SUPPORTING INFORMATION

Non-covalent Cell Surface Engineering with Cationic Graft Copolymers

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MATERIALS AND METHODS.

Synthesis and characterization of cationic copolymers. *PLL-g-PEG₄(OCH₃) Copolymers:* Poly(L-lysine)-graft-poly(ethylene glycol)₄ copolymers were synthesized via active ester coupling between N-hydroxysuccinimidyl (NHS)-ester-functionalized methoxy-PEG₄ (mPEG₄) and primary amines of the poly(L-lysine) (PLL) backbone. Poly(L-lysine)hydrobromide (Sigma Aldrich, St. Louis, MO; M_w=12 kD by MALLS, PDI=1.2 as stated by the supplier) was dissolved at 5 mg/ml in phosphate buffered saline (7.7 mM NaCl, 0.28 mM Na₂HPO₄, pH=7.4) for 30 minutes at room temperature. mPEG₄-NHS (Pierce Biotechnology; Rockford, IL) was dissolved at 250 mM in dry DMSO (Pierce Biotechnology) and slowly added to PLL under vigorous stirring. At 120, 150, and 180 minutes, 10x Dulbecco's phosphate buffered saline (DPBS; Mediatech, Inc., Manassas, VA) was added to the reaction mixture 1:10 by volume, after which the reaction was allowed to proceed for an additional 21 hours. This coupling protocol was empirically determined to yield more efficient grafting of mPEG₄ to PLL than if the constituents were combined in PBS as commonly performed. The product was transferred to dialysis cassettes (Slide-A-Lyzer Dialysis Cassette, 3.5 kD MWCO, Pierce Biotechnology) and dialyzed first against DPBS (pH 7.0, 3 x 24 hours, Mediatech, Inc.) and subsequently against distilled deionized water (3 x 24 hours). The product was then lyophilized until completely dry and stored at -20°C prior to use.

mPEG₄-NHS was added to PLL at various stoichiometric ratios to generate a library of PLL-g-PEG₄ copolymers with variable degrees of backbone modification. The extent of PEG grafting was determined using ¹H NMR (INOVA 600; Varian, Inc., Palo Alto, CA) by taking the ratio of chemical shifts assigned to mPEG linked to lysine (3.15 ppm, m, -CH₂NHC(O)OCH₂-) and ungrafted lysine chains (2.95 ppm, m, -CH₂NH₃⁺). Table S1 summarizes the structural properties of PLL-g-PEG₄ copolymers used in these investigations, including copolymer molecular weight which can be estimated based on the grafting ratio and the molecular weight of PLL and grafted PEG chains¹.

Acetylated poly(L-lysine): A random copolymer consisting of lysine and acetylated lysine monomers (PLL-Acetate) was synthesized in analogous manner to PLL-g-PEG₄ copolymers using sulfosuccinimidyl acetate (sNHS-acetate; Pierce Biotechnology). sNHS-acetate was added to PLL at an appropriate stoichiometric ratio to acetylate 40-45% of lysine repeat units. The product was transferred to dialysis cassettes (Slide-A-Lyzer Dialysis Cassette, 3.5 kD MWCO, Pierce Biotechnology) and dialyzed first against DPBS (pH 7.0, 3 x 24 hours, Mediatech, Inc.), and subsequently against distilled deionized water (3 x 24 hours). The product was then lyophilized until completely dry and stored at -20°C prior to use. The degree of acetylation was determined by ¹H NMR (INOVA 600) by taking the ratio of chemical shifts assigned to acetylated lysine (3.05 ppm, m, -CH₂NHCOCH₃) and unmodified lysine chains (2.95 ppm, m, -CH₂NH₃⁺). Relevant structural properties of PLL-Acetate are summarized in Table S1.

Functionalized PLL-g-PEG Copolymers: Functionalized PLL-g-PEG(R) were synthesized in an analogous manner to PLL-g-PEG₄ copolymers using heterobifunctional poly(ethylene glycol) derivatized with N-

