

Encapsulation of Pancreatic Islets Within Nano-Thin Functional Polyethylene Glycol Coatings for Enhanced Insulin Secretion

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Covalent attachment of polymers to cells and tissues could be used to solve a variety of problems associated with cellular therapies. Insulin-dependent diabetes mellitus is a disease resulting from the autoimmune destruction of the β cells of the islets of Langerhans in the pancreas. Transplantation of islets into diabetic patients is an attractive form of treatment, provided that the islets could be protected from the host's immune system to prevent graft rejection, and smaller numbers of islets transplanted in smaller volumes could be sufficient to reverse diabetes. Therefore, a need exists to develop islet encapsulation strategies that minimize transplant volume. In this study, we demonstrate the formation of nano-thin, poly(ethylene glycol) (PEG)-rich functional conformal coatings on individual islets via layer-by-layer assembly technique. The surface of the islets is modified with biotin-PEG-*N*-hydroxysuccinimide (NHS), and the islets are further covered by streptavidin (SA) and biotin-PEG-peptide conjugates using the layer-by-layer method. An insulinotropic ligand, glucagon-like peptide-1 (GLP-1), is conjugated to biotin-PEG-NHS. The insulinotropic effect of GLP-1 is investigated through layer-by-layer encapsulation of islets using the biotin-PEG-GLP-1 conjugate. The effect of islet surface modification using the biotin-PEG-GLP-1 conjugate on insulin secretion in response to glucose challenge is compared via static incubation and dynamic perfusion assays. The results show that islets coated with the functional PEG conjugate are capable of secreting more insulin in response to high glucose levels compared to control islets. Finally, the presence of SA is confirmed by indirect fluorescent staining with SA-Cy3, and the presence of PEG-peptide on the surface of the islets after treatment with biotin-PEG-GLP-1 is confirmed by indirect fluorescent staining with biotin-PEG-fluorescein isothiocyanate (FITC) and separately with an anti-GLP-1 antibody. This work demonstrates the feasibility of treating pancreatic islets with reactive polymeric segments and provides the foundation for a novel means of potential immunoisolation. With this technique, it may be possible to encapsulate and/or modify islets before portal vein transplantation and reduce transplantation volume significantly, and promote islet viability and insulin secretion due to the presence of insulinotropic peptides on the islet surface. Layer-by-layer self-assembly of PEG-GLP-1 offers a unique approach to islet encapsulation to stimulate insulin secretion in response to high glucose levels.

Introduction

TYPE 1 DIABETES MELLITUS is a disease characterized by the lack of pancreatic islet β -cell function. β cells are responsible for insulin production, and the lack of insulin results in unregulated glucose concentrations in the blood. Clinical islet transplantation is a promising therapy used to restore β -cell function in patients with type 1 diabetes.^{1,2} However, widespread clinical application of islet transplan-

tation remains limited by serious side effects of immunosuppressive therapy necessary to prevent host rejection of transplanted islets.³ The desire to transplant islet tissue without the need for immunosuppression has led to the development of semipermeable microcapsules capable of protecting donor cells from the host immune system while allowing transport of glucose, insulin, and other essential nutrients.⁴⁻⁶ Even though transplantation of microencapsulated islets is a promising method for treating patients

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with type I diabetes, some obstacles such as islet loss,⁷ limited process scalability,⁸ relatively large size of conventional microcapsules,^{5,9,10} number of islets required to achieve normoglycemia, and the deteriorated insulin secretion capability of islets during isolation and encapsulation procedures¹¹ must be addressed to coat a clinically relevant number of islets.

Several approaches, including emulsification,¹² discontinuous gradient density centrifugation,⁸ selective withdrawal,⁷ and interfacial polymerization,¹³ have been used to fabricate 5–50- μm -thick polymeric coatings. The relatively large size of the capsules generated by previous techniques has made it difficult to transplant encapsulated islets into the portal vein of the liver, which is the clinically preferred site for islet transplantation.^{14,15} The large size of encapsulated islets along with the deterioration of the functionality of implanted islets in response to glucose lead researchers to consider functional microcapsules with insulinotropic agents for the purpose of improving the insulin secretion capability of encapsulated islets.^{16–20} Even though the idea of encapsulating islets within functional coatings is promising, this technique, based on droplet generation by extrusion through a needle, produces capsules of constant outer diameters within the range of 400–800 μm .

An attractive alternative to the traditional polymer coatings with defined thicknesses in the micron range is layer-by-layer polymer (LbL) self-assembly. With this technique, it is possible to generate nano-thin films on substrates with diverse geometries.^{21–23} This technique has been used in the literature to protect encapsulated catalase from protease degradation,²⁴ to limit cell adhesion to the proteinaceous surfaces,²⁵ and to elicit specific biochemical responses.²⁶ The methods used to encapsulate enzymes,²⁷ proteins,²⁸ DNA,²⁹ lipid vesicles,³⁰ and drug-loaded nanoparticles³¹ are promising for the coating of cells and tissue to provide immunoisolation. Recently, the formation of nano-thin conformal coatings on individual pancreatic islets using LbL self-assembly of poly(L-lysine)-*g*-poly(ethylene glycol)(biotin) and streptavidin (SA) has shown that islets that were treated with multilayer films performed comparably to untreated controls *in vivo* in a murine model of allogeneic intraportal islet transplantation.³²

The high rate of early islet destruction due to the instant blood-mediated inflammatory reaction has also been recently addressed using LbL self-assembly. Instant blood-mediated inflammatory reaction is a likely cause of both the loss of transplanted tissue and the intraportal thrombosis associated with clinical islet transplantation. Researchers immobilized azido-thrombomodulin,³³ anticoagulant heparin,^{34–36} and urokinase³⁷ directly on the islet surface and highlighted the potential of tissue-targeted chemistry to reduce instant blood-mediated inflammatory reactions induced by the innate immune system.

In this study, we immobilized the insulinotropic ligand GLP-1 directly on the surface of pancreatic islets using layer-by-layer self-assembly of biotin-PEG-NHS, SA, and biotin-PEG-GLP-1 as shown in Figure 1. GLP-1, a potent incretin hormone produced in the L cells of the distal ileum, stimulates insulin gene transcription, islet growth, and neogenesis.^{38,39} GLP-1 immobilized on the islet surface is expected to stimulate insulin secretion in response to high glucose levels, thereby reducing the number of islets required to normalize blood glucose of a diabetic patient, and improving

