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Maintenance of ischemic β cell viability through delivery of lipids and ATP by targeted liposomes

Nicole Atchison^a, Garrett Swindlehurst^b, Klearchos K. Papas^c, Michael Tsapatsis^b, and Efosini Kokkoli^{*,b}

^a Department of Biomedical Engineering, University of Minnesota, Minneapolis, MN, USA

^b Department of Chemical Engineering and Materials Science, University of Minnesota, Minneapolis, MN, USA. Fax: 612- 626-7246; Tel: 612-626-1185

^c Department of Surgery, University of Arizona, Tucson, AZ, USA

Abstract

Islet transplantation is a promising treatment for type 1 diabetes, but despite the successes, existing challenges prevent widespread application. Ischemia, occurring during pancreas preservation and isolation, as well as after islet transplantation, decreases islet viability and function. We hypothesized that the liposomal delivery of adenosine triphosphate (ATP) could prevent the loss of cell viability during an ischemic insult. In this work we use a model β cell line, INS-1 to probe the liposome/cell interactions and examined the ability of liposomes functionalized with the fibronectin-mimetic peptide PR_b to facilitate the delivery of ATP to ischemic β cells. We demonstrate that PR_b increases the binding and internalization of liposomes to the β cells. Unexpectedly, when comparing the ability of PR_b liposomes with and without ATP to protect INS-1 cells from ischemia we found that both formulations increased cell survival. By probing the functional activity of ischemic cells treated with PR_b functionalized liposomes with and without ATP we find that both lipids and ATP play a role in maintaining cell metabolic activity after an ischemic insult and preventing cell necrosis. This approach may be beneficial for preventing ischemia related damage to islet cells, especially in the organ preservation stage.

Introduction

An explosion of studies involving islet transplantation was seen after Shapiro et al.¹ demonstrated insulin independence in seven patients receiving islet transplantations. However, despite the successes,²⁻⁵ this cell based therapy must still overcome many challenges before it can become a widespread option for patients with type 1 diabetes. Some of the primary complications include the shortage of available islets for transplantation and the loss of islet viability before and after transplantation.^{2, 6-9} Ischemia (deprivation of oxygen and nutrients) has been linked to the poor islet viability and function during organ preservation and isolation, as well as after the islets have been transplanted.¹⁰⁻¹⁴ Pancreas preservation prior to islet isolation has been demonstrated to play a large role in islet isolation outcomes.¹⁵ The periods of warm and cold ischemia during the organ storage directly affect the quality of the retrieved islets and therefore the function of the transplanted graft.^{5, 9, 16} Strategies to improve the preservation conditions aim to increase the adenosine triphosphate (ATP) content of the organ and have demonstrated improved islet isolation outcomes.^{15, 17-22} However, loss of islet yield and viability during isolation persists,

requiring multiple pancreata per islet infusion.^{1, 2, 23} This requirement increases costs and risks associated with this procedure and decreases the number of patients that can be treated.²³ Ischemia negatively affects islets after transplantation as well. Isolation disrupts the native vasculature of the islets, which require 7-10 days post-transplant for complete revascularization.²⁴ During this period the islets must rely on diffusion for nutrients and oxygen supply, compromising islet viability and function.²⁵⁻²⁸

Ischemic conditions deplete cells' ATP levels, leading to a series of events that ultimately end in cell necrosis.²⁹ In β cells, the insulin secreting cells of the islets, ischemic conditions also impair the secretion of insulin.^{26, 30} While direct delivery of ATP to ischemic tissues is an appealing idea, the hydrolysis of ATP *in vivo* necessitates a delivery mechanism.^{31, 32} Previous studies on myocardial,³³⁻³⁶ liver,^{31, 32, 37, 38} retina,³⁹ and wound healing⁴⁰⁻⁴² ischemia models have demonstrated the application of liposomal encapsulated ATP (ATPL) for maintaining cell viability. Liposome encapsulation protects the ATP from enzymatic degradation and enhances ATP penetration into the cells. Though much effort has gone into improving the ATP level in preserved pancreata and transplanted islet cells, ATPL delivery has not been attempted. Currently strategies primarily involve improved oxygenation techniques^{12, 17, 21, 43} and anti-apoptosis strategies.⁴⁴ Given the multiple ischemia conditions that β cells are exposed to, ATPL delivery could provide a flexible and beneficial approach for improving pancreas preservation and islet isolation protocols, as well as a treatment option following islet transplantation.

As demonstrated in the drug delivery literature,⁴⁵⁻⁴⁷ functionalizing liposomes with a cell binding ligand improves delivery of the cargo into the cells. Previously, we demonstrated that functionalization with the fibronectin-mimetic peptide PR_b (KSSPHSRN(SG)₅RGDSP) facilitates the binding and internalization of liposomes into porcine islet cells by binding to the $\alpha_5\beta_1$ integrin.⁴⁸ While nontargeted liposomes had little to no internalization into the porcine islet cells, PR_b functionalized liposome internalization was PR_b concentration dependent. The design of PR_b peptide includes a KSS spacer, the RGDSP integrin binding motif, and the $\alpha_5\beta_1$ integrin synergy binding domain, PHSRN.^{49, 50} The two binding domains are separated by a linker (SG)₅ that mimics both the length and hydrophobicity/hydrophilicity ratio found in the amino acids in the native protein.^{51, 52} Addition of a 16 carbon dialkyl tail forms the PR_b peptide-amphiphile ((C₁₆)₂-Glu-C₂-KSSPHSRN(SG)₅RGDSP).⁵² The PR_b peptide binds specifically to the $\alpha_5\beta_1$ integrin with a $K_D = 76.3 \pm 6.3$ nM⁵²⁻⁵⁴ and has outperformed the ubiquitous RGD peptide⁵⁵ in terms of specificity, cell adhesion, cell signalling, and extracellular matrix production.^{52, 54} Additionally, PR_b has proven to be an effective peptide targeting ligand in the delivery of chemotherapeutic loads to $\alpha_5\beta_1$ expressing cancer cells, outperforming both nontargeted and GRGDSP functionalized nanoparticles.^{53, 56-61} PR_b peptide has also been used in the development of tissue constructs, promoting better cell adhesion, proliferation, and signaling.^{62, 63}

In this work our goal is to target the insulin-producing β cell line, INS-1, with PR_b functionalized ATPL under ischemic conditions. INS-1 cells have previously been shown to express the α_5 and β_1 integrins,⁶⁴ and are capable of secreting insulin in response to a glucose challenge.^{65, 66} First, we evaluate the binding and internalization of liposomes containing polyethylene glycol (PEG2000) and functionalized with PR_b into INS-1 cells. The interactions between the liposomes and the cells are investigated for both PR_b functionalized and nontargeted liposomes using flow cytometry and microscopy. The effect of liposomal delivery of ATP to INS-1 cells in an ischemic environment is probed using metabolic assays. We demonstrate that PR_b targeted liposomes provide protection from ischemia through delivery of ATP as well as by supplementing lipids to the compromised cells. Based on these findings, we envision this system implemented into the pancreas

preservation protocol, where it could potentially enhance organ ATP content and functional mass recovery after isolation.

