renal clearance. Furthermore, circular dichroism (CD) spectroscopy was used to probe the effect of PEGylation on the secondary structure of insulin. For mono-PEGylated insulin, its secondary structure was similar to native insulin with only slight changes in α-helix structure and in random coil structure (Fig. 1G and Supporting Information Table S1). However, the α-helix and β-turn structure proportion of di-PEGylated insulin was 92% and 8.5%, respectively, which was significantly different from native insulin. This implied that di-PEG conjugation could improve the storage stability of insulin and potentially affect its activity[20]. Then the effect of di-PEGylation on the *in vitro* activity of insulin was assessed by measuring the extent of phosphorylation of Akt protein, *i.e.*, a downstream event in insulin signaling. As shown in Fig. 1H, the half maximal effective concentration (EC_{50}) of mono-PEGylated insulin (1.1×10^{-7} mol/L) increased by 2.5-fold compared to insulin (4.3×10^{-8} mol/L), while the EC_{50} of di-PEGylated insulin (3.4×10^{-6} mol/L) was 2 orders of magnitude higher than insulin. EC_{50} is used as an indicator of drug potency, which is inversely proportional to the binding affinity of the drug to its receptor[21]. The dramatic change in EC_{50} for di-PEGylated insulin indicated that di-PEGylation would greatly decrease the binding affinity of insulin to its receptor. Meanwhile, the minor variation in EC_{50} for mono-PEGylated insulin could be explained by the fact that the steric hindrance of PEG slightly impacts the binding of insulin to its receptor[13]. Besides EC_{50}, E_{max} (*i.e.*, the maximum effect that a drug can produce irrespective of concentration, and is related to the activation of receptor) is also a pivotal parameter in characterizing drug properties[21]. E_{max} of mono-PEGylated insulin and native insulin were at a comparable level, while di-PEGylated insulin showed 63.5% E_{max} of native insulin, implying it significantly impaired the activation of insulin receptor. Overall, the reduced binding affinity of di-PEGylated insulin to receptor and the efficacy of receptor activation would benefit its half-life and therapeutic index *in vivo*[22].

3.2. *Di-PEGylated insulin exhibits wider therapeutic index*

Typically, the therapeutic index is defined as the level of plasma drug between the lowest dose that produces a therapeutic effect and the highest dose that causes unwanted and dangerous side effects[23,24]. For insulin, the narrow therapeutic index makes it a "lousy" drug, overdose-induced hypoglycemia may lead to a coma or even death. To test the difference in therapeutic index between insulin, mono-PEGylated, and di-PEGylated insulin, three groups of healthy mice were subcutaneously (s.c.) administrated with varied doses of insulin, mono-PEGylated and di-PEGylated insulin, respectively, and their blood glucose was monitored at different time (30 min, 1, 2, 3, 4, 5, and 6 h). As shown in Fig. 2A–D, mice treated with escalating doses of di-PEGylated insulin showed a mild change in the level of blood glucose decline. Notably, all mice didn't suffer from hypoglycemia even at

