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Multifunctional Magnetic Nanocarriers for Image-Tagged siRNA Delivery to Intact Pancreatic Islets

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Abstract

Background—With the ultimate hope of finding a cure for diabetes, researches are looking into altering the genetic profile of the beta cell as a way to manage metabolic dysregulation. One of the most powerful new approaches for the directed regulation of gene expression utilizes the phenomenon of RNA interference.

Methods—Here, we establish the feasibility of a novel technology centered around multifunctional magnetic nanocarriers, which concurrently deliver siRNA to intact pancreatic islets and can be detected by magnetic resonance (MR) and optical imaging.

Results—In the proof-of-principle studies described here, we demonstrate that, following in vitro incubation, magnetic nanoparticles carrying siRNA designed to target the model gene for enhanced green fluorescent protein, are efficiently taken up by murine pancreatic islets, derived from *egfp* transgenic animals. This uptake can be visualized by magnetic resonance imaging (MRI) and near-infrared fluorescence optical imaging and results in down-regulation of the target gene.

Conclusions—These results illustrate the value of our approach in overcoming the challenges associated with genetic modification of intact pancreatic islets in a clinically acceptable manner. Furthermore, an added advantage of our technology derives from the combined capability of our magnetic nanoparticles for siRNA delivery and magnetic labeling of pancreatic islets.

Keywords

magnetic resonance imaging; optical imaging; rna interference; pancreatic islets

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Introduction

In view of the importance of the beta-cell as a core effector of metabolic control and the profound repercussions of beta-cell pathology on human health, the acquisition of tools for the regulation of beta-cell function represents a key research and clinical priority. One very promising and widely explored approach towards this goal involves modification of the gene expression profile of the beta cell. To this end, numerous reports in the literature describe the application of gene transfer in beta-cell derived cell-lines for the study of beta-cell differentiation (1-3), beta-cell function (2,4-7), immunorecognition in the pathogenesis of type 1 diabetes (8), and the mechanisms behind the pathogenesis of type 2 diabetes (9), to name a few. In the context of gene therapy for diabetes, several ideas have shown promise, including stimulation of beta-cell growth, induction of beta-cell differentiation and regeneration, genetic engineering of non-beta cells to produce insulin, and transplantation of engineered beta cells (10).

However, in many cases, the behavior of beta-cell derived lines is not mirrored by that of whole islets (5,11), underscoring the benefit of studying the beta-cell in its native environment. Partially in response to this concern, attempts have been made to transfect/transduce intact islets, using lipid-mediated plasmid delivery (12,13), as well as adeno- (14-17), adeno associated- (18-21), and lentiviral vectors (16,22,23). Gene transfer to intact pancreatic islets is particularly challenging, due to the fact that they exist as clusters of 100–2,000 cells, making physical access to the core of the islet difficult. In general, most studies report that primarily cells localized in the islet periphery become efficiently transduced/transfected (14,16,17,20). With specific relevance to the present study, recombinant adenovirus has been used for the delivery of shRNA to intact pancreatic islets, with the goal of endogenous gene suppression, through the mechanism of RNA interference (24). However, viral or transfection agent-mediated delivery has been directly associated with cytotoxicity (22,25,26) and/or immunogenicity (27), diminishing enthusiasm towards this approach for gene transfer to intact islets, particularly in the context of autoimmune dysfunction, as seen in type 1 diabetes.

The attraction of using RNA interference to silence gene expression in pancreatic islets extends from the relative ease of siRNA delivery using nonviral means, the major prerequisite being delivery of the siRNA duplex to the cytosol. Even though the ensuing silencing effect is transient, it is still relatively long-lasting. In non-dividing or slowly-dividing cells, knockdown can persist for 3-4 weeks (28). Furthermore, the loss of silencing is mainly a function of siRNA dilution, rather than degradation (28), implying that if one designed an approach to retain the siRNA molecule inside the cell for a prolonged time period, they could extend the longevity of the knock-down even further. In agreement with these conclusions, two reports exist of the nonviral liposomal (29) and transfection-agent mediated (30) delivery of siRNA to intact pancreatic islets. The idea behind the latter approach is based on the known capacity of hydrodynamic injection to efficiently deliver siRNA to well-vascularized organs. However, the invasiveness of this method for in vivo transfection makes its clinical application not feasible (31).

