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Polymeric Nanomaterials for Islet Targeting and Immunotherapeutic Delivery

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Abstract

Here we report a proof-of-concept for development of pancreatic islet-targeting nanoparticles for immunomodulatory therapy of autoimmune Type 1 Diabetes. Modified with a unique islet-homing peptide, these polymeric nanomaterials exhibit 3-fold greater binding to islet endothelial cells and a 200-fold greater anti-inflammatory effect through targeted islet endothelial cell delivery of an immunosuppressant drug. Our findings also underscore the need to carefully tailor drug loading and nanoparticle dosage to achieve maximal vascular targeting and immunosuppression.

Keywords

Diabetes; pancreatic islet; nanoparticle; drug delivery; vascular endothelium; inflammation; tissue targeting

Application of the principles of nanotechnology in medicine has led to the development of nanomaterials (e.g. liposomes and polymeric micelles) that can greatly improve the delivery and therapeutic efficacy of drugs by simultaneously increasing drug half-life, reducing toxic side-effects and controlling drug release kinetics¹. Such therapeutic nanomaterials have mostly been used in cancer drug delivery where leaky tumor vessels permit passive targeting of blood-borne nanoparticles to tumor tissue through the enhanced permeability and retention (EPR) effect². The ability of such therapeutic nanomaterials to treat diverse diseases will, however, depend on their ability to selectively target the desired tissue of interest and promote *in situ* tissue normalization¹. Development of such smart nanomaterials will likely create new therapeutic opportunities for the management of intractable diseases where systemic therapies often are limited by a high risk/benefit ratio.

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Supporting Information Available

Detailed experimental procedures, phase images of islet CE cells treated with blank or genistein-loaded (NP-Gen) nanoparticles and quantitative measurement of islet CE cell viability following nanoparticle treatment. This material is available free of charge via the Internet at <http://pubs.acs.org>.

One such disease that is difficult to manage by systemic therapy is Type 1 Diabetes. It is a debilitating and rapidly spreading autoimmune disease where insulin-producing pancreatic islet β cells are progressively destroyed by the body's immune cells, leading to hyperglycemia at clinical diagnosis³. One of the earliest events in the autoimmune destruction of islet β cells (insulinitis) is the adhesion of blood leukocytes to the inflamed islet vascular endothelium, followed by extravasation of the immune cells into the islet parenchyma where they attack the islet β cells^{3,4}. Given that combination of metabolic and autoantibody tests can predict with 90% accuracy the 6-year risk of developing Type 1 Diabetes⁵⁻⁷, managing this debilitating disease at the early stage of leukocyte-islet vessel interaction has emerged as a viable therapeutic strategy⁸. Anti-leukocyte proliferative drugs that reduce the number of circulating leukocytes, such as cyclosporine A and prednisone, have been reported to substantially extend endogenous insulin production while lowering dependence on exogenous insulin treatment^{9,10}. Unfortunately, these systemically-administered drugs have been barred from clinical use due to severe side-effects, including nephrotoxicity¹¹, development of insulin resistance¹², and compromising the body's innate infection-fighting capacity¹³. Thus, development of drug delivery approaches that can selectively inhibit leukocyte-islet vessel interactions without causing any adverse reaction would have important implications for the treatment of insulinitis in subjects that are at high risk of developing Type 1 Diabetes.

Here we describe a proof-of-principle for development of islet-targeting nanoparticles for insulinitis therapy that preferentially bind to islet capillary endothelial (CE) cells and locally deliver an anti-inflammatory agent to inhibit leukocyte adhesion. The active islet-targeting ability of these polymeric nanoparticles is conferred by a unique islet-homing peptide that is conjugated to their surface. These nanoparticles also function as superior drug delivery vehicles, as indicated by a significant increase in the immunosuppressive effect on leukocyte adhesion to islet CE cells exhibited by an encapsulated anti-inflammatory drug. By abrogating the use of deleterious anti-leukocyte proliferative agents and leveraging the outstanding drug delivery properties of nanomaterials, this new islet-targeted immunomodulatory approach may create new therapeutic opportunities for preventing or significantly delaying the onset of Type 1 Diabetes in high-risk individuals.