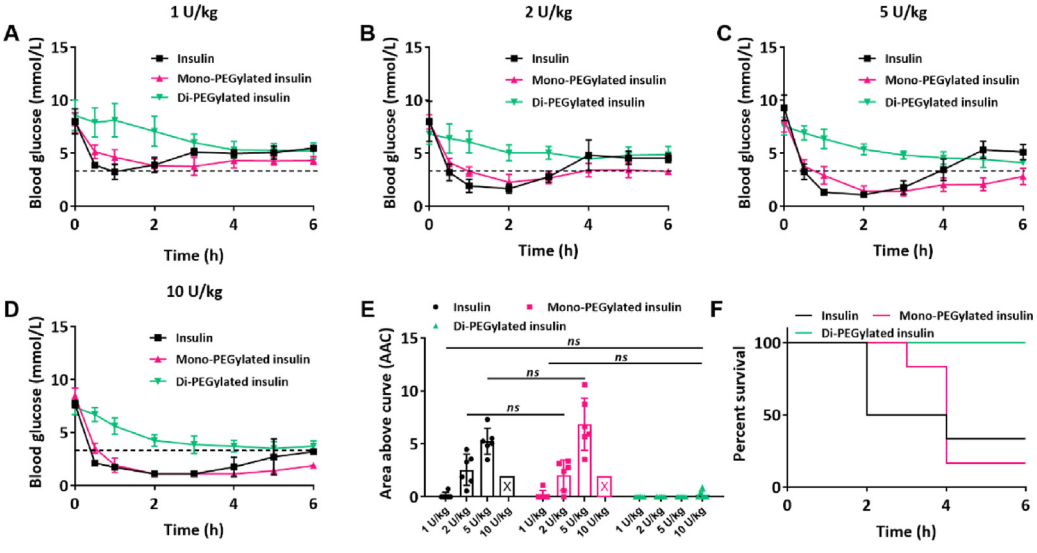


Figure 2 Di-PEGylated insulin improves therapeutic index. Glycemic changes in healthy mice following subcutaneous injection with 1 (A), 2 (B), 5 (C), and 10 U/kg (D) of insulin, mono-PEGylated insulin, and di-PEGylated insulin, results are presented as average ± SD ($n = 6$), only blood glucose values of the surviving mice were recorded at the dose of 10 U/kg, the dotted line was the indicator of hypoglycemia. (E) The area above the glycemic curve and under the hypoglycemia-indicated line was defined as the hypoglycemic effect. (F) Survival percentage of mice treated with insulin, mono-PEGylated insulin, and di-PEGylated insulin at the dose of 10 U/kg. Statistical significance analysis was performed using one-way ANOVA with Tukey's multiple comparisons test, *ns*, not significant.

the high dose of 10 U/kg. However, for insulin and mono-PEGylated insulin, blood glucose-lowering efficacy increased sharply as the dose increased. The mice started to suffer from notable hypoglycemia at the dose of 2 U/kg (Fig. 2B). With further increase in dose, the mice experienced longer and more severe hypoglycemia (Fig. 2C and D). At the dose of 10 U/kg, a majority of mice died from hypoglycemia, 4 in the insulin group and 5 in the mono-PEGylated insulin group (Fig. 2F). The area above curve and under the dotted line (3.3 mmol/L, the indicator of hypoglycemia) was used to quantify the severity of hypoglycemia[18]. As shown in Fig. 2E, the area of 10 U/kg di-PEGylated insulin was comparable to that of 1 U/kg insulin, implying that its maximum tolerated dose was increased tenfold. In contrast, mono-PEGylated insulin didn't show a significant improvement on the maximum tolerated dose, demonstrating the significant difference in the effect of mono-PEGylation and di-PEGylation of insulin on its therapeutic index.

3.3. Di-PEGylated insulin achieves longer duration of glycemic management with better safety

The blood glucose-lowering efficacy of di-PEGylated insulin was evaluated on fasted streptozotocin (STZ)-induced type I diabetic mice. Following s.c. injection with a high dose (15 U/kg) of di-PEGylated insulin, a gradual decrease in blood glucose was observed in mice (Fig. 3A). In contrast, blood glucose of mice treated with insulin or mono-PEGylated insulin dropped quickly to the normoglycemic range within 30 min even at a low dose of 5 U/kg. However, di-PEGylated insulin at the same dose showed a very slow blood glucose-lowering efficacy, with blood glucose dropping to the normoglycemic range after 2 h. Correspondingly, no mice in this group developed hypoglycemia. Even at a high dose (15 U/kg), the glycemic reduction rate of di-PEGylated insulin was slower than insulin or mono-PEGylated insulin during the initial phase. More importantly, only one mouse in di-

