Surface Re-Engineering of Pancreatic Islets with Recombinant azido-Thrombomodulin

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CONTENTS

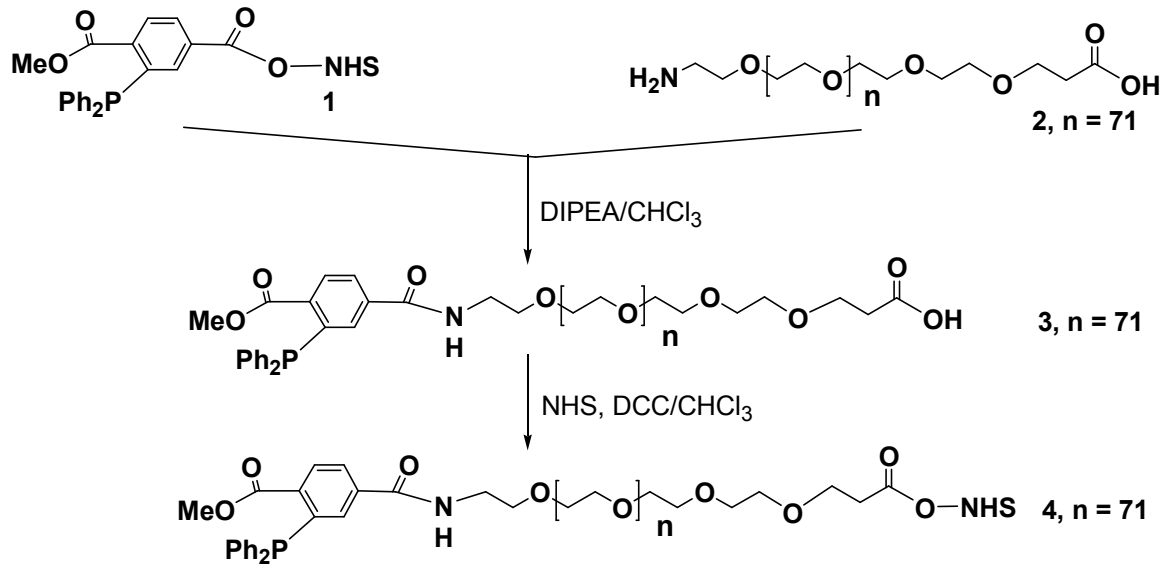
EXPERIMENTAL SECTION

Materials. All chemical reagents were of analytical grade, obtained from commercial suppliers, and used without further purification unless otherwise noted. The methyl-PEG amine 3400 was purchased from Netkar Corp. (Hunstville, AL). All RNA and DNA purification kits were purchased from QIAGEN Inc. (Chatsworth, CA). The sheep monoclonal antibody to human TM, the human TM ELISA kit containing an HRP conjugated sheep monoclonal antibody to human TM, human anti-thrombin, and chromogenic substrate Spectrozyme PCa were purchased from American Diagnostica Inc. (Stamford, CT). Purified recombinant human PC and human alpha-thrombin were obtained from Haematologic Technologies Inc. (Essex Junction, VT).

TM Expression and Purification. Recombinant human azido-thrombomodulin was expressed and purified using methods described elsewhere (1). Briefly, pET39b(+)-TM construct was transformed into the *E. coli* methionine auxotroph B834(DE3) and grown in supplemented M9 minimal medium until the optimum turbidity was reached. Prior to isopropyl-β-D-thiogalactopyranoside (IPTG) induced protein expression, methionine was removed via washing and replaced with the azido-functionalized methionine analogue (2). The recombinant TM was purified from the lysed cell pellet via elution from an immobilized metal-affinity chromatography column containing TALON resin (Sigma, MO) under native conditions using an imidazole gradient. PD10 columns were used to exchange the eluting solvent for dH_2O and the resulting TM was lyophilized until use. For conjugation studies, the lyophilized TM was reconsitituted into fully supplemented RPMI and filter sterilized.

Synthesis of bifunctional poly(ethylene glycol) (PEG) linker: triarylphosphine-PEG-*N*-hydroxysuccinimide (4) (Scheme 1).

