Supporting Information

Light Control of Insulin Release and Blood Glucose Using an Injectable Photoactivated Depot

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Materials and Methods

Materials

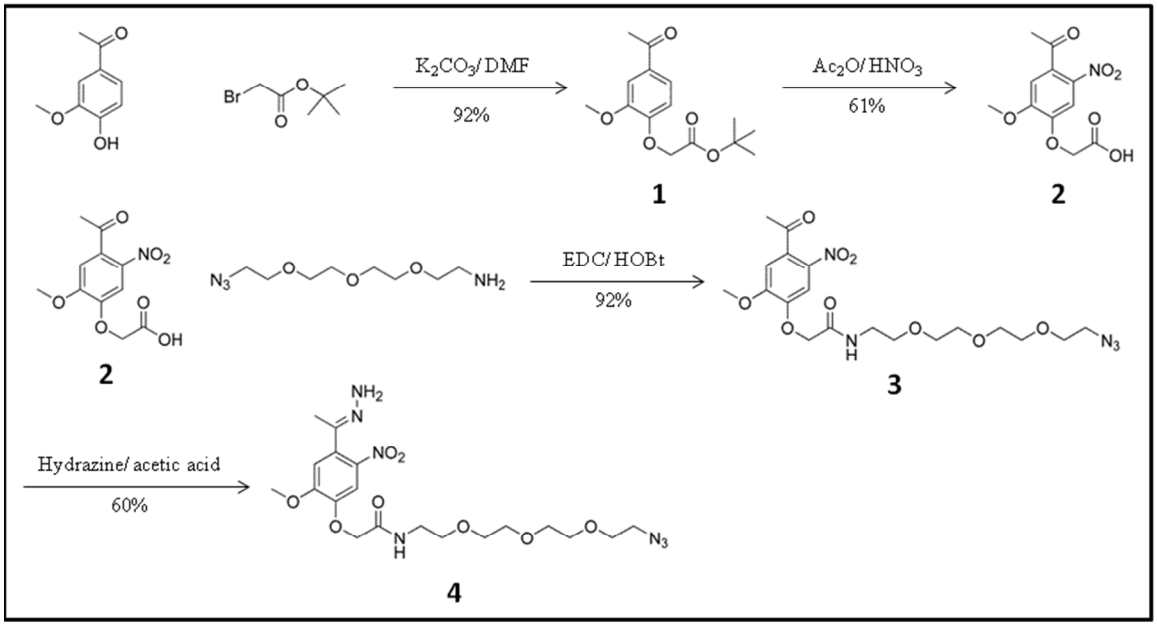
Acetovanillone, t-butyl bromoacetate, nitric acid, 11-azido-3,6,9-trioxaundecan-1-amine, magnesium sulfate, hydrazine monohydrate, manganese dioxide, molecular sieves, HATU and human recombinant insulin were purchased from Sigma Aldrich. DMSO and DMF were stored in molecular sieves containing glass vials and used as stated. DMF, DMSO, DCM, acetonitrile, methanol, ethanol, ethyl acetate, sodium chloride, trifluoroacetic acid, acetic anhydride, 1 N HCl, diethyl ether, sodium bicarbonate and Celite545 were purchased from Fisher Scientific. Additional regents/components were: DBCO acid (Click Chemistry Tools), hydroxybenzotrizole hydrate (Peptide International), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (ThermoFischer Scientific), Tentagel M RAM 10 μm monosized resin (Rapp-Polymere), Vivaspin 500 5000 MWCO PES filter (Sartorius), Microvette 100 μL Li-HEP tubes for blood collection (Sarstedt), ultrasensitive Insulin ELISA kits (Alpco) Flat bottom glass vial insert 100 μL, diameter 3.4 mm (inner), 4.5 mm (outer), height 30.5 mm (Agilent, 5183-2090).

HPLC and Spectrometry

UV-Vis analysis was performed using a USB-2000 fiber optic spectrometer (Ocean Optics, Inc.) with a DT-Mini-B lamp source. HPLC analysis was performed using modular Hewlett Packard Agilent 1050 and 1260 series instruments with attached vacuum degasser, auto sampler and diode array detector. C18 Hypersil (5 μm, 250 × 4.6 mm, Varian) columns were used as stated in the method. HPLC-MS was performed using a modular Agilent 1100 series with DAD coupled with a Q-Trap mass spectrometer (ABI) in a positive ion mode. For HPLC-MS analysis C18 Hypersil (5 μm, 250 × 4.6 mm, Varian) column was used. ESI-MS of small molecules was performed using the Q-Trap mass spectrometer (ABI) instrument in infusion mode. ESI-MS for insulin containing molecules was performed using a 3200 Q-Trap mass spectrometer.

Synthesis of 1, 2, 3, 4, 5, 6, 7 & 8

Compounds 1-4:



Scheme 1: Synthetic scheme of compounds 1, 2, 3, 4.

Compounds **1, 2** were synthesized in two steps as previously described(*1*).

Compounds **3, 4** were synthesized as previously described(*2*)

(5) N-(2-{2-[2-(2-Azido-ethoxy)-ethoxy]-ethoxy}-ethyl)-2-[4-(1-diazo-ethyl)-2-methoxy-5-nitro-phenoxy]-acetamide

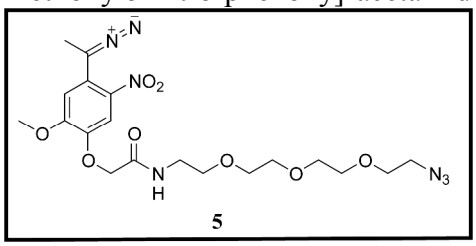


Figure 1. Compound 5

Manganese (IV) oxide (104 mg, 1.23 mmol) was added to a solution of compound **4** (20.7 mg, 43 μmol) in 329 μL of anhydrous dimethyl sulfoxide. This mixture was shaken gently for 45 min. keeping it protected from light with aluminum foil. The red-black mixture was centrifuged and the supernatant was filtered through Celite 545 using a glass-wool plugged glass-pipette. The celite pad was washed with

dimethyl sulfoxide to get a final volume of 1.16 mL. Compound 5 was freshly prepared when required to cage. Compound 5 was used immediately for the caging reaction without further purification. UV/vis (DMSO): λ_{max} : 283 nm, 440 nm.