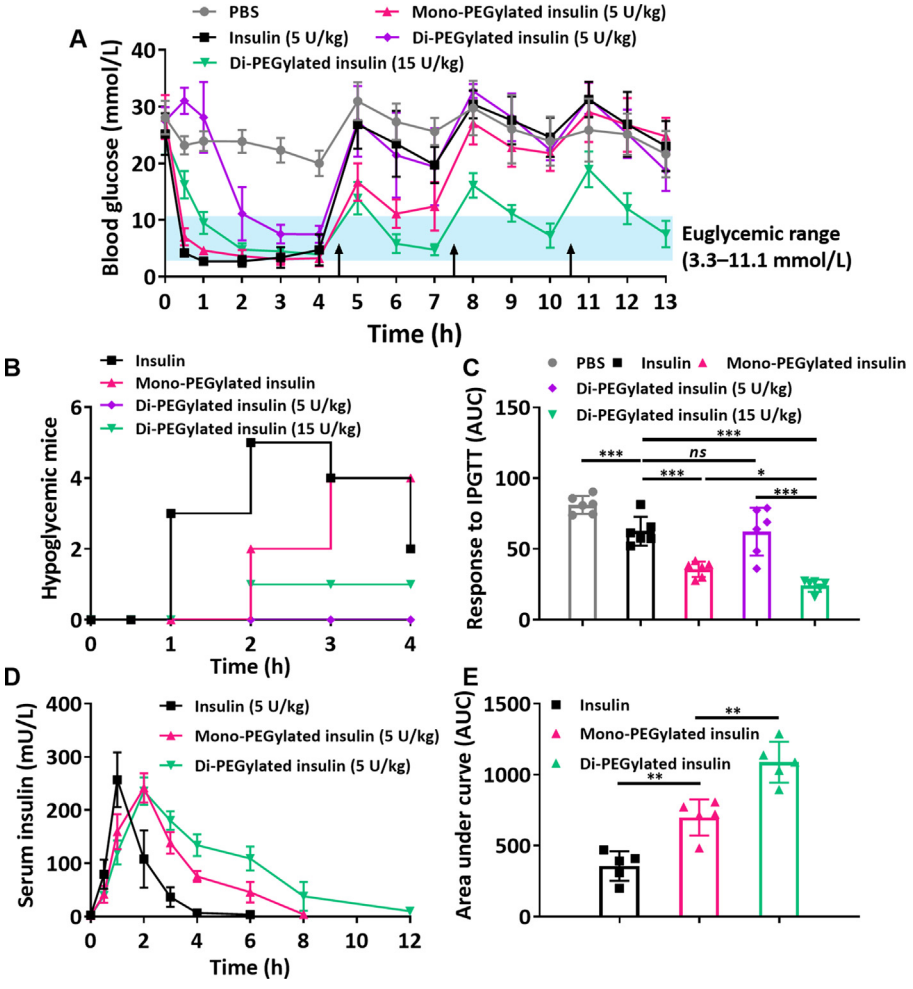


Figure 3 Pharmacodynamics and pharmacokinetics of di-PEGylated insulin. (A) Blood glucose level of STZ-induced diabetic mice following injection with PBS, 5 U/kg insulin, 5 U/kg mono-PEGylated insulin, and di-PEGylated insulin (5 and 15 U/kg). Arrows indicate intraperitoneal glucose tolerance tests (IPGTT) with a glucose dose of 2 g/kg. Data were presented as average ± SD ($n = 6$). (B) The number of mice experiencing hypoglycemia in different groups within 4 h post-injection. (C) The area under curve calculated from the first IPGTT. (D) Serum insulin level of mice following administration of insulin, mono-PEGylated insulin, and di-PEGylated insulin. (E) The quantitative area under curve (AUC) of (D). Data are presented as average ± SD ($n = 5$). Statistical significance analysis was performed using one-way ANOVA with Tukey's multiple comparisons test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

PEGylated insulin (15 U/kg) group experienced mild hypoglycemia (Fig. 3B) and its blood glucose values during the hypoglycemia period, *e.g.*, 3.2 and 3.1 mmol/L, were close to the hypoglycemia threshold (3.3 mmol/L). However, a majority of mice suffered from different degrees of hypoglycemia in the insulin (5 mice) and mono-PEGylated insulin group (4 mice), respectively (Fig. 3B). Moreover, for mice treated with insulin and mono-PEGylated insulin, the blood glucose values of mice suffering from hypoglycemia, *e.g.*, 1.0, 1.5, and 1.8 mmol/L, were much below the hypoglycemia threshold. Typical hypoglycemia symptoms such as hypothermia was also observed in mice treated with insulin and mono-PEGylated insulin.