hydroxysuccinimidyl (NHS)-ester and biotin, *t*-BOC protected hydrazide, or azide. NHS-PEG₄(biotin) was purchased from Pierce Biotechnology (Rockford, IL) and used as received. NHS-PEG₄(*t*-BOC-hydrazide) and NHS-PEG₁₂(azide) were purchased from Quanta Biodesign (Powell, Ohio) and vacuum dried overnight before use to remove trace amounts of residual organic solvent. Heterobifunctional PEGylation reagents were added to PLL at appropriate stoichiometric ratios to generate copolymers with extents of backbone modification between 40 and 50%. The extent of PEG grafting was determined using ¹H NMR (INOVA 600) by taking the ratio of chemical shifts assigned to mPEG linked to lysine (3.15 ppm, m, -CH₂NHC(O)OCH₂-) and ungrafted lysine chains (2.95 ppm, m, -CH₂NH₃⁺). To deprotect hydrazide, *t*-BOC was removed by dissolving PLL-*g*-PEG₄(*t*-BOC-hydrazide) copolymers in 75% (v/v in water) trifluoroacetic acid (TFA; Sigma Aldrich) for 18 hours. TFA was then neutralized by addition of saturated sodium bicarbonate solution, and the product again dialyzed against distilled deionized water (4 x 24 hours) and lyophilized until completely dry. Successful deprotection of the *t*-BOC group was verified by the absence of the *tert*-butyl peak at 1.45 ppm. Table S1 summarizes the structural properties of these copolymers.

Fluorescent labeling of copolymers. To facilitate identification of copolymers on or within pancreatic islets using confocal microscopy, copolymers were labeled with AlexaFluor®488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester (AF488-TFP; Molecular Probes, Eugene, OR) according to manufacturer's instructions. AF488-TFP was added at appropriate stoichiometric ratios to ensure labeling of less than 1% of backbone lysine monomers. Non-reacted dye was removed via dialysis (Slide-A-Lyzer Dialysis Cassette, 3.5 kD MWCO, Pierce Biotechnology, Rockford, IL), and the labeled product was lyophilized until completely dry. The degree of labeling was quantified using UV-vis spectroscopy (Cary 50; Varian Inc., Palo Alto, CA) and determined to be between 0.64% and 0.89%. To facilitate identification of PLL, FITC-labeled poly(L-lysine) (Sigma Aldrich, St. Louis, MO) was used.

Islet isolation and culture. Pancreatic islet isolations were performed as previously described². B10.BR-H2k H2-T18a/SgSnJ (B10) mice (8 weeks old, Jackson Laboratory Bar Harbor, ME) pancreata were removed after distension with collagenase P (1 mg/ml, Roche, Indianapolis, IN) through the common bile duct. Following digestion, islets were purified by a Ficoll-Histopaque discontinuous gradient (Ficoll: 1.108, 1.096, and 1.037; Mediatech Inc., Manassas, VA). Isolated islets were cultured for 48-72 hours at 37°C in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, L-glutamine (2 mM), and penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml) (Mediatech Inc.); media was changed daily.

Treatment of islets with copolymers. Islets (<1000) were placed into 12 mm cell culture inserts with 12 µm pores (Millicell-PCF; Millipore, Billerica, MA). Prior to introduction of polymer solution, islets were washed six times by adding 700 µl serum free RPMI 1640 to the insert, followed by gentle repeated tapping of the insert on a polystyrene surface to facilitate drainage of the wash solution through pores while retaining islets. The insert was placed into a well of a 24 well plate (Corning Inc., Corning, NY) and 700 µl of copolymer solution (80 µM in serum free RPMI 1640) was added to the cell culture insert. After 40 minute incubation in polymer solution, the insert was removed from the well, solution drained through the insert as described above, and islets washed four times as described above to ensure adequate removal of unbound polymer.

Synthesis and characterization of bio- and chemoselective probes. Biotinylated or fluorescently-labeled probes were purchased or synthesized to detect islet surface biotin, hydrazide, and azide groups via confocal microscopy. Biotin groups were detected by incubating islets in Cy3-labeled streptavidin (Cy3-SA; Sigma Aldrich, St. Louis, MO) at 0.1 mg/ml in DPBS for 30 minutes.

