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Long-term Survival of Allograft Murine Islets Coated via Covalently Stabilized Polymers

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Abstract

Clinical islet transplantation (CIT) has emerged as a promising treatment option for type 1 diabetes mellitus (T1DM); however, the anti-rejection drug regimen necessary to mitigate allograft islet rejection is undesirable. The use of polymeric coatings to immunocamouflage the transplant from host immune attack has great potential. We have recently developed alginate and poly(ethylene glycol) (PEG)-based polymers, functionalized with azide and phosphine, respectively, which form spontaneous and chemoselective crosslinks via the bioorthogonal Staudinger ligation scheme. Herein, we explored the utility of these polymers to form immunoprotective, ultrathin coatings on murine primary pancreatic islets. Resulting coatings were nontoxic, with unimpaired glucose stimulated insulin secretion. Transplantation of coated BALB/c (H-2^d) islets into streptozotocin-induced diabetic C57BL/6 (H-2^b) resulted in prompt achievement of normoglycemia, at a rate comparable to controls. A significant subset of animals receiving coated islets (57%) exhibited long-term (> 100 d) function, with robust islets observed upon explantation. Control islets rejected after 15 d (+/- 9 d). Results illustrate the capacity of chemoselectively functionalized polymers to form coatings on islets, imparting no detrimental effect to the underlying cells, with resulting coatings exhibiting significant protective effects in an allograft murine model.

Keywords

immunomodulation; encapsulation; Staudinger ligation; poly(ethylene glycol); alginate

1. Introduction

Type 1 Diabetes Mellitus (T1DM) results from the autoimmune destruction of β -cells within the pancreatic islets of Langerhans. While reasonable management of blood glucose and mitigation of secondary complications can be achieved via exogenous insulin, supplemental agents, and dietary regulation, β -cell replacement can offer physiological glycemic control. Clinical islet transplantation (CIT) represents a viable treatment option for T1DM, whereby recipients of allogeneic cadaveric islets demonstrate stable glycemia, decreased hemoglobin A1c, and elimination of hypoglycemia for multiple years. Long-term (> 5 years) insulin independence, however, has been achieved in only a subset of patients [1].

An impairment to the success of CIT is the requirement of systemic immunosuppressive drugs to dampen allograft rejection and autoimmune β -cell attack. Anti-rejection drugs not only impart a burden to the patient, but also impair engraftment and function of the transplanted islets [2]. Further, current immunosuppressive regimens are unable to completely abrogate allo- and auto-immune attack, resulting in a smoldering immune response leading to islet destruction [3]. Novel approaches seeking to minimize the systemic drug load could not only substantially improve long term function, but also broaden clinical translation.

Encapsulation of islets within polymers is one approach explored to immunoprotect islets, with the goal of eliminating or minimizing the need for systemic anti-rejection drugs. This strategy seeks to camouflage foreign cells from the host via masking direct antigen presentation. Microencapsulation, e.g. the encapsulation of cells within capsules on the scale of 600 – 1000 μm in diameter, is one of the more common encapsulation approaches. Traditional microencapsulation has demonstrated reliable immunoprotection in allograft rodent models in the absence of immunosuppression, exhibiting the great benefits of this approach in mitigating immune attack [4]; however, this micro-scale polymeric coating imparts a significant diffusional barrier, which may result in delays in glucose responsiveness and the generation of nutritional gradients [5]. Further, microscale encapsulation substantially increases the transplant size, hindering the ability to transplant the islets within confined sites.

The generation of ultrathin coatings is an alternative strategy that can alleviate the drawbacks of micro-scale encapsulation by creating immunoprotection without imposing a thick polymeric barrier. Substantially reducing diffusion lengths can retain glucose responsiveness and improve nutritional profiles. Fabrication of these coating is challenging, whereby the materials and coating procedure must be carefully selected for optimal biocompatibility and minimal cytotoxicity. Various methods have been employed to generate ultrathin coatings. Two primary approaches are: surface modification via grafting of poly(ethylene glycol) (PEG); and layer-by-layer assembly. PEGylation of the islet surface is commonly achieved through the use of a PEG (MW > 2,000 Da) terminated with an activated ester, N-hydroxyl-succinimidyl ester (NHS), which readily reacts with free amines

found on the extracellular matrix and/or cell membranes present on the islet periphery. Groups have found benefits of islet PEGylation on dampening inflammation^[6] and enhancing allograft survival^[7]. Additionally, PEG grafting has been used to present additional agents to modulate immune recognition and inflammation, such as albumin, thrombomodulin, and leukemia inhibitory factor (LIF)^[8]. Alternatively, layer-by-layer assembly of polymers on the islet surface may generate more complete coatings. While electrostatic assembly is the most common method used for coating surfaces, the use of polycations on cells is complicated by their well-documented challenges in cytotoxicity^[9] and biocompatibility^[10], although this has been mitigated through approaches such as PEG grafting^[11]. Alternative layer-by-layer approaches include hydrogen bonding^[12], molecular recognition^[13], or combinations thereof^[14]. Evaluation of the immunoprotective properties of these platforms in animal models has been limited to a few selected studies. In Wilson et al., a statistically significant murine allograft protection for their coatings was not observed^[13a]. While immune protection in 70% of coated transplants was detected for coatings developed by Zhi et al., the short duration (> 28 d) of these studies limits full evaluation of their long-term immunoprotective capacity^[15].