Subsequently, three intraperitoneal glucose tolerance tests (IPGTT, 2 g/kg) were performed to test the efficacy in responding to a glucose challenge and duration of glycemic control of di-PEGylated insulin. During all three IPGTT periods, di-PEGylated insulin with a high dose of 15 U/kg could regulate blood glucose back to normoglycemia levels following blood glucose rise. However, insulin and di-PEGylated insulin (5 U/kg) didn't show any efficacy in lowering blood glucose even after the first glucose challenge. Mono-PEGylated insulin showed moderate efficacy but failed to lower blood glucose to normoglycemic levels after the first glucose challenge. The enhanced efficacy of di-PEGylated insulin (15 U/kg) compared to native insulin and mono-PEGylated insulin in terms of glycemic control was confirmed by the calculated area under curve (Fig. 3C). In the following two additional IPGTTs, mono-PEGylated insulin didn't exhibit glucose-lowering effects like insulin. A single dose of diPEGylated insulin could manage blood glucose over 13 h, demonstrating its potential as a twice-daily insulin regimen.

To probe the effect of di-PEGylation on the half-life of insulin, the preliminary pharmacokinetics of different insulin samples were examined. As a negative control, the serum concentration of insulin raised sharply to the peak following injection and then was eliminated rapidly, being essentially undetectable at 4 h (Fig. 3D). However, the time to peak concentration was delayed by approximately 1 h for di-PEGylated insulin and mono-PEGylated insulin. They both exhibited prolonged elimination time compared with native insulin, wherein serum concentration was detected even at 12 h for di-PEGylated insulin. The maximum serum drug concentrations of three insulin samples were at a comparable level, but the area under curve of di-PEGylated insulin was significantly greater than that of insulin and mono-PEGylated insulin (Fig. 3D and E). The improved pharmacokinetics of di-PEGylated insulin could be ascribed to the simultaneously reduced renal clearance and receptor-mediated clearance[12].

3.4. *Di-PEGylated insulin exhibits higher in vitro and in vivo stability*

To probe the effect of di-PEGylation on the *in vivo* fate of insulin, preliminary urine metabolism was performed. After labeling with Cy7, insulin, mono-PEGylated insulin, and di-PEGylated insulin were s.c. administrated into mice and their urine samples collected at predetermined time points were analyzed by *in vivo* imaging system (Fig. 4A and B). Specifically, after reaching the maximum at 2 h, fluorescence signals of the urine sample for the insulin group faded rapidly and were essentially close to the background value at 4 h. However, PEGylated insulin showed longer metabolism time. For example, fluorescence signals could be detected even at 12 h for di-PEGylated insulin. This implied that di-PEGylated insulin could attenuate renal insulin receptor-mediated insulin degradation, as the kidney was the main degradation organ for s.c. injected insulin[25]. Meanwhile, the retention of different insulin