(6) Insulin azide mixture

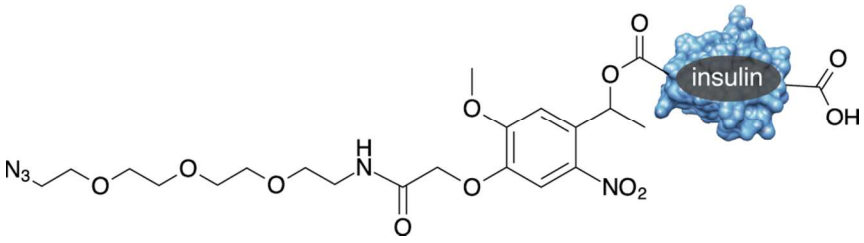


Figure 2. Insulin mono-azide

Freshly prepared compound **5** (approx. 43 µmol) in DMSO (1.16 mL) was added to a solution of human recombinant insulin (0.2 gm, 34.4 µmol) in DMSO (16.04 mL). The mixture was gently shaken for 24 h, protected from light. The mixture was freeze dried and reconstituted in 0.01 N HCl. The sample was then filtered and washed twice with 0.01 N HCl using a spin filter (Vivaspin 500 - 5K MWCO membrane centrifugal filter) by following the provided protocol. This was to remove excess reagents, while retaining the > 5000 mw insulin conjugates. The concentrate then was reconstituted in 0.01 N HCl and freeze dried to remove the solvent.

The mixture of insulin + caged insulin was recovered (203.3 mg) after freeze drying. Insulin, insulin mono-azide and insulin di-azide were analyzed using reversed phase HPLC. Reversed phase HPLC (flow rate 1 mL/min, runtime 30 minutes) solvent A (0.1% TFA in H_2O), solvent B (0.1% TFA in acetonitrile (ACN)), gradient 0% B to 100% B over 30 minutes, post-run 0% B for 5 minutes, C_{18} Hypersil column (5 µm, 250 × 4.6 mm, Varian): retention time (min), relative yield (%); insulin (15.9 min, 62.9%) monoazide insulin (17.2-18.4 min, 33.1%); di-azide insulin (18.7-19.7 min, 3.9%) (Figure 5). The crude insulin-azide mixture was analyzed using direct infusion ESI-MS in 0.01 N HCl (Figure 8). Individual species i.e. insulin, insulin mono-azide and insulin di-

azide were purified from the HPLC and analyzed using direct infusion in ESI-MS for MS characterization. For direct infusion ESI−MS (m/z): $[M]^+$ calculated for insulin, 5808.0; found, 5811.0; $[M]^+$ calculated for insulin mono-azide, 6262.2; found, 6265.0; [M]+ calculated for insulin di-azide, 6716.4; found, 6719.0; for ESI-MS of individually purified peaks; $[M]^+$ calculated for insulin, 5808.0; found, 5804.0; $[M]^+$ calculated for insulin mono-azide, 6262.2; found, 6259.0; $[M]^+$ calculated for insulin di-azide, 6716.4; found, 6712.0 (Figure 9-11). Extinction coefficient calculated ($\varepsilon_{280\ nm}$): insulin (5128 $M^{-1}\ cm^{-1}$), mono-azide (8400 $M^{-1}\ cm^{-1}$), di-azide (11672 $M^{-1}\ cm^{-1}$) (Figure 14-16)

(7) DBCO conjugated resin

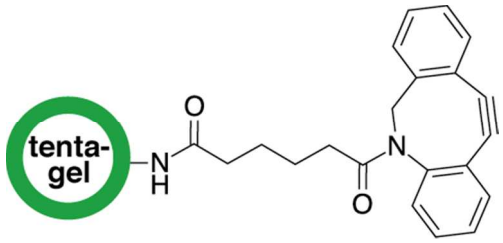


Figure 3. DBCO conjugated tenta-gel

10 µm Tentalgel R RAM resin (85 mg, 17 µmols of amino groups) was washed three times with DMF (600 µL). It was treated three times with 20% piperidine in DMF (700 µL) for 5 minutes each. The resin was then washed with DMF 5 times. A solution of DBCO acid (6 mg, 18 µmols, 250 mM), HATU (6.84 mg, 18 µmols, 250 mM) and N,N-diisopropylethylamine (6.25 µL, 36 µmols, 500 mM) in DMF (72 µL) was shaken for 15 min. This activated solution was added to the washed resin and was gently stirred for 20 h. This resin was washed several times with DMF and then methanol, and then dried under vacuum and stored in 4°C if not used immediately. A small amount was prepared for analysis by with 95% TFA (500 µL) for 90 minutes. The purple colored cleaved product, DBCO amide, was recovered by removing TFA under vacuum and reconstituted the dried product in methanol (100 µL). This product was analyzed first by RP-HPLC (figure 6), and then by reversed phase HPLC-MS (figure 13). HPLC conditions in both case were as follows: Flow rate 0.4 mL/min, runtime 30 minutes. Solvent A (0.1% formic acid in H_2O), solvent B (0.1% formic acid in acetonitrile (ACN)), gradient 0% B to 50%

B over 5 minutes, gradient 50% B to 100% B from 5 to 27 minutes, isocratic 100% B over next 2 minutes, gradient 100% B to 0% B for 1 minute, C18 Hypersil column (5 µm, 250 × 4.6 mm, Varian): retention time (min) 17.8; ESI−MS (m/z): $[MH]^+$ calculated for $C_{19}H_{27}N_5O_9$, 333.1; found, 333.1 (Figure 13).

