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Polymeric Nanomaterials for Islet Targeting and Immunotherapeutic Delivery

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SUPPORTING INFORMATION

Figure S1. ^{1}H-NMR spectrum of the islet-targeting peptide (Pep I; CHVLWSTRKC) displays peaks characteristic of tryptophan (W) residue at 7.2-7.6 ppm, which is used as a reference to confirm successful polymer-peptide conjugation.

Figure S2. Dynamic light scattering analysis reveals that coumarin incorporation into islet-targeting nanoparticles does not alter particle size, with the average particle diameter remaining ~190 nm.

Figure S3. (A) ^{1}H-NMR spectrum of the scrambled peptide (Pep X; CVHWTLSRKC) is similar that of the islet-targeting peptide, with the tryptophan (W) peaks appearing at the expected 7.2-7.6 ppm. (B) Carbodiimide chemistry was used to covalently conjugate the scrambled peptide to the PLGA-b-PEG-COOH block co-polymer. ^{1}H-NMR spectrum of the polymer-peptide conjugate displays peaks characteristic of the peptide's tryptophan (W) residue (arrow), thereby confirming successful peptide conjugation.

Figure S4. Islet CE cells treated with coumarin-loaded nanoparticles were stained with Mitotracker™ red to label mitochondria. Overlay of green (nanoparticle) and red (Mitotracker™) images reveals no detectable colocalization. Scale bar = 10 μm.

Figure S5. (A) Islet CE cells treated with blank or genistein-loaded (NP-Gen) nanoparticles show no apparent changes in cell morphology or loss in cell viability when compared with untreated cells. Cell viability measurement is normalized with respect to untreated islet CE cells. Scale bar = 100 μm. (B) Insulin-producing islet β cells (Min6) treated with unmodified or islet-targeting nanoparticles show no apparent loss in cell viability when compared with untreated cells. Cell viability measurement is normalized with respect to untreated islet β cells.

Pep I

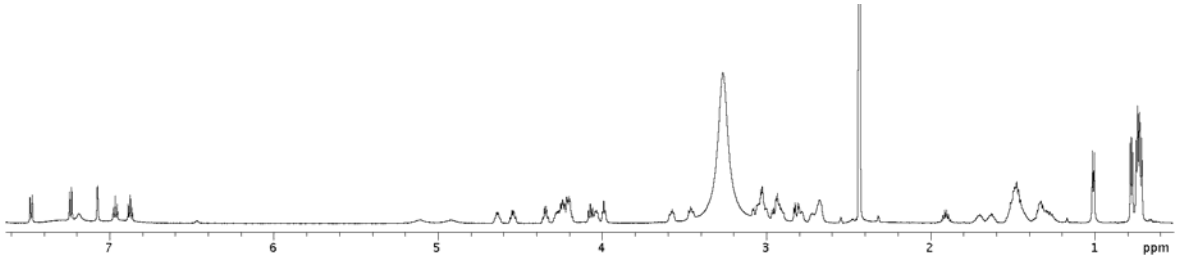


FIGURE S1

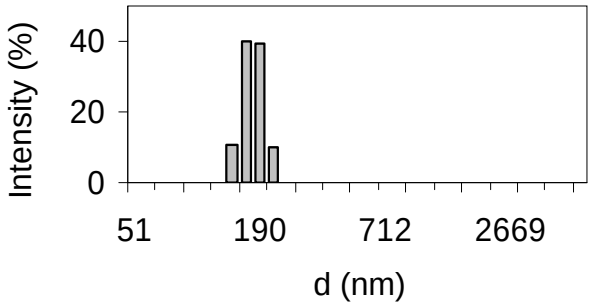
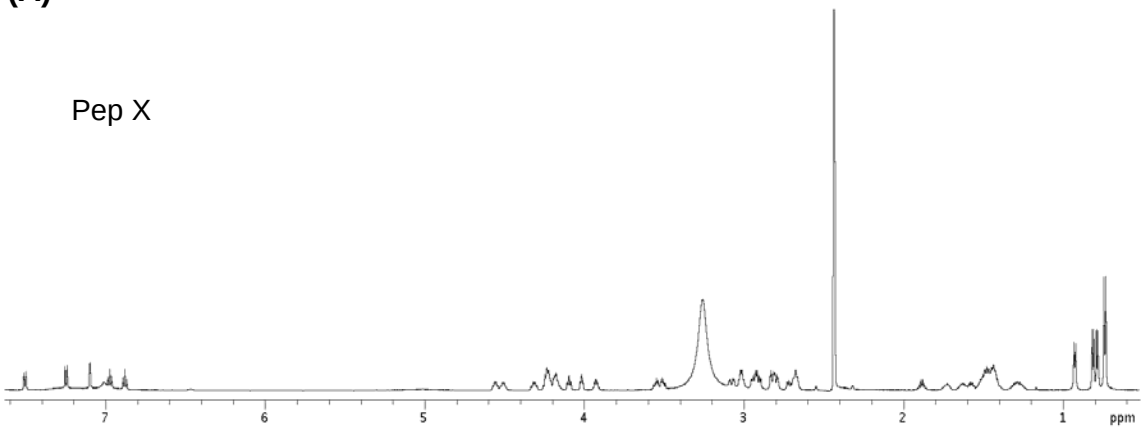