In the present study, we attempted to extend the potential of RNA interference for modification of the gene expression profile of pancreatic islets. Our approach explores the application of superparamagnetic nanoparticulate carriers to deliver siRNA inside the islet cells, coupled with the simultaneous magnetic labeling of the islets allowing for their further tracking by MRI. These studies build upon previous findings by our group, which establish that pancreatic islets avidly take up superparamagnetic nanoparticles, following in vitro incubation, and retain the nanoparticulate label for an extended time-frame (months), without impairment of islet viability and function (32,33). Considering that in these early studies, islet labeling permitted the long-term noninvasive imaging of the islet graft after transplantation, one practical

application of the described approach would involve the delivery of a therapeutic gene, for the knockdown of genes implicated in graft dysfunction, followed by non-invasive imaging.

Materials and Methods

Probe Synthesis

siRNA—The EGFP-targeting siRNA duplex (sense: 5'-CAAGCUGACCCUGAAGUUCdTdT-3'; antisense: 5'-GAACUUCAGGGUCAGCUUGdTdT-3') was designed by Ambion (Austin, Texas) and synthesized by Dharmacon (Lafayette, CO). The siRNA was modified to incorporate a thiol group on the 5' end of the sense strand and the fluorescent dye DY547 on the antisense strand.

Probe synthesis and characterization—The synthetic strategy of the MN-NIRF-siEGFP probe is outlined in Figure 1A. Briefly, a solution of mono-activated Cy5.5 succinimide ester (Amersham Biosciences, Piscataway, NJ) in 20mM sodium citrate and 0.15 M NaCl was allowed to react with the previously dialyzed immunopure, amino-derivatized dextran coated iron oxide (aminated iron oxide, MN-NH₂) at pH 8.5 with constant agitation over a period of 12h at room temperature. The Cy5.5 labeled aminated iron oxide (MN-NIRF) was purified from unreacted dye using a Sephadex™ G-25, PD-10 column (Amersham Biosciences, Piscataway, NJ). MN-NIRF was then conjugated to the heterobifunctional cross-linker N-succinimidyl 3-(2-pyridyldithio) propionate, SPDP (Pierce Biotechnology, Rockford, IL) via the N-hydroxy succinimide ester, followed by purification using a Sephadex™ G-25, PD-10 column (Amersham Biosciences, Piscataway, NJ) in PBS/EDTA, pH 7.5. The advantage of this linker is the affordable chromophore of pyridine-2-thione, which gets released after treatment with a reducing agent (e.g. tris(2-carboxyethyl)phosphine hydrochloride, TCEP, Sigma-Aldrich, St. Louis, MO). The labeling ratio of SPDP groups per nanoparticle can be obtained by measuring the absorbance of pyridine-2-thione at 343 nm ($\epsilon = 8.08 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$). A ratio of 10.5 SPDP molecules per nanoparticle was obtained. The EGFP-targeting siRNA duplex was then conjugated to MN-Cy5.5-SPDP through its 5'-sense thiol group. Prior to conjugation, the disulfide protecting group on 5'-S-S- was deprotected using TCEP according to the manufacturer's instructions. The dsRNA was then reacted overnight (4°C) with the previously activated MN-Cy5.5-SPDP product via the SPDP crosslinker in PBS/EDTA, pH 8, followed by purification using a Quick Spin Column G-50 Sephadex Column (Roche Applied Science, Indianapolis, IN). A ratio of four Cy5.5 molecules per nanoparticle was obtained. The scheme of the resultant probe is illustrated in Figure 1B.