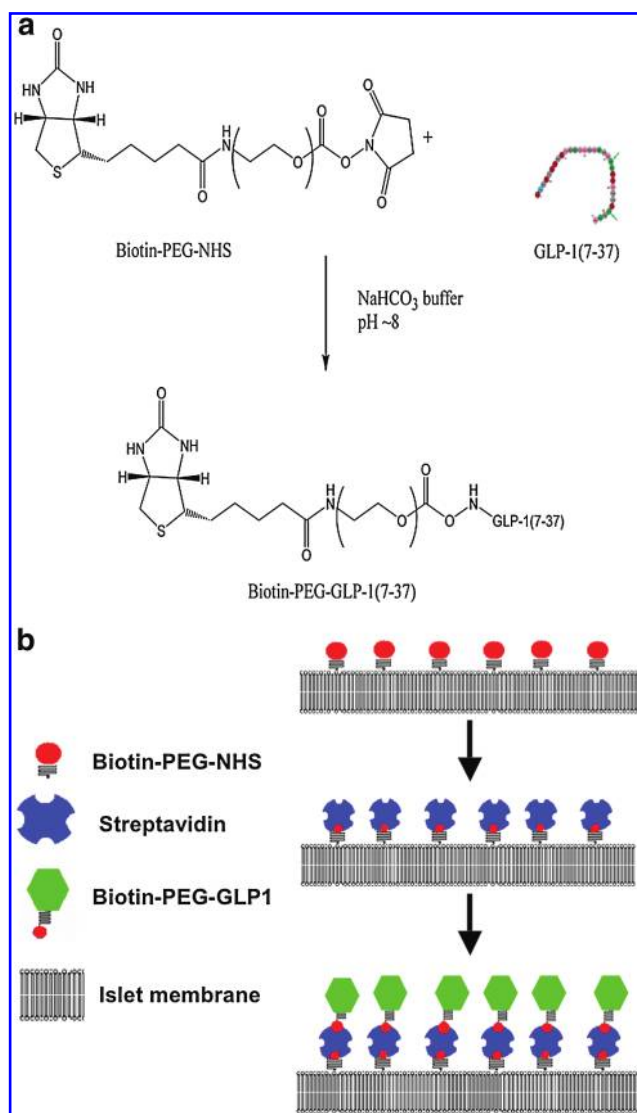


FIG. 1. Schematic illustration of immobilization of glucagon-like peptide-1 (GLP-1) on the surface of an islet. **(a)** Schematic of biotin PEG-GLP-1 synthesis. **(b)** Biotin-PEG-NHS is attached to the islet surface by a covalent reaction between the NHS group with the amine groups of membrane proteins. Next, SA and biotin-PEG-GLP-1(7–37) bind to the islet surface via the high affinity of biotin and SA. PEG, poly(ethylene glycol); NHS, N-hydroxysuccinimide; SA, streptavidin. Color images available online at www.liebertonline.com/ten.

the insulin secretion capability of nanoencapsulated islets. Here we present *in vitro* data showing that pancreatic rat islets that have been coated by layer-by-layer deposition of PEG-biotin, SA, and biotin-PEG-GLP-1 not only have similar viability but also demonstrate enhanced insulin secretion in response to high glucose levels compared to that of untreated islets.

Materials and Methods

Materials

Biotin-PEG-NHS (molecular weight = 5 kDa), biotin-PEG-FITC (molecular weight = 5 kDa), and biotin-PEG (molecular weight = 5 kDa) were purchased from Nanocs, Inc. Sodium

chloride, potassium chloride, sodium bicarbonate, calcium chloride, magnesium sulfate, potassium dihydrogen phosphate, SA, CY3-SA and sodium phosphate, bovine serum albumin (BSA), fluorescein diacetate (FDA), and propidium iodide (PI) were purchased from Sigma. Hank's buffered saline solution (HBSS), Ficoll gradients, and CMRL 1066 were purchased from Mediatech. GLP-1(7–37) (molecular weight = 3.356 kDa) was purchased from Elim Biopharmaceuticals, Roswell Park Memorial Institute (RPMI)-1640 medium was purchased from Invitrogen. Phosphate-buffered saline (PBS) was purchased from Gibco. Fetal bovine serum was purchased from Lonza Biowhitaker. Collagenase was purchased from Roche. Penicillin/streptomycin was purchased from Gibco–Invitrogen. Dialysis membrane cassettes, and M-PER mammalian protein extraction buffer were purchased from Pierce (Thermo Scientific Inc.). Anti-GLP-1, and anti-mouse IgG antibodies were purchased from Abcam Inc.

Synthesis of biotin-PEG-GLP-1

Lyophilized GLP-1 (20 mg) and biotin-PEG-NHS (fourfold in molar excess, 120 mg) were codissolved in 35 mL of sodium phosphate buffer (sodium phosphate, 50 mM, pH 8). The conjugation reaction was carried out for 12 h at room temperature. To remove unreacted biotin-PEG-NHS molecules and unpegylated GLP-1, the reaction is dialyzed twice (molecular weight cutoff 3500 Da) in 3 to 1 ratio of phosphate buffer (50 mM, pH 6).⁴⁰

GLP-1 content in the PEG conjugate was determined by NanoDrop 1000 spectrophotometer (Thermo Scientific). GLP-1 contains one tryptophan residue that strongly adsorbed at a wavelength of 280 nm. A series of GLP-1 and biotin-PEG-GLP-1 solutions in 0.01 N NaOH were used for calibration curves. The absorbance at 280 nm for each sample was acquired as an averaged value from triplicate measurements ($SD \pm 0.03$).

Pancreatic islet isolation

Islets were isolated by a modification of the method described by Rouiller *et al.*⁴¹ as follows. Lewis rats were anesthetized by administration of 0.6 mg/g chloral hydrate intraperitoneally. Mouse pancreatic islets were isolated from male C57/BL6 mice weighing 20–35 g. Mice were not anesthetized; they were sacrificed by cervical dislocation before the procedure. Next, a midline laparotomy was performed. With the common bile duct clamped where it entered the duodenum, the pancreas was inflated with chilled HBSS, containing 5 mM glucose, 0.2% BSA, and 0.25 mg/mL collagenase P in mice (1 mg/mL in rats). The HBSS was bubbled with 95% O₂/5% CO₂ for 20 min before the addition of BSA or collagenase P. The pancreas was removed from surrounding tissues and was transferred to a vessel containing HBSS–collagenase P solution in a 37°C water bath. The vessel remained in the water bath for 8.5 min (12–16 min for rats), and was shaken manually for 30 s after removal from the water bath. After digestion, pancreatic tissue was washed with chilled HBSS and centrifuged. The supernatant was aspirated, and the pellet resuspended in a discontinuous Ficoll gradient consisting of Ficoll's of densities 1.108, 1.096, 1.069, and 1.037. The sample was spun at 800 rpm for 15 min at 4°C. After centrifugation, the islets were harvested from the 1.069–1.096 interface of Ficoll densities. The purified islets

were washed with HBSS and cultured in CMRL 1066 supplemented with 10% fetal bovine serum and 5% penicillin/streptomycin at 37°C in humid conditions with 5% CO₂. The Institutional Animal Care and Use Committee of the University of Chicago approved all animal care and handling protocols.