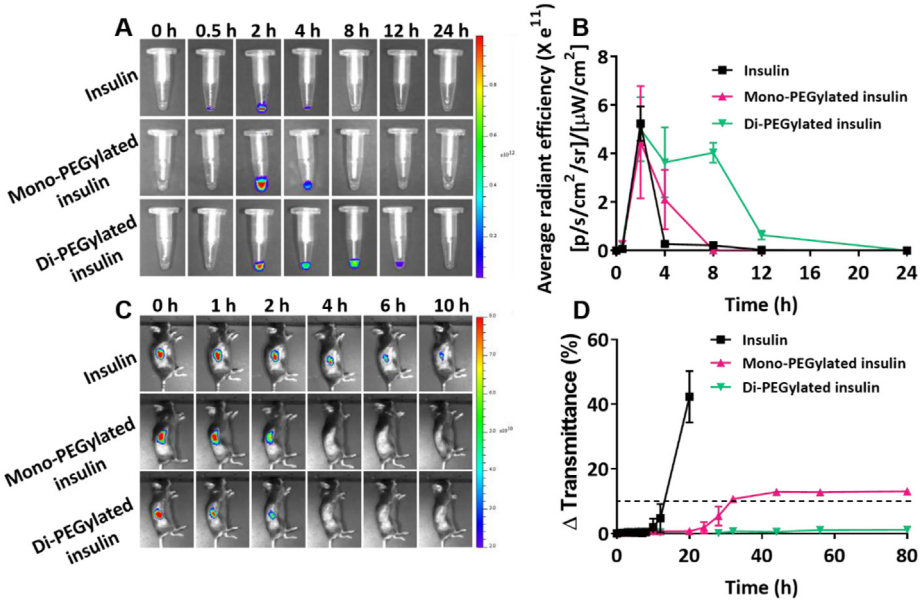


Figure 4 Di-PEGylated insulin exhibits better *in vitro* and *in vivo* stability compared to insulin and mono-PEGylated insulin. (A) Collected urine samples of mice injected with Cy7-labeled insulin, mono-PEGylated insulin, and di-PEGylated insulin and their representative fluorescence images. (B) Fluorescence intensity of urine samples collected at pre-determined time points. Data were presented as average ± SD ($n = 3$). (C) Representative live images of healthy mice following injection with Cy7-labeled insulin, mono-PEGylated insulin, and di-PEGylated insulin. (D) Change in solution transmittance of insulin, mono-PEGylated insulin, and di-PEGylated insulin at pH 7.4 and 37 °C under continuous agitation. Data are presented as average ± SD ($n = 3$).

samples at the injection site was also performed. As shown in Fig. 4C, fluorescence at the injected site disappeared rapidly and was undetectable at 4 h post-injection in both mono-PEGylated insulin and di-PEGylated insulin groups. However, the fluorescence at the injected site of mice that received native insulin injection vanished at a slow rate. The fluorescence retention rates were 50% and 22% at 4 and 10 h post-injection (Supporting Information Fig. S8), respectively. This could be explained by the fact that PEG prevented the aggregation of native insulin at the injection site, which could increase the bioavailability of s.c. insulin medications and avoid the incidence of a pathological condition known as injection amyloidosis[26]. *In vitro* stability test was performed to further demonstrate the effect of di-PEGylation on the stability of insulin. Briefly, a thermal aggregation assay under continuous agitation was used to monitor the variation in the transmittance rate of insulin, mono-PEGylated insulin, and di-PEGylated insulin solution (Fig. 4D and Supporting Information S9). In defining the aggregation time as the variation in transmittance over 10% of the initial transmittance value, native insulin and mono-PEGylated insulin aggregated after 13 and 32 h, respectively. However, di-PEGylated insulin did not show any aggregation throughout the 80-h assay (the change in transmittance was less than 2% after the assay). This superior stability of di-PEGylated insulin reduces its transport and storage expenses and shows great promise in insulin pump therapy, in which insulin formulations need to be stable for several days without refrigeration[27].

3.5. *Di-PEGylated insulin mitigates diabetic nephropathy*

Although the discovery of insulin has transformed diabetes from a lethal disease to a manageable chronic condition, diabetics still need regular injections of insulin to manage their blood glucose to reduce the occurrence and progression of associated complications. Therefore, a two-week experiment (one injection per day) was performed to investigate the long-term therapeutic efficacy of di-PEGylated insulin. Excessive thirst is one of the classic symptoms of diabetes, and diabetics consume much more water per day than healthy people[28]. Daily water consumption of mice treated with di-PEGylated insulin was significantly lower than the negative PBS control group, especially in the second week (Fig. 5A). However, mice in the PBS and insulin groups consumed comparable amounts of water. The glycated serum protein level (*i.e.*, a long-term indicator of average blood glucose)[29] was also measured and the result showed that di-PEGylated insulin markedly reduced the GSP level of diabetic mice as compared to PBS and insulin after 2-week treatment (Fig. 5B). Poor blood glucose management could damage blood vessel clusters in the kidney, resulting in a common