Electrophoresis—The amount of conjugated siRNA was assayed using gel electrophoresis as previously described (34). In order to assess siRNA dissociation from the nanoparticles under reducing conditions, the probe was pre-treated either with 2.5% beta-mercaptoethanol (2-ME) or 100mM dithiothreitol (DTT) for 1 hr. The siRNA standard, untreated probes and the probes treated with reducing agents were electrophoresed on a 20% TBE gel (Invitrogen) at 200V for 45 minutes. After electrophoresis, the gel was stained with 0.5µg/ml ethidium bromide for 30 minutes, and visualized using a Molecular Imager FX scanner (Bio-Rad). The images were quantitated using the software Quantity One, version 4.4.0 (Bio-Rad).

Islet Isolation and Labeling

Pancreatic islets were isolated from Tg(GFPU)5Nagy/J mice (Jackson Laboratories, Bar Harbor, ME). This transgenic strain expresses the enhanced green fluorescent protein (Clontech vector) in all nucleated cells and serves as a source of green-fluorescent cells/tissues. Briefly, the pancreas was perfused through the pancreatic duct with cold Liberase (Roche Applied Science, Indianapolis, IN). Perfused pancreata were removed and incubated for 30 min at 37°C, followed by gravity sedimentation and mechanical filtration. For analysis, islets were hand-

picked from the resultant suspension and cultured in Miami Medium #1 culture media (Cellgro; Mediatech, Herndon, VA), supplemented with 20 mg/l ciprofloxacin hydrochloride (Fisher Scientific, Pittsburgh, PA) and 10 mg/l L-glutathione (Sigma, St. Louis, MO).

For labeling experiments, sixty islets were counted and incubated for 72 hrs with probe in the same medium (50 µg/ml Fe, ~ 39 µg/ml siRNA, in 1 ml medium). After incubation, islets were washed with the culture medium 3 times and used for further studies.

Magnetic Resonance Imaging

For MRI, we prepared islet phantoms by fixing islets in 2% paraformaldehyde and sedimenting them in 0.2 ml-PCR tubes (60 islets/tube). Imaging was performed immediately after phantom preparation using a 9.4T GE magnet (Billerica, MA) equipped with ParaVision 3.0.1 software. The imaging protocol consisted of coronal T2 weighted spin echo (SE) pulse sequences with the following parameters: TR/TE = 3000/8, 16, 24, 32, 40, 48, 56, 64ms, FoV = 3.2×3.2 cm², matrix size 128 × 128, resolution 250 × 250 µm² and slice thickness = 0.5 mm.

Image reconstruction and analysis were performed using Marevisi 3.5 software (Institute for Biodiagnostics, National Research Council, Canada). T2 relaxation times were determined by T2 map analysis of regions of interest drawn around the islet pellet.

Optical Imaging

Optical imaging was performed immediately after the MRI session. Eppendorf tubes containing islets were placed into a whole-body animal imaging system (Imaging Station IS2000MM, Kodak), equipped with band-pass excitation and long-pass emission filters at: 630 nm and 700 nm, respectively, for near-infrared, Cy5.5 imaging, 465 nm and 535 nm for EGFP imaging, and 535 nm and 600 nm for DY547 imaging (Chroma Technology Corporation, Rockingham, VT).

Confocal Microscopy

For fluorescence confocal microscopy of whole islets, islets were fixed in 4% paraformaldehyde. Confocal microscopy was performed in the FITC (EGFP detection, 9L-GFP), Cy3 (siEGFP, DY547 detection), the Cy5.5 channel (Cy5.5 detection), and using Nomarski optics (for cellular/tissue definition). Confocal microscopy was performed using an Axiovert 200M inverted microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with an LSM Pascal Vario RGB Laser Module (Arg 458/488/514 nm, HeNe 543nm, HeNe 633 nm). Images with a slice thickness of < 0.9 µm were acquired in the green, red, and near-infrared channels, as well as using Nomarski optics.

For fluorescence confocal microscopy of islet cryosections, islets were embedded in freezing media and cut into 7 µm-thick sections. Prior to imaging, islet sections were fixed in 4% paraformaldehyde and imaged as described above. Summation projection of all background-corrected confocal slices was produced using LSM 5 Pascal Software (v 3.2 WS). Final images were color-coded green for EGFP, red for DY547, and blue for Cy5.5.