3-(Diphenylphosphino)-4-(methoxycarbonyl)benzoic acid active ester **1** was synthesized, as detailed elsewhere (3). Amine-PEG-Acid **2** was purchased from Nektar Therapeutics (CA). The triphenylphosphine-PEG-Acid **3** was prepared by mixing a 2:1 ratio of **1** (81 mg, 0.176 mmol) and **2** (300 mg, 0.088 mmol) in a solution of 5 mL of DIPEA and 5 mL of $CHCl_3$ under argon at room temperature. After reacting for 48 h, the solvent was evaporated under vacuum. The residue was dissolved in 2 mL of $CHCl_3$ and was precipitated by adding cold ether to yield **3** (300 mg, 91%), which was used directly for the next reaction. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.06 (1H, dd, *J* = 9.1, 3.0 Hz), 7.74 (1H, d, *J* = 9.1 Hz), 7.38-7.20 (11H, m), 6.52 (1H, m) 3.83 (3H, t, *J* = 6.7 Hz), 3.76-3.40 (144H, m), 3.41 (2H, t, *J* = 6.7 Hz), 2.59 (2H, t, *J* = 7.3 Hz). The triphenylphosphine-PEG-Acid **3** (98 mg, 0.026 mmol), dicyclohexylcarbodiimide (DCC) (8 mg, 0.039 mmol), and *N*-hydroxysuccinimide (NHS) (4 mg, 0.039 mmol) were dissolved in $CHCl_3$ (10 mL) under Ar and stirred overnight at room temperature. The solvent was then evaporated under vacuum. The residue was dissolved in 2 mL of $CHCl_3$ and was precipitated by adding cold ether to yield **4**, quantitatively. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.04 (1H, dd, J = 9.1, 3.0 Hz), 7.72 (1H, d, *J* = 9.1 Hz), 7.32-7.20 (11H, m), 6.60 (1H, m), 3.80 (3H, t, *J* = 6.7 Hz), 3.76-3.40 (144H, m), 3.42 (2H, t, *J* = 6.7 Hz), 2.86 (2H, t, *J* = 7.3 Hz), 2.78 (4H, s, -C(=O)-CH2-CH2-C(=O)- from NHS).



Scheme 1. Synthesis of bifunctional poly(ethylene glycol) (PEG) linker – triarylphosphine-PEG-*N*-hydroxysuccinimide

Islet Isolation. Islets were isolated and purified from B10.BR mice using standard techniques by intraductal injection of rodent collagenase (Sigma, MO) (4). After isolation, islets were cultured for 48hrs in RPMI 1640 (Sigma) supplemented with L-Glutamine (Sigma), 100units/mL of penicillin, 100mg/mL of streptomycin (Life Technologies, Grand Island, NY), and 10% FCS (Sigma) and kept within a humidified 37˚C, 5% CO_2/95% air incubator.

PEGylation and chemoselective ligation of recombinant azide-thrombomodulin to mouse islets. After culturing islets for 48hrs, islets were transferred to a 12μm plate insert (Millipore, MA) within a 24 well plate. RPMI media was flushed from the islets in the insert using DPBS (Mediatech, VA) prior to incubation with 4mM NHS-PEG-Phosphine supplemented with 11mM glucose solution in PBS. Islets were incubated in the PEG mixture for 1hr within a humidified 37˚C, 5% CO_2/95% air incubator. After the PEGylation step, the PEG mixture was flushed from the islets using several washes with PBS. The islets were then incubated with the recombinant

azido-thrombomodulin in fully-supplemented RPMI for 1hr within a humidified 37˚C, 5% CO_2/95% air incubator. After TM conjugation, the inserts containing the islets were flushed with several washes of PBS, followed by three more washes with fully-supplemented RPMI.