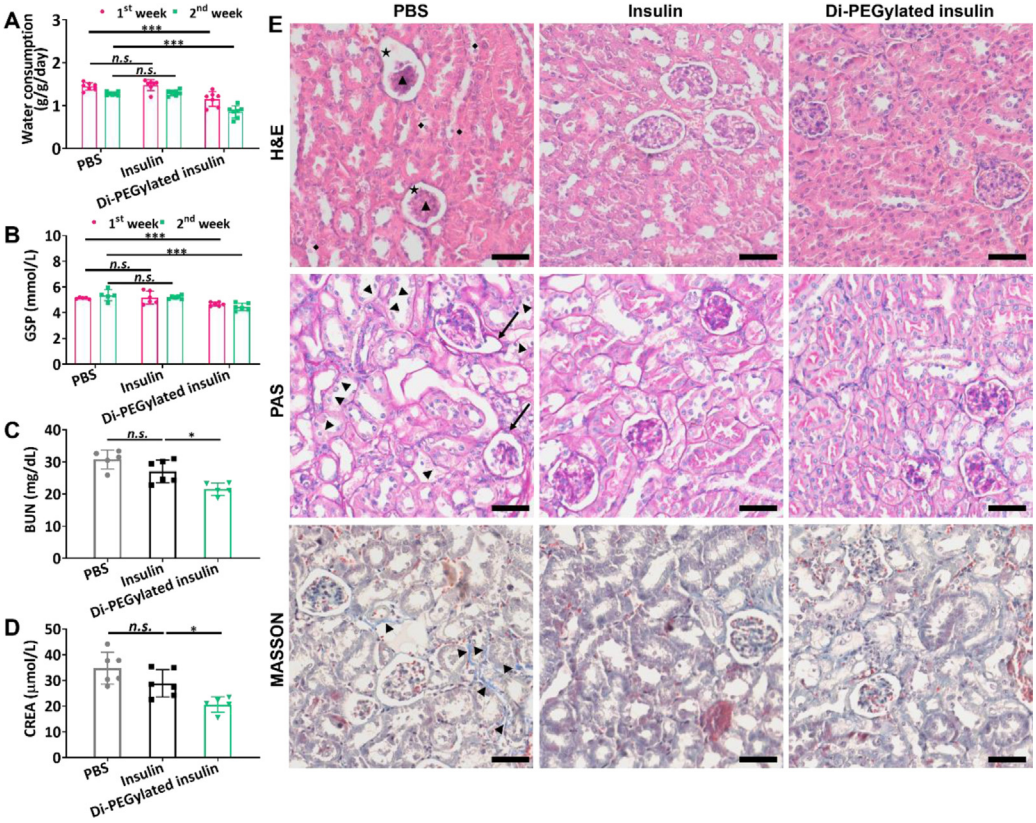


Figure 5 Long-term efficacy of di-PEGylated insulin on diabetic mice. (A) Daily water consumption of mice in different groups treated with PBS, insulin, and di-PEGylated insulin, results are presented as average ± SD ($n = 6$). (B) Glycated serum protein level of mice in different groups, results are presented as average ± SD ($n = 6$). The CREA (C) and BUN (D) values in different groups after treatment, results are presented as average ± SD ($n = 6$). (E) Representative H&E, periodic acid-Schiff (PAS), and Masson's trichrome staining (Masson) images of kidney sections in different groups. The images were taken at 200 × magnification. Scale bar = 50 μm. H&E, urinary space (★), glomeruli (▲), renal tubular cells (◆); PAS, glomerular basement membrane (↑), brush border (▲); Masson, collagen fiber (▲). Statistical significance analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test, $^{**}P < 0.01$, $^{***}P < 0.001$, *ns*, not significant.

