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Supplementary Information for

Glucose Transporter Inhibitor-Conjugated Insulin Mitigates Hypoglycemia

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Supplementary Information Text

Materials and Methods

Materials

All the chemical materials were purchased from Sigma-Aldrich and used as received. Human recombinant insulin was purchased from ThermoFisher Scientific (Catalog Number, A11382IJ). Human red blood cells were purchased from Fisher Scientific. Unless mentioned otherwise, all the reactions were carried out with the protection of argon atmosphere.

Methods

Synthesis of Glut-i2-intermediate 1. *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (3 g, 15.7 mmol) and Boc-2-bromo-*L*-phenylalanine (2 g, 5.8 mmol) were mixed in tetrahydrofuran (40 mL) in an ice bath, followed by the addition of *N,N*-diisopropylethylamine (3 mL, 17 mmol) and 4-dimethylaminopyridine (72 mg, 0.57 mmol). After stirring at room temperature for 30 min, (*S*)-(-)-1-phenylethylamine (1 g, 8.3 mmol) was added and the mixture was stirred for another day. The mixture was concentrated under reduced pressure and purified *via* passing through a silica gel column (hexane:ethyl acetate=50:50) and gave a white solid (2 g, yield 77%). ^{1}H-NMR (400 MHz, DMSO-*d*6): δ 8.18 (d, 1H), 7.55 (d, 1 H), 7.12-7.20 (m, 8 H), 6.90 (d, 1H), 4.89 (m, 1H), 4.26 (m, 1H), 3.02 (m, 1H), 2.87 (m, 1H), 1.19-1.35 (m, 12 H). ^{13}C-NMR (400 MHz, DMSO-*d*6): δ 170.58, 155.46, 144.75, 137.41, 132.78, 131.77, 128.89, 128.60, 127.88, 127.00, 126.29, 124.77, 78.61, 54.38, 48.24, 38.22, 28.55, 22.70.

Synthesis of Glut-i2-intermediate 2. Glut-i2-intermediate 1 (1 g) was dissolved in CH_2Cl_2 (10 mL) containing TFA (3 mL) and stirred for three hours at room temperature. The solvent was removed under reduced pressure. The residual was re-dissolved in toluene, and the solvent was evaporated. This procedure was repeated three times to give the final product (0.71 g, yield 92%). ^{1}H-NMR (400 MHz, DMSO-*d*6): δ 8.19 (d, 2H), 7.53 (d, 2H), 7.16-7.24 (m, 8H), 4.88 (m, 1H), 3.47 (m, 1H), 3.03 (m, 1H), 2.74 (m, 1H), 1.79 (s, 2H), 1.32 (d, 3H). ^{13}C-NMR ((400 MHz, DMSO-*d*6): 173.27, 144.79, 138.53, 132.12，132.77, 128.65, 128.58, 127.90, 126.94, 126.33, 124.78, 55.46, 47.95, 41.49, 22.69.

Synthesis of 2-(4-(allyloxy)phenyl)acetic acid. *p*-Hydroxyphenylacetic acid (5 g, 33 mmol), allyl bromide (10 g, 83 mmol), and K_2CO_3 (11.5 g, 83 mmol) were mixed in DMF (20 mL). The mixture was stirred at 35 ℃ overnight and poured into ethyl ether (200 mL), which was further washed with brine (3×50 mL), dried with Na_2SO_4, filtrated, and concentrated to give the product. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.16 (d, 2H), 6.87 (d, 2H), 6.04 (m, 1H), 5.41 (m, 1H), 5.28 (m, 2H), 4.51 (d, 2H), 3.55 (s, 2H). ^{13}C-NMR ((400 MHz, DMSO-*d*6): δ 173.39, 157.35, 134.25, 130,78, 127.54, 117.73, 114.85, 68.55.

Synthesis of Glut-i2-intermediate 3. (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (3.25 g, 6.2 mmol), 2-(4-(allyloxy)phenyl)acetic acid (1 g, 5.2 mmol), *N,N*-diisopropylethylamine (0.8 g, 6.2 mmol), 4-dimethylaminopyridine (80 mg), and intermediate 1 (0.9 g, 2.6 mmol) were mixed in anhydrous tetrahydrofuran (20 mL) and stirred overnight at room temperature. After removing THF under reduced pressure, the residual was mixed in ethyl acetate (200 mL), washed with HCl (0.1 N, 100 mL), Na_2CO_3 (0.1 N, 2×100 mL), and dried over anhydrous Na_2SO_4. After filtration and solvent evaporation, the residual was purified by passing through a column (eluent, 10% MeOH in CH_2Cl_2) to give the product (0.94 g, yield 70%). ^{1}H-NMR (400 MHz, DMSO-*d*6): δ 8.34 (d, 1H), 8.28 (d, 1H), 7.52 (d, 1H), 7.14-7.24 (m, 8H), 7.02 (d, 2H), 6.80 (d, 2H), 5.98 (m, 1H), 5.38 (d, 1H), 5.23 (d, 1H), 4.88 (m, 1H), 4.67 (m, 1H), 4.50 (s, 2H), 3.32 (m, 2H), 3.04 (m, 1H), 2.89 (m, 1H), 1.29 (d, 3H). ^{13}C-NMR (400 MHz, DMSO-*d*6): δ 170.64, 170.16, 157.16, 144.73, 137.12, 134.29, 132.78, 131.65, 130.38, 128.87, 128.74, 128.60, 127.85, 126.94, 126.22, 124.70, 117.71, 114.74, 68.54, 52.50, 48.26, 41.65, 38.27, 22.85.

Synthesis of Glut-i2-NH_2. Glut-i2-intermediate 3 (0.52 g, 1 mmol), cysteamine (0.15 g, 2 mmol) and benzoin methyl ether (0.023g, 0.1 mmol) were mixed in DMSO (5 mL) under an argon atmosphere. The mixture was exposed to UV light for 10 min. The DMSO was removed *via* lyophilization and the residual was purified *via* passing through a silica column (10% MeOH in CH_2Cl_2, containing 1% TEA) to give the product (0.35 g, yield 60%). ^{1}H-NMR (400 MHz, DMSO-*d*6): δ 8.36 (d, 1H), 8.26 (d, 1H), 7.50 (d, 1H), 7.12-7.24 (m, 8H), 7.03 (d, 2H), 4.88 (m, 1H), 4.65 (m, 1H), 3.98 (m, 2H), 3.31 (m, 2H), 3.04 (m, 1H), 2.88 (m, 1H), 2.60-2.66 (m, 4H), 2.48 (m, 2H),1.91 (m, 2H), 1.29 (d, 3H). ^{13}C-NMR (400 MHz, DMSO-*d*6): δ 170.69, 170.20, 157.50, 144.73, 137.14, 132.77, 131.69, 130.42, 128.87, 128.64, 128.60, 127.84, 126.94, 126.23, 124.70, 114.57, 66.37, 52.59, 48.30,38.27, 34.93,29.41, 27.87, 22.85.