In our laboratory, we have explored the potential of chemoselective polymers to form robust hydrogels for cellular encapsulation^[16]. We have functionalized polymers with agents capable of undergoing bioorthogonal reactions, i.e. spontaneous, chemoselective, aqueous schemes that proceed in cell compatible conditions and involve non-native chemical handles. We are particularly interested in the Staudinger ligation scheme, whereby an azide and phosphine, specifically 1-methyl-2-diphenylphosphino-terephthalate (MDT), react to form a stable amide bond. This scheme is not only bioorthogonal; the resulting covalent bond is highly stable^[17]. We have previously reported the use of Staudinger ligation for achieving bioorthogonal covalent stabilization of alginate hydrogels^[16a-c]. In these studies, alginate was functionalized with pendant alkyl azide groups utilizing a linear poly(ethylene glycol) (PEG) linker to form alginate azide (Alg-N₃). The addition of a PEG polymer terminated with 1-methyl-2-diphenylphosphino-terephthalate (MDT) resulted in amide bond linkage. We have assessed cytocompatibility of the crosslinking via encapsulation of rodent and human islets^[16c].

Herein, we explored the potential of MDT and azide functionalized PEG and alginate polymers to form ultrathin coatings, via dip coating of idealized beads or pancreatic islets in polymers expressing complementary functional groups (Figure 1). We subsequently evaluated the effects of these coatings on islet viability and function. Further, we assessed the immunoprotective capacity of these coatings via transplantation into a full MHC mismatched murine model (BABL/c; H-2^d islets into C56BL/6; H-2^b streptozotocin-induced diabetic recipients).

3. Results and Discussion

3.1 Coating of polymers on idealized surfaces and islets

Using biocompatible PEG and alginate-based polymers, functionalized to undergo bioorthogonal crosslinking, we evaluated the potential of these polymers to form ultrathin coatings on idealized spheres or primary pancreatic islet cell clusters. Coatings were made

via subsequent incubation of polymers with alternating functional groups, MDT or N₃, resulting in covalent linkage between polymeric layers (Figure 1A). The base coating was achieved via PEG grafting of free amines, using a bi-functional NHS-PEG-N₃. Due to the lack of chemoselectivity of the reaction of the activated ester, this incubation step was performed in a buffered saline solution. Incubation of the azide-functionalized surface with MDT-functionalized 4-arm PEG (4PEG-MDT) resulted in covalent crosslinking between the two polymers. ALG-N₃ was used as the final incubation step, resulting in covalent linkage between the PEG and the alginate layers. Given the chemoselectivity of the Staudinger ligation reaction (Figure 1B), incubation steps 2 and 3 were performed in full culture media.

Resulting coatings were evaluated via confocal microscopy. For these studies, the terminal ALG-N₃ was labeled with carboxyfluorescein prior to coating. As shown in Figure 1C–D, uniform, ultrathin coatings were observed on the aminated glass beads. Coated beads were stable for over 6 months, when maintained within a buffered saline solution. Control beads, treated with NHS-PEG-N₃ and FITC-ALG-N₃, but not the interim layer of 4PEG-MDT, did not exhibit strong fluorescence, supporting the chemoselectivity of the reaction (see Supporting Information Figure S1).

The capacity of these polymers to coat murine islets was subsequently evaluated. While coated and uncoated islets were indistinguishable under standard microscopy (Figure 2, A–B), fluorescent labeling of the terminal alginate layer illustrate a substantial and ultrathin coating on islets incubated with all three polymer layers (Figure 2C). As highlighted in Figure 2D, the coating remains on the islet periphery, with no observed intracellular and minimum intraislet infiltration. Given that polymer endocytosis leads to decreased cell viability^[11b], retention of the polymers to the apical surface is advantageous. Variation in coating uniformity on the islet is noted, in contrast to idealized surfaces. Imaging analysis of coatings found coating uniformity at $78 \pm 17\%$, with a variance from up to 98% to as low as 49%. Variation in coating uniformity is likely due to inhomogeneity in the surface extracellular matrix (ECM) of the islet cells following isolation. Given that the base layer of this coating strategy is NHS-based PEGylation, variation of surface amine groups, specifically those present within ECM, results in non-uniform coatings^[18]; likely leading to overall variation in the deposition of subsequent layers. Despite the lack of complete and uniform encapsulation via this strategy, the polymeric coating on the dynamic cell cluster appears stable, with retention of coating following 5 days of culture (Figure 2E). Control islets, treated with NHS-PEG-N₃ and FITC-ALG-N₃ but not the interim layer of 4PEG-MDT exhibited minimal fluorescence (Figure 2F), supporting the chemoselectivity of the coating.