(8) Insulin conjugated resin

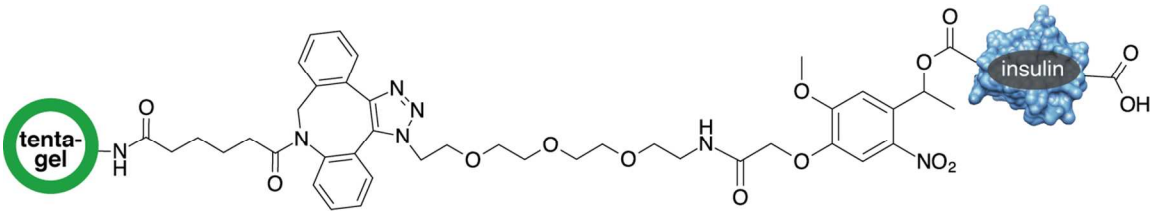


Figure 4. Insulin conjugated resin

25 mg DBCO conjugated resin **(7)** was washed three times with 500 µL DMF and five times with 500 µL MilliQ water. In another tube, 11.2 mg of insulin azide mixture **(6)** was dissolved in 200 µL of 0.01 N HCl and the pH was brought to 7.5 using 1 N NaOH until it became a clear solution. This insulin azide solution was then mixed with the DBCO conjugated resin and stirred at 550 rpm at 37°C for 48 hours. After the reaction, it was washed multiple times with sterile PBS to wash off any unbound insulin. Supernatant analysis (before and after the reaction) was performed using reversed phase HPLC using the HPLC gradient used for **6**. The disappearance of insulin azide with respect to insulin from supernatant was measured to calculate amount immobilized on the resin. The integration area of insulin & insulin azide at 280 nm on HPLC chromatogram was normalized with its extinction coefficient to quantify the amount disappeared (Figure 7).

The click reaction was analyzed by chemical cleavage of the rink amide linkage. Insulin conjugated resin **7** (3.75 mg) was washed five times with methanol followed by ether, dried under vacuum. The dried resin was treated with 500 µL 95% TFA for 90 minutes. After removing the TFA by evaporation, the cleaved product was reconstituted in 0.01N HCl and used for ESI-MS characterization.

TFA cleavage product: ESI−MS (m/z): [M]+ calculated for insulin-triazole linker, 6593.4; found, 6597.0 (Figure 12).

This reaction was done in multiple replicates on this scale. The loading of insulin monoazide on the resin was calculated to be within the range of 14-18 nmoles/ mg in different parallel runs.

In-vitro photolysis of Insulin conjugated Tentagel resin

140 nmoles of insulin conjugated on the resin was washed multiple times with PBS. For the photolysis studies, it was suspended in 100 µL PBS. The photolysis was performed in Agilent glass vial insert (catalog # 5183-2090). The irradiation was performed by placing the glass vial insert directly above 365 nm Nischia LED. The irradiation was performed twice (for two minutes each) from t=0 to t=2 min and t=65 to t=67 min. The sample was vortexed for 5 min and centrifuged before taking sample at each time point. 25 µL volume was removed and replaced with same volume of fresh PBS. The amount of insulin released was quantitated using HPLC from insulin standard curve. The solution of the final time point was desalted with 5k MWCO centrifugal spin filter and infused into MS to get molecular weight. The control sample was treated identically except light was blocked by aluminum foil from reaching the sample.

Analytical results

HPLC chromatograms

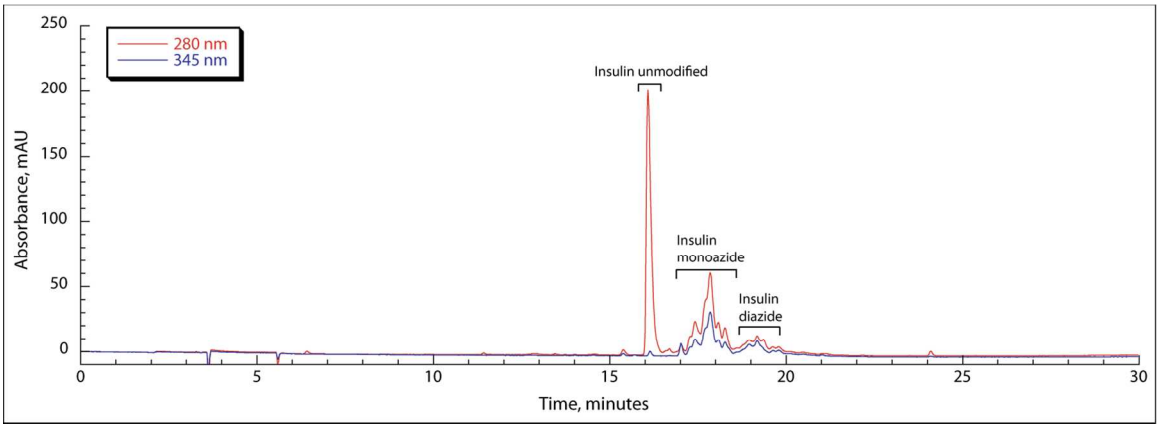


Figure 5: HPLC chromatograms of product **6.** Chromatograms at 280 nm (red) and 345 nm (blue) shown.

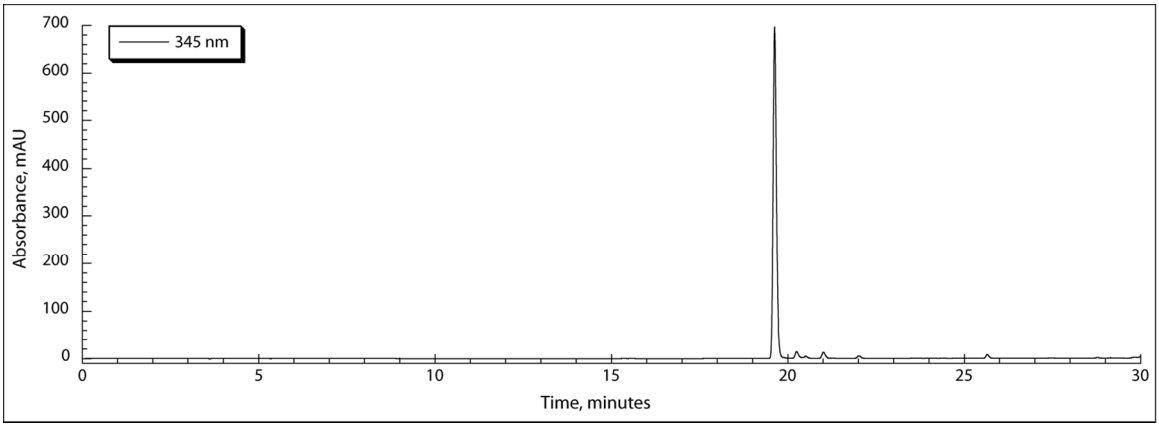


Figure 6: HPLC chromatograms of DBCO amide from the TFA cleavage of **7** represented at 345 nm