Synthesis of *i*-insulin. Insulin (50 mg) dissolved in PBS (50 mL) was treated with EDTA (500 mM, 500 μL) and cooled in an ice bath. The pH value of the insulin solution was adjusted to 8.3. Traut's reagent (20 mg) dissolved in PBS (100 mM, pH 7.2, 2 mL) was added to this solution. The solution was incubated in the ice bath for six hours. The unreacted Traut's reagent was removed by ultracentrifugation and PBS washing (3×10 mL) to give a wet white precipitate (insulin-SH). At the same time, Glut-i2-NH_2 (20 mg) was reacted with SMCC (8 mg) in DMSO (4 mL) containing PBS (100 mM, pH=7.2, 0.4 mL) for two hours. The obtained insulin-SH was added to the Glut-i2-SMCC solution and the resultant clear solution was incubated at room temperature overnight. After dialysis in deionized water (3×4 L) and lyophilization, the obtained product was further purified using HPLC (XBridge OBD prep column, reversed-phase, 5 μm spherical hybrid, 19 mm×150 mm) and a mixed eluent containing 0.1% TFA (30% to 40% acetonitrile in H_2O in 5 min, and 40% for another 5 min).

To confirm the A1 modification, *i*-insulin was completely reduced by dithiothreitol in the presence of guanidine hydrochloride. A species at ~1650 m/z that possibly corresponded to the singly modified alpha chain (~3297 Da) was observed (Fig. S7A). The peptide was collected for MSMS. MSMS was performed by nano-electrospray ionization using a Fourier-transform ion cyclotron resonance MS in a positive ion mode (LTQ FT Ultra; Thermo). The only observable charge state (2+) of the modified chain was analyzed at standard and extended mass range. Mass spectrum was analyzed in Qual Browser software (Thermo) and fitted to human insulin sequence with ProSightPC software (version 4.0; Thermo). The result supports the conclusion that this modified chain corresponded to the singly modified alpha chain with the modification (918.28 Da) at the only primary amine available at the *N*-terminus.

Synthesis of Cy5-labeled *i*-insulin. Sulfo-Cy5-NHS (1.2 mg) and *i*-insulin (10 mg) were mixed in DMSO (1 mL) containing PBS (10 mM, pH=7.4, 0.1 mL) for two hours. After dialysis in DI water (4 L) and lyophilization, Cy5-labeled *i*-insulin was obtained.

Preparation of erythrocyte ghosts. Red blood cell suspension (40 mL) was centrifuged at 2000×g for 20 min, and the supernatant was replaced with fresh PBS (4 ºC). This procedure was repeated three times. The obtained packed red cells were hemolyzed with 30 folds of 0.05 isotonic balanced PBS (4 ºC) for 10 min. The mixture was centrifuged at 5000×g for 20 min and washed until the supernatant was colorless. The prepared erythrocyte ghosts were resuspended in 0.05 isotonic balanced PBS at a required density and kept at 4 ºC for subsequent use within one week.

Confocal laser scanning microscopy (CLSM) observation of the binding of *i*-insulin to Glut. Cy5-labeled *i*-insulin (1 μM) was added to a PBS solution containing erythrocyte ghosts at a density of 2×10^9/mL and incubated for 30 min before observation with Zeiss LSM 880 confocal system with Airyscan and a Zeiss Z.1 light-sheet microscopy. Cy5 filter (excitation wavelength: 633 nm, emission filter: 650-701 nm) was set for observation.

CLSM observation of the binding and release kinetics of *i*-insulin. Cy5-labeled *i*-insulin (1 μM) was added to erythrocyte ghost solution (2×10^9/mL), and a drop of the solution (2 μL) was added immediately onto a plain glass microscope slide and covered with a piece of cover glass. The two pieces of glass were slightly pressed to push the extra solution out, and the sample was observed at timed intervals using 40 × lens with the cover glass-side down. The release kinetics was observed *via* dilution. PBS with the same osmotic pressure was used for dilution.

CLSM observation of the glucose-concentration dependent binding of *i*-insulin to erythrocyte ghosts. The Cy5-*i*-insulin-treated erythrocyte ghosts (2×10^9/mL) were allocated to four Eppendorf tubes (100 μL), followed with the addition of a glucose solution (300 mg/mL) for the required concentrations (0, 400, 800, 1600 mg/dL). After 10 min, the samples were observed using confocal microscopy.

The change of fluorescence intensity of the supernatant solution. The Cy5-labeled *i*-insulin (1 μM) was added to the erythrocyte ghosts (2×10^9/mL, 3 mL), which was evenly allocated to three tubes. At each predetermined time point, the solution (50 μL) was withdrawn and centrifuged at 2000×g for 10 min, and the fluorescence intensity of the supernatant solution (20 μL added to 80 μL DMSO) was measured using a microplate reader (excitation wavelength: 650 nm, emission wavelength: 700 nm). The fluorescence intensity at 0 min was set as 100%.