Assessement of islet viability and function. Islet viability was visualized using confocal microscopy and the LIVE/DEAD viability/cytotoxicity assay kit (Invitrogen, CA), which employs a calcein AM and ethidium homodimer staining protocol to distinguish between live and dead cells. Islets were imaged under 10 and 20X magnification and gross alterations in viability were assessed by examination of two color staining. Islet function was evaluated by glucose-stimulated insulin secretion under static incubation. Briefly, ten islets aliquots were distributed into plate inserts within 24-well plates and pre-incubated with Krebs buffer (KRBB: 99mM $NaCl_2$, 5mM KCl, 1.2mM KH_2PO_4, 1.2 $MgSO_4$, 2.6mM $CaCl_2$, 26mM $NaHCO_3$, and 0.2% BSA) supplemented with basal glucose levels, 60mM for 1hr within a humidified 37˚C, 5% CO_2/95% air incubator. Following pre-incubation, inserts were rinsed and incubated with KRBB containing basal glucose, 60mM, for 1hr within a humidified 37˚C, 5% CO_2/95% air incubator, followed by another rinse and incubation with KRBB containing secretory glucose level, 300mM, for another hour within a humidified 37˚C, 5% CO_2/95% air incubator. Samples were collected at the beginning and end of each incubation period. Insulin levels were determined using a mouse insulin ELISA (ALPCO, NH). Each experiment was run in quintuplicate and a minimum of three independent experiments were preformed for each islet condition. Insulin function is expressed as the glucose-stimulated insulin secretion ratio (basal secretion level/glucose stimulated secretion level) and normalized by islet DNA content. DNA content was measured (Quanti-it DNA Quantification Kit, Invitrogen, CA) following extraction of DNA (DNeasy Purification Kit, Qiagen, CA).

Assessment of surface levels of islet-bound recombinant thrombomodulin. Three methods were employed to verify the conjugation of the recombinant azido-thrombomodulin to the islet surface. First, confocal images were collected using an S•Tag peptide FITC tag (1:500 dilution in PBS; Emd Biosciences, CA) to label the S•Tag fusion protein on the recombinant azido-thrombomodulin protein. Thrombomodulin conjugation was also confirmed via slot blot analysis using Bio-dot SF (BioRad, CA). Briefly, two-hundred islets from each treatment group were counted into centrifuge tubes. Islets were lysed in 200 μL of 50 mmol/L Tris-HCl containing 150 mM NaCl, 0.5% Triton X-100, and 0.5mM EDTA and disassociated using a rotameter. The lysed cell solution was then loaded into the slot blot and onto nitrocellulose membranes. Following overnight blocking in 5% nonfat dry milk in PBS, the membranes were then treated with a sheep anti-human TM monoclonal antibody (1:100; American Diagnositca) and detected with an anti-sheep horseradish peroxidase (HRP) conjugated antibody (1:15,000, BioRad). Membranes were developed using an ECL Western blotting detection kit (Amersham Biosciences, U.K.). As a third approach, a modified cell-based ELISA method was developed using an HRP-conjugated sheep anti-human TM monoclonal antibody (American Diagnostica). Two-hundred islets were counted into centrifuge tubes and incubated for 1 hr on ice in PBS containing 0.2% BSA and the HRP-conjugated anti-TM antibody. Islets were then washed extensively with PBS, followed by incubation with perborate/3,3',5,5'-tetramethylbenzidine (TMB) substrate. After a 30 min reaction period, the color development was halted via addition of sulfuric acid. The islets were gently spun down, the supernatant removed, and the absorbance of the supernatant measured at 450 nm. Absorbance was correlated to the amount of bound antibody using a standard curve.

Measurement of recombinant azido-thrombomodulin activity. The enzymatic activity of surface-bound thrombomodulin was determined by measuring the production of activated protein C (APC). In brief, 200 islets were incubated in the presence of 1 μM human protein C, 1 nM thrombin, 50 mM $CaCl_2$, 100 mM $NaCl_2$, 0.1% (wt) BSA in 20 mM Tris-HCI solution, pH 7.4. After 1 hr, production of APC was terminated by the additional of antithrombin III. Thirty microliter samples were collected and APC detected by the addition of Spectrozyme PCa (0.4 μM) for 1 hr, followed by determining absorbance at 405nm. Absorbance measurements were quantified by a standard curve using purchased APC (American Diagnostica).

Clotting time assay. Treated or untreated islets were washed in PBS and 50 islets dispensed into centrifuge tubes. Islets were gently spun down, the supernatant was removed, and islets reconstituted in 50 μL, pre-warmed human plasma (STA System Control, Diagnostica Stago, NJ) in a 96 well plate. Immediately thereafter, 50 μL of warmed 0.025M $CaCl_2$ was added to each well. Time to clot formation was measured by monitoring absorbance at 405 nm. For each group, the half-maximal absorbance average was calculated from a minimum of six separate wells.

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