Surface modification of islets

Islets were removed from culture media after 24 h in culture and were suspended in 11 mM glucose/PBS solution (pH 7.4) to provide an energy source for the islets during the treatment. Islets were not modified in the culture medium because it contains amino acids, proteins, and other compounds that could react with biotin-PEG-NHS.⁴²

The islet suspension was used to solubilize biotin-PEG-NHS, resulting in a 0.15 mg/mL solution. Biotin-PEG-NHS reacted with the amine groups present on the islet surface. The biotin-PEG-NHS was added directly to a solution of the islets in the 11 mM glucose in PBS solution. After the biotin-PEG-NHS was solubilized by the cell suspension, the cells were incubated at 37°C in a humidified 5% CO₂ incubator for approximately 10 min with occasional agitation. The islets were pelleted and the supernatant was removed. Next, the islets were incubated in a solution of 0.01 mg/mL of SA (or avidin) in PBS/11 mM glucose for 7 min at room temperature, and again were rinsed by changing the solution three times.

Finally, 0.15 mg/mL biotin-PEG-GLP-1 in PBS/11 mM glucose was allowed to bind to the SA coating for 7 min at 37°C. The islets were finally rinsed by changing the solution three times.

Islet viability

Islet viability was tested before and after surface modification to determine the effects of the procedure on cell viability. The standard FDA/PI method was used to evaluate islet viability. FDA is cleaved in viable cells to liberate a fluorescent molecule, while PI, a fluorescent molecule, is excluded from viable cells but enters nonviable cells. Briefly, groups of 100 control (unmodified) and modified islets were randomly picked. Islets were incubated for 30 min in an FDA/PI working solution. After rinsing away excess FDA/PI with PBS, the islets were exposed to excitation wavelengths corresponding to the absorption wavelengths of the dyes FITC (480/30, 535/40) and Texas Red (560/55, 645/75). Images were acquired via a Zeiss Axiovert 200 microscope using an Orca ER camera controlled by Improvion software. Islet viability assays were performed on mouse islets that were kept in culture for 1 day after surface modification. Previous studies employed this assay in observing the viability of coated islets, because this technique does not require the destruction of the coating as common metabolic activity assays such as the methylthiazol tetrazolium assay would.^{43–45}

In vitro islet function

Islet function was determined by quantifying insulin release after a static incubation glucose challenge assay. Islets were exposed to low- and high-glucose solutions for equal periods of time, and the amount of insulin secreted in

response to each glucose concentration was determined. RPMI solutions containing 3.3 mM and 16.7 mM glucose solutions and 0.1% BSA were prepared. Three or four sets of 10 islets were incubated in each of the solutions at 37°C for 30 min with slight agitation. At the end of the incubation period, the supernatant was collected and frozen at -20°C for later analysis using a standard insulin ELISA. Results are reported as the mean \pm standard error of the mean.

In vitro perfusion of islets

Untreated and treated islets were perfused using an automated perfusion system (Bio rep[®] Perfusion System V 3.03; BioRep). A low pulsatility peristaltic pump pushed Krebs-Ringer-bicarbonate buffer containing 0.1% w/v essentially fatty acid-free BSA (Sigma; pH 7.4) through columns harboring 30 islets immobilized in Bio-Gel P-4 Gel (BioRad) and 100 μ L/min. Except where otherwise noted, glucose concentration was adjusted to 3.3 mM for a basal glucose level, and 16.7 mM for a high glucose level for all experiments. Islets were perfused for 10 min at basal 3.3 mM glucose (after an initial period of 30 min equilibration in low-glucose solution), and then perfused with the Krebs-Ringer-bicarbonate solution containing 16.7 mM glucose for 20 min. After the 16.7 mM glucose stimulation, a washout perfusion with the 3.3 mM glucose buffer was performed for 20 min. The perfusate was collected every minute in an automatic fraction collector that was designed for a multiwell plate format. The chambers that contain the islets and the perfusion solutions were kept at 37°C in a built-in temperature-controlled unit; to preserve the integrity of the analytes in the perfusate, the perfusate in the collecting plate was kept at <4°C. After perfusion of the islets, mammalian protein extraction reagent was used to completely lyse the islets and release all of the intracellular insulin. All effluent perfusate samples obtained throughout the study were stored frozen at -20°C until the insulin ELISA was performed to measure insulin concentration. The amount of insulin released at each time point was then normalized by the total insulin content in each group to calculate the fraction of total insulin release.

Fluorescent staining of islets

The supernatant of the purified islets was removed, and the islets (about 100) were suspended in 500 μ L of 11 mM glucose solution in PBS to provide an energy source to the islets during the treatment. Islets were not modified in the culture medium because it contains amino acids, proteins, and other compounds that could react with biotin-PEG-NHS.⁴² The islet suspension was biotinylated by incubating the islets for 60 min at 37°C in PBS solution at 0.15 mg/mL. Biotin-PEG-NHS reacted with the amine groups present on the islet surface. The biotin-PEG-NHS was added directly to a solution of the islets in the 11 mM glucose in PBS solution. The islets were incubated at 37°C in a humidified 5% CO₂ incubator for approximately 15 min with occasional agitation.

The islets were pelleted and the supernatant was removed. Next, to demonstrate the presence of biotin on islets, islets from the previous step were incubated with 500 μ L of 0.01 mg/mL of Cy3-SA for 30 min. This was used to identify accessible biotin groups. Control islets that were not modified with biotin-PEG-NHS were also incubated with only Cy3-SA. Islets were rinsed by changing the medium three

times.³⁶ Finally, 0.15 mg/mL biotin-PEG-FITC was allowed to bind the SA coating for 10 min at 37°C. The islets were finally rinsed by changing the medium three times. Fluorescent emission was observed with a confocal microscope.

Confirmation of the presence of GLP-1 on PEG-GLP-1-coated islet surface

The presence of GLP-1 on the surface of islets after coating was determined using an anti-GLP-1 antibody. After layer-by-layer assembly of the functional PEG coating, islets were cultured overnight, and subsequently fixed in 10% formalin. After 24 h of fixation in formalin, islets were placed in 10 mM tris(hydroxymethyl) aminomethane (TRIS)/1 mM ethylenediaminetetraacetic acid at 60°C for 24 h to remove methylene bridges formed during fixation, which can mask antibody binding sites (this procedure is referred to as epitope retrieval). The next day, islets were washed twice in PBS/1% BSA to remove epitope retrieval solution. Next, islets were placed into a PBS solution containing 1% BSA and 1 μ g/mL mouse anti-GLP-1 antibody (Abcam) and incubated at room temperature for 2 h. The primary antibody solution was removed by washing the islets twice in PBS/5% BSA, after which islets were incubated in PBS/5% BSA for 10 min. Islets were then placed in a secondary antibody solution (PBS/5% BSA containing 0.5 μ g/mL goat anti-mouse IgG conjugated to FITC, also from Abcam) and incubated at room temperature for 1 h. Finally, islets were washed twice with PBS and exposed to an excitation wavelength corresponding to the absorption wavelength of FITC (495 nm). Images were acquired via an Olympus IX71 inverted fluorescence microscope using an Olympus CCD camera controlled by Olympus Micro Suite software. Two sets of controls confirmed specific binding of the anti-GLP-1 antibody. The first control was a group of islets selected randomly from the set of modified islets used in the experimental group described above. This group was incubated only in secondary antibody (after 2 h incubation in PBS/1% BSA). The second control was a group of unmodified islets incubated in both primary and secondary antibody as described in this section.