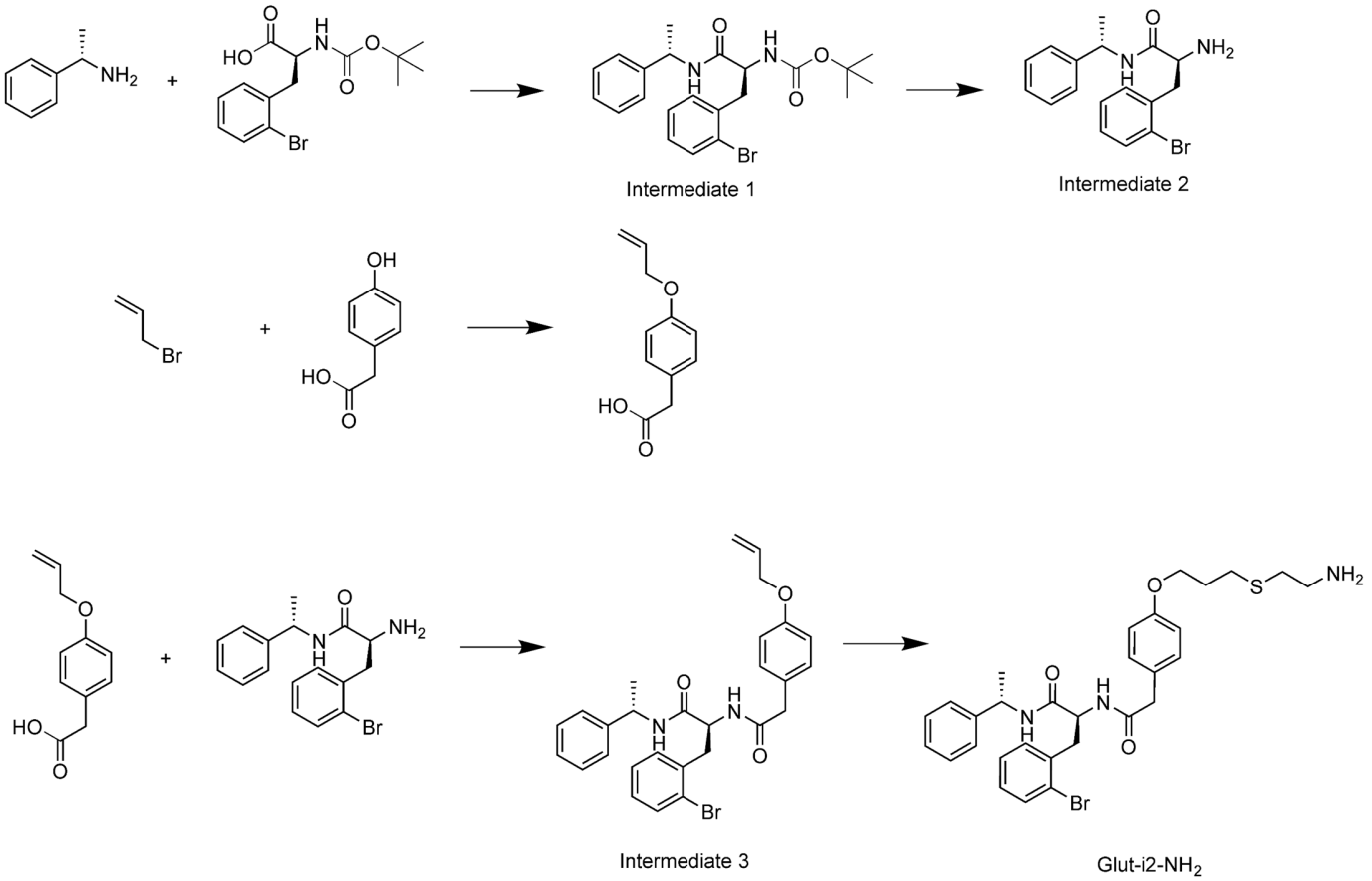
Blood glucose regulation of *i*-insulin using chemically-induced type 1 diabetic mice. The animal study protocol was approved by the Institutional Animal Care and Use Committee at North Carolina State University and the University of California, Los Angeles. Diabetic C57BL/6J mice (chemically-induced) were purchased from Jackson Laboratory. Diabetic mice (n=5) were treated with *i*-insulin (6 mg/kg), native insulin (1.5 mg/kg) or PBS subcutaneously. The blood glucose levels were monitored using a glucose meter (ACCU-CHEK). Living imaging of the mice treated with subcutaneously-injected Cy5-labeled insulin or *i*-insulin was measured by an IVIS Spectrum imaging system (Perkin Elmer).

Intraperitoneal glucose tolerance test. Diabetic mice (n=5) treated with subcutaneously injected *i*-insulin (6 mg/kg) or native insulin (1.5 mg/kg) were intraperitoneally administrated with a glucose solution (1.5 g/kg) three hours posttreatment. The blood glucose levels were monitored by an ACCU-CHEK glucose meter. The insulin levels in plasma (n=4) were measured using an ELISA kit following the provided protocol (Invitrogen, USA).

Hypoglycemia induction on healthy mice. Healthy C57BL/6J mice (n=5) were subcutaneously injected with *i*-insulin (3 mg/kg) or native insulin (0.75 mg/kg) in PBS. Blood glucose levels were monitored using a glucose meter (ACCU-CHEK).

Host immune response to *i*-insulin. Both diabetic and healthy mice (n=4 or 5) were treated with *i*-insulin continuously for two weeks. Each mouse received one injection per day. The dose was set as 6 and 3 mg/kg for diabetic and healthy mice, respectively. The serum was collected after one- or two-week treatment using serum separator tubes (Lot No. BD 365967). The serum was stored at -80ºC before measurement. The IgG and IgM levels were measured using ELISA purchased from Abcam (Lot No. ab157719 (IgG) and ab215085 (IgM)).

Statistical analysis. The data were shown here as mean ± SD or mean + SD. One-way analysis of variance (ANOVA) with Tukey post-hoc tests were used to calculate the significance as indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



Scheme S1. Synthetic route of Glut-i2-NH_2.

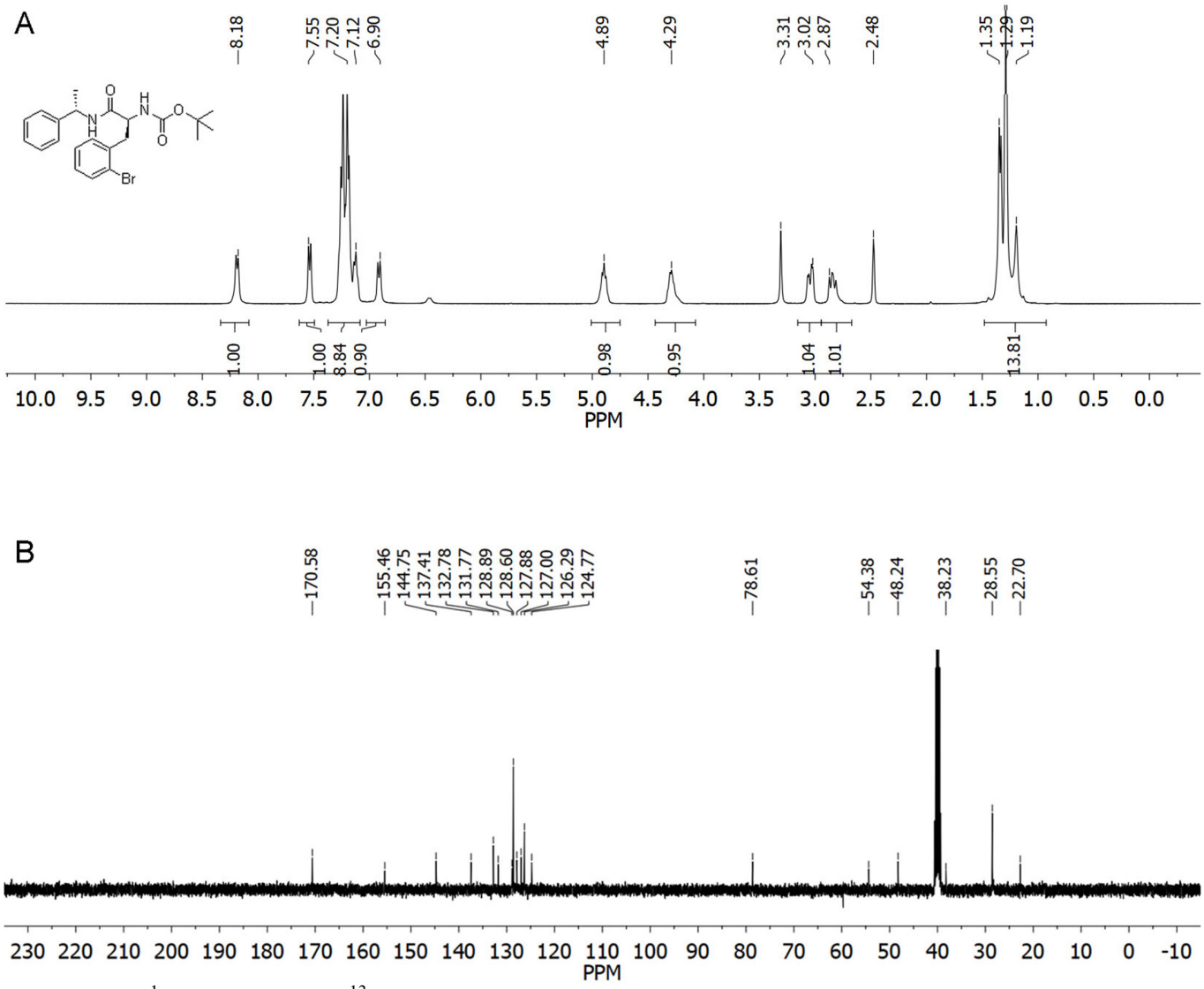


Fig. S1. The ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of Glut-i2-intermediate 1.