FIGURE S2

(A)



(B)

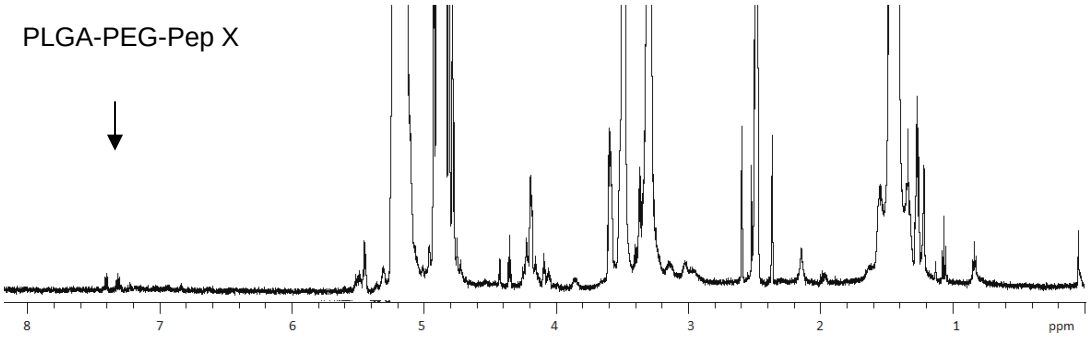


FIGURE S3

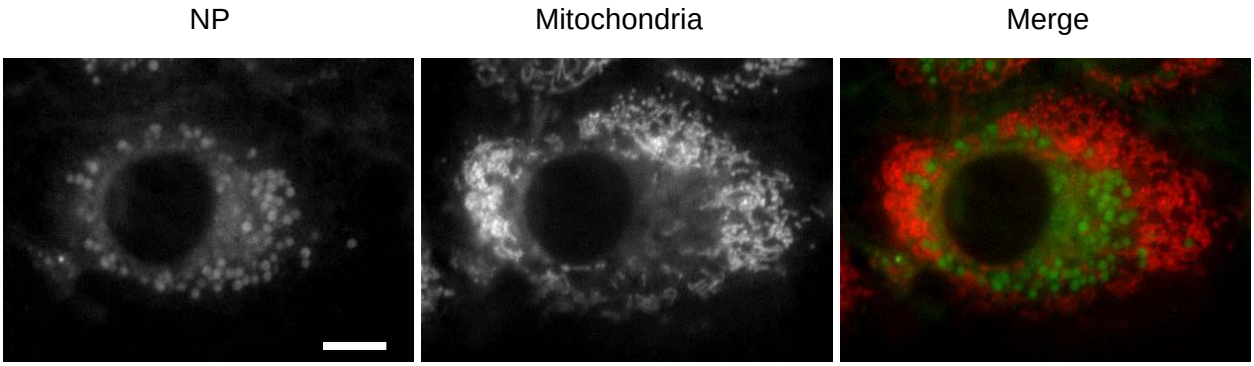
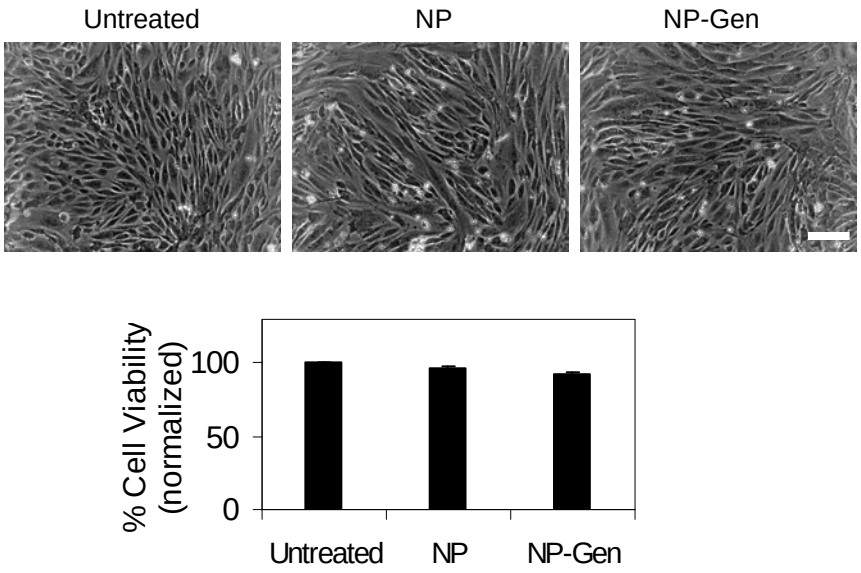