Experimental

Materials

The PR_b peptide sequence (KSSPHSRN(SG)₅RGDSP) was purchased in crude form from the Oligonucleotide and Peptide Synthesis Facility at the University of Minnesota. The PR_b peptide-amphiphile (C₁₆)₂-Glu-C₂-KSSPHSRN(SG)₅RGDSP (referred to as PR_b throughout) was synthesized as described previously.^{49, 52, 67, 68} Lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-2-phosphoethanolamine-N-(methoxy(polyethylene glycol)-2000) (ammonium salt) (DPPE-PEG2000, referred to as PEG2000), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (DPPE-RHOD, referred to as RHOD), 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine (DC(8,9)PC), and cholesterol were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Extrusion assembly and 100 nm polycarbonate membranes were obtained from Avestin, Inc. (Ottawa, Canada). WST-1 assay was purchased from Clontech Laboratories, Inc. (Mountain View, CA). Calcein (excitation, 494; emission, 517), hoechst 33342 nucleic acid stain, and Prolong Gold Antifade reagent were purchased from Invitrogen, Inc. (Carlsbad, CA). The bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Fischer Scientific (Rockford, IL). Fetal bovine serum (FBS) was purchased from Atlas Biologicals, Inc. (Fort Collins, CO). The insulin ELISA was purchased from Mercodia Inc. (Uppsala, Sweden). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Liposome Preparation and Characterization

Liposomes composed of DPPC, cholesterol, PEG2000, and PR_b were prepared as described previously.^{48, 56, 58, 60, 69} Briefly, solutions of lipids, amphiphiles, and cholesterol were mixed in a round bottom flask and dried under argon to create a thin film. Solutions were combined at concentrations of (62-x-y):35:3:x:y mol% of DPPC:cholesterol:PEG2000:PR_b:RHOD, where x is the mol% of the PR_b peptide-amphiphile and y is the mol% of the fluorescently labeled lipid RHOD.

DC(8,9)PC-PEG liposomes were made by combining 99 mol% DC(8,9)PC and 1 mol% PEG2000. The lipid films were hydrated with 2 mM calcein in HBSE buffer (10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4), HBSE buffer, Tris buffer (10 mM Tris, 136.4 mM KCl, 13.6 mM NaCl, pH 7.4), or 400 mM ATP in HBSE buffer, freeze-thawed five times, and then extruded 21 times through two stacked 100 nm polycarbonate membranes. Liposomes were filtered over a Sepharose CL-4B gel filtration column to remove unencapsulated material and stored at 4-8°C. After gel filtration, DC(8,9)PC-PEG liposomes were cooled to 4°C and placed in a precooled glass dish. The lipids were polymerized by crosslinking for 10 minutes in a CL-1000 UV Crosslinker (UVP LLC, Upland, CA). Polymerization was monitored by a change in absorbance spectrum and visual color change. The absorbance spectrum was acquired on a Synergy H1 plate reader (Biotek, Winooski, VT).

Liposome phosphorous concentration was determined using the phosphorus colorimetric assay described elsewhere.⁷⁰ PR_b concentration was determined using the BCA assay according to the manufacturer's protocol. The ZetaPALS zeta potential analyzer (Brookhaven Instruments, Holtsville, NY) was used to determine both zeta potential and diameter of the liposomes by dynamic light scattering (DLS). Encapsulated ATP concentration was determined using reversed phase high-performance liquid

chromatography (HPLC). Liposomes were lysed with 0.4% (v/v) sodium dodecyl sulfate (SDS) in distilled water. The chromatography was performed on an Xterra Prep MS C₁₈ 150*10 mm stainless steel column (Waters Corp., Milford, MA) packed with 5 µm beads. The isocratic elution was run at room temperature with the 96/4 (v/v) mixture of 0.1 M KH₂PO₄ buffer, pH 6.0, and methanol at a flow rate of 1 mL/min. The UV absorbance was detected at 254 nm on an Agilent 1100 series HPLC (Santa Clara, CA). ATP standards were run each time liposome formulations were evaluated.

Cell Culture

INS-1 cells were cultured in RPMI-1640 medium containing 11.1 mM glucose and supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37°C in a humidified 5% CO₂ environment until they were 80-100% confluent and subsequently passaged.

Microscopy

INS-1 cells were plated at 5*10⁵ cells/well in 12 well plates containing poly-l-lysine coated coverslips and allowed to adhere overnight. Liposomes (containing 2 mM calcein or rhodamine-labelled lipid) were added at 250 µM lipid concentration in complete media at the indicated temperature and incubated for 1 hour at 4 or 37°C. For 4°C incubation, plates were placed in a refrigerator. Cells were washed twice with phosphate buffered saline (PBS) and prepared for imaging. For calcein liposome imaging cells were fixed with 4% paraformaldehyde in PBS and the nuclei stained with 2 µM Hoechst 33342. Cells were then mounted with Prolong Gold Antifade reagent. INS-1 cells incubated with liposomes containing rhodamine-labelled lipids were stained with Hoechst 33342 immediately after washing with PBS and the coverslips inverted onto a glass slide containing a small amount of PBS. Cells were imaged immediately. The Olympus Fluoview FV1000 confocal laser scanning microscope at the University Imaging Center at the University of Minnesota was used for all confocal studies. INS-1 cells treated with 2 mM calcein liposomes (as indicated above) were also imaged with fluorescence microscopy. Cells were processed as above, without fixation. Cells were immediately imaged using an EVOS_{fl} microscope (AMG, Bothell, WA) equipped with DAPI, GFP and Texas-Red filter sets.