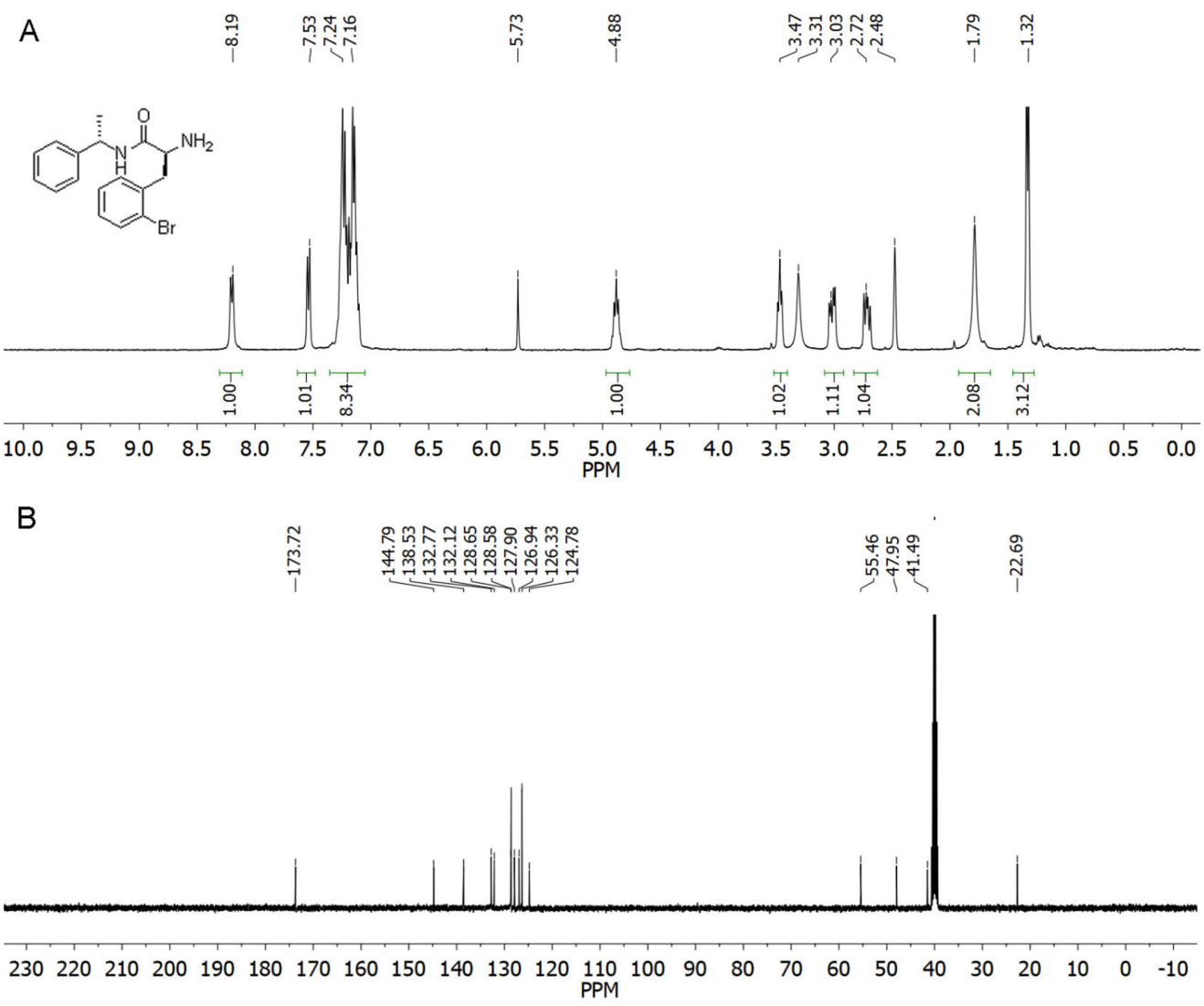


Fig. S2. The ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of Glut-i2-intermediate 2.