In situ apoptosis detection

To investigate whether the described treatment was associated with cellular toxicity in pancreatic islets, we performed a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay (Apoptag Peroxidase In Situ Apoptosis Detection kit, Chemicon International, Temecula, CA) according to the manufacturer's protocol. Islet cryosections were examined using a Nikon Eclipse 50i microscope. Images were acquired using a CCD camera (SPOT 7.4 Slider RTKE; Diagnostic Instruments, Sterling Heights, MI) and analyzed using SPOT 4.0 advanced version software (Diagnostic Instruments, Sterling Heights, MI).

Consecutive sections were stained with hematoxylin & eosin (H&E) and analyzed by light microscopy.

Flow Cytometry

To analyze probe distribution in islet cells, we performed FACS analysis. Islets were dissociated by the addition of cell dissociation buffer (Gibco-BRL, Carlsbad, CA) (prewarmed at 37°C) followed by a 2-min incubation at room temperature. The islet suspension was pipetted up and down repeatedly to ensure mechanical disruption, followed by the addition of cold RPMI containing 10% FBS. Islets were then washed, fixed in 2% paraformaldehyde, and subjected to FACS analysis in the FL2 channel (siEGFP, Dy547), FL4 channel (MN, Cy5.5) and the FL1 channel (EGFP), using using a FACSCalibur (Becton Dickinson, San Diego, CA, USA) equipped with the Cell Quest software package (Becton Dickinson, San Diego, CA).

Gene Expression Analysis

To evaluate the silencing effect of siRNA in islets after incubation with the probe, we performed qRT-PCR analysis. Total RNA was extracted from pancreatic islets using the RNeasy Mini kit, according to the manufacturer's protocol (Qiagen Inc., Valencia, CA). Relative levels of EGFP mRNA were determined by real-time quantitative RT-PCR (TaqMan protocol). TaqMan analysis was performed using an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, CA). The PCR primers and TaqMan probe specific for EGFP mRNA were designed using Primer Express software 1.5. Primer and probe sequences were as follows:

Forward primer, 5'-GTCCGCCCTGAGCAAAGA-3'; Reverse primer, 5'-CACGAACTCCAGCAGGACC-3'; TaqMan Probe 5'-FAM-ACGAGAAGCGCGATCACA-TAMRA-3'. Eukaryotic 18S rRNA TaqMan PDAR Endogenous Control reagent mix (PE Applied Biosystems, Foster City, CA) was used to amplify 18S rRNA as an internal control, according to the manufacturer's protocol.

Statistical Analysis

Data were expressed as means $\pm$ SD. Statistical differences were analyzed by Student's (equal variances) or Welch's (unequal variances) t-test (SigmaStat 3.0; Systat Software, Richmond, CA). A value of $P < 0.05$ was considered statistically significant.

Results

Characterization of MN-NIRF-siEGFP

As described above, MN-NIRF-siEGFP consists of superparamagnetic iron oxide nanoparticles, conjugated to the near-infrared fluorescent dye, Cy5.5, and bearing siRNAs targeting enhanced green fluorescent protein (*egfp*) as a model gene (Fig. 1B). MN is typically taken up into the cell by endocytosis and resides in the endosome. In order to mediate effective RNAi, the siRNA needs to undergo dissociation from the MN complex, followed by endosomal escape and entry into the cytosol, where RISC assembly and target mRNA degradation take place. Furthermore, this dissociation and relocalization of the siRNA need to occur with a favorable stoichiometry, so that sufficient cytosolic concentrations of siRNA are achieved for maximal knock-down. To evaluate the capacity of the siRNA molecule to dissociate from the core nanoparticle we performed gel electrophoresis experiments of MN-NIRF-siEGFP under reducing conditions, following incubation in two different kinds of reducing buffers (beta-mercaptoethanol and dithiothreitol, DTT). Dissociation of the siRNA from MN at a ratio of 7 siRNAs per MN crystal was achieved (Fig. 1C).