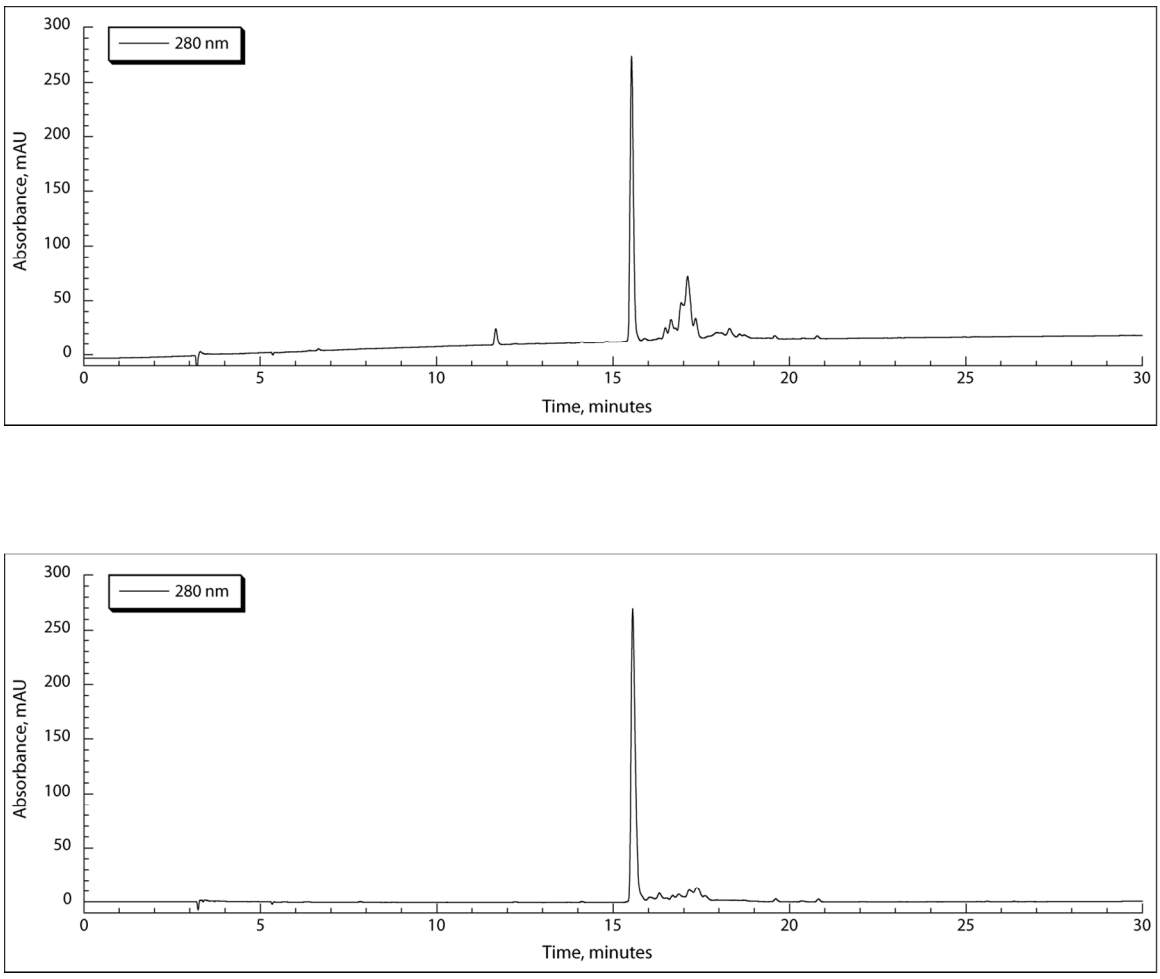


Figure 7: HPLC chromatograms of analysis of the supernatant **6** before the click reaction (top chromatogram) and analysis of the supernatant after 48 h of the reaction (bottom chromatogram) at 280 nm. Chromatogram indicates that insulin azide was consumed during the reaction and hence only unmodified insulin is remaining.

ESI-MS analysis

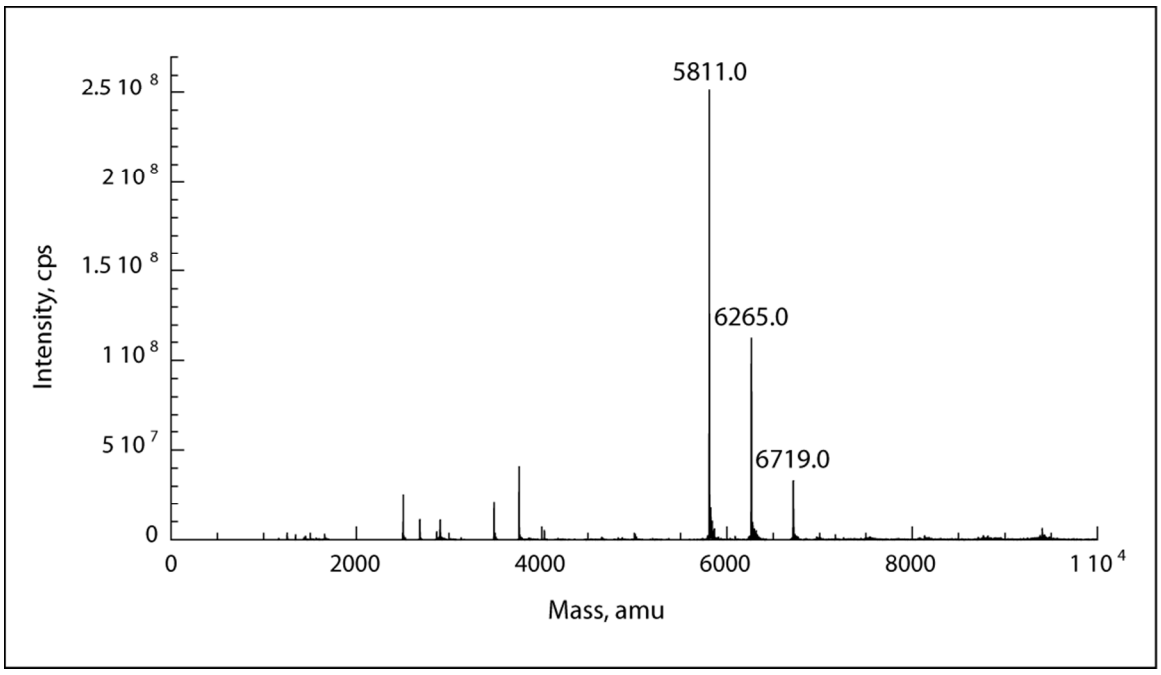


Figure 8: ESI-MS of **6** showing mixture of unmodified insulin with mass of 5811.0 (expected mass = 5808.0), insulin monoazide with mass of 6265.0 (expected mass = 6262.2) and insulin diazide with mass of 6719.0 (expected mass = 6716.4).