Live/Dead Imaging

INS-1 cells were seeded at 5*10⁵ cells/well of a 96 well plate and incubated for approximately 40 hours. Cells were washed twice with PBS followed by addition of medium and treatments. For ischemic conditions cells were incubated in serum free medium without glucose supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin (referred to as glucose depleted minimal medium). Liposomes were added at a concentration of 250 µM lipids and free ATP was added at 200 µM. Cells were incubated for 6 to 9 hours under anoxic conditions (0% O₂). Anoxic conditions for all experiments were established by placing cells in a water jacketed chamber filled with 95% N₂, 5% CO₂ and connected to a circulating water bath maintained at 37°C. After the anoxic period the media was replaced with complete medium and the cells were stained with 1 µM calcein AM and 150 nM propidium iodide (PI). After 45 minute incubation the cells were imaged with a Nikon Eclipse TE 300 inverted light microscope (Nikon Inc., Tokyo, Japan) equipped with a Nikon high pressure mercury arc lamp or the Advanced Microscopy Group (AMG) EVOS_{fl} integrated light microscope. For the Nikon Eclipse, a FITC filter (excitation BP465-495, emission BP515-555; Nikon 96107M B-2E/C C12353) was used to collect calcein AM fluorescence and a rhodamine filter (excitation BP528-553, emission 590LP; Nikon DM575 G-2A) was used to collect PI fluorescence. Images were captured using a SPOT RT CCD

camera (Diagnostic Instruments, Sterling Heights, MI) and Metamorph imaging software (Molecular Devices Corp., Sunnyvale, CA). For the EVOS[®]fl, the GFP light cube (excitation 470/22 nm, emission 510/542 nm) was used to collect calcein AM fluorescence and the Texas Red light cube (excitation 585/529 nm, emission 624/640 nm) was used to collect propidium iodide fluorescence.

Flow Cytometry

For investigation of calcein liposome binding to INS-1 cells and temperature dependence of rhodamine-labelled lipid transfer, the INS-1 cells were trypsinized (0.25% trypsin +0.1% EDTA) and resuspended in complete medium at a concentration of 1×10^6 cells/mL. Cells were incubated with liposomes at a lipid concentration of 250 μ M on a rotary shaker for 1 hour at either 4 or 37°C. For 4°C incubation, plates were placed in a refrigerator. Following the incubation period cells were pelleted and washed twice with ice-cold PBS. Flow cytometry was carried out immediately. For blocking experiments, the same protocol was followed except the cells were incubated with 500 μ g/mL free PR_b peptide for 30 minutes before the addition of liposomes.

For the time course studies of rhodamine-labelled lipid transfer from liposomes to INS-1 cells, cells were seeded in 24 well plates at 3×10^5 cells/well and incubated overnight. Cells were washed twice with PBS and incubated with liposomes at 250 μ M total lipid in glucose depleted minimal medium. Plates were incubated in either normal conditions (normoxic, 21% O₂) or anoxic conditions (as described in the Live/Dead Imaging section) for the indicated period. The media was then removed and placed in a microcentrifuge tube and the cells were trypsinized and added to the microcentrifuge tube. The cells were pelleted and washed twice with ice-cold PBS. The cells were resuspended in ice-cold PBS and flow cytometry analysis carried out immediately. All flow cytometry analyses were performed at the flow cytometry core in the Cancer Research Center of the University of Minnesota.

Metabolic Activity

To test the dependence of lipid concentration on the ability of liposomes to protect INS-1 cells from ischemia and to compare ATP liposomes to other liposome formulations, INS-1 cells were seeded at 5×10^5 cells/mL in 96 well plates and incubated for approximately 40 hours. Cells were washed twice with PBS and incubated with 3.6 μ M free PR_b peptide, 120 μ M free ATP, a combination of free PR_b and free ATP at 3.6 and 120 μ M respectively, or liposomes at 100, 200, or 300 μ M lipid concentration in glucose depleted minimal medium. Free PR_b concentration was determined based on the average concentration of PR_b presented on the surface of PR_b-PEG liposomes. The plates were incubated for 6 to 9 hours in an anoxic environment. A normoxic control sample was prepared in the same fashion but was incubated in complete medium in the normal environment for the duration of the experiment. The media was replaced with 100 μ L complete medium to provide substrates for metabolism⁷¹ and 10 μ L of WST-1 reagent was added to each well. The plates were incubated for 3 hours under normal conditions. Metabolic activity was monitored by measuring the absorbance of the metabolized WST-1 reagent at 450 nm with a reference wavelength of 650 nm to account for background. The data are presented normalized to the normoxic sample using the following formulas:

$$\text{mean OD}_{\text{sample}} = \text{OD}_{450} - \text{OD}_{650}$$

$$\% \text{ Metabolic Activity} = \frac{\text{mean OD}_{\text{sample}} - \text{mean OD}_{\text{blank}}}{\text{mean OD}_{\text{normoxic control}} - \text{mean OD}_{\text{blank}}} * 100$$

Glucose Stimulated Insulin Secretion

INS-1 cells were seeded at 5×10^5 cells/well of a 96 well plate and incubated overnight. Wells were washed with PBS and incubated with liposomes at $300 \mu\text{M}$ lipid concentration or free ATP in glucose depleted minimal medium. Cells were then incubated in an anoxic environment for 6 hours. A normoxic control sample was collected by incubating in a normal environment (37°C , $5\% \text{CO}_2$) and using complete media during the 6 hour incubation. Following 6 hour incubation, the medium was replaced with complete medium and the cells were cultured under normal conditions for the 16 or 24 hours. After the culture period a glucose stimulated insulin secretion (GSIS) test was performed. The media was replaced with 2.8 mM glucose in Krebs buffer (25 mM HEPES, 115 mM NaCl, 24 mM NaHCO_3 , 5 mM KCl, 1 mM $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, 0.1% w/v BSA, 2.5 mM $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, pH 7.4 and incubated for 30 minutes in a normal environment. Media was then replaced with either 2.8 mM or 15 mM glucose in Krebs buffer. Cells were incubated with glucose solutions for 1 hour in a normal environment. Following incubation, media was collected to measure insulin concentration via ELISA. Cells were lysed and total protein measured using the BCA assay. Data are displayed as the ratio of the insulin secretion (*Ins*, ng insulin/mg protein/hr) at 15 mM glucose to 2.8 mM glucose.