Imaging Cellular uptake and silencing efficacy of MN-NIRF-siEGFP in EGFP-expressing pancreatic islets

Mouse pancreatic islets expressing EGFP were incubated with MN-NIRF-siEGFP, buffer, or a control probe devoid of siRNA (MN-NIRF) for 72 hrs. The delivery of the probes was assessed by magnetic resonance imaging (MRI) and near-infrared optical (NIRF) imaging (Fig. 2). MRI revealed a significant ($p < 0.01$) drop in the T2 relaxation times of the islets after incubation with MN-NIRF or MN-NIRF-siEGFP compared to unstained islets (buffer), indicating that we can obtain a semi-quantitative estimate of the accumulation of the probe in islets by MRI (Fig. 2A). NIRF optical imaging confirmed the MRI findings. There was a bright near-infrared signal, representative of probe accumulation, in the islets incubated with MN-NIRF and MN-NIRF-siEGFP but not in unstained islets (Fig. 2B). In addition, a direct indicator of siRNA accumulation in the islets was obtained by optical imaging in the red channel. Since the siRNA molecule is labeled with the red dye DY547, fluorescence in the red channel could be detected only in the islets incubated with MN-NIRF-siEGFP (Fig. 2B). Finally, to evaluate the extent of *egfp* silencing, we also imaged the islets by optical imaging in the GFP channel. There was a noticeable reduction in the level of green fluorescence in islets incubated with MN-NIRF-siEGFP compared to controls, indicating effective silencing (Fig. 2B).

Validation of cellular uptake and silencing efficacy of MN-NIRF-siEGFP in EGFP-expressing pancreatic islets by flow cytometry

To confirm our imaging results regarding probe uptake and the RNAi-mediated reduction in EGFP fluorescence in the islets incubated with MN-NIRF-siEGFP, we performed FACS analysis. Pancreatic islets were incubated with MN-NIRF-siEGFP, buffer, or the control probe MN-NIRF for 72 hrs, washed and dissociated into a single-cell suspension. FACS analysis from two separate experiments showed that approximately 40% of the islet cells were labeled with the probe (Fig. 3A) and that there was an approximately 38% reduction in EGFP signal after incubation with the probe, compared to the control MN-NIRF probe (Fig. 3B). These initial experiments confirmed the observed reduction in EGFP fluorescence observed by imaging.

Validation of cellular uptake, silencing efficacy, and lack of cytotoxicity of MN-NIRF-siEGFP in EGFP-expressing pancreatic islets by microscopy

Further confirmation of probe delivery and silencing efficacy was obtained by microscopy. First, we performed confocal microscopy on fixed whole islets incubated with MN-NIRF-siEGFP, buffer, or MN-NIRF. Islets were imaged in the green (EGFP), red (DY547, siEGFP), and near-infrared (Cy5.5, MN) channels. Fig. 4 shows accumulation of both the MN-NIRF-siEGFP and MN-NIRF probes in pancreatic islets (Cy5.5 channel). However, only islets incubated with the MN-NIRF-siEGFP probe fluoresced in the red channel indicating the presence of the dye on the anti-sense strand of the siRNA. Most importantly, we observed a noticeable loss in EGFP fluorescence in islets incubated with the MN-NIRF-siEGFP probe compared to control islets, demonstrating functionality of the probe and successful mediation of target gene silencing (Fig. 4A).

For a more accurate evaluation of probe accumulation and silencing on a cell per cell basis, we performed confocal microscopy of frozen islet sections. This method revealed the presence of abundant levels of MN-NIRF-siEGFP in the islet cells. This result corresponded to a reduction in the level of EGFP fluorescence, relative to controls, indicating silencing of the target protein (Fig. 4B).