Results

Synthesis of biotin-PEG-GLP-1

The synthesis of biotin-PEG-GLP-1 conjugate was performed as shown in Figure 1a. Unreacted GLP-1 (3.356 kDa) was removed by dialysis (molecular weight cut off = 3.5 kDa). The GLP-1 content of the conjugate was confirmed by UV spectrophotometry. To confirm that the absorbance at 280 nm was sensitive enough to quantify the amount of GLP-1 in the biotin-PEG-GLP-1 conjugate, the UV spectra were scanned from 200 to 400 nm. Calibration curves showed the correlations between the concentrations of GLP-1 or biotin-PEG-GLP-1 and absorbance at 280 nm. As a result, it was estimated that the biotin-PEG-GLP-1 conjugate contained 15% (by weight) of GLP-1, which represents a molar ratio of GLP-1: biotin-PEG-GLP-1 as 0.4:1.

Surface modification of islets

Purified islets were suspended in 11 mM glucose solution in PBS. To provide an energy source to the islets, glucose

solution (11 mM) in PBS was used during surface modification steps. A nano-thin membrane composed of SA and biotin-PEG-GLP-1 on islet surfaces using biotin-PEG-NHS and layer-by-layer deposition techniques is shown in Figure 1b. NHS groups of the biotin-PEG-NHS polymer molecule were covalently bound to membrane proteins through amide bonds, causing PEG chains terminated with biotin to extend into solution.⁴⁶ Under physiological conditions biotin and SA interact to form a stable conjugate with an association constant of $\sim 10^{15} \text{ M}^{-1}$.⁴⁷ Figure 2a shows spectral-fluorescence confocal images of untreated islets. The frame on the left shows the confocal fluorescence micrograph of the same field of view as the differential interference contrasting image on the right. The absence of fluorescence on the left of Figure 2a shows that there is no islet autofluorescence. To demonstrate binding of SA to biotin-PEG-NHS bound to islets, Cy3-labeled SA was used. Incubation of islets with biotin-PEG-NHS bound to their surface facilitated the specific binding of Cy3-SA to the islet surface as shown in Figure 2b. Red (Cy3) fluorescence emission observed from the cross-sectional views indicates that conjugation occurred at the peripheries of the islets.

The final step of islet surface modification involved incubation of biotin-PEG-NHS and SA-treated islets further in the biotin-PEG-GLP-1 conjugate. The last step of conjugation was characterized by biotin-PEG-FITC. FITC-labeled biotin-PEG bound to SA molecules on the surface that allowed direct observation. Fluorescence emission observed from FITC indicated the presence of FITC-PEG-biotin on the islet surface (Fig. 2c).

Clear fluorescence observed at the peripheries of islets (Fig. 2) along with the differential interference contrasting microscope images shown in Figure 3 indicates that GLP-1 can be introduced onto the surface of islets using treatment of islets in biotin-PEG-NHS, SA, and biotin-PEG-GLP-1 without damaging islet morphology.

Islet viability

Figure 4 shows the results of the standard FDA/PI assay performed on the islets cultured 24 h after surface modification. There were 10 samples for both control and treated groups of islets. Each sample contained approximately 10 islets. The standard visual assessment placed both the control and the treated islets in the 90% to 100% viability range. Quantitative comparisons of the areas of the green and red regions suggested that percent viability of the control islets and modified islets was 97% and 99%, respectively.

In vitro islet function

As a result of islet encapsulation, the potential for damage to the islet basal membrane exists, or diffusive transport of essential nutrients and hormones (such as insulin) across the capsule may be impeded, potentially resulting in decreased islet viability or function.⁴⁸ Therefore, islet function was assessed by static incubation after encapsulation of islets within the nano-thin PEG-GLP-1 coating. Islet function was assessed *in vitro* by measuring insulin release in response to a change in glucose concentration. The first group of columns represents untreated and PEG-GLP-1-coated islets incubated in 3.3 mM glucose solution. The 3.3 mM glucose is referred to as a low glucose or basal-level glucose. The second group of

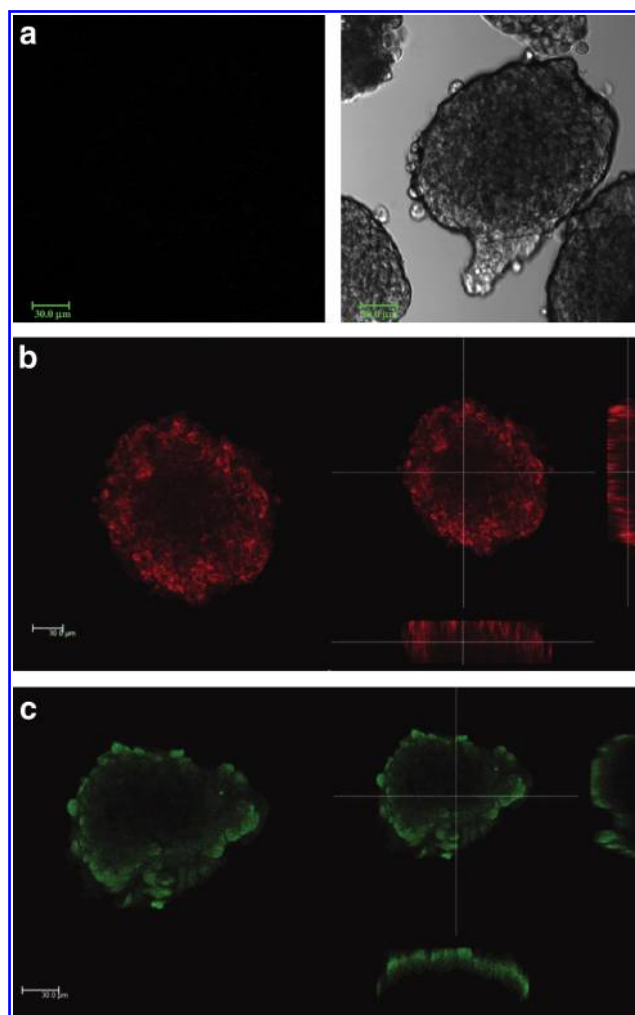


FIG. 2. Spectral-fluorescence confocal images of islets showing the surface modification steps. (a) An untreated control islet. The frame on the right employs differential interference contrasting (DIC) microscopy to image the islet. The frame on the left shows the confocal fluorescence micrograph of the same field of view as the DIC image on the right. The absence of fluorescence on the left shows that there is no islet autofluorescence. (b) Islets treated with NHS-PEG-biotin and then with SA-Cy3 demonstrated a fluorescent signal. The frame on the right shows horizontal and vertical cross-sectional views of an islet. (c) Islets treated with NHS-PEG-biotin, then with SA, and finally with NHS-PEG-FITC demonstrated fluorescence emission from FITC on the islet surface. The frame on the right shows horizontal and vertical cross-sectional views of an islet. Scale bars indicate 30 μm (a–c). FITC, fluorescein isothiocyanate.