$$\text{Stimulation Index} = (\text{Ins}_{15 \text{ mM}}) / (\text{Ins}_{2.8 \text{ mM}})$$

Statistical Analysis

Significance was determined using a Student's t test. Data analysis was performed using JMP Pro 9 software (SAS Institute Inc.). Significance was determined at $p < 0.05$. Significance is indicated throughout as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results and Discussion

In a previous report we demonstrated that PR_b functionalized liposomes showed effective binding and internalization into porcine islet cells and theorized that these functionalized liposomes could provide a benefit by delivering molecules of interest to insulin producing β cells.⁴⁸ In this study we are using a model β cell line, INS-1, to probe liposome-cell interactions and the delivery of ATP. By using a model cell line in this study we are able to avoid the convoluting effects of islet viability varying from batch to batch. First we investigated the effect of functionalizing liposomes with PR_b on the binding and internalization of the nanoparticles into INS-1 cells. We have previously demonstrated that the concentrations of both PR_b and PEG2000 affect the binding of the liposomes to cells.⁵⁵ PEG2000 imparts stealth properties of the liposomes and has been shown to decrease protein adsorption and increase circulation time.⁷² All liposomes in this study include 3 mol% PEG2000 and approximately 1.5-3.6 mol% PR_b on average in functionalized samples. Dynamic light scattering was used for characterization and demonstrates diameters ranging on average from 113-134 nm for the liposomes used in these studies. Full characterization (size, zeta potential, PR_b concentration and ATP encapsulation) and nomenclature information is shown in Table S1 in the Supporting Information. To investigate the effect of PR_b functionalization on liposome binding, liposomes containing 2 mM calcein functionalized with or without PR_b were incubated with INS-1 cells for 1 hour at either 4°C or 37°C and imaged with confocal or fluorescent microscopy. The cell viability is not

affected at 4 °C.⁷³ As shown in the confocal micrographs of Figure 1A, when liposomes without PR_b (PEG CAL) are incubated at 4 °C or 37 °C, there is no detectable binding and internalization to the INS-1 cells. However, liposomes functionalized with PR_b (PR_b-PEG CAL) (Figure 1A, bottom row) show binding to the outside of the cells at 4 °C and are internalized inside the cells at 37 °C. The lack of binding by PEG CAL liposomes represents the stealth ability imparted by the PEG2000. These results are in agreement with other studies that suggest PR_b improves the binding capability of stealth liposomes compared to nontargeted liposomes.^{48, 56, 58, 60} Additionally, previous studies by our lab on PR_b functionalized stealth nanoparticle trafficking have indicated uptake through an $\alpha_5\beta_1$ mediated endocytosis pathway rather than fusion or non-specific binding.^{53, 60, 74} This is in agreement with Figure 1A, which shows that liposomes merely adhered to the cells and did not internalize when incubated at 4 °C, a temperature at which endocytosis is mostly inhibited while fusion is still possible,⁷⁵⁻⁷⁷ but are internalized at 37 °C. To demonstrate the widespread difference between binding and internalization of PR_b-PEG CAL and PEG CAL liposomes, fluorescent images were taken at a lower magnification. Figure 1B demonstrates that at 37 °C the PR_b-PEG CAL liposomes have much higher binding than the PEG CAL liposomes throughout the sample. Further, flow cytometry was used to get a quantitative assessment of liposome binding and to probe PR_b specificity. In addition to incubating PEG CAL and PR_b-PEG CAL liposomes for 1 hour at 4 °C and 37 °C, cells were also incubated with 500 $\mu\text{g/mL}$ free PR_b peptide for 30 minutes prior to PR_b-PEG CAL addition to block the integrin binding sites. Figure 1C displays the histograms from a typical flow cytometry analysis. The histograms for the PR_b functionalized liposome samples are shifted farther to the right than the other samples. This indicates a higher level of fluorescence, and therefore liposome binding, in these samples. The mean fluorescence intensities from the histograms of multiple experiments are combined in Figure 1D. A higher intensity represents increased calcein liposome binding to the cells. As demonstrated in the confocal micrographs, samples treated with PR_b-PEG CAL liposomes have a significantly higher fluorescent intensity at both 4 °C and 37 °C than PEG CAL liposomes, which show virtually no binding at either temperature. Cells that are blocked with free PR_b peptide prior to liposome addition show significantly less binding compared to the unblocked PR_b-PEG CAL liposomes at both 4 °C or 37 °C. This indicates that the free PR_b is blocking the binding of the PR_b-PEG CAL liposomes to the cells. This is in agreement with other work that has demonstrated that PR_b is specific to the $\alpha_5\beta_1$ integrin using both antibody and peptide blocking assays as well as lack of binding to cells with low levels of $\alpha_5\beta_1$.^{53, 58, 60, 61, 74}

Having demonstrated that PR_b is necessary to internalize the liposomes into the INS-1 cells, we next investigated the ability of PR_b functionalized liposomes to deliver ATP to ischemic INS-1 cells and the effect of this delivery on cell viability. Ischemic conditions were achieved by incubating cells in glucose depleted minimal medium in a 37 °C, 95% N₂, 5% CO₂ environment. Cells treated with buffer only (control), free ATP, PR_b-PEG HBSE, or PR_b-PEG ATPL were exposed to 6 hours of ischemia followed by live/dead staining. As shown in Figure 2, control samples (Figure 2A) showed the highest loss of cell viability, followed by free ATP samples (Figure 2B). Unexpectedly, cells treated with PR_b-PEG liposomes containing both HBSE buffer (Figure 2C) and ATP (Figure 2D) had better cell viability and a more spread morphology than the control. Results were similar with cells exposed to liposomes containing Tris buffer (Figure S1). Treatment with DPPC alone (not as liposomes) did not show the same level of protection (Figure S2). The ability of the liposomes containing only buffer to protect the cells from ischemia was surprising and warranted further investigation.