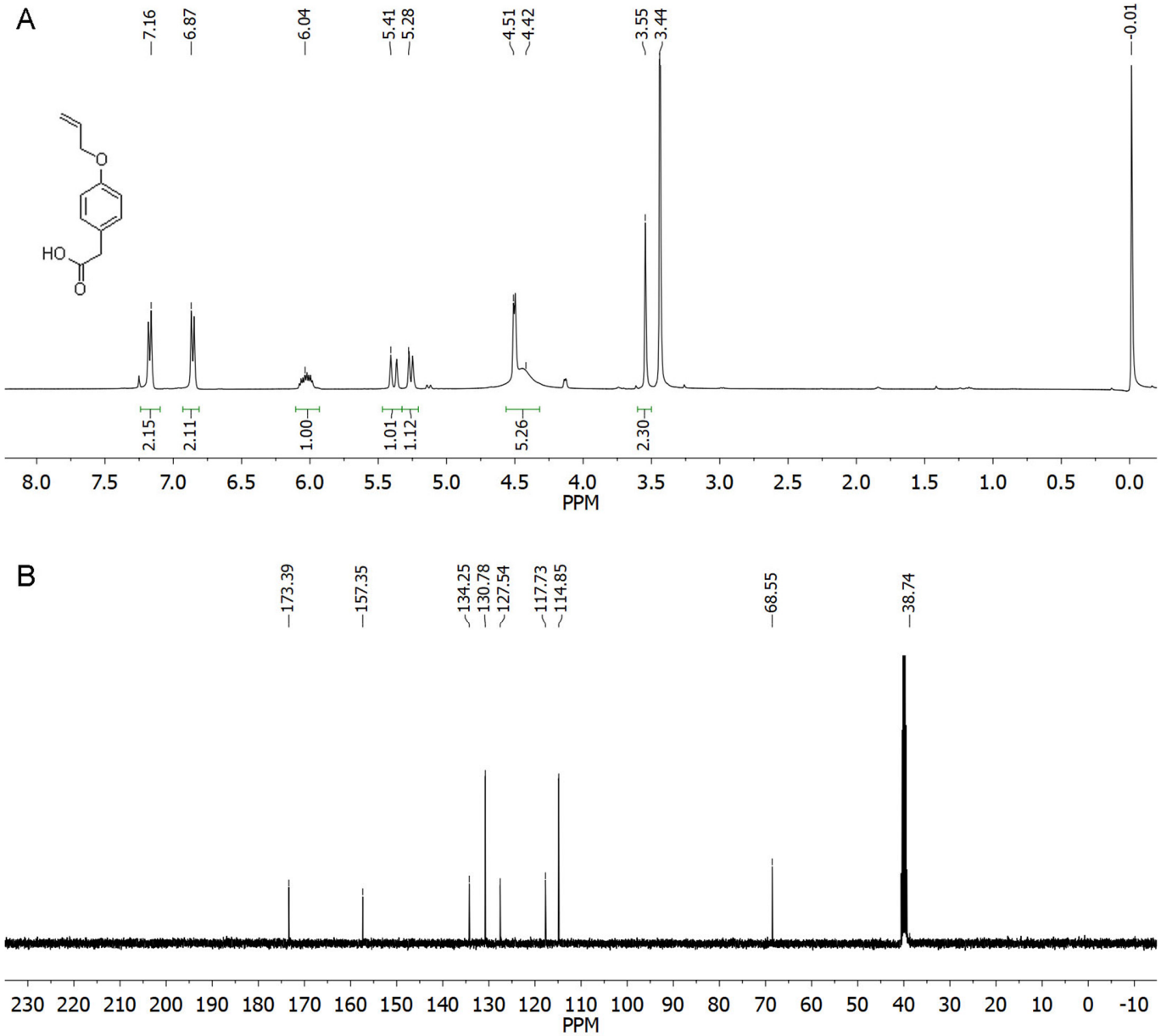


Fig. S3. The ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of 2-(4-(allyloxy)phenyl)acetic acid.

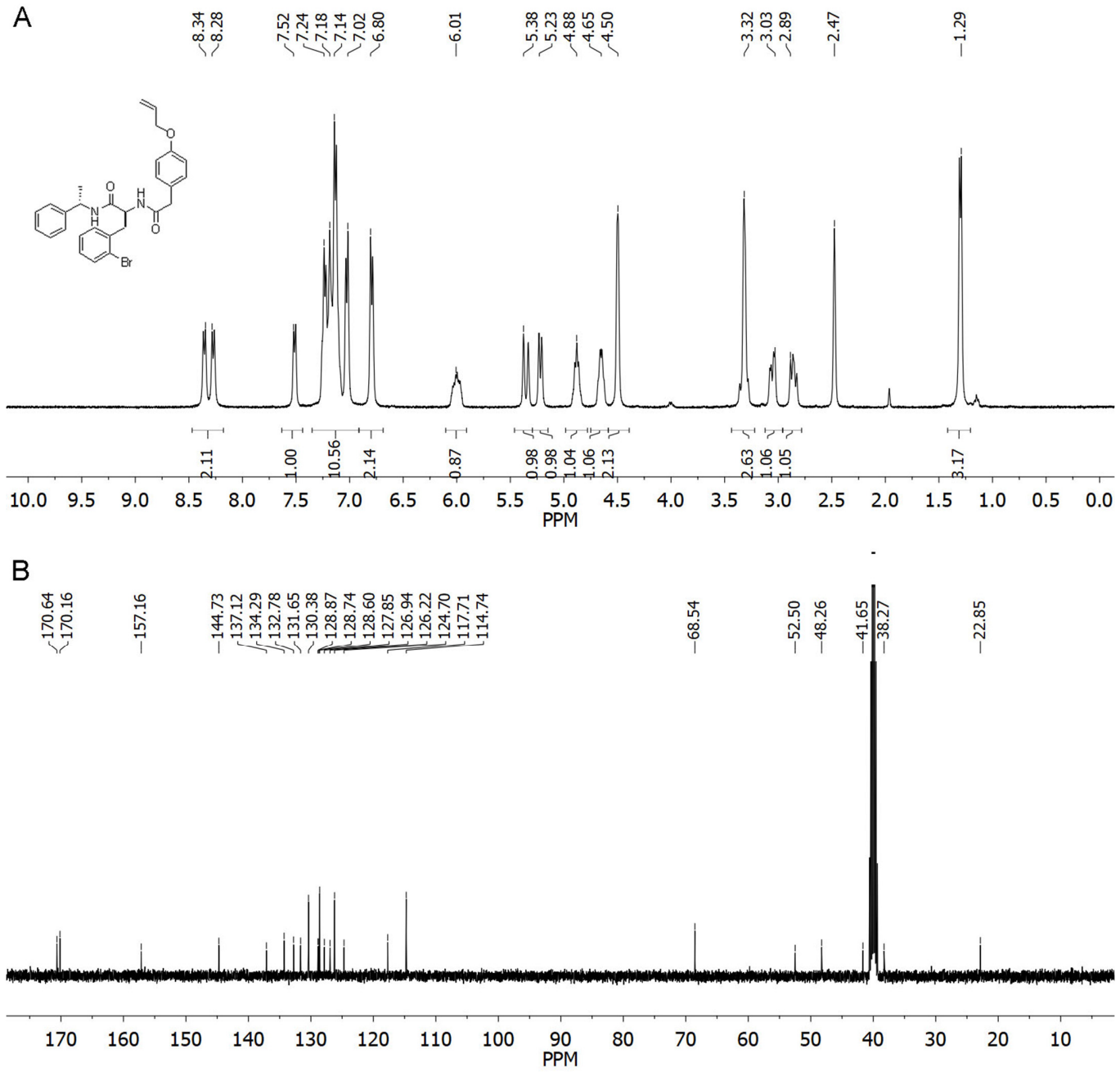


Fig. S4. The ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of Glut-i2-intermediate 3.