columns represents the islets incubated in 16.7 mM glucose solution, referred as high-glucose solution. For both sets of islets there was a low level secretion of insulin to the basal glucose solution and a higher response to the high-glucose solution. The insulin response to the 16.7 mM glucose solution was higher for PEG-GLP-1-coated islets (Fig. 5). This shows that islets coated with multiple layers of biotin-PEG-NHS/SA/biotin-PEG-GLP-1 exhibit higher function in terms of insulin secretion than untreated islets in response to glucose stimulation.

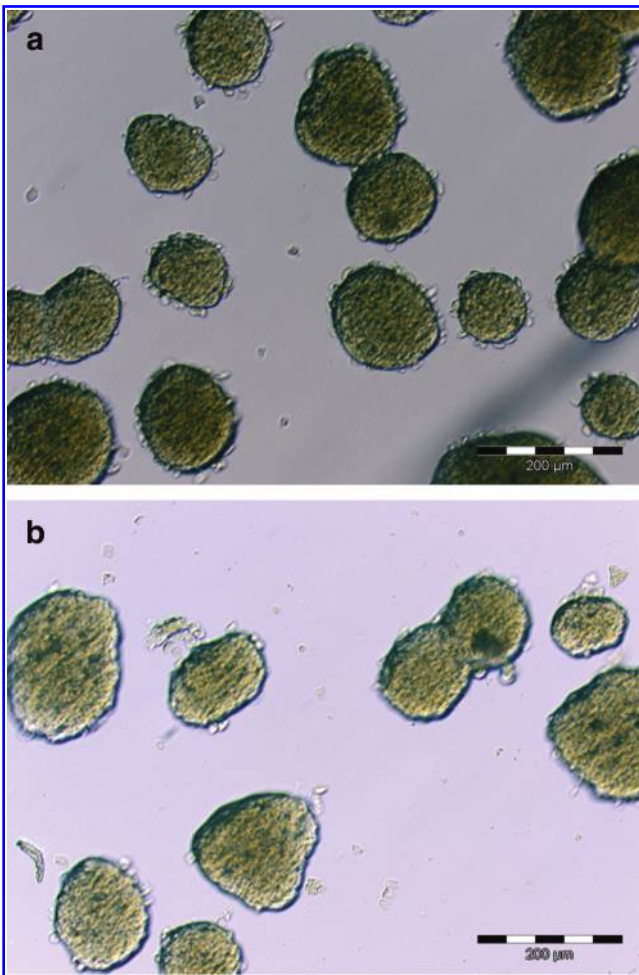


FIG. 3. DIC microscopic images of (a) untreated islets and (b) islets that were treated with biotin-PEG-NHS/SA/biotin-PEG-GLP-1. Color images available online at www.liebertonline.com/ten.

In vitro perfusion of islets

We compared the insulin released by untreated and PEG-GLP-1-coated islets as a function of time in response to a varying glucose concentration by means of a standard perfusion assay. For the analysis of insulin in perfusion experiments, insulin secretion for each group of islets has been quantitatively compared, where the amount of insulin released from each sample was normalized by total insulin content. After the islets were perfused, mammalian protein extraction reagent was used to completely lyse the islets and to release all of the intracellular insulin. The amount of insulin released at each time point then was divided by the total insulin in each group to calculate the fraction of total insulin release. The result demonstrated that the modified islets exhibited a higher response to glucose challenge, in terms of insulin secretion, compared to untreated islets after 1 day in culture. Figure 6a represents insulin release per minute as a fraction of total insulin to demonstrate this observation. Both sets of islets showed a response to the high-glucose solution of an increase in insulin release, where coated islets demonstrated enhanced insulin secretion in response to high glucose compared to the control islets (Fig. 6a). In addition, the ab-

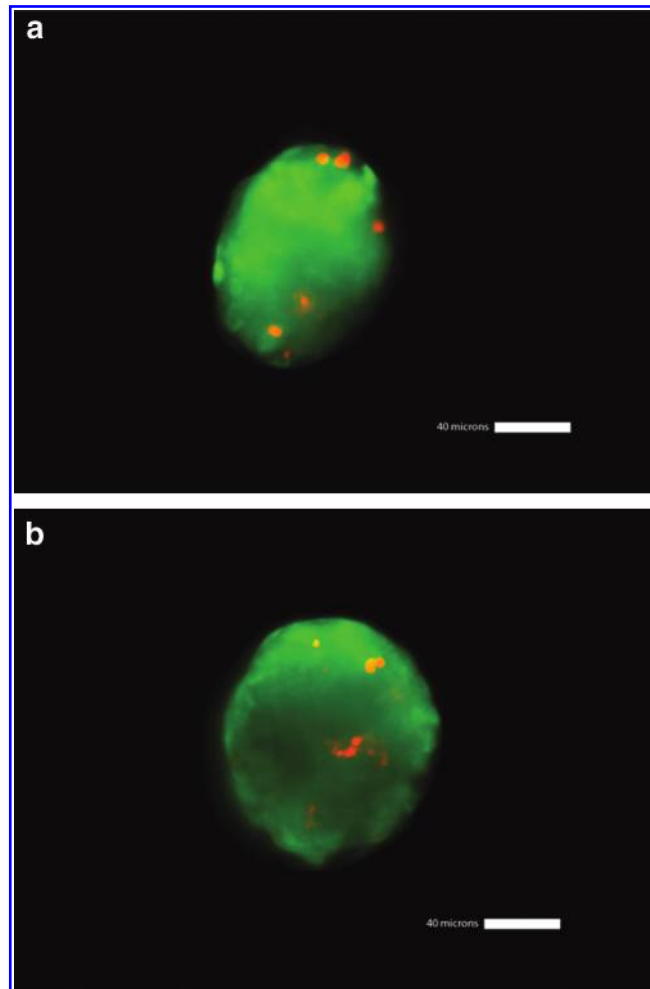


FIG. 4. Islet viability was preserved after layer-by-layer self-assembly of the biotin-PEG-NHS/SA/biotin-PEG-GLP-1 coating. Confocal microscope images of (a) untreated and (b) biotin-PEG-NHS/SA/biotin-PEG-GLP-1-coated mouse islets stained with FDA (green, viable cells) and PI (red, nonviable cells). Scale bars indicate 40 μm (a, b). FDA, fluorescein diacetate; PI, propidium iodide.