With these studies we also tested the hypothesis that after cellular uptake, the siRNA molecule would dissociate from the MN complex and enter the cytosol for successful initiation of the RNAi pathway. We expected that since the Cy5.5-labeled MN complex was conjugated to the

sense strand of the siRNA through a cleavable bond, while the antisense strand was labeled with DY547, dissociation of the siRNA from MN, endosomal escape and migration to the cytoplasm would be evident on confocal microscopy as the relocalization of DY547-signal from the endosomal to the cytoplasmic compartment. Efficient dissociation of the siRNA from MN was suggested by the poor co-localization between fluorescence in the near-infrared channel, representative of the Cy5.5 label on MN, and the red channel, representative of the DY547 label on the siRNA (Fig. 4B and inset). The punctate Cy5.5 fluorescence indicated endosomal accumulation of MN. By contrast, the DY547 fluorescence appeared diffuse and distributed throughout the cytoplasm, suggesting endosomal escape and cytosolic re-distribution of the siRNA, following dissociation from MN (Fig. 4B, inset). These results are in agreement with previous studies, in which the mechanism of siRNA dissociation from a delivery complex and its subsequent endosomal escape, was explored for siRNA delivery to cells (31, 35, 36).

Light microscopy on frozen islet sections stained by the TUNEL method revealed no differences in levels of apoptosis between unstained islets and islets incubated with MN-NIRF or MN-NIRF-siEGFP, suggesting that the treatment is not associated with cytotoxicity (results not shown). This strengthened the validity of the observed silencing effect and supported the potential clinical applicability of the method.

Validation of the silencing efficacy of MN-NIRF-siEGFP in EGFP-expressing pancreatic islets by quantitative RT-PCR

Final validation of *egfp* knock-down was obtained by quantitative RT-PCR. Over three independent experiments, we observed a significant ($72\pm 27\%$, $p = 0.04$) reduction in target mRNA transcript levels in islets treated with MN-NIRF-siEGFP compared to control islets, incubated with MN-NIRF (Fig. 5). Since the mechanism of RNAi operates at the post-transcriptional level, this represents the most direct assessment of the efficacy of silencing mediated by our probe.

Discussion

The beta-cell, as the cell type which governs glucose homeostasis, is a central effector of the pathogenesis of both type 1 and type 2 diabetes. Type 1 diabetes is caused by the autoimmune destruction of beta cells, whereas in type 2 diabetes, deficiency in insulin production, insulin resistance in peripheral tissues, or both have been implicated in the development of hyperglycemia (37). As islet transplantation becomes an acceptable clinical modality for restoring beta-cell mass, there is a critical need for non-invasive assessment of the fate of islet grafts. At the same time, gene therapy through the mechanism of RNA interference holds promise for silencing genes implicated in islet loss and improving islet graft performance. From a purely research perspective, the elucidation of the factors responsible for proper islet function and the interactions between them is key for the ultimate design of alternative novel therapies for diabetes.

With the experiments described here, we attempt for the first time to combine therapy and noninvasive imaging with pancreatic islets as a target organ. We have synthesized a novel multifunctional probe, which, in addition to its capability to deliver gene therapy in the form of siRNA, can also serve as an imaging contrast agent capable of detecting and following the fate of labeled pancreatic islets. This approach is based on a previously developed by us method for labeling pancreatic islets with superparamagnetic iron oxides and their tracking in vivo by magnetic resonance imaging (MRI) (32,33,38). The therapeutic aspect of our approach is founded upon our prior experience in synthesizing dual-purpose probes for siRNA delivery to cancer cells with subsequent in vivo imaging (39). The added value in combining the siRNA delivery and imaging capabilities within a single nanoparticle module derives from the fact

that by conjugating the siRNA to the imaging “label” we can monitor noninvasively and quantitatively the bioavailability of the therapeutic agent. Using our approach, we could directly correlate graft fate with the time course and abundance of the therapeutic molecule or potentially identify new transcriptional targets, associated with enhanced graft survival, from a better-informed perspective.