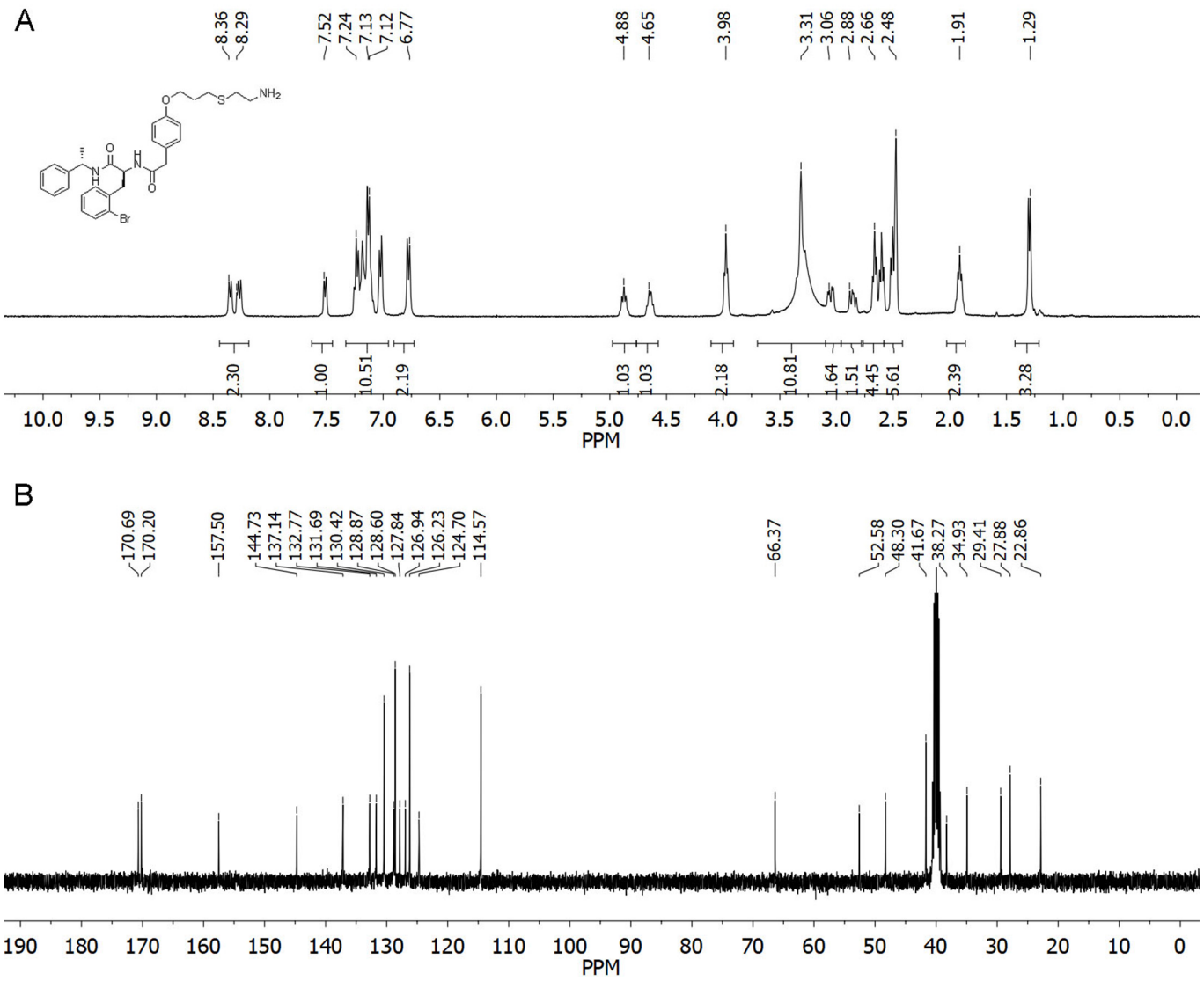


Fig. S5. The ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of Glut-i2-NH$_2$.

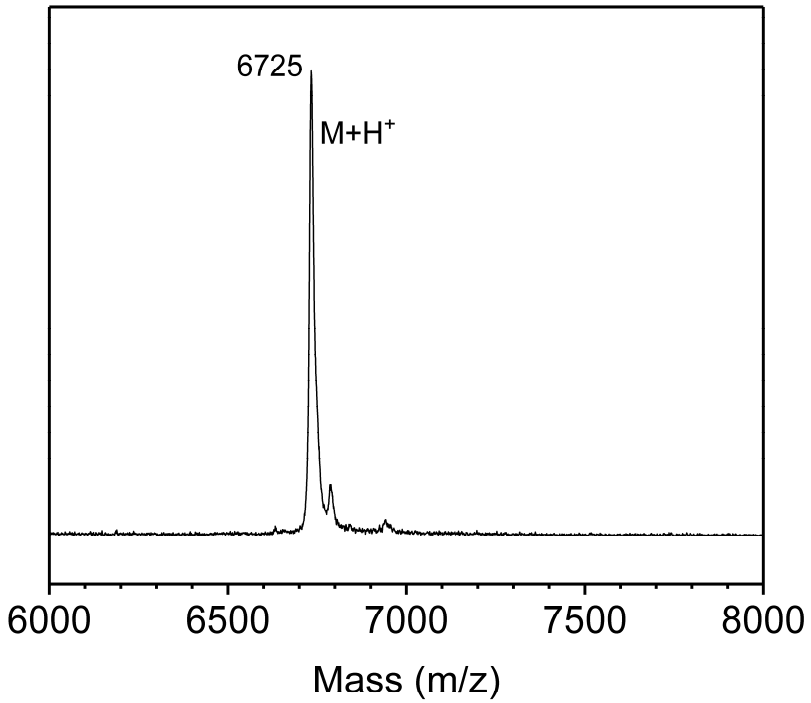


Fig. S6. MALDI-TOF mass spectrum of *i*-insulin. MALDI-TOF is the abbreviation of matrix-assisted laser desorption ionization-time of flight.

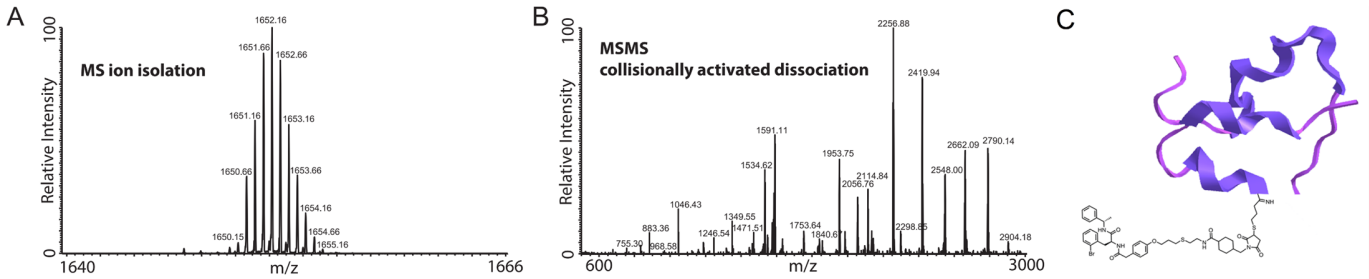


Fig. S7. Validation of the modification site of *i*-insulin. (A) The precursor ion isolation used for the dissociation experiment. (B) The product ion spectrum after collisionally activated dissociation. (C) Schematic of the structure of *i*-insulin.

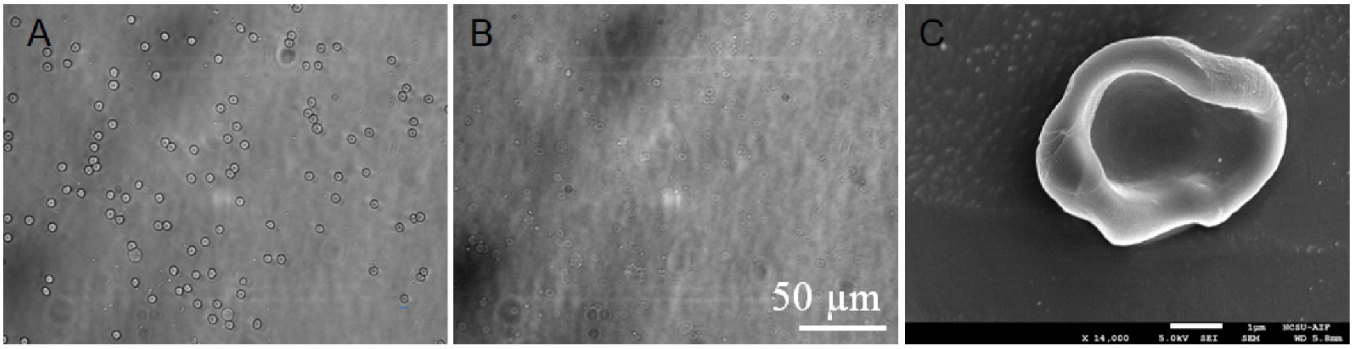


Fig. S8. Representative polarizing microscopy images of red blood cells (A) and erythrocyte ghosts (B). Scale bar, 50 µm. (C) The representative Cryo-SEM image of erythrocyte ghosts. Scale bar, 1 µm. The Cryo-SEM image was taken on a JEOL JSM-7600F SEM outfitted with a cryogenic transfer system and stage.