sence of a time delay in the response as compared with control islets suggests that the coating presented a negligible barrier to the diffusion of glucose and insulin. To confirm that the increase in insulin secretion capability is due to the presence of GLP-1 on the islet surface but not due to the presence of PEG, we performed perfusion experiments that include additional sets of islets coated with biotin-PEG and SA-biotin-PEG. The results are presented in Figure 6b, which demonstrates that the presence of GLP-1 on the islet surface is critical and that islets coated with biotin-PEG or SA-biotin-PEG secrete insulin at levels similar to control islets. Therefore, islets coated with GLP-1 secrete more insulin than the islets in the other groups. One additional observation that can be made about the insulin secretion profiles is that they are representative of patterns typical to the species (rat and mouse, respectively) exhibited under control conditions, as shown in previous studies.^{42,49}

Fluorescent staining of islets

Three-dimensional reconstruction of serial optical sections of islets coated with biotin-PEG-NHS/SA/biotin-PEG-GLP-1

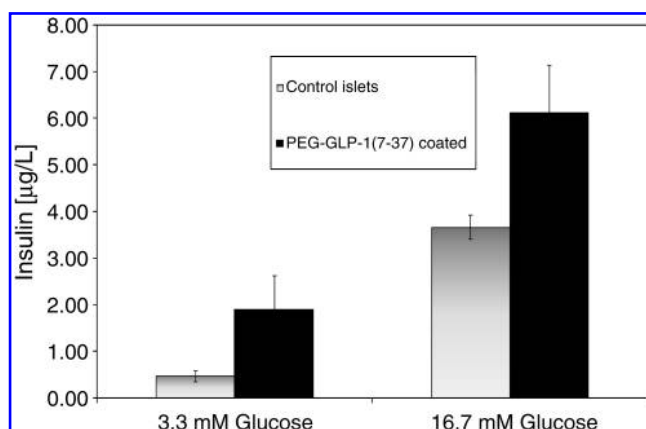


FIG. 5. Islet function is enhanced after the formation of a biotin-PEG-NHS/SA/biotin-PEG-GLP-1 coating on the islet surface. Treated islets secrete more insulin compared with untreated islets in response to a high glucose level (16.7 mM). Data points represent mean \pm standard error, for a minimum of three independent measurements.

film demonstrated that the film conforms to roughness on the islet surface and is grossly uniform. Figure 7 shows confocal microscope series images of a coated islet, where each image is rotated $\sim 10^\circ$ from the previous image.

Confirmation of the presence of GLP-1 on PEG-GLP-1-coated islet surface

We confirmed the presence of GLP-1 on PEG-GLP-1-coated islets by means of an anti-GLP-1 antibody test. The first group of islets consisted of PEG-GLP-1-coated islets (islets coated with NHS-PEG-biotin/SA/biotin-PEG-GLP-1) that were treated with primary and secondary antibodies as described in the Materials and Methods section. Two additional groups of islets were used as controls to prove that the fluorescence observed in the experimental group was the result of specific binding of anti-GLP-1 antibody to GLP-1 bound to the islet surface, and not as a result of nonspecific binding of either the primary or the secondary antibody to either treated or untreated islets. Figure 8 shows the results of the antibody experiment for control islets and PEG-GLP-1-coated islets. Figure 8a represents fluorescence images obtained from either of the control groups. This image, revealing no fluorescence at an exposure time of 360 ms (the same exposure time used to obtain the image in 8b), indicates that neither the primary nor the secondary antibody bound nonspecifically to islets in an appreciable amount. Figure 8b demonstrates green fluorescence as a result of specific binding of anti-GLP-1 to GLP-1 attached to the surface of coated islets.

Discussion

One of the critical issues of the clinical applications of the bioartificial pancreas is the large size of implants. Even though the blood glucose level can be normalized by 20,000 islets/kg body weight,⁵⁰ islet isolation procedures and conventional islet encapsulation demand a larger volume. Moreover, early graft loss occurs after purified islets are infused into the portal vein of the liver, which is the clinically

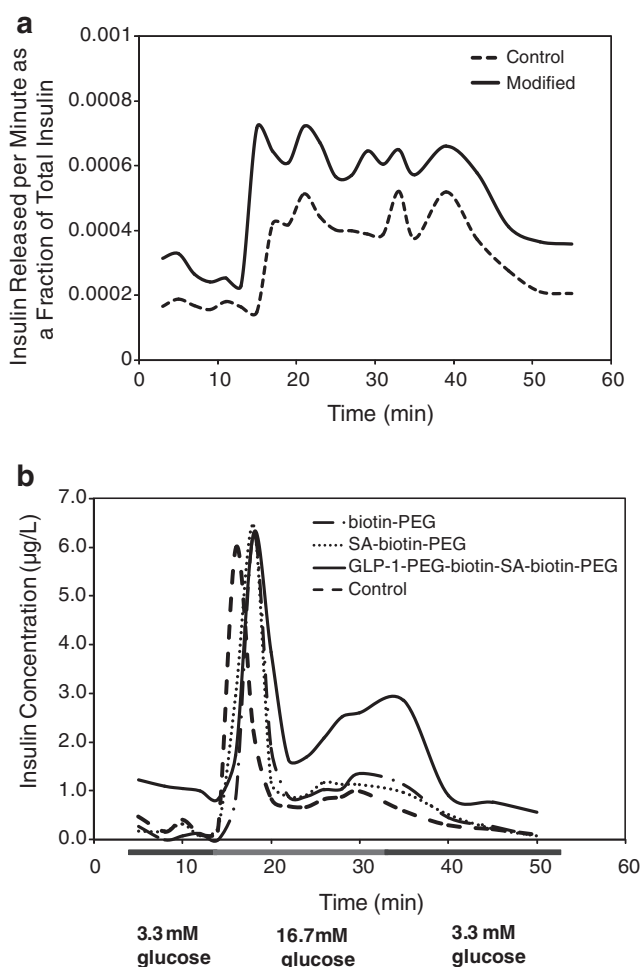


FIG. 6. (a) Dynamic insulin response to glucose stimulation of coated and uncoated islets. Insulin secretion is enhanced after formation of biotin-PEG-NHS/SA/biotin-PEG-GLP-1 coating on the surface of rat islets. Insulin is normalized as a fraction of total insulin. (b) Insulin secretion is similar for murine control islets and for islets coated with an SA-biotin-PEG sequence. Insulin secretion is higher after the third step of surface modification, in which GLP-1 is bound to the islet surface.

preferred site for islet transplantation. Despite encouraging early results of the Edmonton protocol, and hence the potential of islet transplantation,^{51,52} most conventional microcapsules are not suitable for transplantation into microvascular beds because of their large diameter.⁵³⁻⁵⁶ It has been shown in one study with animal models that intraportal infusion of 420 μm microparticles results in dangerous elevation of intraportal pressure,⁵⁴ and in another study impaired engraftment was demonstrated for islets encapsulated in 350- μm -diameter alginate/ Ba^{2+} transplanted into the portal vein.⁵⁵ The reason for impaired engraftment of islets and elevation of intraportal pressure was attributed to the occlusion of small and medium-sized portal venules and subsequent islet hypoxia. Clearly, encapsulation strategies for transplantation of islets into the portal vein must minimize transplant volume and improve islet functionality.