FIGURE S4

(A)


Untreated
NP
NP-Gen
% Cell Viability (normalized)
100
50
0
Untreated
NP
NP-Gen

(B)

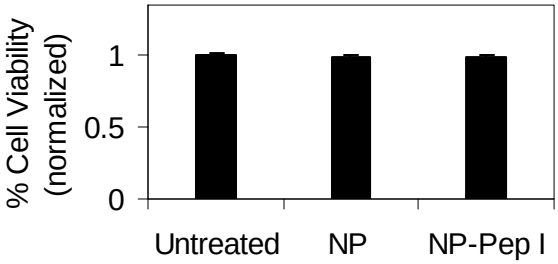

% Cell Viability (normalized)
1
0.5
0
Untreated
NP
NP-Pep I

FIGURE S5

Experimental Procedures

Polymer Nanoparticle Formulation

PLGA-PEG-COOH (75:25, PLGA M_W: 17000 kDa, PEG M_w: 3400 Da, Advanced Polymer Materials Inc., QC, Canada), was dissolved in DMSO at a concentration of 1 mg/ml in DMSO. To enable visualization of the polymeric nanoparticles, a fluorescent dye, Coumarin 6 (Sigma Aldrich, MO, USA), was added to the polymer/DMSO solution at ~0.1 % by weight of the co-polymer. Coumarin-loaded nanoparticles were obtained by a simple solvent displacement method that involved dialyzing the polymer-dye solution against water at room temperature. The morphology and size distribution of the nanoparticles were characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS), as described below.

Nanoparticle-Peptide (NP-Pep) Conjugation

The cyclic islet-homing peptide CHVLWSTRC (Pep I), discovered previously using phage display[16], and the scrambled sequence CVHWTLSRKC (Pep X) were synthesized by Tufts University Peptide Core Facility, MA, USA (>98% purity, as determined by Mass Spectroscopy). During peptide synthesis, a lysine residue (K) was inserted between the arginine and cysteine residues of Pep I to facilitate its covalent conjugation to PLGA-PEG-COOH using the carbodiimide chemistry. Peptides were either directly conjugated to the polymer chain prior to nanoparticle formation or functionalized to the surface of the preformed nanoparticles using the conventional EDC/NHS chemistry. Briefly, the carboxyl end group of the PLGA-PEG-COOH was first activated with 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS) or N-hydroxysuccinimide (NHS) at a 1:5:10 molar ratio for 2 hours at room temperature either in phosphate buffer saline (PBS) or DMSO, depending on whether the modification was done to the nanoparticle surface or polymer chain. The pre-activated polymer/nanoparticles were then reacted with Pep I or Pep X in a 2:1 molar ratio for 4 hours at room temperature either in PBS or in PBS/DMSO, as per the reaction scheme. The reactions were then purified by either centrifugation/wash or simple dialysis. The polymer-

peptide conjugation reaction was confirmed by Nuclear Magnetic Resonance (NMR) spectroscopy. ^{1}H NMR spectral measurements were performed using a Variance 600 MHz VNMRS spectrometer (Agilent, Palo Alto, CA, USA) in d-DMSO at 25°C. The polymer-peptide conjugation efficiency was calculated by comparing the area under the tryptophan and lactide –CH peaks.