The live/dead assay provides a snapshot of the cells' states but a metabolic assay, such as the WST-1 assay, provides a better indication of their viability. This assay was used to

investigate how the PR_b-PEG liposomes (encapsulating HBSE or Tris buffer) were providing protection to the ischemic INS-1 cells. Figure 1 demonstrates that nontargeted liposomes do not internalize into the INS-1 cells and that PR_b facilitates internalization. However, given that the PR_b-PEG HBSE liposomes used in Figure 2 and PR_b-PEG Tris liposomes used in Figure S1 only deliver buffer, it was unclear what interaction provided the benefit. Therefore, we compared the nontargeted PEG liposomes to PR_b-PEG liposomes encapsulating the HBSE or Tris buffer. Liposomes were delivered to INS-1 cells at varying lipid concentrations and incubated in ischemic conditions for 6-9 hours. The WST-1 assay was used to probe their metabolic activity following this period. INS-1 cells treated with liposome formulations had significantly higher metabolic activity than control cells treated only with buffer (Figure 3). Furthermore, metabolic activity increased with increasing amounts of added lipid for both the PR_b functionalized and nontargeted samples. There was no significant difference between the metabolic activities of samples treated with PEG or PR_b-PEG and encapsulating the HBSE or Tris buffer at each lipid concentration. There was also no significant statistical difference between the HBSE and Tris formulations at each lipid concentration for both the PEG and PR_b-PEG liposomes. While the liposome treatments do increase the metabolic activity over ischemic controls, the mean activity is still less than normal controls (100%). This is expected due to changes in gene expression and metabolism resulting from the period of ischemia.^{30, 78} Considering that we have demonstrated that liposomes without PR_b do not internalize (Figure 1), it is possible that a mechanism that does not involve binding may be involved in the protection shown in Figure 3, as both PR_b-PEG (that internalize) and PEG liposomes (that do not internalize) show similar protection independent of the encapsulated buffer.

Under ischemic conditions phosphatidylcholine degradation can lead to a loss in cell membrane integrity.⁷⁹ We hypothesized that the liposomes provide excess lipids to the ischemic cells and maintain the membrane integrity to prolong cell survival. By supplying the lipids the liposomes could be working in the plug and seal method, originally proposed by Khaw et al. in 1995.⁸⁰ In this scheme, entire liposomes physically plug lesions created in the membrane during ischemia, maintaining the membrane integrity crucial for cell viability. In the work by Khaw et al., the liposomes were targeted to cytoskeletal myosin by functionalization with an anti-myosin antibody, allowing the liposomes to bind to myosin exposed by the membrane lesions and plug the holes in the membrane. They found that anti-myosin targeted liposomes provided more protection than nontargeted liposomes. Here, the plug and seal theory seems unlikely as the cytoskeleton targeting capability is not included in the liposomes used in this study. Another mechanism could include transfer of individual lipids from the liposomes to the cells during incubation. The transfer of lipids could supplement the ischemic cells with additional lipids to replace the degraded ones. Lipids have been shown to transfer from vesicles to the air/water interface⁸¹ and to cells.⁸² Perhaps a similar mechanism leads to transfer of lipids from the liposomes to the ischemic β cells, supplementing those lost due to degradation. To investigate this theory, we included 1 mol% lissamine rhodamine B DPPE lipid (RHOD) in the liposome formulations and encapsulated HBSE buffer (PEG HBSE* and PR_b-PEG HBSE*). These liposomes were then incubated with INS-1 cells in normoxic (21% O₂) or anoxic (0% O₂) environments at 37 °C. The amount of fluorescent lipids transferred to the cells was monitored by measuring the fluorescence emitted from the cells after incubation and washing via flow cytometry. Figure 4 shows a representative data set for the anoxic environment (Figure 4A) and the cumulative mean fluorescence intensity data for both the anoxic (Figure 4B) and normoxic (Figure 4C) environments. The flow histogram for a normoxic environment experiment is shown in Figure S3 of the Supporting Information. From Figure 4 it is evident that the RHOD lipids are transferring to the INS-1 cells from both PEG-HBSE* and PR_b-PEG HBSE* liposomes. Under both conditions, and at each time point, the mean fluorescence from the cells is significantly greater than the autofluorescence of the cells. Additionally, the

fluorescence transferred to the cells increases with longer incubation periods. Figure 1 indicates that PEG HBSE liposomes do not bind or internalize into the INS-1 cells so the fluorescence transferred in the PEG HBSE* instance is not likely from binding of the liposomes to the cells, but rather from the transfer of lipids from the liposomes to the cells. It is important to note that the fluorescence intensities measured in Figure 1 and Figure 4 are not directly comparable as they are different fluorescent markers.

To further investigate this result we measured the temperature dependence of the RHOD lipid transfer. INS-1 cells were incubated with both PEG HBSE* and PR_b-PEG HBSE* for 1 hour at 4 °C and 37 °C. Cells were visualized by confocal microscopy and analysed via flow cytometry. Cells were imaged without fixation due to artifacts appearing in the RHOD localization when samples were fixed⁸³ (Figure S4). Figure 5A displays the confocal images and demonstrates that when cells were incubated with the liposomes at 4 °C the fluorescence from the rhodamine labelled lipids (shown in red) was primarily on the periphery of the cells. When cells were incubated with the liposomes at 37 °C the fluorescence was primarily located on the periphery and in a punctate pattern within the cell, most likely in the membranes of different cellular organelles. These images indicate that the rhodamine labelled lipids are likely located in the membrane components of the cells. Flow cytometry was used to quantify the fluorescence transfer (Figure 5B, representative experiment shown in Figure S5). The transfer of RHOD to the cells at 37 °C was significantly greater than the transfer at 4 °C for both formulations. At 4 °C the lipids in the bilayer of the liposomes are closer to a gel state and therefore less likely to be able to escape the bilayer. There was no statistical difference in mean fluorescence intensities of the two formulations at both 4 °C and 37 °C. The lack of difference between the PEG HBSE* and PR_b-PEG HBSE* fluorescence transfer in both Figure 4 and Figure 5 indicates that binding of the liposomes is not necessary for lipid transfer. To confirm that nontargeted liposomes containing rhodamine labelled DPPE were not binding or internalizing, liposomes containing the rhodamine labelled lipid were made with 2 mM calcein encapsulated inside (PEG CAL* and PR_b-PEG CAL*). These liposomes were incubated with the INS-1 cells for 1 hour and imaged with confocal microscopy. Samples treated with PEG CAL* liposomes demonstrate fluorescence from rhodamine but there is no detectable fluorescence from the calcein channel (Figure 6, top row) indicating that the rhodamine lipid transfer is happening without the binding and internalization of the liposomes. In contrast, samples treated with PR_b-PEG CAL* liposomes have significant fluorescence from the calcein and rhodamine fluorophores (Figure 6, bottom row), indicative of liposomes that have bound to the cells. Similar results were found in conditions with glucose depleted minimal medium (Figure S6). This figure confirms that inclusion of the rhodamine labelled DPPE lipid does not change the binding and internalization characteristics of the PR_b functionalized and nontargeted liposome formulations. The combination of Figure 1 and Figure 6 demonstrates that PR_b functionalized liposomes are able to bind and internalize into INS-1 cells only via specific interactions, while nontargeted liposomes do not internalize (i.e., no nonspecific internalization or liposome fusing). This is demonstrated by the lack of calcein cargo present in cells incubated with nontargeted liposomes in both Figures 1 and 6. However, lipid transfer is occurring from both liposome formulations and therefore is not dependent on liposome binding and internalization into the cells.