3.2 Cytocompatibility of Coating on Islets

The effect of the coating procedure on islet viability and function was assessed 24 hr after coating. Coated islets (islets incubated with all three polymers) were compared to both unmanipulated and manipulated (islets exposed to coating procedure and washes but not polymers) controls. For clarity, only manipulated controls are shown (see Supporting Information Figure S2 for unmanipulated controls)

Islets were evaluated for viability via live/dead imaging and MTT metabolic activity. Live/dead confocal imaging was performed to visualize cell viability. If the coating procedure was cytotoxic, a large degree of peripheral cell death would be observed. As shown in Figure 3A–B, the proportion of dead to viable cells appears consistent among groups, indicating the benign nature of the procedure and the cytocompatibility of the polymers. MTT metabolic assessment further supports this cytocompatibility (Figure 3C), where no detrimental effects of the coating, or its procedure, on islet metabolic activity was observed. Of note, metabolic activity of coated islets was elevated, when compared to control ($P = 0.044$). This trend was observed for all trials conducted (3 independent islet isolations).

Islet function following coating was assessed via glucose-stimulated-insulin-secretion. Islets were incubated to varying concentrations of glucose (low/high/low) in 1 hr intervals. As shown in Figure 3D, islet responsiveness to the glucose challenge was not detrimentally affected by the presence of the coating. Insulin secretion under high glucose (stimulation) was comparable to controls, while insulin secretion under low glucose (basal) was slightly lower ($P = 0.006$). Low insulin secretion under basal glucose levels, when robust stimulatory insulin secretion is observed, is not indicative of poor function, impaired sensing, or impeded glucose or insulin diffusion, as defective beta cells trend to elevated basal secretion^[19]. Insulin stimulation indices (high/low 1) for the islets groups were 5.01, and 7.56 for control and coated groups, respectively. This elevation in the stimulation index is primarily due to decreased basal secretion for the treated group.

3.3 Function of Coated Islets within full MHC mismatched murine model

Following assurance of appropriate islet function and viability following coating, islets were implanted within full MHC mismatched murine recipients to provide a stringent evaluation of their immunoprotective properties. BALB/c islets were divided into three groups: control (n=8); PEGylated (n=8); and 3-layer coated (n=7). Control islets were simply maintained in culture prior to transplantation. PEGylated islets were treated with NHS-PEG-CH₃ to generate a single PEG coating. This control was used to evaluate the contribution of the base PEG layer in providing immunoprotection. Islets coated with 3 polymeric layers were treated as previously described, where islets were incubated in subsequent solutions of NHS-PEG-N₃, 4armPEG-MDT, and ALG-N₃. Islets were transplanted 24 hr after coating, for a total of 3 d post-isolation. Islets (750 IEQ) were loaded within the kidney capsule. This islet transplant mass, based on pooled islets, was within the standard dosage used to achieve prompt and consistent diabetes reversal for uncoated islets in murine allograft models^[20]. Transplantation of coated islets within this confined kidney capsule transplant site was feasible due to the insignificant effects of the coating on the overall transplant volume.

Efficient stabilization to normoglycemia was observed for diabetic recipients of 3-layer coated islets with a mean reversal time of 6 ± 4.3 d, an average statistically insignificant from control or PEGylated islets at 4 ± 2.2 d and 2 ± 1.3 d, respectively. Graft duration is summarized in Figure 4A. All recipients of control islets rejected the islets, as indicated by a return to hyperglycemia, with an average rejection time of 15 ± 9.3 d. Recipients of PEGylated islets rejected (100%) with an average rejection time of 16 ± 3.9 d; illustrating that the base PEG coating did not provide immunoprotection from allograft rejection for this

particular allograft model. For recipients receiving 3-layer coated islets, 4 of the 7 grafts exhibited stable blood glucose levels for the duration of the study, for a success rate of 57%. The three rejected grafts destabilized at 16 ± 0.6 days. The average nonfasting blood glucose levels for successful (n=4) and rejected (n=3) 3-layer coated grafts is shown in Figure 4B (see Supplementary Figure S3 for full blood glucose summary for all transplants). Successful long-term grafts exhibited stable blood glucose levels throughout the duration of the study (> 100 days), with the exception of 1 graft, which was euthanized on day 92 due to an infection unrelated to the islet graft. After over 100 days, survival nephrectomies of the graft bearing kidney was performed in the remaining grafts. For all explants, restoration to the diabetic state was observed, verifying that normoglycemia was due to the transplanted islets.

Histological evaluation was performed for all explanted grafts. Representative H/E and insulin stained sections of 3-layer coated islets from functional grafts, explanted over 100 d post-transplant (Figure 4C–D), and control islets, explanted 19 d post-transplantation (Figure 4E–F), are shown. For 3-layer coated islet grafts that exhibited long-term function, robust islets with a high degree of insulin are found over 100 days post-transplant. This is in stark comparison to control grafts, which were completely devoid of islets, with no positive insulin staining, 19 d post-transplant. Masson's Trichrome images also highlight the healthy engraftment of the 3-layer coated islets, with no fibrotic tissue observed for functioning grafts (Figure 5A–B). Of note, insulin positive islets were also observed in failed 3-layer coated grafts, explanted 19 d post-transplanted, indicating a degree of protection provided by the coating (see Supporting Data Figure S4).