Genistein-loaded Nanoparticles (NP-Gen) and Drug Release Kinetics

Genistein (EMD Chemical, USA) was added to a 1 mg/ml PLGA-PEG-COOH solution (in DMSO) at 1%, 5% or 50% by weight of the co-polymer. Following 30 minutes of mixing, the genistein-polymer mix was dialyzed against water at room temperature to obtain genistein-loaded nanoparticles (NP-Gen) and remove excess genistein. The size distribution of NP-Gen was characterized by dynamic light scattering (DLS). To measure the release kinetics of incorporated genistein, 250 µg of NP-Gen was suspended in 1.5 ml PBS and incubated at 37^{o}C for pre-determined time durations (n=4 per time point). At the end of each time point, NP-Gen nanoparticles were pelleted by spinning down the suspension at 10,000 rcf for 10 min and the pellets were dissolved in acetonitirile/methanol for UV-Vis spectral measurement of residual genistein using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, CA, USA). The O.D. measurements were converted into concentration using a standard curve generated from pure genistein of known concentrations (n=3 per concentration). To measure total drug loading, freshly prepared NP-Gen was immediately dissolved in acetonitirile/methanol for UV-Vis spectral measurement (n=3).

Transmission Electron Microscopy (TEM)

A JEOL 1400 TEM microscope (JEOL, Peabody, MA, USA) was used to characterize the morphology of the nanoparticles. About 5 µl of nanoparticle solutions was added onto Formvar 400 mesh copper grids. After ~5 minutes, the excess solution was wicked by filter paper and the sample was washed with H_2O. Next, the sample was stained with 0.75% uranyl formate (Polysciences Inc, PA, USA) and air dried for 5 min prior to imaging.

Dynamic Light Scattering (DLS)

A zeta particle size analyzer (Malvern instruments, UK) operating with a HeNe laser, and a 173° back scattering detector was used to determine the size distribution of the nanoparticles. Samples were prepared at 100 µg/ml in water and filtered through a 0.8 µm or 5 µm filter prior to the dynamic light scattering measurement (n=3 per condition). Malvern instrument software or Microsoft Excel was used to analyze the acquired data.

Nanoparticle (NP) binding and uptake

Mouse pancreatic islet CE cells were a gift from Judah Folkman while the mouse skin CE cells were isolated from the dermis of TRAMP mice, as previously described[S1]. CE cells were cultured on gelatin-coated tissue culture dishes, grown in culture medium composed of low glucose DMEM, 10% fetal bovine serum, 10% Nu Serum IV, basic fibroblast growth factor (6 ng/ml), heparin salt (0.1 mg/ml), 1% insulin-transferrin-selenium and antibiotic/mycotic mixture, and were used between passages 12-19. For nanoparticle binding and uptake studies, CE cells were grown on gelatin-coated glass coverslips and NP-Pep I or NP-Pep X nanoparticles suspended in cell culture medium at 10 µg/ml were added to cells for 30 minutes at 37^{o}C (n=3). Next, the unbound nanoparticles were removed by PBS rinsing and the cells were fixed with 4% paraformaldehyde (PFA). Fluorescent images of the samples were acquired with a Nikon Eclipse TE 2000-E microscope (Nikon, Japan) fitted with a CoolSnap HQ digital camera (Photometrics). The fluorescence intensity of cell-bound nanoparticles (n>30 per condition) was measured using IP Lab imaging software (Becton Dickinson, NJ, USA). To evaluate percent nanoparticle uptake, unbound coumarin-loaded nanoparticles were collected and the cells washed twice with PBS to remove nanoparticles loosely bound to cell surface. Quantitative florescence measurement of unbound nanoparticles was performed using a PTI QM40 Fluorometer (PTI-FL) (Photon Technology International, NJ, USA) and compared with a calibration curve (obtained using coumarin-loaded nanoparticles of various concentrations) to estimate the amount of unbound nanoparticles. The percentage of bound nanoparticles was

determined by subtracting the amount of unbound nanoparticles from the initial amount added to cells, and measurements were performed in quadruplicates.

To determine whether cell-bound nanoparticles were internalized by an endocytic mechanism, CE cells treated with nanoparticles were stained with Lysotracker™ red or Mitotracker™ red CMXRos (Invitrogen, CA, USA) to label intracellular acidic organelles (lysosomes and endosomes) or mitochondria, respectively. Quantitative analysis of the degree of colocalization between green (nanoparticle) and red (acidic organelles) fluorescent images was performed using Volocity® imaging software (PerkinElmer, MA, USA).