The data to this point indicate that the lipids are playing a large role in protecting the β cells from ischemia. However, to strengthen this theory we used polymerizable liposomes as a negative control. These liposomes are composed of 99 mol% 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine (DC(8,9) PC) and 1 mol% PEG2000. The DC(8,9)PC-PEG liposomes contain HBSE buffer and are 144 ± 65 nm in diameter. The DC(8,9)PC lipid is able to form crosslinks in the bilayer when exposed to UV light.⁸⁴⁻⁸⁸ Figure S7A demonstrates the schematic of the liposome formation and Figure S7B shows the absorbance

and color change indicative of liposome polymerization. The lipids in polymerized liposomes should have a greatly reduced ability to transfer to the cells during incubation, thus serving as a negative control for comparison with the non-crosslinked liposomes used in this study which have demonstrated lipid transfer to the cells. When the DC(8,9)PC-PEG liposomes are incubated with INS-1 cells exposed to ischemic conditions, there is no significant change in metabolic activity compared to the anoxic control (Figure S7C). This demonstrates that without lipid transfer, the polymerized liposomes are unable to protect the cells from ischemia. Therefore, this strengthens our hypothesis that the lipids transferred to the cells from non-crosslinked liposomes do impart some protection to the ischemic β cells.

Next we investigated if ATP delivery via targeted liposomes could provide additional functional benefits when compared to liposomes without a payload. INS-1 cells were treated with free ATP, free PR_b peptide, a combination of free PR_b peptide and ATP, or different liposome formulations (PEG HBSE, PEG Tris, PEG ATPL, PR_b-PEG HBSE, PR_b-PEG Tris, and PR_b-PEG ATPL) and exposed to 6 hours of ischemic conditions. The concentration of free PR_b peptide was determined based on the average concentration presented on the surface of PR_b functionalized liposomes used in this study. The metabolic activity of these cells was then compared to normally cultured cells (set as 100% metabolic activity). Figure 7 demonstrates that the metabolic activity of the cells is increased over the ischemic control sample for free ATP and liposomal treatments. Free PR_b peptide alone does not increase metabolic activity of the ischemic INS-1 cells over controls. Free ATP provides a similar benefit as the PEG HBSE, PEG Tris, PEG ATPL, PR_b-PEG HBSE and PR_b-PEG Tris treatments. Comparison between liposomes formulated with HBSE and Tris buffers only (PEG HBSE, PEG Tris, PR_b-PEG HBSE and PR_b-PEG Tris) demonstrates that the buffer encapsulated by the liposome does not impact the ability of the liposome formulation to increase metabolic activity, further implicating lipid transfer as the protective mechanism. Overall, the formulation functionalized with PR_b and encapsulating ATP (PR_b-PEG ATPL) performs significantly better than other liposome treatments. Though the effects of free ATP and lipids do not appear to be additive, liposomal delivery of ATP via the use of targeted stealth liposomes does increase the metabolic activity of the ischemic cells. The ability of PR_b functionalized liposomes to deliver ATP into cells, as well as transfer lipids, provides an additional benefit compared to nontargeted liposomes which can only transfer lipids to the ischemic cells. We also explored the effect of free ATP, PR_b-PEG HBSE, and PR_b-PEG ATPL treatments on the ability of INS-1 cells exposed to ischemia to secrete insulin in response to a glucose challenge. After the 6 hours of ischemic treatment, the cells were cultured normally for 16 or 24 hours and then tested for insulin secretion response. Cells were cultured for extended times to allow the insulin response to rebound.¹³ Insulin response was inhibited immediately after ischemic conditions but started to recover after longer culture periods (data not shown). As shown in Figure 8, normoxic control cells have between a 2 and 3 fold increase in insulin secretion when the glucose concentration changes from 2.8 to 15 mM. However, cells exposed to ischemic conditions have a reduced response to the increase in glucose concentration. Untreated cells have a stimulation index of about one, meaning that the insulin secretion is the same at both 2.8 and 15 mM glucose. The PR_b-PEG HBSE liposomes and free ATP treatments increase the stimulation index of the ischemic cells over the ischemic control, however only the PR_b-PEG ATPL treated samples after 24 hours of culture are significantly higher than the ischemic control at the same time point. Though PR_b-PEG ATPL treatment slightly increases the insulin response of ischemic cells, the values are still well below normoxic controls. This is likely due to the changes in gene expression that occur in β cells exposed to ischemic environments.³⁰ Ischemia leads to activation of the hypoxia inducible factor 1alpha (HIF-1 α) transcription factor which switches glucose metabolism from aerobic oxidative phosphorylation to anaerobic glycolysis in β cells.^{30, 78, 89, 90} This is accomplished through changes in genes controlling glucose uptake, glycolysis, and pyruvate utilization.³⁰ The

delivery of ATP and lipids as shown in this work is able to maintain INS-1 cell viability during ischemic insult, however it does not appear sufficient to prevent the changes associated with HIF-1 α expression. The ability of the ATP and lipid delivery to maintain viability and prevent the cells from undergoing necrosis could potentially allow the cells time to recover from the ischemic exposure in terms of glucose stimulated insulin secretion. Further work, which is beyond the scope of this paper, is needed to properly address this point.