In histological evaluation of long-term functioning coated grafts, two unique observations were noted: 1) the presence of intra-islet vascularization; and 2) pockets of mononuclear accumulation. As shown in Figure 5A–B, trichrome staining of these long-term functional grafts highlight intra-islet vascularization with the presence of red blood cells. This observation demonstrates host cell infiltration within the coated islet post-transplantation; indicating loss of the cell barrier function of the coating. The exact mechanism as to why these allogeneic grafts continue to be accepted by the host following loss of the coating barrier is unclear; however, evaluation of the immunological response of the host to these grafts may assist in elucidating some of the immune players in this response.

For long-term functioning coated islet grafts, the complete absence of lymphocytic infiltrate was observed in most sections; however, for selected areas within the graft, pockets of mononuclear accumulation were noted, as indicated by dense nuclei staining (Figure 5C). The infiltrate appeared to remain at the periphery of the functional islets, where the adjacent islets were intact and functional with no notable intra-islet infiltrate. This pattern of pockets of mononuclear accumulations adjacent to the grafted islets has been commonly detected in long-term grafts when pharmacological agents are employed^[21]. Immunostaining of T cells, as well as T regulatory cells was performed for these sections to evaluate the general immune response (Figure 5D). T cell lymphocytes (CD3⁺) dominated the lymphocytic regions. Within these T cell-rich pockets, a high degree of FoxP3⁺ stained cells was also observed (Figure 5D, **inset**), indicating a strong presence of T regulatory cells. The presence

of these cells may provide insight as to why active lymphocytic attack of the islet was not observed.

4. Discussion

Strategies to form ultrathin coatings on cellular spheroids offer the potential to mask immunological recognition and attack, while preserving efficient mass transport to the underlying graft cells, as well as minimization of overall transplant volumes. In this study, polymeric coatings were created on islets via bioorthogonally reactive biomaterials. Without a dependence on polymer net charge for layer deposition, the cytotoxicity and lack of biocompatibility commonly observed when polycations are used for electrostatic layer-by-layer coatings were avoided. Further, electrostatic layer-by-layer formation results in coatings bound via non-covalent interactions. As such, disintegration of these layers is common in unfavorable conditions, such as mechanical stress or swift changes in the ionic environment^[22]. Given the dynamic viable cell surface and the harsh *in vivo* environment, it is envisioned that the stability of such coatings *in vivo* is extremely limited^[6]. Therefore, the employment of covalent stabilization of coatings represents a viable strategy for the generation of ultrathin coatings, with minimal perturbations on islet viability.

Our approach is unique in its use of chemoselectively functionalized polymers to generate covalently stabilized coatings. Functionalization of polymers with complimentary Staudinger ligation reactive groups permits for the formation of covalent bonds with minimal perturbation to the biological environment. Reactions occur spontaneously without the need for additional reagents or environmental (e.g. pH, temperature) changes. Resulting bonds are highly stable, with cytocompatible reaction by-products. This ligation approach is in contrast to alternative methods employing free radical polymerization and/or initiators for covalent ligation. While these approaches may be highly effective for micron-scale coatings, it is envisioned that the combination of an ultrathin layer and the inherent sensitivity of islets to environmental perturbations could result in significant peripheral cell loss^[23].

Following the transplantation of coated islets, a significant increase in immunoprotection was observed. Results found enhanced protection of islets from immune attack, resulting in a significant percentage of allogeneic islets exhibiting long-term function. Grafts that failed (n=3) did so on the later end of the rejection timeline (i.e. 19–20 days post-transplant). Histological analysis of failed grafts demonstrated insulin positive cells and intact islets, in stark comparison to control islet transplants where no insulin reactive cells were observed at identical explant time points. These results indicate that graft failure may be correlated to the variation in coating uniformity, whereby failed grafts may have received a larger portion of islets only partially coated; resulting in greater islet graft loss and concomitant destabilization of normoglycemia.

The incompleteness of coatings prior to transplant, as well as the presence of functional vessels within the islet, indicate that the observed long-term immunoprotection was not due to the presence of a cell-isolating barrier. Recent studies indicate that the presence of a polymeric brush on the cell surface may mask inflammatory proteins and immunoreactive agents via charge reduction and attenuation of cell:cell and ligand:receptor interactions,

resulting in decreased generalized inflammation and immunorecognition^[24]. Thus, the polymeric coating generated, while not immunoisolating long-term, may mask the islet surface in a manner that reduces general inflammatory and immunological responses to the allograft transplant.

While the exact mechanism by which these coatings impart long-term immunoprotection remains unclear, immunohistochemical analysis of the explanted grafts provides some indication of the immunological response to the graft. The peri-islet mononuclear accumulations observed in these grafts have been highlighted in several promising immunomodulatory approaches, although the nature of this nonpathogenic infiltration has not been fully elucidated^[21b, 25]. The increased presence of CD3⁺FoxP3⁺ T regulatory cells (Tregs) within the pockets of lymphoid cells in long-term functioning grafts indicates a regulatory component to the observed protective effects of the coatings. Inducible Tregs (e.g. regulatory T cells induced in the periphery) have been associated with enhanced allograft survival via tolerance pathways^[26]. The mechanism by which these coatings may result in the increased presence of T regulatory cells is a focus of future studies.

