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A Forskolin-Conjugated Insulin Analog Targeting Endogenous Glucose-Transporter for Glucose-Responsive Insulin Delivery

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Abstract

Insulin administration for management of diabetes is accompanied by hypoglycemia, which is expected to be mitigated by glucose-responsive smart insulin that has self-regulation ability in response to blood glucose level (BGL) fluctuation. Here, we have prepared a new insulin analog by modifying insulin with forskolin (designated as insulin-F), a glucose-transporter (Glut) inhibitor. *In vitro,* insulin-F is capable of binding to Glut on erythrocyte ghosts, which can be inhibited by glucose and cytochalasin B. Upon subcutaneous injection in type 1 diabetic mice, insulin-F maintains the BGLs below 200 mg/mL for up to 10 hours, and achieves 20 hours with two sequential injections. Meanwhile, insulin-F also binds to endogenous Gluts. Upon a glucose challenge, the elevated level of glucose competitively replaces and liberates insulin-F that binds to Glut, restoring BGLs to the normal range rapidly.

Introduction

Diabetes mellitus is prevailing globally with an estimation of 425 million people worldwide affected in 2017, and the number may rise to 625 million in 2045.[1, 2] The general method for treating type 1 and advanced type 2 diabetes includes multiple and daily exogenous insulin injections or continuous exogenous insulin infusion relied on continuous glucose monitoring.[3, 4] However, insulin administration *via* subcutaneous injections is inevitably accompanied by the risk of hypoglycemia. Narrow therapeutic window and passive absorption mode attribute to this potentially lethal symptom.[5] Conversely, in healthy individuals, plasma insulin levels follow the fluctuation of the BGLs in real-time, and the insulin levels spike at mealtimes.[6] Therefore, commercialized insulin analogs, which are

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Conflicts of interest

J.W. and Z.G. have applied for patents related to this study.

mainly categorized into postprandial and basal insulin, have been developed and injected in sequence to mimic the daily major fluctuation of insulin levels. However, repeated injections bring about low adherence and complexity of administration, while hypoglycemia is still unavoidable.[5] Glucose-responsive insulin analog or formulation that can self-modulate its activity or release kinetics has the potential to address these issues.[7–15] Therefore, various types of glucose-responsive insulin delivery systems relying on phenylboronic acid,[16–21] glucose oxidase,[22–27] and glucose-binding molecules[28–31] have been developed. However, it remains challenging to prepare a long-acting insulin analog that can tightly regulate BGLs with embedded glucose-responsive performance.

Recently, we demonstrated a new strategy for developing glucose-responsive insulin by conjugating glucose transporter inhibitor to insulin.[32] Here, we have prepared an insulin analog (designated as insulin-F) *via* conjugating human recombinant insulin to a new glucose-transporter inhibitor, forskolin,[33, 34] to endow insulin-F the capability of binding to Gluts. The introduction of forskolin reduces the water-solubility of insulin and prolongs its retention after subcutaneous injections, therefore providing sustained release of insulin-F from the injected depot to achieve a prolonged BGL regulation ability as compared with native insulin. In addition, dissolved insulin-F can bind to Gluts on living cells, generating an *in vivo* "reservoir" that is able to sense the fluctuation of BGLs. Upon potential food intake, the elevated level of blood glucose enhances the Glut-binding capability of glucose, which subsequently replaces the insulin-F that binds to Glut. Consequently, insulin-F is released from Gluts into the surrounding environment, promoting the clearance rate of blood glucose and regulating blood glucose to normal levels rapidly (Fig. 1A).

Experimental

Materials

Forskolin was purchased from LC Laboratories. Succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) was purchased from Chem Impex Int'L Inc. Sulfo-cyanine5 NHS ester (sulfo-Cy5-NHS) was purchased from Lumiprobe. Human recombinant insulin was purchased from ThermoFisher Scientific (Catalog No. A11382IJ). Phosphate buffered saline (PBS) (pH=7.4) was purchased from GIBCO. Deionized (DI) water was prepared in the lab using Aqua Solution®. Other chemical reagents used in this study were purchased from Sigma-Aldrich. Human insulin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Invitrogen.