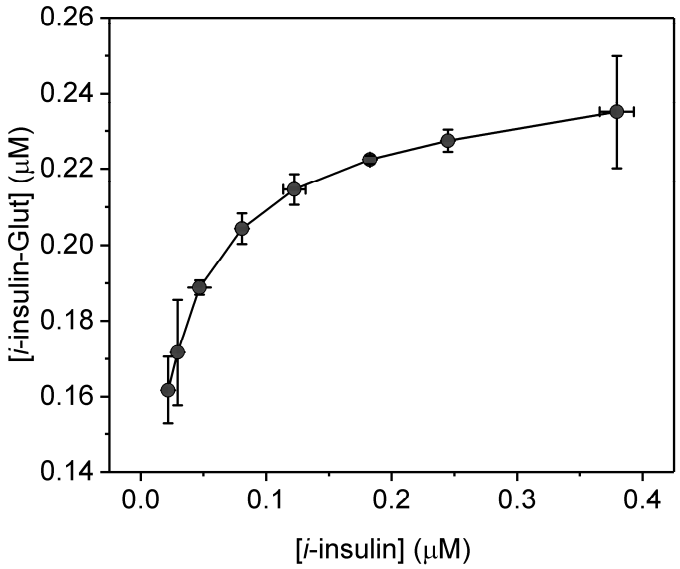


Fig. S9. [*i*-insulin-Glut] as a function of [*i*-insulin]. *i*-insulin was labeled with sulfo-Cy5. Data are presented as mean ± SD (*n*=3).

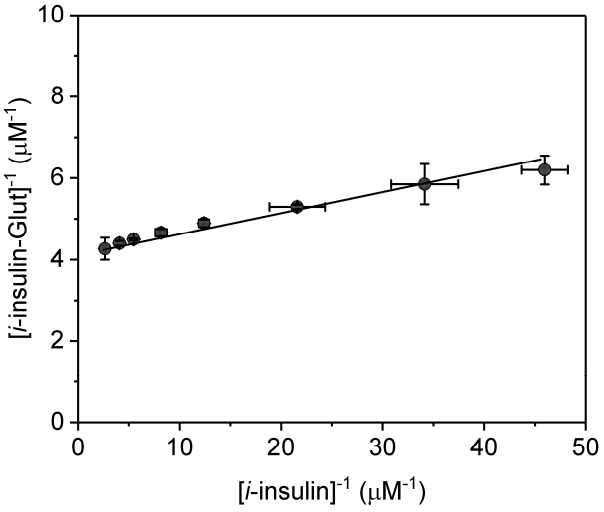


Fig. S10. The reciprocal of [*i*-insulin-Glut] as a function of the reciprocal of [*i*-insulin]. Data are presented as mean ± SD (*n*=3).

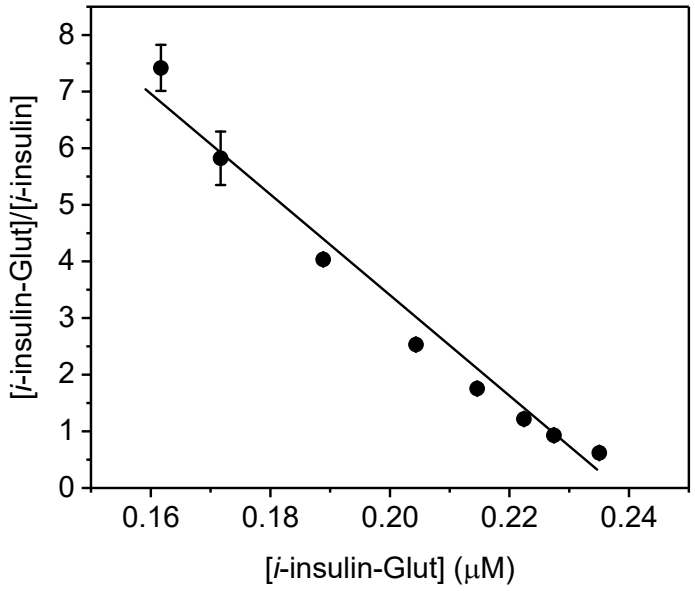


Fig. S11. The ratio of [*i*-insulin-Glut] to [*i*-insulin] as a function of [*i*-insulin-Glut]. Data are presented as mean ± SD (*n*=3).

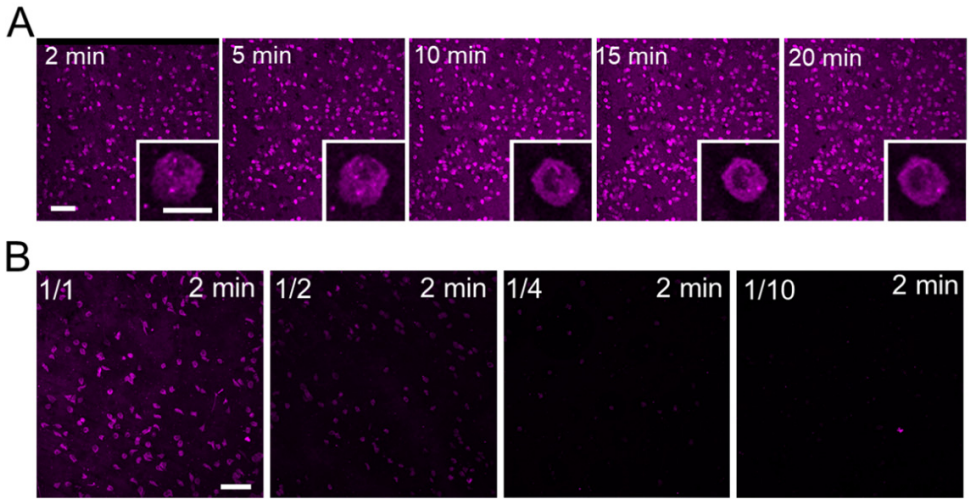


Fig. S12. *In vitro* binding or releasing of *i*-insulin. (A) Representative confocal microscopy images of erythrocyte ghosts over time upon treatment. *i*-insulin labeled with sulfo-Cy5 was set as 1 μM. Scale bar, 50 μm. Insets, the representative enlarged images of erythrocyte ghosts. Scale bar, 5 μm. (B) Representative confocal microscopy images of erythrocyte ghosts upon dilution. *i*-insulin-treated erythrocyte ghost solution was diluted to 1/2, 1/4, and 1/10 of the original density and observed within 2 min. Scale bar, 50 μm. All the images were taken by CLSM.