For flow studies, microfluidic channels were prepared from polydimethylsiloxane (PDMS) using conventional soft lithography[S2]. A master mold was designed using a CAD program and prepared by utilizing 80 micron thick features formed using a cutter plotter (CE5000, Graphtec, CA). The PDMS channels were sealed with a glass microslide (170 μm thick) using plasma bonding. Microfluidic devices were then sterilized using oxygen plasma and coated with fibronectin (50 μg/ml for 30 min) to support cell adhesion. Each PDMS device comprises two identical channels (80 μm high x 500 μm wide x 30 mm long), as shown in Figure 3. Islet CE cells (> $2x10^6$ cells/ml) were introduced to one microchannel, followed by introduction of skin CEs, at a similar density, to the second channel in the same device. The devices were then placed in a tissue culture incubator (37°C, 5% CO_2) and the cells were allowed to adhere under static conditions for 2 hr. Following the static incubation, culture medium was infused at a flow rate of 50 uL/hr using a conventional syringe pump (Braintree Scientific, Braintree, MA). The cells were cultured in the devices for 1- 2 days until cell monolayer was formed. Nanoparticle suspension (10 μg/ml in culture medium) was then infused for 30 min through the channels at a flow rate of 800ul/hr (wall shear stress of ~2 dyne/cm^2). Unbound nanoparticles were flushed away by infusing PBS through the channels at the same flow rate for more than 10 min. The samples were then fixed by infusing PFA for 5 min. Phase contrast and fluorescence

microscopic images of cells and bound nanoparticles were acquired using a Nikon Eclipse TE 2000-E microscope (Nikon, Japan) fitted with a CoolSnap HQ digital camera (Photometrics).

Leukocyte Adhesion Assay

After islet CE cells were grown to confluence on gelatin-coated 24-well plates, the culture medium was replaced with low-serum assay medium composed of low glucose DMEM, 5% fetal bovine serum and antibiotic/mycotic mixture. Free genistein or genistein-loaded nanoparticles (NP-Gen) were then added to the confluent cells in assay medium to obtain a final free genistein concentration of 10, 50 or 100 μM or NP-Gen concentration of 10 μg/ml. Free genistein was added to cells for 18 hours prior to leukocyte adhesion while the NP-Gen solution was removed after 30 minutes, followed by rinsing with PBS and addition of fresh assay medium. After 18 hours of treatment with free genistein or bound NP-Gen, islet CE cells were stimulated with tumor necrosis factor-α (TNF; 10 ng/ml) for 5 hours. Separately, leukocytes were isolated from freshly collected mouse blood using a red blood cell lysis buffer (BD Pharm Lyse™, Becton Dickinson, NJ, USA), as per the manufacturer's instructions, and labeled with a green fluorescence live cell tracker dye (Green CMFDA, Invitrogen, CA, USA). After 5 hours of stimulation, TNF solution was removed from islet CE cell cultures and fluorescently-labeled leukocytes added at a density of 200,000 cells/well. After 30 minutes of leukocyte/islet CE cell interaction, the leukocyte suspension was removed and the islet CE cells were rinsed 3-4 times with PBS prior to PFA fixation. Fluorescent images of labeled leukocytes were acquired using a Nikon Eclipse TE200 microscope (Nikon, Japan) fitted with a Spot RT Monochrome camera (Diagnostic Instruments, MI, USA) and leukocyte adhesion was quantified using Image J software (NIH).

Cell Viability

Islet CE cells or insulin-producing islet β cells (Min6) were grown to confluence in 96-well plates, following which they were either left untreated or treated with blank or genistein-loaded (NP-Gen) nanoparticles (10 μg/ml) for 18 hours. CellTiter-Blue® reagent was then added to

each well and, following 4 hour incubation at 37^{o}C, the fluorescence signal was measured using a fluorescence multiwell plate reader (Victor3™, PerkinElmer, MA, USA). All fluorescent intensity measurements were then normalized with respect to the untreated islet CE or islet β (Min6) cells.

Statistical Analysis

All data are obtained from multiple replicates, as indicated in the respective procedures, and expressed as mean ± SEM. Statistical significance was determined using analysis of variance (ANOVA; InStat®, GraphPad Software Inc.). Results were considered significant if $p<0.01$.

References

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2. Xia, Y.; Whitesides, G. M. *Annu. Rev. Mater. Sci.* **1998,** 28 (1), 153-184.