To detect cell surface hydrazide groups, islets were incubated with fluorescein-labeled alginate-aldehyde at 2 mg/ml in DPBS for 60 minutes at room temperature. Aldehyde groups were introduced into alginate as previously described³. Briefly, sodium alginate (UP LVM, MW ~75 kD; NovaMatrix, Sandvika, Norway) was dissolved at 10 mg/ml in molecular grade water and 0.25 M sodium metaperiodate (Sigma Aldrich) in water was added at 0.10 equivalents with respect to uronate repeat units. After 24 hours, the reaction was quenched with 10 equivalents excess ethylene glycol (Sigma Aldrich), and the product dialyzed (Slide-A-Lyzer Dialysis Cassette, 3.5 kD MWCO, Pierce Biotechnology) 3 x 24 hours against distilled deionized water and lyophilized until completely dry. The extent of alginate oxidation was quantified as previously described³. Ten-fold excess of *tert*-butyl carbazate (Sigma Aldrich) was reacted with oxidized alginate for 24 hours. The amount of unreacted *tert*-butyl carbazate was determined by the addition of trinitrobenzenesulfonic acid (TNBS) solution (Sigma Aldrich), and measuring the absorbance of the colored complex formed at 334 nm. The degree of oxidation was determined to be ~10.2%, corresponding to ~20 aldehyde groups per 100 monomer repeat units. To facilitate identification of oxidized alginate on islets with confocal microscopy, fluorescent labeling was achieved through thiosemicarbazone bond formation between aldehyde groups and fluorescein-5-thiosemicarbazide (Sigma Aldrich). Oxidized alginate (10% oxidation) was dissolved at 5 mg/ml in phosphate buffered saline (Mediatech, Inc.) and fluorescein-5-thiosemicarbazide was added at an appropriate stoichiometric ratio to ensure labeling of no more than 5% of aldehyde groups. After reaction for 24 hours, non-reacted dye was removed via gel filtration (PD-10, GE Healthcare, Piscataway, NJ). The fluorescent conjugate, F-Alg(CHO), was lyophilized and stored protected from light at -20°C. Degree of fluorescent labeling was quantified by UV-vis spectroscopy (Cary 50; Varian Inc.) and determined to be less than 1%.

To detect cell surface azide groups, islets were incubated with cyclooctyne-PEG_{3.4kD}-biotin (CyO-PEG-biotin) at 100 μM in DPBS for 60 minutes at room temperature, followed by detection of biotin groups using Cy3-SA as described above. CyO-PEG-biotin was synthesized by reaction between a heterobifunctional biotin-PEG_{3.4kD}-NHS conjugate (Laysan Bio, Arab, AL) and a cyclooctyne-PEG₃-amine (Scheme 1). The cyclooctyne-PEG₃-amine was synthesized as follows: the activated Boons cyclooctyne **1** was synthesized by following the literature protocol.⁴ The activated cyclooctyne **1** (25 mg, 0.064 mmol) and triethylamine (26 μL, 0.192 mmol) were dissolved in DMF (0.5 mL). To this solution was added BocNH-PEG₃-NH₂ (Quanta Biodesign, 0.078 mmol, 1.2 eq) and stirred at room temperature for 24 h. The resulting yellow solution was purified by chromatography over silica gel (5 % MeOH/CH₂Cl₂) to afford the intermediate cyclooctyne-PEG₃-NHBoc (25 mg, 68 %). Characterization data for the intermediate: ¹H-NMR (400 MHz, CDCl₃) : δ = 7.48 (d, *J* = 7.2 Hz, 1H), 7.34 – 7.26 (m, 6H), 5.61 (br s, 1H), 5.47 (s, 1H), 4.94 (br s, 1H), 3.67 – 3.51 (m, 13 H), 3.32 – 3.29 (dd, *J* = 5.6, *J* = 3.2 Hz, 1H), 3.20 (m, 2 H), 3.14 (dd, *J* = 5 Hz, *J* = 15 Hz, 1H), 2.87 (dd, *J* = 3.2 Hz, *J* = 14.8 Hz, 1H), 1.71 – 1.81 (m, 4H), 1.42 (s, 9 H); ESI-HRMS calculated for [M + H]⁺: 567.30648, found: 567.30658. This intermediate was subsequently dissolved in dichloromethane (1 mL) and cooled to 0 °C. To this solution was added methanolic solution of 1M HCl (3 mL) and stirred at room temperature. The reaction was monitored by TLC and after 12 h, the reaction mixture was concentrated under reduced pressure to obtain **2** in quantitative yield (21 mg). ¹H-NMR (400 MHz, CD₃OD): δ = 7.53 (d, *J* = 7.2 Hz, 1H), 7.36 – 7.29 (m, 7H), 5.40 (s, 1H), 3.61 – 3.49 (m, 12 H), 3.20 (m, 2 H), 3.04 (m, 2H), 2.86 (br s, 2H), 2.79 (dd, *J* = 14.8 Hz, *J* = 2.8 Hz, 2H), 1.90 (m, 1H) 1.71 – 1.81 (m, 3H); ESI-HRMS calculated for C₂₇H₃₅O₅N₂ [M + H]⁺: 467.2540, found: 467.25334.