As building block for these nanomaterials, we used an amphiphilic poly(D,L-lactide-co-glycolide) –block –poly(ethylene glycol) (PLGA-b-PEG-COOH) co-polymer that spontaneously self-assembles in aqueous solution to form nanoscale particles (Figure 1A). Both PLGA and PEG are FDA-approved for use in a variety of clinical products^{14,15}; thus their block co-polymer is expected to be safe for use in humans. To render these nanoparticles suitable for active islet targeting, we employed conventional carbodiimide chemistry to covalently conjugate to PLGA-b-PEG-COOH a cyclic peptide sequence (CHVLWSTRC; Pep I) that was previously identified to home specifically to pancreatic islet microvessels¹⁶ (Figure 1A). Pre-functionalizing the constitutive co-polymer blocks prior to their self-assembly into nanoparticles simplifies their optimization and large-scale production^{17,18}. Successful polymer-peptide conjugation was confirmed by the appearance of a peptide-derived tryptophan peak in the ¹H-NMR spectrum (Figure 1B and Figure S1 in Supporting Information). Further analyses of the areas under the tryptophan and lactide -CH peaks revealed that 3 out of 10 polymer chains were modified with the islet-homing peptide. The size distribution profile of these nanoparticles determined by dynamic light scattering revealed an average diameter of 190 ± 40 nm, which was independently confirmed by transmission electron microscopy (Figure 1C).

To demonstrate their ability to selectively bind to pancreatic islet vessels, we added nanoparticle-peptide (NP-Pep I) conjugates loaded with the fluorescent dye coumarin, to cultured mouse islet and skin CE cells. Coumarin incorporation did not alter nanoparticle

size, as indicated by dynamic light scattering (Figure S2 in Supporting Information). Quantitative measurement of fluorescence intensity of nanoparticle-treated cells revealed 3-fold greater binding of NP-Pep I conjugates to islet CE cells compared to the skin cells ($p < 0.001$; Figure 2A). The preferential binding of these nanomaterials to islet CE cells is attributed to the unique islet-targeting Pep I sequence since nanoparticles modified with an identical-sized scrambled peptide (CVHWTLRKC; Pep X) at a similar surface density (~ 3 out of 10 polymer chains modified with the peptide) did not exhibit islet CE cell binding specificity (Figure 2A and Figure S3 in Supporting Information). Past work has implicated EphA4 receptors as mediators of this preferential NP-Pep I binding as the immunofluorescent staining pattern of EphA4 colocalizes with Pep I distribution in islets *in vivo*¹⁶.

Quantitative fluorescence measurement of unbound nanoparticles revealed that approximately 32% of the added nanoparticles were taken up by islet CE cells. To determine whether and how these bound nanoparticles were taken up by islet CE cells, we stained the NP-Pep I-bound islet CE cells with LysoTracker™ or Mitotracker™ to label intracellular acidic lysosomes/endosomes or mitochondria, respectively. Overlay of green (NP-Pep I) and red (LysoTracker™ or Mitotracker™) fluorescence images revealed ~100% co-localization of nanoparticles with acidic organelles (Figure 2B), whereas there was no colocalization with mitochondria (Figure S4 in Supporting Information). These data strongly suggest that these nanomaterials are taken up via endocytosis and processed within intracellular endocytic compartments.

To mimic the binding of NP-Pep I conjugates to islet vessels *in vivo* where islet CE cells will encounter these nanomaterials under flow, we designed a simple microfluidic device where islet and skin CE cells were grown in parallel channels and fluorescently-labeled NP-Pep I conjugates were flowed over them at a physiological flow rate (2 dyne/cm²; Figure 3). Quantitative analyses of fluorescence intensity revealed that, similar to static culture conditions, NP-Pep I conjugates exhibited a 3-fold greater binding affinity for islet CE cells compared to skin CE cells ($p < 0.001$; Figure 3), thus, confirming the robust islet-homing capability of these engineered nanoparticles.