complication of diabetes, *i.e.*, diabetic nephropathy[30]. Herein, the renal function makers (creatinine, CREA, and blood urea nitrogen, BUN) were measured to investigate whether long-term administration of di-PEGylated insulin could alleviate diabetic nephropathy. As shown in Fig. 5C and D, mouse CREA and BUN level in the di-PEGylated insulin group were significantly lower than PBS and insulin group, implying that Di-PEGylated insulin could provide some relief from diabetic nephropathy. Furthermore, H&E, periodic acid-Schiff (PAS), and Masson's trichrome staining (Masson) were used to confirm the ameliorative effect of di-PEGylated insulin on kidney injury at the pathological level (Fig. 5E). As a negative control, representative H&E images of PBS-treated mice showed classical diabetic pathology, *e.g.*, shrunken glomeruli, wide irregular urinary space, and vacuolated renal tubular cells[31]. While in treated groups (insulin or di-PEGylated insulin), these pathological features were somewhat attenuated (such as normal glomeruli and narrow irregular urinary space), particularly di-PEGylated insulin. For PAS staining, thicker glomerular basement membrane and incomplete brush border were noticed in mice treated with PBS or insulin[31]. While in the di-PEGylated insulin group, most renal tubules were presented with completed brush border and glomerulus were seen with preserved glomerular basement membrane. Examinations of Masson staining of mice treated with PBS showed noticeable collagen fibers around renal tubules, while the amount of collagen fiber was decreased in mice treated with insulin or di-PEGylated insulin[32].

3.6. *High dose of di-PEGylated insulin exhibits excellent biosafety*

Finally, the systemic toxicity of the high dose of di-PEGylated insulin was investigated on healthy mice. After 2-week administration (one injection per day, the doses of insulin and di-PEGylated insulin were 1 and 3 mg/kg, respectively), no significant difference in body weight was observed in mice of PBS,