With these studies, we establish the feasibility of nanoparticle-based image-tagged siRNA delivery to pancreatic islets. Against the background of these findings, it would now be possible to devise similar dual-purpose imaging and delivery systems to answer specific biological questions. As an example, single or multiple genes can be targeted both simultaneously and over time and the ensuing effects monitored in the context of siRNA bioavailability. Our findings also lay the groundwork for future studies, in which genes implicated in islet graft loss (allo-rejection, islet cell death, autoimmune attack, etc.) can be targeted pre-transplant in order to improve graft outcome. Besides limiting immunorecognition, siRNA delivery in situ has potential in immunotherapy/immunomodulation of type 1 diabetes. For example, it is known that there is persistence of autoimmunity well after “most” of the beta cells are destroyed and that this autoimmunity can be reactivated (40). In this scenario, siRNA-based therapy targeting elements of cytokine signaling would be appropriate. Another example derives from the recent finding that therapy in the context of autoimmunity is most effective under conditions that foster occupation of the target organ lymphocyte niche by nonpathogenic, low-avidity clonotypes (41). RNAi directed towards modifying the lymphocytic pool, as an immunomodulatory intervention would be very valuable towards this goal. In all cases, the inherent imaging capabilities of our approach would permit the noninvasive tracking of the MN-siRNA label and its relationship to therapeutic outcome.

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Figure 1.
A. Synthetic scheme of the MN-NIRF-siEGFP probe. B. Schematic representation of the MN-NIRF-siEGFP probe. C. Gel electrophoresis of MN-NIRF-siEGFP demonstrating dissociation of ~7 siRNAs per nanoparticle under reducing conditions.

Figure 2.

Magnetic resonance (A) and optical imaging (B) of EGFP-expressing pancreatic islets. A. MRI revealed a drop in T2 relaxation times in the presence of the MN-NIRF or MN-NIRF-siEGFP label ($p < 0.01$, $n = 3$). B. NIRF optical imaging permitted the detection of the Cy5.5 label on MN-NIRF and MN-NIRF-siEGFP in islets, following in vitro incubation. Optical imaging in the red channel allowed the direct detection of the DY547-labeled siRNA in islets incubated with MN-NIRF-siEGFP. Optical imaging in the green channel suggested a relative reduction of EGFP fluorescence in islets incubated with MN-NIRF-siEGFP.

Figure 3.

Flow cytometry of dissociated EGFP-expressing pancreatic islets. A. Approximately 40% of the islet cells were labeled with the probe as evident from flow cytometric analysis in the FL2 (DY547) and FL4 (Cy5.5) channels. B: Representative FL1 histogram showing a 38% shift in EGFP fluorescence in islets incubated with MN-NIRF (blue) or MN-NIRF-siEGFP (red) indicating efficient silencing of the target gene. C. Quantitation of the FACS analysis data showing a significant ($p = 0.03$) reduction in EGFP fluorescence in dissociated islets incubated with MN-NIRF-siEGFP relative to controls incubated with MN-NIRF. The data represent a summary of two independent experiments.

Figure 4.

Confocal microscopy of pancreatic islets. A. Confocal microscopy of whole fixed pancreatic islets incubated for 72 hrs with MN-NIRF (top) or MN-NIRF-siEGFP (bottom). Note that both probes were taken up by the islets, but only the MN-NIRF-siEGFP probe produced signal in the red channel, consistent with siRNA incorporation. There was a noticeable reduction in EGFP fluorescence in islets incubated with MN-NIRF-siEGFP, indicating that the probe was functional. B. Confocal microscopy of frozen islet sections. Cy5.5 fluorescence was seen in cells from islets incubated with MN-NIRF and MN-NIRF-siEGFP, indicating probe accumulation in both. However, red fluorescence corresponding to siRNA was only seen in cells from islets incubated with MN-NIRF-siEGFP. EGFP fluorescence was noticeably weaker

in cells from islets incubated with MN-NIRF-siEGFP, indicating efficient silencing. The poor co-localization between NIR (MN-NIRF) and red (siRNA) fluorescence served as evidence of successful siRNA dissociation from MN. While Cy5.5 fluorescence appeared endosomal, DY547 fluorescence appeared cytosolic, suggesting endosomal escape of the siRNA (inset).

Figure 5.

Real-time semi-quantitative RT-PCR of *egfp* expression in islets incubated with MN-NIRF-siEGFP or the control MN-NIRF probe. There was a significant ($p = 0.04$, $n = 3$, Welch's t-test) reduction in *egfp* expression levels following incubation with MN-NIRF-siEGFP. The data represent a summary of three independent experiments.