The results from these analyses demonstrate that both the lipids and the ATP provide benefits to the ischemic cells by maintaining cell viability, although they can only marginally improve insulin secretion. The delivery of ATP via PR_b functionalized stealth liposomes affords the best performance of all the treatments in terms of maintaining metabolic activity of β cells exposed to ischemia. The protection provided by free ATP in these experiments is promising; however in an *in vivo* setting these results would be unlikely due to the rapid degradation of free ATP *in vivo*.^{31, 32} Therefore, *in vivo* a bigger benefit would likely be seen for the PR_b-PEG ATPL (PR_b functionalized stealth liposomes loaded with ATP) compared to free ATP due to the ability of the liposomes to protect the ATP from degradation.³⁵

Conclusions

We have demonstrated the dual effect of treating ischemic β cells with PR_b targeted ATP liposomes. PR_b was shown to be necessary to internalize stealth liposomes into INS-1 cells and therefore could promote liposomal ATP delivery. Unexpectedly, INS-1 cells were partially protected from ischemic conditions by liposomes containing only buffer. Further investigation revealed that the lipids composing the liposomes play a role in maintaining cell membrane integrity and decreasing cell necrosis. We hypothesize that the lipids are transferring from the liposomes to the cells, however an in depth analysis of this mechanism is outside the scope of the current work. PR_b targeted ATP liposomes were able to promote better cell metabolic activity than both free ATP and PR_b targeted liposomes containing only buffer. These results demonstrate that both the ATP and the lipids play a role in ischemic β cell protection, however they cannot completely prevent the changes that result in decreased insulin secretion. We hypothesize that the activation of HIF-1 α is leading to changes in gene expression that the ATP and lipids cannot prevent. However, by maintaining cell viability, these treatments may lead to better recovery of glucose responsiveness. Additionally, PR_b-PEG liposomes could be utilized to deliver alternative cargos specifically designed to interact with the HIF-1 α cascade. The interactions of INS-1 α cells with the PR_b-PEG ATPL described here will provide insight into the protection of islets from the myriad of ischemic conditions faced throughout isolation and transplantation. The ability of the PR_b targeted ATPL to prevent necrosis and maintain metabolic activity in ischemic β cells is promising for applications in pancreas preservation prior to islet isolation. As mentioned previously, current work in pancreas preservation is attempting to increase the ATP content of the organ. PR_b functionalized liposomes could facilitate the delivery of ATP and provide the additional benefit of lipid delivery. Improvement in preservation and isolation outcomes could lead to widespread application of islet transplants from a single donor, reducing both costs and risks associated with this procedure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Notes and references

1. Shapiro AMJ, Lakey JRT, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV. *New. Engl. J. Med.* 2000; 343:230–238. [PubMed: 10911004]
2. Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, Lakey JRT, Shapiro AMJ. *Diabetes.* 2005; 54:2060–2069. [PubMed: 15983207]
3. Hering BJ, Kandaswamy R, Ansite JD, Eckman PM, Nakano M, Sawada T, Matsumoto I, Ihm S-H, Zhang H-J, Parkey J, Hunter DW, Sutherland DER. *J. Am. Med. Assoc.* 2005; 293:830–835.
4. Robertson RP. *Diabetes.* 2010; 59:1285–1291. [PubMed: 20508222]
5. Hering BJ, Kandaswamy R, Harmon JV, Ansite JD, Clemmings SM, Sakai T, Paraskevas S, Eckman PM, Sageshima J, Nakano M, Sawada T, Matsumoto I, Zhang HJ, Sutherland DER, Bluestone JA. *Am. J. Transplant.* 2004; 4:390–401. [PubMed: 14961992]
6. Beck J, Angus R, Madsen B, Britt D, Vernon B, Nguyen KT. *Tissue Eng.* 2007; 13:589–599. [PubMed: 17518605]
7. Rother KI, Harlan DM. *J. Clin. Invest.* 2004; 114:877–883. [PubMed: 15467822]
8. Emamaullee JA, Shapiro AMJ. *Diabetes.* 2006; 55:1907–1914. [PubMed: 16804057]
9. Shapiro AMJ, Ricordi C. *Am. J. Transplant.* 2004; 4:295–298. [PubMed: 14961980]
10. Linn T, Schmitz J, Hauck-Schmalenberger I, Lai Y, Bretzel RG, Brandhorst H, Brandhorst D. *Clin. Exp. Immunol.* 2006; 144:179–187. [PubMed: 16634789]
11. Moritz W, Meier F, Stroka D, Giuliani M, Kugelmeier P, Lehmann R, Candinas D, Gassmann M, Weber M, Surgery T. L. *Laboratories and V. Physiology. FASEB J.* 2002; 16:745–747. [PubMed: 11923216]
12. Nadithe V, Mishra D, Bae YH. *Biotechnol. Bioeng.* 2012; 109:2392–2401. [PubMed: 22447333]
13. Emamaullee JA, Liston P, Korneluk RG, Shapiro AMJ, Elliott JF. *Am. J. Transplant.* 2005; 5:1297–1305. [PubMed: 15888033]
14. Lazard D, Vardi P, Bloch K. *Diabetes Metab. Rev.* 2012; 28:475–484.
15. Tanioka Y, Sutherland DE, Kuroda Y, Gilmore TR, Asaheim TC, Kronson JW, Leone JP. *Surgery.* 1997; 122:435–441. [PubMed: 9288151]
16. Brandhorst D, Brandhorst H, Hering BJ, Federlin K, Bretzel RG. *J. Mol. Med.* 1999; 77:93–95. [PubMed: 9930937]
17. Hering BJ, Matsumoto I, Sawada T, Nakano M, Sakai T, Kandaswamy R, Sutherland DER. *Transplantation.* 2002; 74:1813–1816. [PubMed: 12499907]
18. Matsuda T, Suzuki Y, Tanioka Y, Toyama H, Kakinoki K, Hiraoka K, Fujino Y, Kuroda Y. *Surgery.* 2003; 134:437–445. [PubMed: 14555931]
19. Lakey JRT, Tsujimura T, Shapiro AMJ, Kuroda Y. *Transplantation.* 2002; 74:1809–1811. [PubMed: 12499905]
20. Scott Iii WE, O'Brien TD, Ferrer-Fabrega J, Avgoustiniatos ES, Weegman BP, Anazawa T, Matsumoto S, Kirchner V. a. Rizzari MD, Murtaugh MP, Suszynski TM, Aasheim T, Kidder LS, Hammer BE, Stone SG, Tempelman LA, Sutherland DER, Hering BJ, Papas KK. *Transplant.* 2010; 42:2016–2019.
21. Scott Iii WE, Weegman BP, Ferrer-Fabrega J, Stein SA, Anazawa T, Kirchner VA, Rizzari MD, Stone J, Matsumoto S, Hammer BE, Balamurugan AN, Kidder LS, Suszynski TM, Avgoustiniatos ES, Stone SG, Tempelman LA, Sutherland DER, Hering BJ, Papas KK. *Transplant. P.* 2011; 42:2011–2015.
22. Suszynski TM, Rizzari MD, Scott WE III, Tempelman LA, Taylor MJ, Papas KK. *Cryobiology.* 2012; 64:125–143. [PubMed: 22301419]
23. McCall M, Shapiro AMJ. *Cold Spring Harb. Perspect. Med.* 2012; 2:1–16.