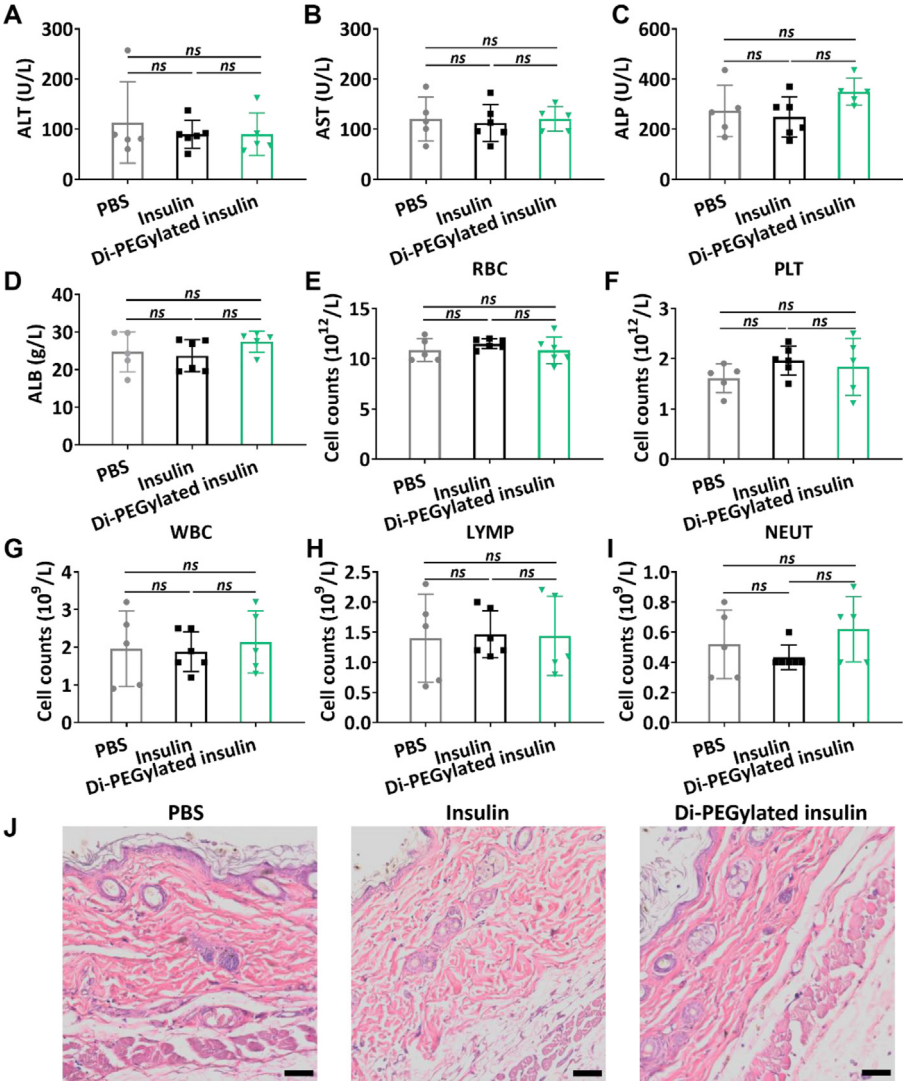


Figure 6 *In vivo* biosafety study of Di-PEGylated insulin. (A−D) Mouse serum ALT, AST, ALP, and ALB concentration after the treatment, results are presented as average ± SD (n = 5 or 6). (E−I) Full blood count examination of red blood cell (RBC), platelet (PLT), white blood cell (WBC), lymphocyte (LYMP), and neutrophil (NEUT), results are presented as average ± SD (n = 5 or 6). Statistical significance analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test, *ns*, not significant. (J) H&E staining of the skin at the injection site. The images were taken at 200 × magnification. Scale bar = 50 μm.

insulin, and di-PEGylated group (Supporting Information Fig. S10). Liver function markers, alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and serum albumin (ALB), were at a comparable level in the three groups after the long-term treatment (Fig. 6A−D). Full blood count results showed that there was no noticeable change in blood cell counts (*e.g.*, red blood cell, platelet, and white blood cell) between all three groups (Fig. 6E−I). No significant inflammatory cell infiltration was observed in the skin at the injection site in all three groups of mice (Fig. 6J). Consistent with the PBS group, mice treated with a high dose of di-PEGylated insulin did not show any detectable morphological lesions in the H&E images of the heart, liver, spleen, lung, and kidney (Supporting Information Fig. S11), indicating the excellent biosafety of di-PEGylated insulin at this high dose.

4. Conclusions

In conclusion, we have developed an approach to broaden the therapeutic index of insulin that reduces the risk of hypoglycemia during its use. A kind of di-PEGylated insulin that could simultaneously reduce renal clearance and receptor-mediated clearance was synthesized and characterized, confirming that the PEGylated sites were Lys B29 and Gly A1 (involving receptor binding area) of insulin and that di-PEGylation significantly sacrificed the *in vitro* bioactivity of insulin. Moreover, di-PEGylated insulin not only exhibited improved pharmacokinetics compared to mono-PEGylated insulin but also a 10-fold increase in its maximum tolerated dose. Long-term administration of di-PEGylated insulin with a high dose could mitigate diabetic nephropathy without noticeable system toxicity. The effects of polymer length and type on the therapeutic index and long-acting efficacy of insulin need to be further studied. This conceptual platform may be also applied to other protein drugs with intrinsic narrow therapeutic index through a modified protocol based on the same design mechanism, which is essential to unleash their full therapeutic potential.

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Author contributions

Zhipeng Zeng, Mingqiang Li, and Yongming Chen conceived and designed the experiments. Zhipeng Zeng conducted all the experiments. Runcheng Tan, Shi Chen, and Haolin Chen helped with the animal experiments and data analysis. All authors discussed the results. Zhipeng Zeng and Yongming Chen outlined and wrote the paper. Zhijia Liu and Lixin Liu helped revise the paper. Yongming Chen developed the concept and supervised the study. All authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2024.02.022.

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