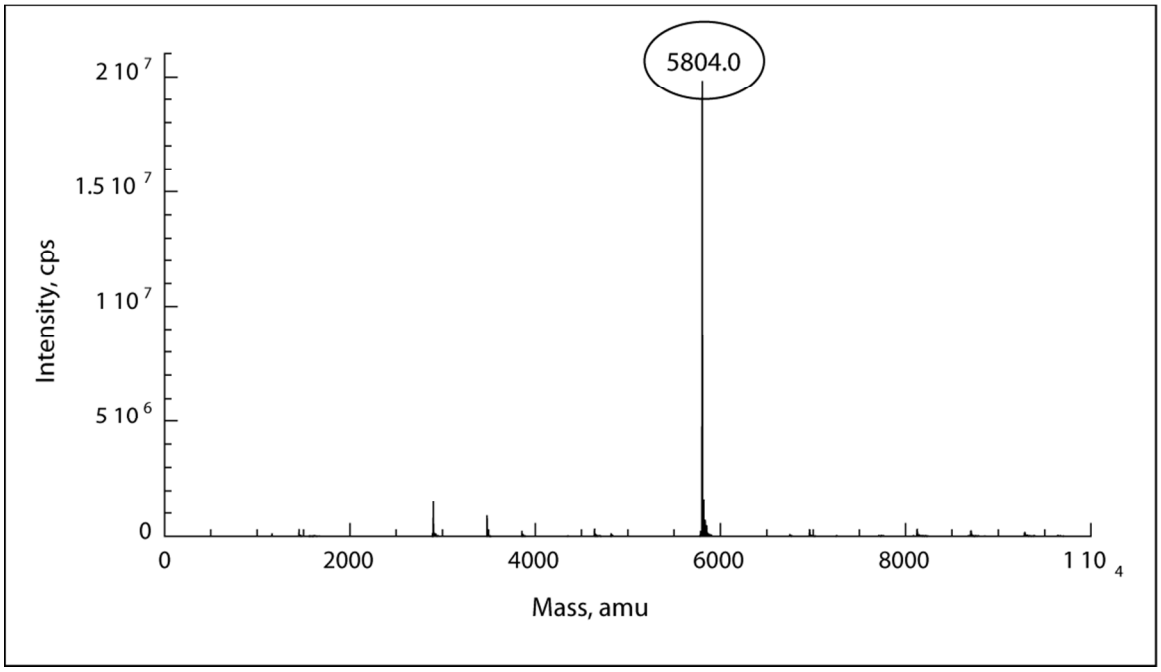


Figure 9: ESI-MS of unmodified insulin purified during HPLC run of **6** (shown in figure 5, elution time 16 minutes), observed mass = 5804.0 (expected mass = 5808.0)

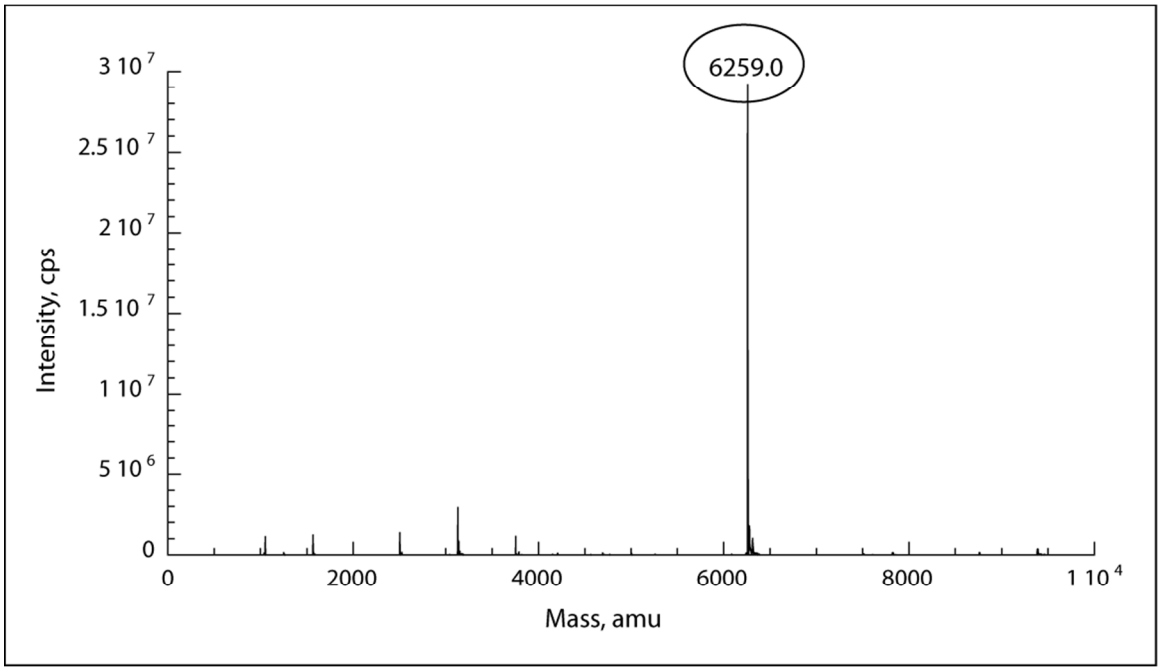


Figure 10: ESI-MS of insulin monoazide purified during HPLC run of **6** (shown in figure 5, elution time between 17.2 – 18.4 minutes) observed mass = 6259.0 (expected mass = 6262.2)