Comparison of the observed benefit of our polymeric coatings to short-course pharmacologic agents highlights the potent effects of these coatings in dampening immune rejection. Numerous short-course (e.g. < 20 d) pharmacological treatments, targeted to inhibit T cell activation, migration, and/or proliferation, have been screened in murine allograft models (e.g. α -LFA-1, cyclosporine, rapamycin, CTLA4-Ig, and CD40L mAb). In all, the majority of these approaches resulted in moderate immune protection, with 30–50% of grafts exhibiting long-term (>100 day post-transplant) function, particularly in this stringent allograft model (e.g. Balb/c islets into C57Bl/6J mice^[21]). The combination of pharmacological therapies enhanced protective effects; however, the systemic treatment of multiple immunosuppressive agents increases toxicity and side effects. The use of polymeric coatings, however, provides a less risky approach to dampen immunological attack. The combination of polymer grafting with short-course immunosuppression has shown a highly synergistic effect in enhancing long-term graft survival^[27]. Thus, the combination of these coatings with a low dose, short-course immunosuppressive regimen may prove to be an optimal approach to enhancing graft survival, while minimizing clinical risk.

A challenge with the use of these specific polymers for coating, however, is the miscibility of the 4ArmPEG-MDT polymer. 4ArmPEG-MDT exhibits phase separation over time, due to the hydrophobic nature of the MDT. This phase separation presents challenges in islet coating, likely contributing to the observed incomplete coatings. The flexibility of the PEG arms further decreases layer efficiency, as aggregation of MDT groups to minimize free energy is expected. This is supported by the inability of these coatings to undergo consistent layer-by-layer deposition on idealized aminated surfaces. While single layers could be formed, demonstrating the chemoselectivity of the reaction, building of polymeric layers using 4ArmPEG-MDT and alginate-N₃ on planar surfaces was not feasible. On islets, however, coating was observed, indicating that surface topography, the density of reactive groups, and surface charge of the islet may play a role in enhancing polymer deposition. Finally, the time duration of the procedure prevents the efficient deposition of multiple layers, which is desirable to enhance the uniformity and integrity of coating. We have

recently published exploratory studies evaluating coatings generated using similar bioorthogonal chemistry, but utilizing polymers with dense, restricted end groups, via hyperbranching and dendritic polymers^[28]. Future studies seek to evaluate the immunoprotective capacity of these coatings in allograft rodent models.

5. Conclusion

Immunoprotective coatings were formed on primary pancreatic islet cell surfaces via incubation with bioorthogonal polymers functionalized with Staudinger ligation reactive groups. This method resulted in coatings on islets that did not adversely affect cell viability or function. The resulting coating imparted immunoprotective properties, whereby ~60% of grafts exhibited long-term function in fully MHC mismatched murine models. Explantation of grafts revealed highly engrafted islets, with minimal peri-islet lymphocytic infiltrate, as well as an increased presence of T regulatory cells.

Experimental Section

General Reagents and Polymers

Sodium alginate (PRONOVA UP VLVG, Mw <75 kDa, G/M ratio = 2.03, Batch BP-0711-09) was purchased from NovaMatrix. The 1-ethyl-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Advanced Chemtech. The H₂N-4 armPEG (Mw 11,000 g/mol) and H₂N-PEG-tBoc (Mw 3,600 g/mol) were purchased from Laysan Bio, Inc. NHS-PEG-N₃ (Mw 3,400 Da) and NH₂-PEG-Flourecein (Mw 3,400) were purchased from Nanocs. Aminated glass beads (30–50 µm) were purchased from Polyscience. Triethylamine was purchased from Across Organics. Thin layer chromatography (TLC) was performed on Baker-flex Silica Gel IB-F cut into 12×25 mm sheets using 9/1 (v/v) of dichloromethane/methanol as developing solvent and I2 for spot visualization. All other filter, solvents, and chemical reagents including the including 2-(Diphenylphosphino)terephthalic acid 1-methyl 4-pentafluorophenyl diester Mw 530.38) and NH₂-PEG₅₂₆-N₃ (Mw 526.62 g/mol) were purchased from Sigma-Aldrich.

Polymer Synthesis

Final polymers used for coatings were synthesized and purified in-house. Alginate-PEG-N₃, was fabricated as previously described [21]. In brief, 300 mg of ultrahigh purity sodium alginate (VLVG) was dissolved in 15 mL de-ionized water. 82 mg N-hydroxysuccinimide (NHS), 373 mg 2-(N-morpholino)ethanesulfonic, 500 mg of 1-ethyl-(dimethylaminopropyl)carbodiimide hydrochloride were sequentially added to the stirred solution, followed by 350 mg N₃-PEG₅₂₆-NH₂ (M_w 526 g/mol). Following a 20 min incubation, 200 µL of 6 M NaOH was slowly added to the solution and stirred for 120 min under Ar atmosphere. Purification was achieved via 4 d dialysis (10,000 MWCO membrane) against 1000 mL water, which was changed three times a day. During the first 3 d, NaOH (10 µL of 6 M) and NaCl (400 µL of 4.26 M) were added twice daily to the alginate-containing solution. The remaining solution was filtered through a 0.45 µm membrane, and lyophilized. FITC-Alginate-PEG-N₃, was fabricated as previously described [21]. In brief, 200 mg of ultrahigh purity sodium alginate (VLVG) was dissolved in 10 mL de-ionized water, followed by the addition of 56 mg N-hydroxysuccinimide (NHS), 248 mg 2-(N-