Conformal coatings of islets have emerged as an attractive technique to reduce void volume relative to conventional

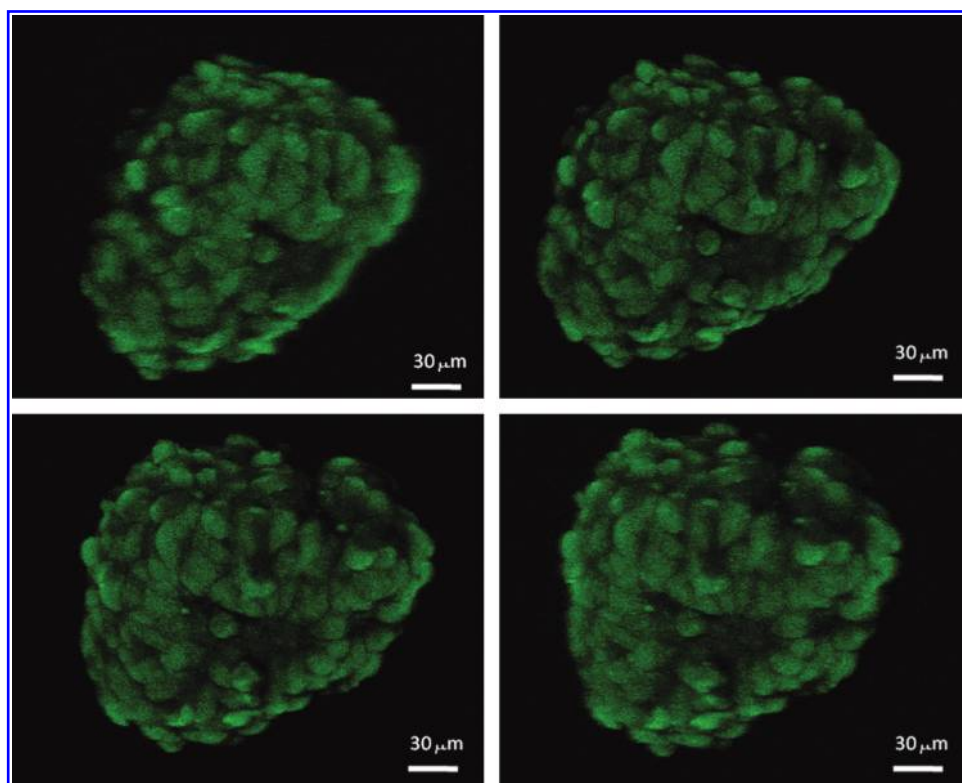


FIG. 7. Three-dimensional reconstruction of confocal microscope images of an islet coated with biotin-PEG-NHS/SA/biotin-PEG-FITC multilayer. Each image is rotated $\sim 10^\circ$ from the previous (left to right, top to bottom). Color images available online at www.liebertonline.com/ten.

microcapsules to generate membranes of submicron or nanoscale thickness. Acceptance rates of transplants in cellular therapy have been observed to increase due to the enclosure of cell surface antigens within a polymeric membrane or immobilization of bioactive substances on cell surfaces through synthetic polymers. Several groups have demonstrated that immobilization of PEG chains to the islet surface provides a mechanism of preventing, or at least attenuating

host response to both allo-⁵⁶⁻⁶⁰ and xenografts.^{61,62} For example, covalent coupling of PEG to amines of cell surface proteins or carbohydrates, or by direct insertion of PEG-lipid into the cell membrane through hydrophobic interaction has been applied to form a thin polymer layer on islet surfaces to provide immunoisolation. It was also reported that graft survival can be improved by immobilizing heparin or thrombomodulin to the islet surface to inhibit blood coagu-

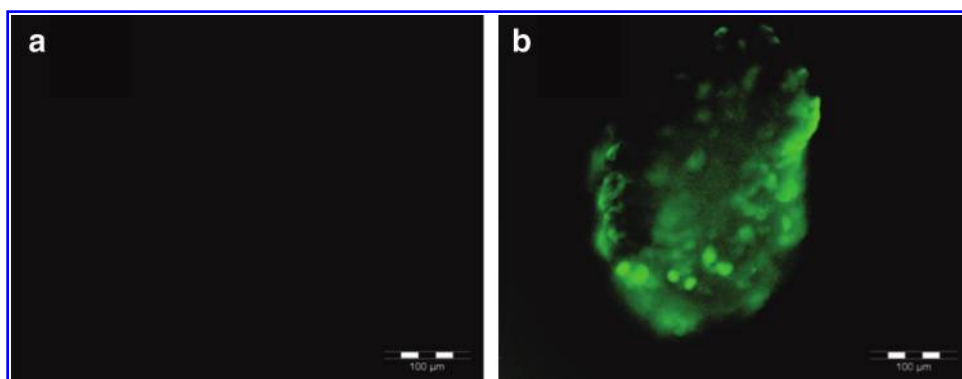


FIG. 8. Fluorescent microscope images of islets in (a) control groups: the first control was a group of islets selected randomly from the set of modified islets that contain GLP-1 attached to the surface. This group was incubated only in secondary antibody. The second control was a group of unmodified islets incubated in both primary and secondary antibody. (b) The experimental group in anti-GLP-1 antibody experiments. Islets were selected randomly from the set of modified islets that contain GLP-1 attached to the surface. This group was incubated both in primary and in secondary antibody. These images indicate specific staining of GLP-1 attached to the surface of modified islets, which cannot be seen in control groups. Color images available online at www.liebertonline.com/ten.

lation and reduce islet-mediated thrombogenicity. Despite these encouraging improvements in the graft survival by surface modification techniques, biofunctionalization of PEG-rich nano-thin layers with insulinotropic ligands to promote islet viability and insulin secretion has not been addressed to further reduce transplantation volume.

Our study utilizes the layer-by-layer self-assembly method to generate a PEG-rich nano-thin multilayer film of NHS-PEG-biotin/SA/biotin-PEG-GLP-1. The use of a biofunctional multilayer film is a novel approach due to the attachment of the insulinotropic peptide GLP-1(7–37) through the layer-by-layer self-assembly process. GLP-1 is immobilized to the outermost surface to enhance insulin secretion, promote islet viability, and reduce the number of islets required to achieve normoglycemia. The hypothesis was that by employing the technique of layer-by-layer self-assembly to attach GLP-1 to the islet surface, it may be possible for GLP-1 to bind to its receptor on the islet surface and enhance insulin secretion in response to stimulation with high glucose. Physically two possibilities for GLP-1-GLP-1 receptor binding exist. The GLP-1 molecule extending in solution may be able to bind to the receptors on the cell to which it is attached. Or, alternatively, the GLP-1 molecule may be able to bind to the receptors of adjacent cells. In either case, the same effect is achieved.