Preparation of insulin-F

Forskolin-NH_2 was prepared from forskolin and 1,7-Heptanediamine according to literature.[35] For the synthesis of insulin-F, Forskolin-NH_2 (10 mg) and SMCC (4 mg) were dissolved in DMSO (1 mL) containing 0.1 mL PBS (10×, pH 7.4). The mixture was incubated at room temperature for 6 hours to give Forskolin-SMCC and used without purification. Meanwhile, insulin (10 mg) was dissolved in 1 mL of PBS containing EDTA (10 mM), with the pH adjusted to 8.3 using $NaHCO_3$ powder, and the mixture was cooled in an ice bath. Then, 2-iminothiolane (4 mg) dissolved in PBS (10×, pH=7.4, 0.4 mL) was added to the iced insulin solution. This reaction was further incubated in an ice bath for 6 hours before centrifugation

(Beckman Coulter, Avanti J-E, 7000 rpm) using the centrifugal filters (MilliporeSigma™, MWCO=3 kDa) and washing with PBS (1×, pH=7.4, 2 mL) three times to give insulin-SH. At last, forskolin-SMCC and insulin-SH were mixed and incubated overnight. After dialysis against DI water (four liters), a white precipitate was collected *via* centrifugation and purified *via* preparative high-performance liquid chromatography.

Cyanine5-labeled insulin-F (designated as Cy5-insulin-F) or insulin (designated as Cy5-insulin) was prepared *via* mixing sulfo-cyanine5 NHS ester (sulfo-Cy5-NHS) (1 mg) with insulin-F (10 mg) or insulin (10 mg) in a mixed solvent composed of DMSO (1 mL) and PBS (10×, pH=7.4, 0.1 mL). After dialysis (MWCO=3.5 kDa) in DI water (3 × 4 litres) and subsequent lyophilization, Cy5-insulin-F or Cy5-insulin was obtained.

Confocal microscopy observation of Rhodamine B-loaded erythrocyte ghosts

Rhodamine B dissolved in hypotonic solution (0.1 mg/mL) was used for preparing the erythrocyte ghosts. After washing with PBS several times, rhodamine B-loaded erythrocyte ghost suspension was dropped onto a glass microscope slide and covered with a microscope cover glass. The observation was performed on a ZEISS LSM 880 confocal microscope with the emission wavelength ranging from 566 to 638 nm (excited at 543 nm).

Confocal microscopy observation of erythrocyte ghosts incubated with Cy5-insulin-F

Erythrocyte ghosts (2×10^9/mL) was treated with Cy5-insulin-F (1 μM) overnight before observation using confocal microscopy without washing. The treated erythrocyte ghost suspension (2 μL) was dropped onto a glass microscope slide and covered with a microscope cover glass slide with a gentle press. The RBC ghosts were then observed using a 40× lens with the emission wavelength ranging from 650–700 nm (excited at 633 nm).

Binding kinetics of insulin-F to erythrocyte ghost

The binding of insulin-F to erythrocyte ghost was studied *via* adding concentrated Cy5-insulin-F to erythrocyte ghost suspension (2×10^9/mL, 0.1 mL) at a final concentration of 1 μM. The suspension was observed at timed intervals using confocal microscopy. Meanwhile, the fluorescence intensity of the supernatant was recorded using a microplate reader (excitation wavelength was 650 nm; emission wavelength was 700 nm).