morpholino)ethanesulfonic acid. Subsequently, 464 mg of 1-ethyl-(dimethylaminopropyl)carbodiimide hydrochloride was added to the solution with stirring for 5 minutes, followed by 224 mg N_3 -PEG₅₂₆-NH₂ (M_w 526 g/mol) and 150 mg fluorescein-PEG-NH₂ (3,400 Mw), with incubation for 20 min under agitation. Finally, 200 μ L of 6 M NaOH was slowly added to the solution and stirred for 90 – 120 min under Ar atmosphere. Purification was achieved via 4 d dialysis (10,000 MWCO membrane) against 1000 mL water, which was changed three times a day in a dark environment. During the first 3 d, NaOH (10 μ L of 6 M) and NaCl (400 μ L of 4.26 M) were added twice daily to the alginate-containing solution. The remaining solution was filtered through a 0.45 μ m membrane, and lyophilized. Note that the reaction and purification were in a dark environment to protect the Fluorescein from light.

4ArmPEG-MDT was fabricated via dissolving 200 mg H₂N-4arm-PEG₁₁₀₀₀ in 1 mL anhydrous dimethylformamide (DMF) under Ar flow. Subsequently, 39.2 mg of 2-(Diphenylphosphino)terephthalic acid 1-methyl 4-pentafluorophenyl diester was added to the solution and stirred until complete mixing, followed by the addition of 7.4 μ L triethylamine to the solution. After 1 h under Ar atmosphere, the reaction was tested for completion using the Kaiser Test. The product was precipitated with 12 mL cold diethyl ether (Et₂O) and collected by centrifugation at 0 °C. It was re-dissolved and re-crystallized in 12 mL 200 proof ethanol (warmed to 35 °C to dissolve, cooled in ice-water bath to precipitate, collected precipitate by centrifugation at 0 °C). The precipitate was washed twice with 12 mL Et₂O, collected by centrifugation at 0 °C, and dried under reduced pressure for at least 2 days. All polymer purity was assessed via ATR-FT-IR and mass spectroscopy.

Animals

All animal studies were reviewed and approved by the University of Miami Institutional Animal Care and Use Committee. All procedures were conducted according to the guidelines of the Committee on Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources (National Research Council, Washington DC). Animals were housed within microisolated cages in Virus Antibody Free rooms with free access to autoclaved food and water at the Department of Veterinary Resources of the University of Miami. The Preclinical Cell Processing and Translational Models Core at the Diabetes Research Institute performed the rodent islet isolations, diabetes induction, and animal monitoring. Male BALB/c (H-2^d) mice, between 10–12 wks of age (Jackson Laboratory; Bar Harbor, Maine), were used as islet donors. Male C57BL/6J (H-2^b) mice, between 7–9 wks of age (Jackson Laboratory), were used as transplant recipients. Mice were rendered diabetic by intravenous administration of 200 mg/kg streptozotocin (STZ; Sigma, St. Louis, MO) freshly dissolved in citrate buffer and were used as recipients of allogeneic islets only if overtly diabetic upon three consecutive readings of non-fasting blood glucose levels > 350 mg/dL, using portable glucose meters (OneTouchUltra2; Lifescan, Milpitas, CA) [29].

Islet Isolation and Culture

Islets were obtained from donor mice via mechanically-enhanced enzymatic digestion followed by density gradient purification, as previously described [29]. Islet purity was

assessed by dithizone (Sigma) staining, and the islets were counted and scored for size using an algorithm for the calculation of the 150 μm diameter islet equivalent (IEQ) number. The DRI Preclinical and Translational Models Core performed all islet isolations. Islets were cultured in completed CMRL 1066-based medium (Mediatech), which is CMRL 1066 supplemented with 10% fetal bovine serum (FBS; Sigma), 20mM Hepes Buffer, 1% penicillin-streptomycin (Sigma), and 1% L-glutamine (Sigma). Islets were cultured for 48 h prior to coating.

Coating of Glass Beads and Islets

The coating procedure for both glass beads and islets were identical. Polymeric solutions used to generate coatings were: 1) NHS-PEG- N_3 for PEGylation; 2) 4armPEG-MDT for the intermediate layer; and 3) ALG- N_3 for terminal layer. Polymer solution 1 consisted of: 4 mM NHS-PEG- N_3 in 11 mM glucose in DPBS, pH 7.7–7.8. Polymer solution 2 consisted of: 2.0 mM MDT-4 arm PEG in complete CMRL1066 culture media, pH 7.3–7.4. Polymer solution 3 consisted of: 1.0 mM ALG- N_3 in complete CMRL1066 culture media, pH 7.3–7.4. All solutions were filter sterilized prior to use. All incubation steps were conducted at 37°C in a standard humidified incubator. Beads or islets were first rinsed three times with DPBS (10 mL) in a 15 mL conical prior to incubation with first layer. Beads or islets (< 2500 IEQ per tube) were resuspended in 2 mL of solution 1 for 45 mins to form the base PEG layer. Following rinsing with complete CMRL1066 culture media (10 mL) three times, beads or islets were resuspended in 2 mL of solution 2 for 1 hr. Following rinsing with complete CMRL1066 culture media (10 mL) three times, beads or islets were then resuspended in 2 mL of solution 3 for 2 hr. Beads or islets were then rinsed an additional 3 times in complete CMRL1066 culture media prior to incubation in final culture solution; saline for beads and complete CMRL for islets.