During insulinitis, blood leukocytes infiltrating the pancreatic islets produce various cytokines, such as tumor necrosis factor- α (TNF) and interleukin-1 β , that render islet CE cells more adhesive, thereby creating a self-amplifying loop of autoimmune β cell destruction^{3,19}. To demonstrate the promise of islet-targeting nanoparticles as a therapeutic intervention for insulinitis, we tested their ability to inhibit leukocyte adhesion to TNF-stimulated islet CE cells *in vitro* using a leukocyte-endothelial cell adhesion assay that is widely used as an inflammation model for both basic mechanistic and drug discovery studies^{20, 21}. In the present study, we used genistein, a protein tyrosine kinase inhibitor that is known to impair leukocyte binding to TNF-stimulated endothelial cells²², as a model drug for incorporation within the islet-targeting nanoparticles.

We first confirmed the anti-inflammatory effect of genistein by showing that islet CE cells treated with soluble genistein exhibit dose-dependent inhibition of leukocyte adhesion (Figure 4A). Next, genistein was incorporated into the nanoparticles at 5% by weight of the added copolymer and its release kinetics were evaluated over a 48-hour period. UV-Vis spectral measurements revealed a typical temporal profile with an initial burst release of genistein lasting approximately 8 hours followed by a gradual release over 48 hours, with approximately 75% of the incorporated drug being released over two days (Figure 4B). These drug release kinetics are similar to those reported previously for hydrophobic drugs incorporated within PLGA-PEG-COOH co-block polymers of similar molecular weight¹⁸. From our drug release measurements, we also determined that the incorporation efficiency

of genistein was ~40% (w/w). Thus, these islet-targeting nanomaterials can be used as highly efficient, controlled drug delivery vehicles.

To demonstrate the therapeutic efficacy of the genistein-loaded islet-targeting nanoparticles (NP-Gen) for insulinitis treatment, we treated islet CE cells with NP-Gen or blank nanoparticles (10 µg/ml) for approximately 18 hours prior to TNF stimulation and quantified the adhesion of fluorescently-labeled leukocytes. Less than half the number of leukocytes ($p < 0.001$) adhered to islet CE cells treated with NP-Gen compared to those treated with blank nanoparticles or untreated cells (Figure 4C), and there was no detectable change in cell viability (Figure S5 in Supporting Information). Thus, the decrease in leukocyte adhesion to the islet CE cells produced by NP-Gen resulted from the immunosuppressive effect of genistein. In addition, the islet-targeting nanoparticles did not exert any detrimental effect on insulin-producing islet β cells (Figure S5 in Supporting Information), thus further supporting the high therapeutic potential of these islet-targeting nanoparticles.

Importantly, the finding that only 32% of the nanoparticles are taken up by islet CE cells indicates that the amount of genistein required to produce 50% inhibition of leukocyte adhesion to islet CE cells is approximately 200-fold lower when incorporated within nanoparticles (0.064 µg/ml genistein) than when added in free solution (13.5 µg/ml genistein). This profound increase in the therapeutic efficacy when this drug is presented within the NP-Gen formulation appears to be due to the rapid preferential uptake of these nanoparticles by the islet CE cells, which likely creates a higher intracellular genistein concentration than that caused by free diffusion of soluble genistein. The superior therapeutic efficacy of these islet-targeting nanomaterials can potentially be leveraged to achieve site-specific delivery and potent local effects of powerful anti-inflammatory drugs (e.g. cyclosporine A and prednisone) that are currently limited in their clinical use because they cause severe side effects upon systemic administration.