To synthesize cyclooctyne-PEG_{3.4kD}-biotin **3**, a solution of cyclooctyne-PEG₃-amine **2** (10 mg, 0.021 mmol) dissolved in 4 mL chloroform/triethylamine (1:1) was added to biotin-PEG_{3.4kD}-NHS (87 mg, 0.025 mmol) and the reaction mixture was stirred at room temperature. After 24 h, the solvent was removed under reduced pressure to obtain a yellow oil and to this oil was added 10 mL of cold ether and the

cyclooctyne-PEG_{3.4kD}-biotin was precipitated out as a yellow solid (84 mg, quantitative). ¹H-NMR (400 MHz, CDCl₃): δ = 7.48 (d, *J* = 7.2 Hz, 1H), 7.34 – 7.26 (m, 6H), 5.46 (br. s, 1H), 5.38 (br. s, 1H), 4.48 (dd, *J* = 5.2 Hz, 8.0 Hz, 1H), 4.31 (dd, *J* = 5.6 Hz, *J* = 8.0 Hz, 1H), 3.79 – 3.14 (m, 134 H), 2.90 (*J* = 5.2 Hz, *J* = 13.5 Hz, 1H), 2.56 (m, 4 H), 2.44 (t, *J* = 6.0 Hz, 1H), 2.20 (m, 1H), 1.18 – 1.43 (m, 20 H).

Scheme 1. Synthesis of cyclooctyne-PEG_{3.4kD}-biotin conjugate

Confocal microscopy. Confocal microscopy (Zeiss LSM 510 META; Carl Zeiss, Inc., Thornwood, NY) was used to identify fluorescently labeled copolymers and probes on or within pancreatic islets. A representative population of islets selected at random was placed in silicon isolators (Grace Bio-Labs, Bend, OR) adhered to glass coverslips (Fisher Scientific) containing serum free RPMI 1640. Coverslips were then placed on the microscope stage and images were captured within 15-90 minutes of treatment with fluorescently-labeled probes. In some instances, islets were incubated with 8 μ M Hoechst 33342 (Molecular Probes, Eugene, OR) for 60-90 minutes before or after incubation with polymers to allow individual cell nuclei within islets to be clearly identified. All experiments performed using Hoechst staining were repeated and results confirmed in the absence of staining to ensure that nuclear staining did not confound findings.

Assessment of islet viability. Islet viability was assessed as previously described⁵ with some modifications. Briefly, islets were incubated in DPBS (Mediatech, Inc.) containing 4 μ M calcein AM and 8 μ M ethidium homodimer-1 (Molecular Probes) for one hour, and a representative number of individual islets (35-50) were imaged with two-channel confocal microscopy using a 20x objective as described above. Confocal micrographs were analyzed using MATLAB® (The MathWorks, Natick, MA) to quantify

the number of pixels corresponding to fluorescent emission from live (green) and dead (red) cells. Viability is expressed as the percentage of fluorescent pixels associated with emission from live cells.

Quantification of streptavidin immobilized on islet surface. To compare islet biotinylation achieved through covalent modification of cell surface amine groups and adsorption of biotin-derivatized PLL-*g*-PEG copolymers, islets were incubated with either NHS-PEG₄(biotin) or PP-biotin at equimolar concentration of biotin (1.4 mM in DPBS) for one hour. Following biotinylation, islets were incubated in a 1:50 mixture (by mass) of horseradish peroxidase (HRP)-labeled streptavidin (HRP-SA; Zymed Laboratories, Inc., San Francisco, CA) and streptavidin (Pierce Biotechnology) at 0.1 mg/ml in DPBS for 30 minutes. After rinsing non-bound streptavidin, groups of 30-50 islets were placed into wells of a 96 well plate. The microplate was briefly centrifuged to settle islets, supernatant was removed, and 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) solution (1-StepTM Ultra TMB-ELISA, Pierce Biotechnology) was added to islets. Microwell plates containing islets and TMB were placed on a plate shaker (800 m^{-1} , MS1 Minishaker; IKA, Wilmington, NC) at room temperature for 20 minutes, upon which time 50 μ L 2M H₂SO₄ was added to quench the reaction. Microwell plates were briefly centrifuged to settle islets, 100 μ L of solution was transferred to a fresh well, and absorbance was recorded at 450 nm using a microplate reader. The amount of streptavidin immobilized on islets was quantified using a standard curve relating absorbance at 450 nm to known concentrations of soluble SA-HRP.

Statistics. Tests for statistical significance between the means of two groups were conducted with the Student's t-test (two-tailed, homoscedastic). Tests between three or more groups were conducted with the one-way ANOVA followed by the Tukey HSD test.