In vivo evaluation of treatment efficacy in diabetic mice

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of North Carolina State University and University of California, Los Angeles and approved by the Animal Ethics Committee of North Carolina State University and University of California, Los Angeles. Diabetic mice were purchased from the Jackson Laboratory. The mice were normally fed during the whole experiment. Diabetic mice were allocated to three groups with the treatment of insulin-F, native insulin and PBS. The doses of insulin-F and native insulin were set to 6 and 1.5 mg/kg, respectively. The BGLs of diabetic mice were measured before and during treatment until their BGLs returned to the original ones utilizing a glucose meter (ACCU-CHEK).

For the four weeks long diabetes treatment experiment, the diabetic mice were treated daily for the first two weeks, and every two days for the second two weeks at 6 mg/kg. The dosage was determined based on our preliminary experiment that after two-week treatment, insulin-F only needed to be injected every two days to maintain the BGL in the normal range. The live imaging of mice treated with Cy5-insulin-F or Cy5-insulin were performed on an IVIS Spectrum imaging system (Perkin Elmer).

Multiple intraperitoneal glucose tolerance tests (IPGTTs)

Multiple IPGTTs were performed in diabetic mice after 4, 10, and 20 hours post-treatment of insulin-F. The glucose was given at 2 g/kg. To evaluate IPGTT-stimulated insulin release, plasma was collected at timed intervals, and measured by an insulin ELISA kit.

Results and Discussion

Forskolin, a well-studied glucose transporter inhibitor[36] and a dietary supplement[37], was incorporated with an amino group using the method reported to obtain Forskolin-NH_2.[35] Forskolin-NH_2 was subsequently reacted with SMCC while human recombinant insulin was treated with 2-iminothiolane. The subsequent mixing of these two compounds provided insulin-F. The obtained insulin-F has a molecular weight of 6692.02 g/mol as measured with matrix-assisted laser desorption/ionization with a time-of-flight analyzer (MALDI-TOF) (Fig. S1 in the ESI†), indicating that one Forskolin-NH_2 has been conjugated to one insulin molecule. The circular dichroism (CD) spectrum of insulin-F was similar to that of native insulin (Fig. S2 in the ESI†).

The binding of insulin-F to glucose transporter was evaluated in the erythrocyte ghost, a widely used carrier of glucose transporter.[38] Erythrocyte ghosts were prepared according to literature,[38] and the retained membrane integrity of red blood cells was confirmed by confocal microscopy (Fig. 1B) and cryo-scanning electron microscopy (cryo-SEM) (Fig. 1C). The erythrocyte ghosts were maintained in a hypotonic solution and used within two weeks. Insulin-F was then labeled with sulfo-Cy5-NHS to provide Cy5-insulin-F. After incubating erythrocyte ghosts with Cy5-insulin-F overnight, an intense fluorescence intensity was observed on the membranes (Fig. 1D), whereas the surrounding solution also showed fluorescence with a slightly lower intensity. In contrast, the fluorescence intensity of erythrocyte ghosts was much weaker when they were incubated with Cy5-labeled native insulin (Fig. S3 in the ESI†).

The binding kinetics of insulin-F toward erythrocyte ghosts was further studied. Upon the addition of Cy5-insulin-F to the erythrocyte ghost suspension, the fluorescence on the erythrocyte membrane was negligible within two minutes (Fig. 2A). Then, the fluorescence intensity on membranes was increased over time that the profile of erythrocyte ghost became clear after 70 min incubation. Meanwhile, the fluorescence intensity in the supernatant solution was decreasing over time (Fig. S4 in the ESI†). This binding rate of insulin-F to Glut was slower than that of the previously reported *i*-insulin.[32] Furthermore, the specific binding of Cy5-insulin-F to Glut on erythrocyte ghost was evaluated *via* adding glucose or

†Electronic supplementary information (ESI) available.

cytochalasin B,[34] a standard inhibitor of Glut to Cy5-insulin-F treated erythrocyte ghosts (Fig. 2B). After coincubation with cytochalasin B (10 μM) and glucose (1800 mg/dL) (Fig. 2C), the fluorescence intensity was significantly decreased.