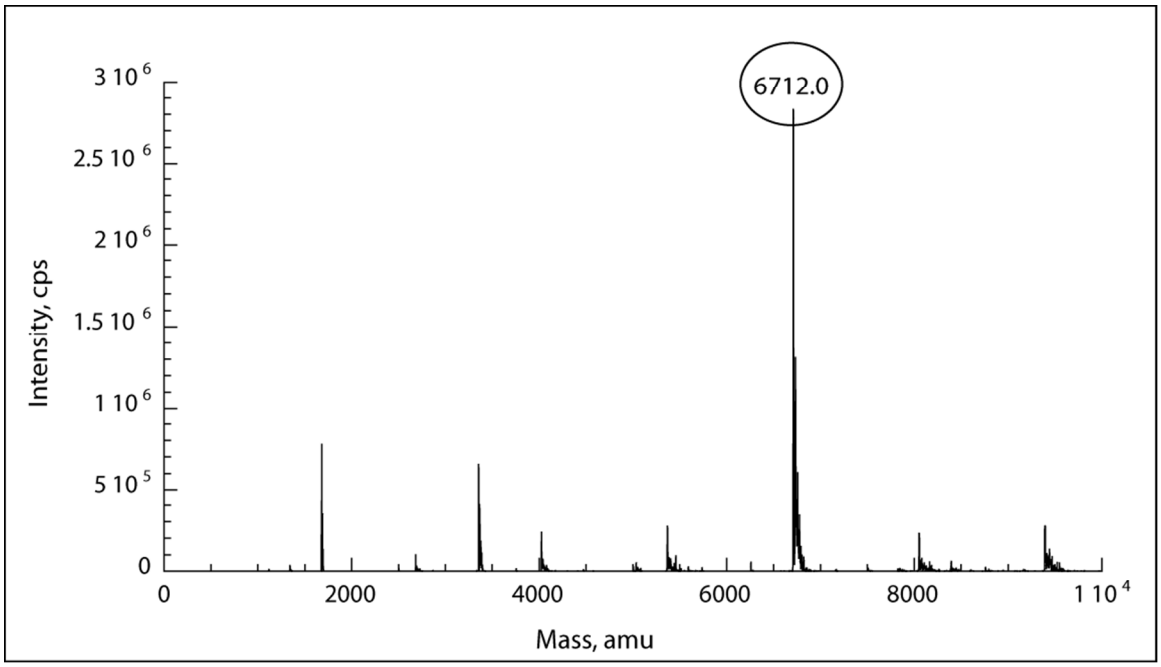


Figure 11: ESI-MS of insulin diazide purified during HPLC run of **6** (shown in figure 5, elution time 18.7 – 19.7 minutes) observed mass = 6712.0 (expected mass = 6716.4)

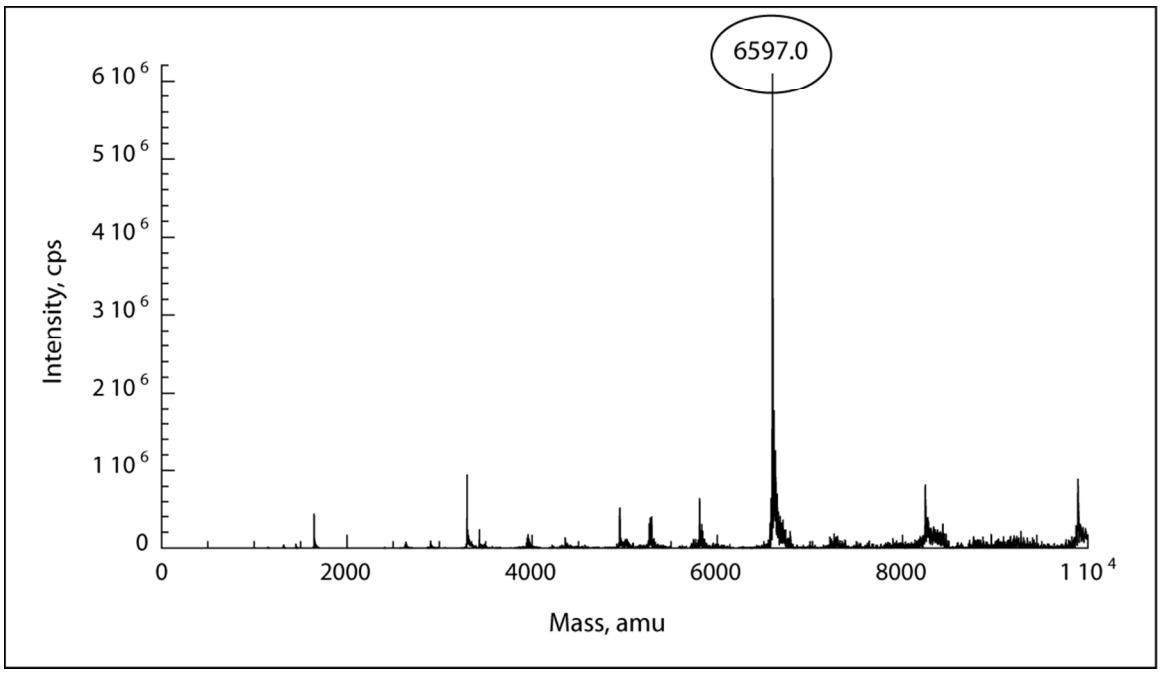


Figure 12: ESI-MS of TFA cleavage of **8** showing mass of 6597.0 (expected mass = 6593.4)