We used a confocal microscope to view fluorescently stained islets as a qualitative test to demonstrate the presence of biotin-PEG, SA, and biotin-PEG-GLP-1. Confocal microscope images revealed evenly distributed fluorescence, showing that PEG-GLP-1 coating covers the entire surface. Further, cross sections of islets demonstrated that PEG-GLP-1 is mainly bound to the outer region of the islet surface. Confocal micrograph images show surface-modified islets in the micron scale, but this is not an indication of a microthick coating around islets. Rather, it shows the islet as a cluster of many cells that are bonded together and surrounded with a basal membrane, and that it was possible with this technique to modify the surface of not only the basal membrane but also individual cells located at the outer region of an islet. Therefore, it should be noted that surface modification of islets has been achieved to produce a conformal coating at the nano scale. Also, this test does not indicate how much PEG or PEG-GLP-1 is at the surface or whether surface-grafted PEG and PEG-GLP-1 will be stable over the anticipated lifetime of the graft. However, we can conclude that islet surface modification steps were successful and that PEG-GLP-1 was attached to islets at the end of the modification process.

The viability of rat islets after the surface modification process was investigated using the FDA/PI method. Studies have shown that the incorporation of PEG into organ preservation solutions improved viability, and reduced ischemic injury due to its prevention of cell swelling and interaction with lipids in the cell membrane.^{63,64} GLP-1 has also an established history of preserving morphology and function and inhibiting apoptosis when added to freshly isolated human islets in the culture medium.⁶⁵ Therefore, the presence of PEG and GLP-1 on islet surfaces may allow protection of islets by similar mechanisms. In fact, the viability of surface-modified islets was not found to be significantly different from control islets, and that control islets and treated islets remained 97% and 99% viable, respectively.

The biological function was observed through both static incubation and dynamic glucose challenge assays. In the static incubation experiment, each sample contained a different sets of islets, and the amount of insulin secreted in response to low (3.3 mM) and high glucose levels (16.7 mM) for each set was measured. In the dynamic perfusion experiment, the same set of islets was exposed to different buffers (low glucose, high glucose) and hence dynamic perfusion experiments are physiologically more relevant than static incubation experiments in terms of assessing islet function. GLP-1-dependent regulation of glucose homeostasis is well established in the literature. It is known to stimulate insulin gene transcription and proinsulin biosynthesis in β -cells of pancreatic islets. Importantly, an additional observation is that the incretin action of GLP-1 is dependent on glucose concentration creating a feedback mechanism that prevents hypoglycemia as a result of GLP-1-stimulated over-secretion of insulin. As a result of these biological effects, it was clearly observed from the static incubation and dynamic perfusion experiments (Figs. 5 and 6) that islets that had been coated with the GLP-1-containing nano-thin capsule demonstrated enhanced insulin release in response to high glucose levels. One additional conclusion that can be made from the perfusion experiment is that the islets that had been treated with the PEG-rich GLP-1 functional coating did not exhibit a time-delay in their response to changes in extracellular glucose concentration when compared with control islets.

The presence of GLP-1 on the surface of PEG-GLP-1-coated islets was confirmed with an anti-GLP-1 antibody test. From this experiment, with one experimental and two control groups, we were able to conclude that GLP-1 was in fact present on the surface of modified islets (Fig. 8). Conclusions as to whether the orientation of GLP-1 is facing the islet or extending away from the islet cannot be drawn, but this experiment supports the indirect fluorescent staining experiment where FITC was bound to the islet surface, and provides further evidence that GLP-1 is bound to the islet surface. The possibility exists that GLP-1 is present on the islet surface due to direct binding of GLP-1 with its receptor; however, it is reasonable to assume that due to the high binding constant of SA and biotin, the orientation of GLP-1 is directed into solution. Further, since β cells in an islet are close to one another, GLP-1 molecules may stimulate the insulin secretion activity of the β cell to which they are bound, or that of adjacent β cells.

As presented in this study, covalent conjugation of PEG-GLP-1 to islet surfaces through layer-by-layer self-assembly is a unique strategy used to enhance the insulin secretion capability of islets. None of the current methods that allow covalent conjugation of PEG to islets used insulinotropic ligands bound via PEG-rich layers to promote viability and enhance insulin secretion. With this approach it would also be possible to immobilize more than one ligand through the use of different PEG-ligand conjugates (such as heparin, pituitary-adenylate cyclase activating polypeptide, laminin, and collagen type IV⁶⁶). This provides the ability to produce multifunctional barriers that may be able to biologically influence not only the encapsulated cells but also extracapsular cells such as host cells after implantation of capsules.

The unique properties of the PEG structure enable the molecules to act as a steric barrier on the islet surface.

However, surface PEG immobilization techniques that rely on PEGylation with only one layer may provide limited immunoisolation due to the lack of a highly cross-linked film. Multilayer films, on the other hand, generate PEG-rich networks and hence may improve immunoisolation properties compared with a PEG monolayer. Moreover, the multilayered structure used in this work allows integration of biotin and SA-linked PEG molecules within the film, which may provide better immunoisolation compared with single-step molecule immobilization. The capability of the nanocapsule to exclude components of the immune system was not the objective of this study, and our future work will focus on the immunoisolation capability of this PEG-rich nano-thin coating *in vitro* and *in vivo*.

Conclusion

The insulinotropic peptide GLP-1(7–37), which binds to its receptor on the surface of islets and increases insulin secretion in response to high glucose, was immobilized on islet surfaces through layer-by-layer assembly of biotin-PEG-NHS/SA/biotin-PEG-GLP-1. The functional islet coating produced in this work addresses the islet transplantation obstacles of intraportal pressure elevation during transplant and donor shortages, by reducing the number of islets required in a transplant to achieve normoglycemia. In addition to reduced transplantation volume and lower hepatic pressures, the GLP-1-functionalized nano-thin coating may significantly promote the viability of pancreatic islets. This technique can be used to encapsulate islets within nano-thin PEG coatings functionalized with GLP-1 and antiinflammatory molecules (thrombomodulin and heparin) for the purpose of immunoisolation, enhanced insulin secretion, and attenuation of inflammatory response. In conclusion, this technique improves upon current methods used to modify islet surfaces and has potential to further minimize necessary islet transplant volumes, while also improving islet functionality.

Acknowledgments

This study was supported by grants from the State of Illinois College of Engineering at Koç University in Turkey, and the Turkish Academy of Sciences-L'oreal (TUBA-L'OREAL) Young Women in Science 2009 Award to S.K.

Disclosure Statement

No competing financial interests exist.

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Received: September 24, 2009

Accepted: February 16, 2010

Online Publication Date: March 18, 2010