Insulin-F was further evaluated in type 1 diabetic mice induced by streptozotocin (STZ). The subcutaneous retention of insulin-F was studied using live imaging. After subcutaneous injection, the fluorescence intensity of Cy5-insulin-F as well as the plasma insulin was decreasing over time but remained detectable even after 48 hours (Fig. 3A and Fig. S5 in the ESI†). In contrast, the fluorescence of subcutaneously injected Cy5-insulin vanished within 10 hours post-injection (Fig. 3A). Next, the blood glucose regulation ability of insulin-F was studied. The diabetic mice were allocated to three groups with the treatment of insulin-F, native insulin, and PBS. Upon subcutaneous injection, insulin-F was capable of maintaining BGLs of diabetic mice at the normal range (< 200 mg/dL) for more than 10 hours (Fig. 3B), whereas native insulin could only maintain the normoglycemic state for less than 4 hours (Fig. 3B). A second injection of insulin-F or native insulin was further given three hours post-treatment when normoglycemic conditions had been acquired for diabetic mice treated with either native insulin or insulin-F. Native insulin induced an obvious decrease in BGLs to around 50 mg/dL (Fig. 3C). In contrast, a mitigated hypoglycemic state was observed for insulin-F-treated diabetic mice. Notably, an extended normoglycemic state longer than 20 hours was observed in diabetic mice receiving the second injection of insulin-F (Fig. 3C).

Multiple IPGTTs were performed at 4, 10, and 20 hours after diabetic mice were treated with insulin-F. Blood glucose spikes were observed for both healthy and diabetic mice upon glucose administration (Fig. 3D). The blood glucose of both insulin-F-treated diabetic mice and healthy mice returned to the original levels within two hours, suggesting the ability of insulin-F to regulate the blood glucose levels tightly. Also, an IPGTT-triggered insulin-F release into blood was observed, which could help enhance the blood glucose regulation ability of insulin-F (Fig. 3E).

A four-week-long study was performed to evaluate the long-term diabetes treatment efficacy of insulin-F. Insulin-F was treated every day at a dose of 6 mg/kg for the first two weeks and every two days at the same dose for the following two weeks. The BGLs of diabetic mice were maintained at the normal range during the whole period (Fig. S6 in the ESI†). The toxicity of insulin-F toward liver and kidney was also evaluated *via* performing analysis of serum biochemical indices, including the serum levels of albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN), and creatinine (CREAT) after one-week, two-week, and four-week continuous treatment (Fig. 4). All the biochemical indices had negligible change except ALP. The ALP levels of diabetic mice were around 220 U/L, while the normal ones were around 90 U/L. The constant treatment of insulin-F induced a gradual decrease of ALP levels over time to a healthy range after four weeks of treatment. Meanwhile, no significant change was observed for blood cell counting of RBC, WBC, PLT, EO and BASO after four-week treatment of insulin-F, while the NEUT counting was increased which may be attributed to the repeated subcutaneous injections of insulin-F