HPLC-MS analysis

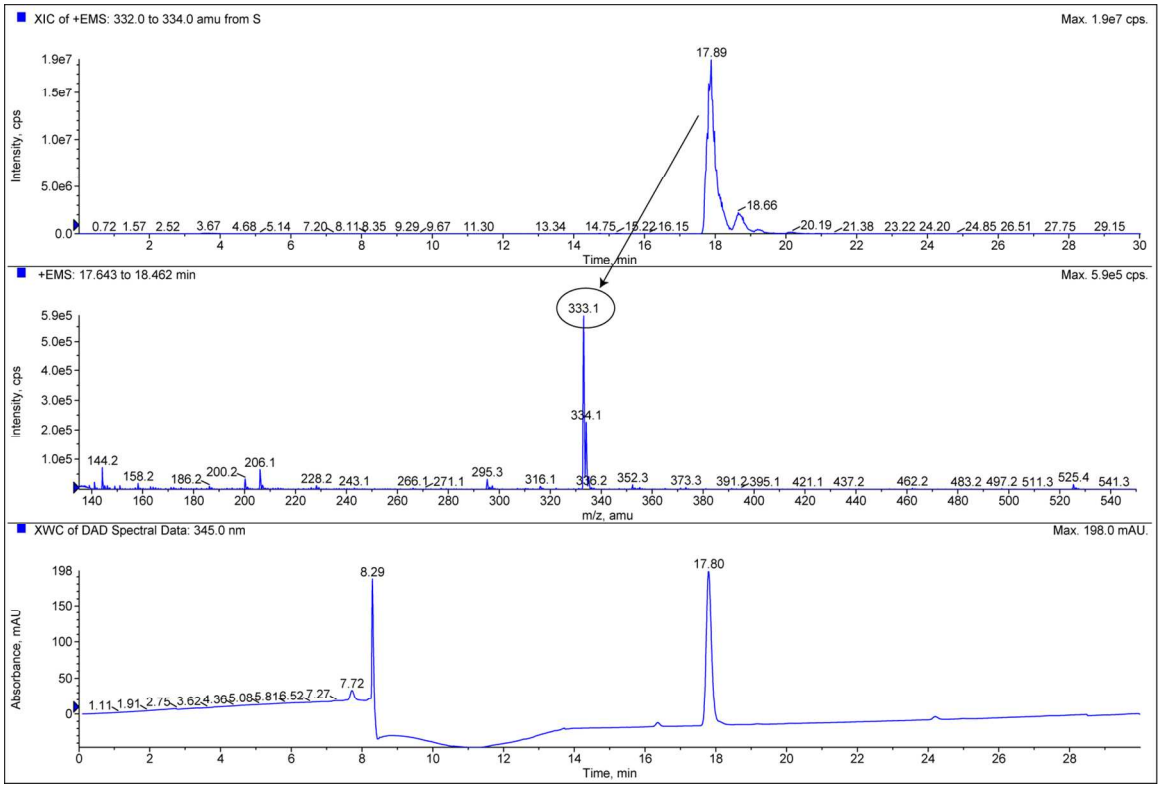


Figure 13: HPLC-MS of TFA cleavage of product **7** represented three panes in the following order from top to bottom, extracted ion chromatogram (XIC) between 332 to 334.0 amu, enhanced multiple spectrum (EMS) of peak from 17.643 – 18.462 min and extracted wavelength chromatogram (XWC) at 345 nm. ESI−MS (m/z): $[MH]^+$ calculated for $C_{19}H_{27}N_5O_9$, 333.1; found, 333.1