To determine whether greater immunosuppression can be achieved by administering a higher dose of islet targeting NP-Gen nanoparticles, we treated islet CE cells with increasing concentrations of NP-Gen and evaluated its effect on inhibition of leukocyte adhesion. Surprisingly, we found that higher NP-Gen concentrations were less effective in inhibiting leukocyte adhesion to islet CE cells (Figure 5a). This observation was counterintuitive as the levels of intracellular genistein, and thus its inhibitory effect, were expected to increase with increasing concentrations of NP-Gen. We, therefore, reasoned that perhaps the polymeric nanoparticles were themselves pro-inflammatory at higher concentrations, thus neutralizing the anti-inflammatory property of genistein. Indeed, TNF-stimulated islet CE cells that were treated with blank nanoparticles at higher concentrations (50 µg/ml) exhibited greater leukocyte binding than those treated with a lower dose (10 µg/ml) of nanoparticles or left untreated (Figure 5a). Thus, nanoparticle concentration appears to be an important determinant of vascular inflammation, thereby underscoring the need to optimize nanoparticle dosage for immunomodulatory therapy.

We next looked to see if the immunosuppressive effect of NP-Gen could be regulated by varying genistein loading in the nanoparticles. When TNF-stimulated islet CE cells were treated with NP-Gen containing 1%, 5% and 50% (w/w) genistein, greater inhibition of leukocyte adhesion was observed with increasing genistein loading (Figure 5b). However, in addition to enhancing the anti-inflammatory effect of NP-Gen, the increased drug loading also produced an overall increase in NP-Gen size. Specifically, the relatively homogeneous (d_{avg} 190 nm) nanoparticle population seen at low (0% and 1%) genistein loading was gradually replaced by a biphasic distribution at higher genistein concentrations (Figure 5c). For instance, at 5% genistein loading, two distinct NP-Gen populations emerged - one showing a characteristic peak at 190 nm and the other NP-Gen population exhibiting a peak

size of 458 nm. Increasing genistein loading to 50% (w/w) caused a further increase in overall NP-Gen size, with one fraction averaging at 615 nm and a secondary peak at 2300 nm.

This drug dose-dependent control of nanoparticle size has important implications for their effectiveness as vascular-targeting agents as it determines their rate of clearance from blood as well as their vascular retention and extravasation properties. For example, past studies have shown that particles larger than 500 nm are likely to get phagocytosed by circulating macrophages²³ whereas particles smaller than 200 nm typically extravasate from hyperpermeable vessels that are characteristic of ischemic tissues and tumors^{2, 24}. Since inflammation is also marked by an increase in vascular permeability²⁵, nanoparticles that range between 200–500 nm will likely be most suitable for active vascular targeting by virtue of their maximal vessel retention and minimal phagocytic clearance properties. Based on these design criteria, the 5% NP-Gen nanoparticles appear to be the preferred formulation for active islet vessel targeting and immunotherapeutic delivery as they combine potent anti-inflammatory effects with optimal dimensions.

In summary, we have demonstrated a proof-of-concept for islet-targeting nanoparticles that could potentially provide a new therapeutic intervention for insulinitis in individuals that are at high risk of developing Type 1 Diabetes. In addition to exhibiting selective islet-targeting capability, these nanomaterials offer a tremendous advantage over systemic drug delivery due to their ability to elicit a similar immunosuppressive response with a 200-fold lower drug concentration. However, these studies also emphasize that maximizing the immunotherapeutic potential of these nanomaterials will require precise control of both their physicochemical properties and administered dose.