Conclusions

Glucose-responsive insulin delivery formulations or insulin analogs have the potential to change the complicated guidelines of insulin administration and may greatly improve the treatment outcomes. In this study, we conjugated forskolin, which is a glucose transporter inhibitor and health supplement, to native insulin to achieve a glucose-responsive insulin analog, *via* reversible binding to endogenous glucose transporters in a glucose-dependent manner. *In vitro*, insulin-F was able to bind to glucose transporter on erythrocyte ghost membranes, which could be inhibited by glucose and cytochalasin B. *In vivo,* upon subcutaneous injection in type 1 diabetic mice, insulin-F formed a depot under skin, which released insulin-F into systemic circulation and regulated blood glucose within the normal range for more than 10 hours. Moreover, a second injection performed three hours post-treatment induced negligible hypoglycemia. Multiple IPGTTs further confirmed that insulin-F could quickly return the BGLs to normal range even at 20 hours after treatment, attributed to the long retention of insulin-F at the subcutaneous depot and the glucose-responsive release of insulin-F from endogenous Gluts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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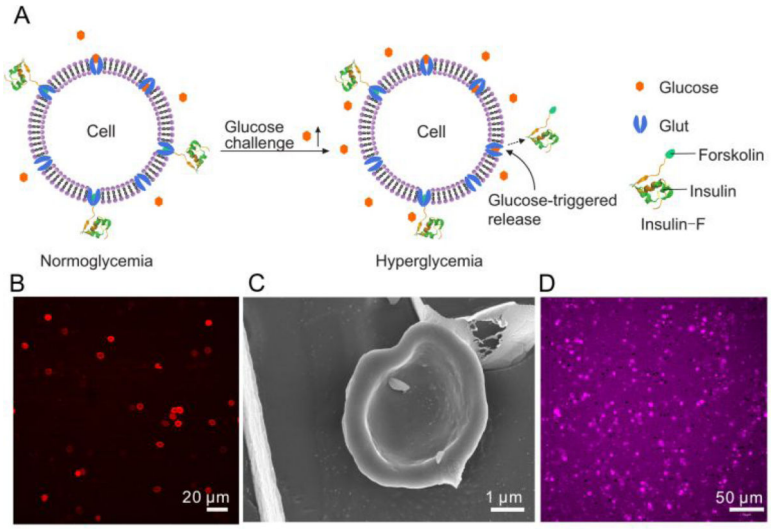

Figure 1.

Glut-mediated glucose-responsive insulin delivery system. (A) Schematic of glucose-triggered insulin-F release upon glucose challenge. After subcutaneously injected, insulin-F binds to endogenous Gluts, and a “reservoir” of insulin-F is formed *in situ.* When BGL increases, insulin-F is competitively replaced by glucose and released from Glut into the milieu. (B) A representative confocal microscopy image of erythrocyte ghosts. The erythrocyte ghosts were loaded with rhodamine B. Scale bar, 20 μm. (C) A representative cryo-SEM image of the erythrocyte ghost. Scale bar, 1 μm. (D) A representative confocal microscopy image of erythrocyte ghosts treated with Cy5-insulin-F overnight. The concentration of Cy5-insulin-F was set to 1 μM.

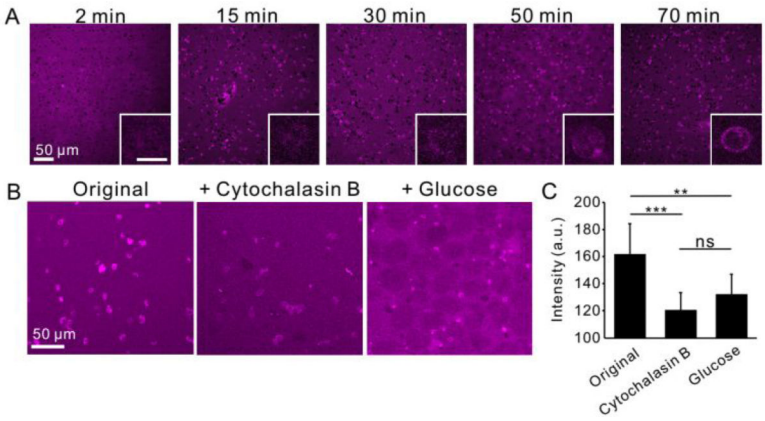

Figure 2.

In vitro study of insulin-F binding to erythrocyte ghosts. Insulin-F was labeled with sulfo-Cy5. (A) Kinetics study of the binding of insulin-F toward erythrocyte ghosts. The suspension was observed at 2, 15, 30, 50 and 70 min after the addition of Cy5-insulin-F to erythrocyte ghost suspension. Scale bar, 50 μm. Insets are enlarged representative images. Scale bar, 5 μm. (B) Representative images of erythrocyte ghosts treated with Cy5-insulin-F in the presence of cytochalasin B and glucose. The concentrations of cytochalasin B and glucose were 10 μM and 1800 mg/dL, respectively. (C) The fluorescence intensity on the membranes was analyzed using ImageJ. Data were are presented as mean ± S.D. (n=10). One-way ANOVA was used to calculate the P-value, using a Tukey post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns, not significant.