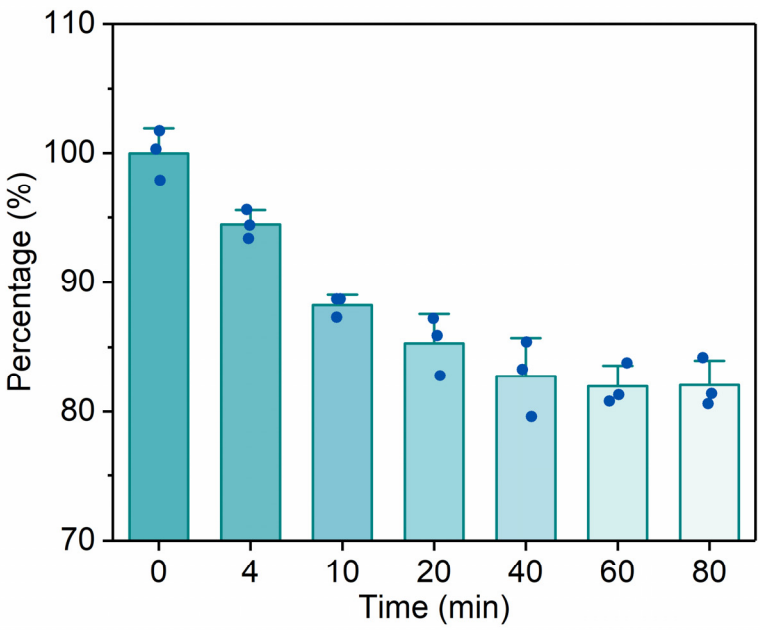


Fig. S13. The binding kinetics study. The fluorescence intensity of the supernatant was recorded. The averaged intensity at 0 min was set as 100%. Data are presented as mean + SD (*n*=3).

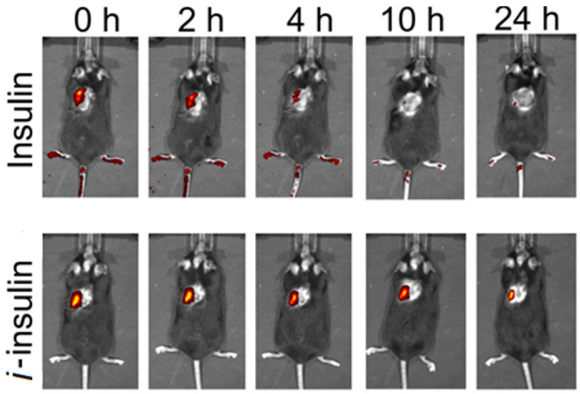


Fig. S14. The living imaging of mice treated with *i*-insulin or native insulin. *i*-insulin and native insulin were both labeled with sulfo-Cy5.

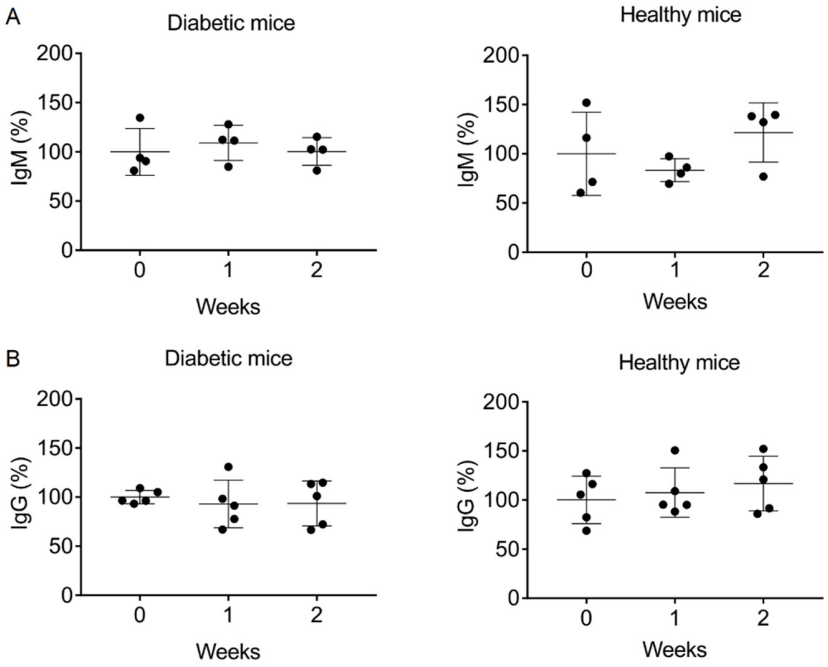


Fig. S15. The host response of mice to continuous treatment with *i*-insulin. Panel A represents the change of IgM levels, while panel B represents the change of IgG levels. The time points of observing the immune response were set as one and two weeks from the first day of treatment as shown in the x-axis. 0 week indicated the time point before treatment. The dose was set as 6 and 3 mg/kg for diabetic and healthy mice, respectively. All the mice received one injection per day. The serum was collected from both diabetic and healthy mice before or after one- or two-week continuous treatment. The serum collected from mice before treatment was used as control and set as 0 week. Data are presented as mean ± SD (n=4 or 5).

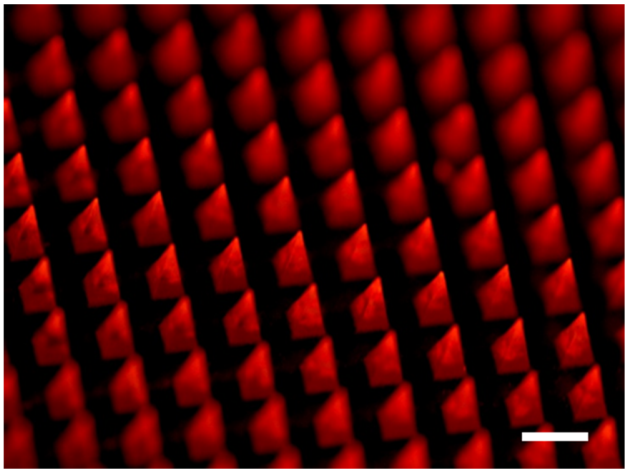


Fig. S16. Representative fluorescence image of microneedle array patch loaded with *i*-insulin. Sulfo-Cy5 was used to label *i*-insulin (shown in red). Scale bar, 600 μm.

$$\frac{[i-insulin-Glut]}{[i-insulin]} = \frac{[Glut_0]}{K_d} - \frac{[i-insulin-Glut]}{K_d} \quad \text{(S1)}$$

or

$$\frac{1}{[i-insulin-Glut]} = \frac{K_d}{[Glut_0][i-insulin]} + \frac{1}{[Glut_0]} \quad \text{(S2)}$$

Eqs. S1-2. [Glut$_0$] is the total concentration of Glut. K_d is the dissociation constant.