To realize the full impact of the field of nanomedicine, smart nanoscale materials will need to be developed that can selectively home and deliver therapeutic payloads to any tissue and organ of interest. The success of such tissue-targeted nanotherapeutics will depend on: 1) identification of unique tissue-targeting moieties (peptides, aptamers, antibodies) using high-throughput techniques, such as phage display, 2) design of long-circulating nanoparticles of appropriate size using biodegradable polymers, and 3) identification of pathological conditions that will particularly benefit from this active targeting strategy. The incidence of diabetes has reached epidemic proportions worldwide. Thus, nanomaterials that can selectively home to pancreatic islets are of particular interest as they have implications not only for diabetes therapy, as demonstrated in this study, but also for early diagnostic screening of pre-diabetic subjects through incorporation of imaging agents that can permit real-time monitoring of pancreatic islets. Stem and progenitor cells are also being increasingly explored as therapeutics for pancreatic islet regeneration^{26, 27}. Thus, by conjugating the islet-targeting nanomaterials to the surface of these regenerative cells, it may be possible to selectively guide them to diseased islets following systemic delivery, thereby substantially improving their islet engraftment and therapeutic efficacy in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic representation and physicochemical characterization of islet-targeting nanomaterials. (A) Carbodiimide chemistry was used to covalently conjugate islet targeting peptide (CHVLWSTRKC) to the amphiphilic PLGA-b-PEG-COOH block co-polymer, which undergoes spontaneous self-assembly in aqueous solutions to form nanoparticles (B) $^1\text{H-NMR}$ spectrum of polymer-peptide conjugate displays the peaks characteristic of tryptophan (W) residue in the peptide (arrow), which is absent in the unmodified polymer. (C) Transmission electron microscopy (TEM) and dynamic light scattering analysis reveal that the islet-targeting nanoparticles have an average diameter of 190 ± 40 nm. Scale bar on TEM image = 200 nm

Figure 2.

Nanoparticle binding to islet capillary endothelial (CE) cells in static culture. (A) Under static culture conditions, nanoparticles displaying islet-targeting peptide (CHVLWSTRKC; Pep I) exhibit a 3-fold increased binding ($***p<0.001$) to islet CE cells compared to skin CE cells, as shown in the representative fluorescent images of coumarin-loaded nanoparticles and quantified in the bar graph. This preferential binding to islet CE cells is specific to Pep I since the scrambled peptide (PepX) containing the same amino acids does not exhibit any binding preference. Scale bar = 20 μ m (B) Islet CE cells treated with coumarin-loaded nanoparticles were stained with lysotracker™ red to label acidic organelles. Quantitative analysis of green (nanoparticle) and red (lysotracker) images reveals approx. 100% colocalization; Mx (green colocalizing with red) = 97.86%; My (Red colocalizing with green) = 99.99%

Figure 3.

Nanoparticle binding to islet capillary endothelial (CE) cells under flow. To mimic nanoparticle binding to islet endothelium *in vivo*, islet and skin CE cells were cultured in parallel microfluidic channels and the NP-Pep I and NP-Pep X suspensions (10 ug/ml) were flown over them at a wall shear rate of 2 dyne/cm². Quantitative analyses of fluorescence images showed NP-Pep I binding specificity similar to that seen under static conditions (***) ($p < 0.001$). Scale bar = 100 μ m

Figure 4.

Immunosuppressive effect of islet-targeting nanomaterials. (A) Genistein, a pharmacologic inhibitor of receptor tyrosine kinase, blocks leukocyte binding to TNF- α -stimulated islet CE cells in a dose dependent manner (** $p < 0.001$). (B) Genistein was encapsulated into NP-Pep I nanoparticles at 5% (w/w) and drug release measured over 48 hours. (C) Islet CE cells treated with genistein-loaded islet-targeting nanoparticles (NP-Gen; 10 $\mu\text{g/ml}$) 18h prior to TNF- α stimulation exhibit significant inhibition in leukocyte binding (** $p < 0.001$)

Figure 5.

Regulation of therapeutic and physicochemical properties of NP-Gen as a function of drug and nanoparticle dosage. (A) NP-Gen concentration of 10 μg/ml produced the most significant inhibition (***p<0.001) in leukocyte/islet CE cell binding while higher doses were progressively less effective. This trend is attributed to the pro-inflammatory effect of nanoparticles, as indicated by increased leukocyte binding to islet CE cells treated with higher doses of blank NP-Pep I nanoparticles. (B) Increasing genistein loading into NP-Gen nanoparticles results in a significantly greater immunosuppressive effect, as indicated by a marked decrease in leukocyte binding to islet CE cells. (C) NP-Gen nanoparticles loaded with increasing amounts of genistein exhibit a progressive increase in their average size.