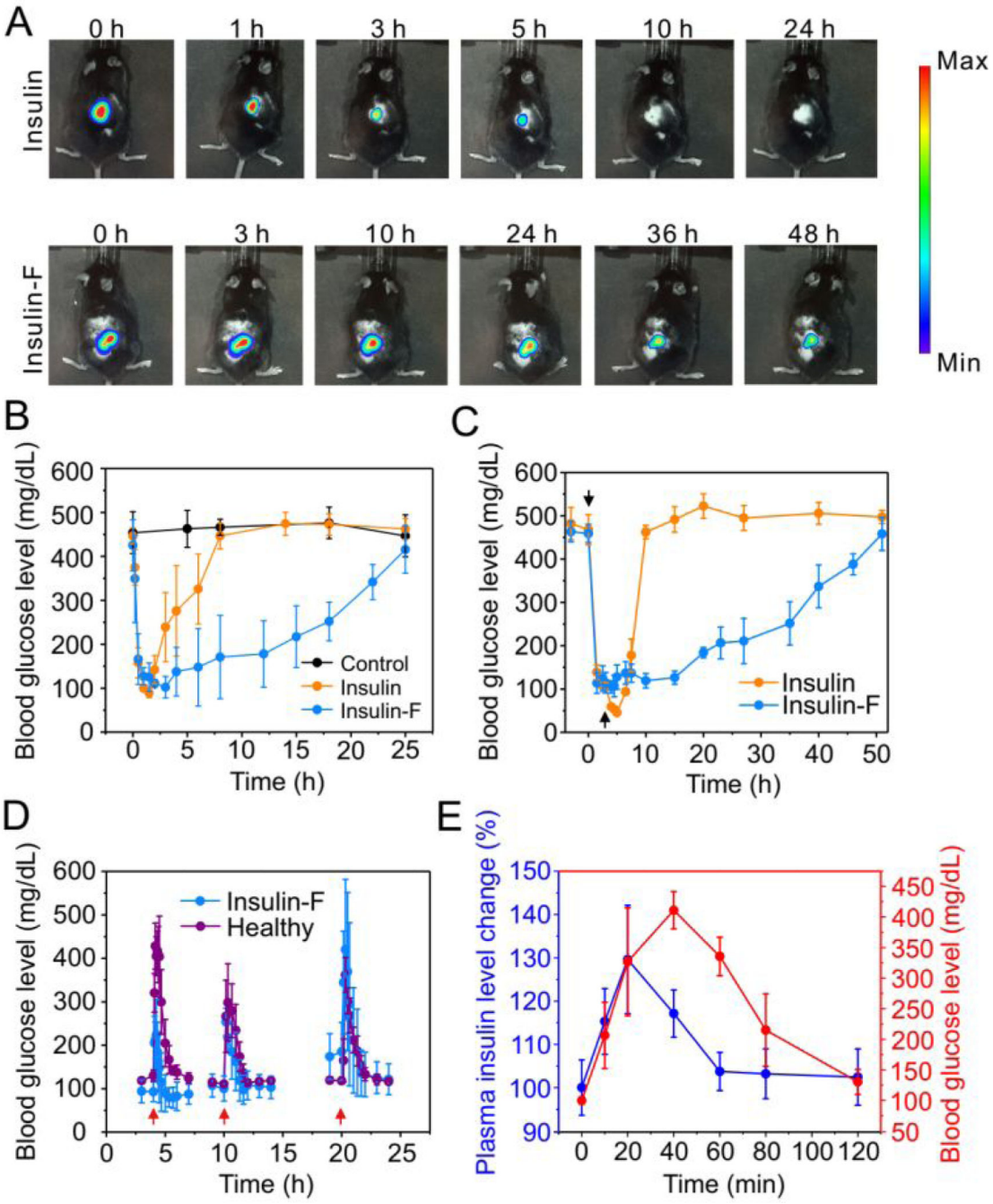

Figure 3.