Table S1. Structure of polycations employed in this work

Polymer ID	PEG _n (n)	PEG End Group	% Lysine Modified	Estimated MW ^a (Da)
PLL	n/a	n/a	0	7,400
PP-OCH ₃ (a)	4	OCH ₃	15	9,320
PP-OCH ₃ (b)	4	OCH ₃	26	10,700
PP-OCH ₃ (c)	4	OCH ₃	36	11,960
PP-OCH ₃ (d) ^b	4	OCH ₃	43	12,850
PLL-Acetate	0	n/a	45	8,400
PP-biotin	4	Biotin	45	19,730
PP-hydrazide	4	Hydrazide	47	17,610
PP-N ₃	12	Azide	40	21,510

a: $MW_{\text{copolymer}} = MW_{\text{PLL}} + (MW_{\text{PLL}}/MW_{\text{Lys}})(x^{-1})(MW_{\text{PEG(R)}})$, excludes contribution from Br⁻, non-approximated grafting ratio used for calculation. Molecular weight of PLL-HBr starting material (includes contribution of Br⁻ counterion) = 12 kD MW by MALLS, 1.2 M_w/M_m. **b:** Referred to as PP-OCH₃ in manuscript text.

SUPPLEMENTAL DATA.

Supporting Figure 1. PLL-Acetate exerts significant cytotoxicity towards pancreatic islets and localizes intracellularly. A non-PEGylated analog of PP-OCH₃, PLL-Acetate, was synthesized by acetylating 40-45% of lysine monomers (a). Unlike PP-OCH₃, incubation with PLL-Acetate results in significant decrease ($p < 0.01$) in islet viability (b), indicating a dependence on grafted PEG chains in reducing the toxicity of PLL-*g*-PEG copolymers. In accord with this observed cytotoxicity, confocal microscopy of islets incubated with AF488-labeled PLL-Acetate demonstrates intracellular localization of the polymer (c); scale bar: 50 μm (left panel), 10 μm (right panel).

Supporting Figure 2. Assessment of islet viability after 40 min incubation with PLL-*g*-PEG₄(biotin), PLL-*g*-PEG₄(hydrazide), and PLL-*g*-PEG₁₂(azide) demonstrates that appropriately structured functionalized copolymers do not significantly ($p > 0.05$) influence islet viability relative to untreated controls.

Supporting Figure 3. Representative confocal micrographs of islets incubated with PP-OCH₃ followed by incubation with (a) Cy3-labeled streptavidin, (b) cyclooctyne-PEG-biotin (followed by biotin detection with Cy3-streptavidin), and (c) F-Alg(CHO). Negligible non-specific binding of probes was observed; scale bar: 50 μ m.

Supporting Figure 4. Biotin and hydrazide groups may be simultaneously displayed through co-adsorption of functionalized PLL-*g*-PEG copolymers. Islets were incubated in a mixture of PP-biotin and PP-hydrazide (each at 0.5 mg/ml), and subsequently incubated with F-Alg(CHO) (top panels), Cy3-SA (middle panels), or a mixture of the two (bottom panels). Two-channel confocal microscopy was used to detect Cy3 (left panels) and fluorescein (right panels). Representative confocal micrographs demonstrate simultaneous display of both biotin and hydrazide moieties on the islets surface; scale bar: 50 μ m.

Supporting Figure 5. Representative confocal images overlaid on bright field micrographs of islets treated with PP-biotin (a) and NHS-PEG₄(biotin) (b) and subsequently with Cy3-labeled streptavidin. Islets treated with NHS-PEG₄(biotin) presented an irregular morphology (b), whereas islets incubated with PP-biotin (a) maintained the smooth border characteristic of isolated and cultured murine islets; scale bar: 50 μ m.

NMR SPECTROSCOPY AND MASS SPECTROMETRY.

PLL-*g*-PEG₄(OCH₃): ~15% PEG grafting

PLL-*g*-PEG₄(OCH₃): ~25% PEG grafting

PLL-*g*-PEG₄(OCH₃): ~35% PEG grafting

PLL-*g*-PEG₄(OCH₃): ~40% PEG grafting (PP-OCH₃)

PLL-Acetate

PLL-*g*-PEG₄(biotin): PP-biotin

PLL-*g*-PEG₄(hydrazide): PP-hydrazide

PLL-*g*-PEG₁₂(azide): PP-N₃

Cyclooctyne-PEG₃-NHBoc

FT9906_081015112301 #37-39 RT: 1.10-1.16 AV: 3 NL: 2.86E7
T: FTMS + p APCI corona Full ms [200.00-600.00]

Cyclooctyne-PEG₃-amine

FT9907_081015113623 #8-12 RT: 0.21-0.33 AV: 5 NL: 8.78E7
T: FTMS + p APCI corona Full ms [200.00-600.00]

Cyclooctyne-PEG_{3.4kD}-biotin

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