Coating Visualization

For image studies of the resulting coating, a fluorescently labeled ALG- N_3 , CF-ALG- N_3 , was used for the polymer 3 coating. Prior to imaging, islets were counterstained with Hoechst nuclei stain. Beads or islets were then placed on a glass cover slip and visualized via a Leica SP5 Inverted Confocal Microscope. Single plane images and merged multi-slice images (4–8 μm thickness; 8–15 slices per image; 1024×1024; 20x objective) were collected. Coating uniformity was assessed via Metamorph (MetaMorph, Molecular Devices, Sunnyvale, CA) analysis of merged multi-slice images of the FITC labeled coating. Green channel images (n=9) were converted to binary, the islet was selected as the region of interest, and pixels within the inclusion threshold (35–255) were measured. Due to the lateral and z-axis resolution, only gaps in coatings above ~15 μm^2 were detectable. Uniformity was expressed as % positive pixels within the islet region of interest.

Cell Viability and Metabolic Assessment

For all assessments, three groups were evaluated: 1) unmanipulated controls; 2) manipulated controls, e.g. controls exposed to procedural washes and incubations but no polymers; and 3) islets coated with three layers of polymers. Assessments were conducted 24 hr after coating (3 days after isolation).

Cell viability was visualized via LIVE/DEAD Viability/Cytotoxicity Assay Kit (Invitrogen) and imaged through a Leica SP5 Inverted Confocal Microscope. Islets were briefly rinsed in HBSS once and incubated for 60–90 min in a DPBS composed of 4 μ M calcein AM and 8 μ M ethidium homodimer-1 (EthD-1) as previously described [16c]. Islets were then rinsed in DPBS and placed on a cover slip for visualization via a fluorescence scanning confocal microscope. Images were collected using a Leica SP5 spectral confocal inverted microscope. Multi-slice images (4–8 μ m thickness) were collected and compiled using 3-D projection function and Leica software.

For quantitative assessment of metabolic activity of the islets, CellTiter 96 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (Promega, Madison, WI) was used. Triplicates of 150 islets from each group were loaded into 48-well plates containing medium with 0.5 mg/ml MTT (Promega) for 1 h 15 min at 37 °C. The stop/solubilization solution was then added, and the islets were incubated overnight within humidified 37 °C, 5 % CO₂/95 % air incubator to completely dissolve the formazan crystals. From each well, triplicates of 120 μ L of solution were transferred to a 96-well plate and the absorbance of the final solution was read at 570nm–620nm using a Molecular Devices Spectra-Max 500 plate reader.

The functional insulin secretion rate of islets was determined via static glucose-stimulated insulin release (GSIR) assay, as previously described [30]. Groups were sequentially incubated for 1 hr intervals in glucose solutions Low1→High1→Low2, with low (40 mg/dL) and high glucose (300 mg/dL) concentrations using a column method. Eluate following incubations was collected in tubes and stored at – 80 °C for later analysis. Insulin was quantified using the Mercodia Murine Insulin ELISA (Winston Salem, NC).

Islet Transplantation and Graft Assessment

Under general anesthesia (isoflurane USP; Baxter, Deerfield, IL), islets (700 – 800 IEQ) were transplanted in the kidney subcapsular space, as previously described [29]. Islets were transplanted 24 hr after coating (3 days after isolation). Three groups were tested: controls (n=8); islets PEGylated via NHS-PEG-CH₃ (n=8); and 3-layer coated islets treated with NHS-PEG-N₃/4armPEG-MDT/ALG-N₃ (n=7). The PEGylated islet group was PEGylated via 45 min incubation in 4 mM NHS-PEG-N₃ (11 mM glucose in DPBS, pH 7.7–7.8). Islets were washed three times with PBS prior to overnight incubation in culture media. The 3-layer coated islet group was coated as described above. Islets were coated 48 hr after isolation and transplanted 24 hr after coating. Normoglycemia of the recipients was defined as stable non-fasting glycemic levels < 200 mg/dL for 2 consecutive readings. Mice that remained hyperglycemic for over 10 d following transplant were considered as primary nonfunction, euthanized, and censored from analysis. Graft rejection was defined as functional grafts that exhibited diabetes recurrence, glycemic levels > 300 mg/dL for over 3 days. Animals maintaining a functional graft long term (>100 days) underwent a survival nephrectomy of the graft-bearing kidney to rule out residual function of the native pancreas.