The *in vivo* study of insulin-F in diabetic mice. (A) Live imaging of diabetic mice treated with Cy5-insulin-F and Cy5-insulin. (B) BGLs of diabetic mice treated with insulin-F and native insulin. Data are presented as mean ± S.D. (n=5). PBS-treated ones were used as control. (C) The BGLs of diabetic mice treated with two sequential injections. A second injection was given at three hours after the first treatment. Each data point indicates mean ± S.D. (n=5). Black arrows indicate the injection of insulin-F. The doses were set to 6 and 1.5 mg/kg for insulin-F and native insulin, respectively in Figures A-C. (D) Multiple IPGTTs.

Glucose (2 g/kg) was given at 4, 10, and 20 hours posttreatment. The dose of insulin-F was set to 10 mg/kg. Each data point indicates mean ± S.D. (n=5). Red arrows indicate the injections of glucose. (E) The IPGTT-triggered insulin release. Glucose (2 g/kg) was given at 6 hours posttreatment. The dose of insulin-F was set to 6 mg/kg. Blood was collected before and after glucose administration at timed intervals. Data are shown as mean ± S.D. (n=5).

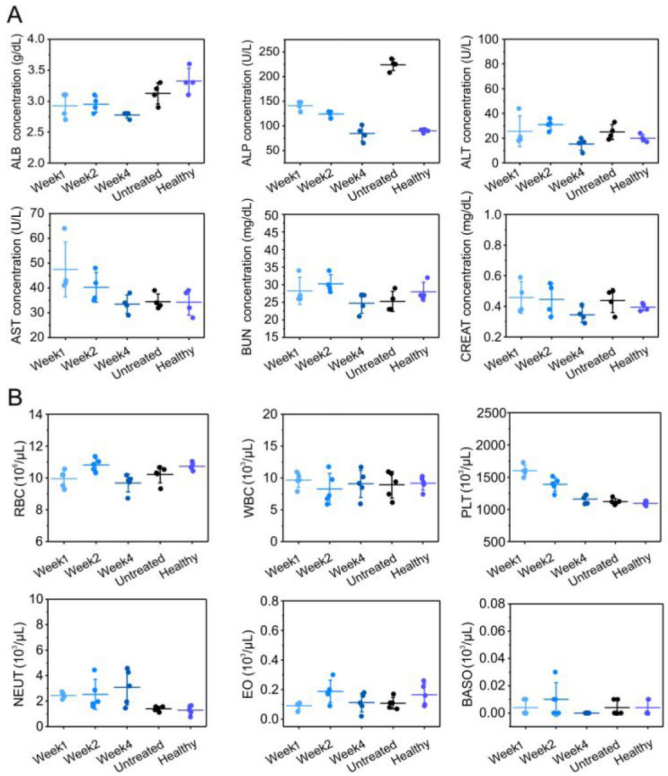

Figure 4.

Biocompatibility evaluation of insulin-F in diabetic mice. (A) Serum biochemical indices in diabetic mice treated insulin-F. The serum was collected using a serum separation tube. (B) Complete blood count study of diabetic mice treated with insulin-F. Insulin-F was injected subcutaneously at a dose of 6 mg/kg every day for the first two weeks. Then, it was used every two days for the second two weeks. Both untreated diabetic mice and healthy mice were used as controls. Data points are shown individually and as mean ± S.D. (n=4). RBC, red blood cell; WBC, white blood cell; PLT, platelet; NEUT, neutrophil; EO, eosinophil; BASO, Basophil.