UV-vis spectra analysis

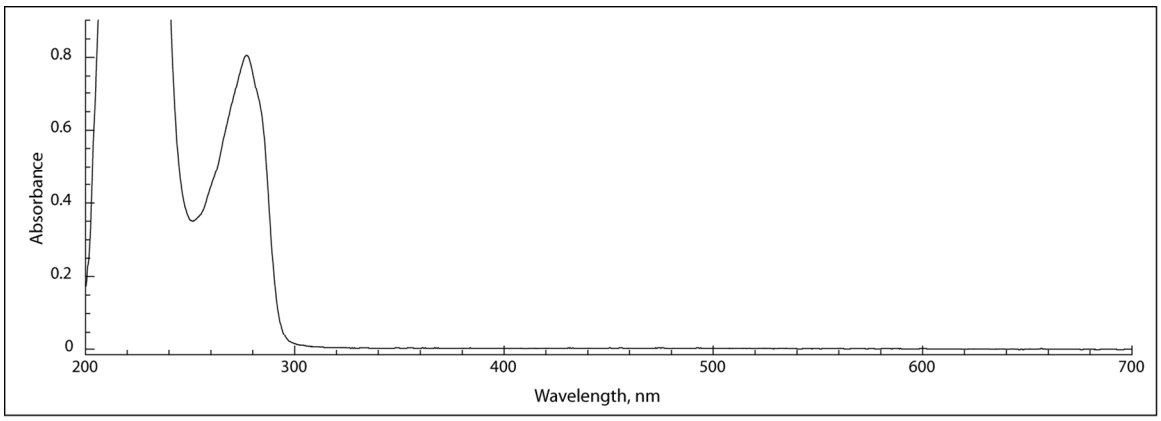


Figure 14: UV-vis spectrum of insulin in DMSO

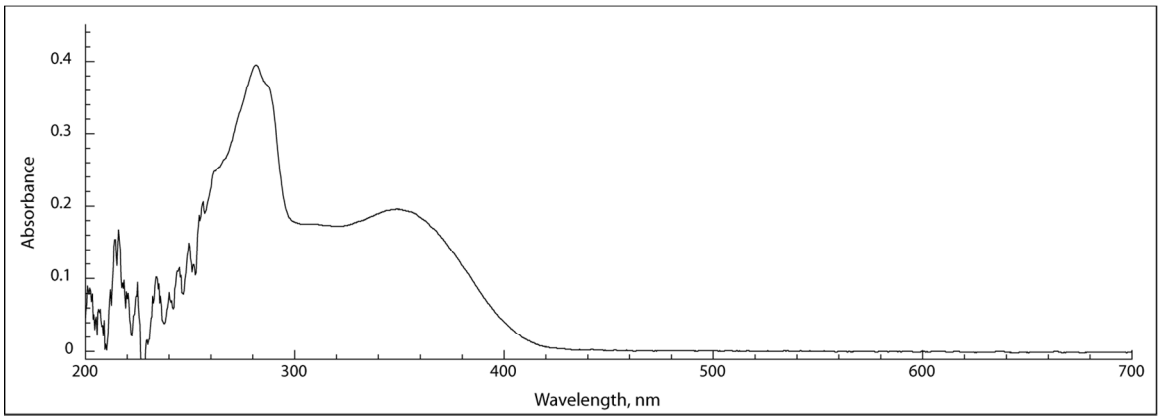


Figure 15: UV-vis spectrum of insulin monoazide in DMSO

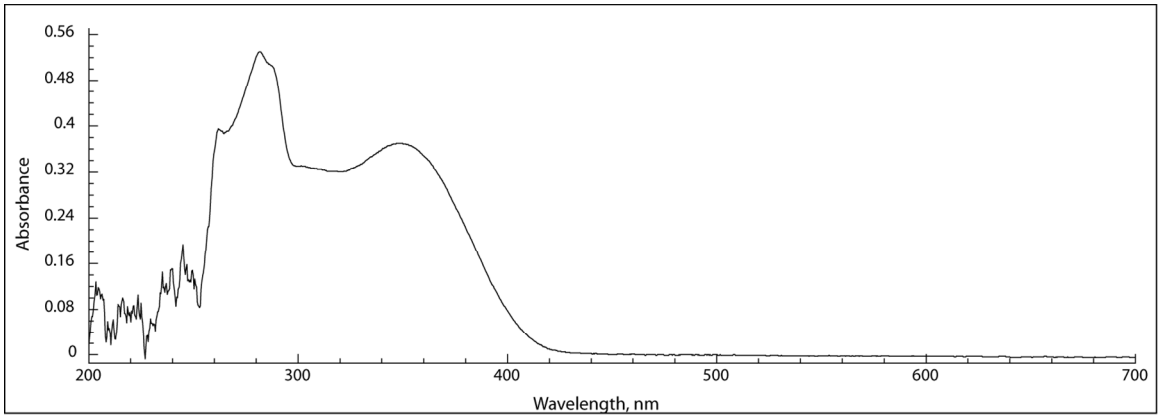


Figure 16: UV-vis spectrum of insulin diazide in DMSO

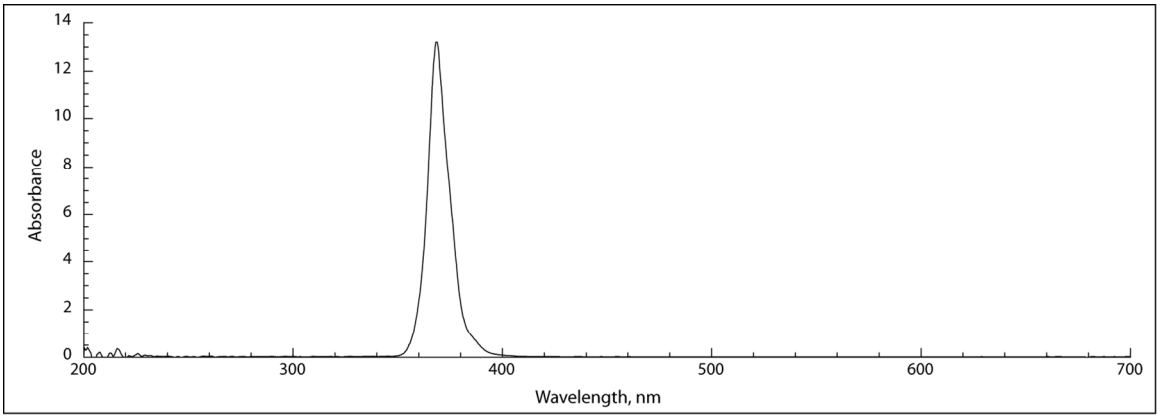


Figure 17: UV-vis emission of LED light source

In-vivo testing of insulin PAD

Light source

The light source was constructed from a Nichia NCSU033B LED, with a 365nm peak irradiation. This was driven by a 6.5 V power source using a current limiting power resistor. The light source holds the LED ~0.32cm from the skin surface, and the measured absolute irradiance at the skin was 0.71 W/cm^2. The absolute irradiance of the light source was determined using a calibrated USB2000 spectrophotometer (Ocean Optics) and a CC-3-UV-S cosine corrector via an optic fiber. The Spectrasuite software was used to analyze absolute irradiance in the range of 350-400 nm that brackets the LED output.

Animals

Spague Dawley male rats (250-300 grams) were obtained from Harlan Laboratories (Indianapolis, IN). Chemical diabetes was induced by treatment with 65mg/kg Streptozotocin (Sigma-Aldrich, St. Louis, MO). Diabetes was defined as blood glucose concentrations >250mg/dl on 3 consecutive days using a One Touch II glucometer and blood obtained from the tail vein.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health 8th Edition. The protocol was approved by the University of Missouri Kansas City Institutional Animal Care Use Committee protocol #1401.

Intradermal injection of PAD material

Rats were anesthetized with isoflurane gas using a precision vaporizer. The upper backs of rats were shaved prior to injection of the PAD materials. Injections of ~80 ul of PAD material were made using a ½ cc syringe and a 27 gauge needle. Due to limitations of the volume of material that can be injected into the dermal layer of skin at one time, 2 injections (40ul each) of PAD materials were made side by side. The compact LED light

source was anchored to the skin over the injection sites by 2 small dots of superglue. Rats' body temperature and hydration was maintained though out the experiment.

Insulin analysis by ELISA

Blood samples were collected in Microvette 100 µL Li-HEP tubes (Sarstedt) from the tail-vein using a glass capillary. After collecting all time points from an experiment, the samples were centrifuged at 5000 rpm for 2 minutes. The supernatant was removed at stored at -20°C until the ELISA analysis was performed. The ultrasensitive human insulin ELISA kit (Alpco, Salem, NH) was used according to the manufacturer's instructions.

Glucose measurements

Glucose measurements were made using One Touch II Lifescan glucometer and strips (Johnson & Johnson, Milpitas, CA) using plasma from blood samples obtained from the tail vein.

Statistical Analysis

The two sample unequal variance t-test was used to calculate p-value between the experimental and control groups (Excel).

References

1. C. P. Holmes, Model Studies for New o-Nitrobenzyl Photolabile Linkers: Substituent Effects on the Rates of Photochemical Cleavage. *Journal of Organic Chemistry* **62**, 2370 (1997).
2. P. K. Jain, D. Karunakaran, S. H. Friedman, Construction of a Photoactivated Insulin Depot. *Angew Chem Int Ed Engl*, (Dec 3, 2012)