Histological analysis of explanted grafts was conducted following fixation in 10 % formalin buffer, whereby grafts were then paraffin-embedded and sectioned into 10 μ m thick sections. Graft morphology was assessed via hematoxylin and eosin staining and Masson's

Trichrome. Immunofluorescence was used to image islets and immunological response. Staining for insulin was performed using polyclonal primary Guinea pig anti-insulin (1:100; DAKO, A0564) and a secondary goat anti-guinea pig AlexaFluor-568 conjugated antibody (for single insulin staining) or goat anti-guinea pig AlexaFluor-647 conjugated antibody (for sequential insulin staining with CD3 and FoxP3) (Molecular Probes). Lymphocytic infiltrates were detected via polyclonal primary rabbit anti-mouse CD-3 (1:50; Cell Marque) and secondary goat anti-rabbit AlexaFluor-488 conjugated antibody (1:400; Invitrogen). T regulatory cells were detected via rat anti-mouse FoxP3 (1:100, eBioscience, clone FJK-16s) and secondary goat anti-rat AlexaFluor-568 conjugated antibody (1:400; Invitrogen). For CD3, FoxP3, and insulin staining, CD3 and FoxP3 antibodies were incubated overnight, followed by incubation with complementary secondary antibodies prior to insulin staining (1 hr) and secondary staining. Nuclei were counterstained using DAPI. All results were compared to isotype controls (primary antibody omitted) to ensure specificity of detection. All histological images were collected using a Leica SP5 Inverted Confocal Microscope.

Statistical Analysis

All islet comparisons between in vitro groups (e.g. controls to coated) were made using the same islet preparation. A minimum of three independent replicate measurements were made for each assay. The number of replicates is indicated in the figure legends, and results are expressed as the mean $\pm$ SD. A minimum of three independent experiments (e.g. three islet isolations) were performed for each assessment, with graphs summarizing results from a representative experiment. For in vivo experiments, three independent transplants were performed, whereby controls were included in all transplant sets to minimize effects of islet isolation. Statistical analyses for islet viability and insulin secretion experiments used multi-factor analysis of variance, with single tailed T-tests for comparison between individual groups. For time to reversal analysis (% normoglycemia), Mantel-Cox (logrank) test was performed to evaluate differences between groups. Differences were considered significant when $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic of coating scheme (A) for aminated beads and islet cell clusters. The primary layer is achieved via conjugation between free amines on the bead or islet surface and the activated ester group on the bi-functional NHS-PEG-N₃ polymer. The second layer is achieved via addition of 4-arm PEG-MDT, covalently linked in place via Staudinger ligation. The terminal layer is achieved via addition of ALG-N₃, covalently linked in place via Staudinger ligation. Schematic of the Staudinger Ligation scheme (B), whereby azide and MDT react to form an amide bond. Confocal microscopy images (C–D) of idealized aminated glass spheres 10 d after multi-layer assembly of NHS-PEG-N₃/4armPEG-MDT/Alg-N₃ coatings; single plane (C, D) and multi-slice projection (inset) images illustrate uniformity and scale of coating. Fluorescence intensity profile from coated bead (E). Spatial position of intensity profile illustrated by white line (in D). Scale = 50 μm

Figure 2.

Assembly of 3-layer coatings on murine islets. Coated islets were treated with NHS-PEG-N₃/4armPEG-MDT/Alg-N₃, whereby coatings were detected via FITC labeling of the terminal ALG-N₃ layer. Microscope images of control (A) and coated (B) islets after coating procedure, illustrating ultrathin nature of coating (scale = 150 μm). Multi-slice projection confocal images of coated BALB/C mouse islets 48 hrs after coating (C) (scale = 50 μm). Single slice confocal image of islet periphery, illustrating extracellular assembly of coating (D) (scale = 20 μm). Multi-slice projection confocal image of coated islet 5 d after coating (E) (scale = 50 μm). Control islets, treated with NHS-PEG-N₃ and FITC-ALG-N₃ but not the interim layer of 4armPEG-MDT (F) (scale = 50 μm). Confocal images treated with Hoechst nuclei counterstain (blue).

Figure 3.

Assessment of murine islet viability and function 24 hrs following coating assembly. Coated islets were treated with NHS-PEG-N₃/4armPEG-MDT/Alg-N₃, while control islets were exposed to washing steps but not the polymers. Representative live/dead multi-slice projection confocal microscopy images of control (A) and coated (B) islets; (green=viable; red=dead). Metabolic activity, per MTT metabolic assay, (C) and glucose stimulated insulin secretion (GSIS) (D) of murine islets indicate high cytocompatibility of coating. *P < 0.05 Scale bar = 50 μ m

Figure 4.

Murine islets coated with NHS-PEG-N₃/MDT-4armPEG/Alg-N₃ polymers exhibit significant improvement in overall long-term tolerance in mismatched murine allograft transplants. A significant percentage of 3-layer coated islet grafts (n=7) exhibited long-term function, as demonstrated by % normoglycemia (A), compared to control islet grafts (n=8) or PEGylated islet grafts (n=8) ($P=0.0015$). Blood glucose levels (B) of individual diabetic recipients of 3-layer coated islets. *Censored graft at d 92 due to euthanization for infection. Representative histopathological images of coated (C–D) and control (E–F) islets, stained via H/E (C & E) and insulin (red, D & F). Scale bar = 100 μm

Figure 5.

Explants of long-term functioning, >100 d, grafts of murine islets treated with NHS-PEG-N₃/4PEG-MDT/Alg-N₃ polymers stained via Masson's trichrome (A–B) or immunostained for insulin (white); CD3 (green); and FoxP3 (red). Insets are higher magnification images of lymphocytic infiltrate. Arrows highlight vascularization. Scale bar = 50 μ m