24. Menger MD, Jaeger S, Walter P, Feifel G, Hammersen F, Messmer K. *Diabetes*. 1989; 38:199–201. [PubMed: 2463196]
25. Giuliani M, Moritz W, Bodmer E, Dindo D, Kugelmeier P, Lehmann R, Gassmann M, Groscurth P, Weber M. *Cell Transplant*. 2005; 14:67–76. [PubMed: 15789664]
26. Dionne KE, Colton CK, Yarmush ML. *Diabetes*. 1993; 42:12–21. [PubMed: 8420809]
27. Ryu GR, Lee M-K, Lee E, Ko S-H, Ahn Y-B, Kim J-W, Yoon K-H, Song K-H. *Biochem. Bioph. Res. Co.* 2009; 386:356–362.
28. Suszynski TM, Avgoustiniatos ES, Stein SA, Falde EJ, Hammer BE, Papas KK. *Transplant. P.* 2011; 43:3221–3225.
29. Michiels C. *Am. J. Pathol.* 2004; 164:1875–1882. [PubMed: 15161623]
30. Cantley J, Grey T, Maxwell PH, Withers DJ. *Diabetes Obes. Metab.* 2010; 11:159–167. [PubMed: 21029313]
31. Korb V, Tep K, Escriou V, Richard C, Scherman D, Cynober L, Chaumeil J, Dumortier G. *Crit. Rev. Ther. Drug.* 2008; 25:305–345.
32. Tep K, Korb V, Richard C, Escriou V, Largeau C, Vincourt V, Bessodes M, Guellier A, Scherman D, Cynober L, Chaumeil J-C, Dumortier G. *J. Lipos. Res.* 2009; 19:287–300.
33. Hartner WC, Verma DD, Levchenko TS, Bernstein EA, Torchilin VP. *WIREs Nanomed. Nanobiotechnol.* 2009; 1:530–539.
34. Levchenko, TS.; Hartner, WC.; Verma, DD.; Bernstein, EA.; Torchilin, VP. Vol. 605. Humana Press; Totowa, NJ: 2010. p. 361-375.
35. Verma DD, Levchenko TS, Bernstein EA, Torchilin VP. *J. Control. Release.* 2005; 108:460–471. [PubMed: 16233928]
36. Verma DD, Hartner WC, Levchenko TS, Bernstein EA, Torchilin VP. *Pharma. Res.* 2005; 22:2115–2120.
37. Neveux N, De Bandt JP, Chaumeil JC, Cynober L. *Scand. J. Gastroentero.* 2002; 37:1057–1063.
38. Hayashi K, Ochiai T, Ishinoda Y, Okamoto T, Maruyama T, Tsuda K, Tsubouchi H. *J. Gastroen. Hepatol.* 1997; 12:249–256.
39. Dvorianchikova G, Barakat DJ, Hernandez E, Shestopalov VI, Ivanov D. *Mol. Vis.* 2010; 16:2882–2890. [PubMed: 21203410]
40. Chiang B, Essick E, Ehringer W, Murphree S, Hauck MA, Li M, Chien S. *Am. J. Surg.* 2007; 193:213–218. [PubMed: 17236849]
41. Chien, S. *Liposomes, Methods in Molecular Biology*. Vol. 605. Humana Press; Totowa, NJ: 2010. p. 377-391.
42. Wang J, Wan R, Mo Y, Li M, Zhang Q, Chien S. *Am. J. Surg.* 2010; 199:823–832. [PubMed: 20609726]
43. Witkowski P, Liu Z, Guo Q, Poumian-Ruiz E, Cernea S, Herold K, Hardy MA. *Transplant. P.* 2005; 37:3398–3401.
44. Ah Kim H, Lee S, Park J-H, Lee S, Lee B-W, Ihm SH, Kim T-I, Kim SW, Ko KS, Lee M. *J. Drug Target.* 2009; 17:242–248. [PubMed: 19558363]
45. Pangburn TO, Petersen MA, Waybrant B, Adil MM, Kokkoli E. *J. Biomech. Eng.* 2009; 131:074005. [PubMed: 19655996]
46. Pearce TR, Shroff K, Kokkoli E. *Adv. Mater.* 2012; 24:3803–3822. [PubMed: 22674563]
47. Levine RM, Scott CM, Kokkoli E. *Soft Matter.* 2013; 9:985–1004.
48. Atchison N, Fan W, Papas KK, Hering BJ, Tsapatsis M, Kokkoli E. *Langmuir.* 2010; 26:14081–14088. [PubMed: 20704278]
49. Mardilovich A, Kokkoli E. *Biomacromolecules.* 2004; 5:950–957. [PubMed: 15132686]
50. García AJ, Schwarzbauer JE, Boettiger D. *Biochemistry.* 2002; 41:9063–9069. [PubMed: 12119020]
51. Craig JA, Rexeisen EL, Mardilovich A, Shroff K, Kokkoli E. *Langmuir.* 2008; 24:10282–10292. [PubMed: 18693703]
52. Mardilovich A, Craig JA, McCammon MQ, Garg A, Kokkoli E. *Langmuir.* 2006; 22:3259–32564. [PubMed: 16548586]

53. Pangburn TO, Bates FS, Kokkoli E. *Soft Matter*. 2012; 8:4449–4461.
54. Shroff K, Pearce TR, Kokkoli E. *Langmuir*. 2012; 28:1858–1865. [PubMed: 22149259]
55. Kokkoli E, Mardilovich A, Wedekind A, Rexeisen EL, Garg A, Craig JA. *Soft Matter*. 2006; 2:1015–1024.
56. Demirgöz D, Garg A, Kokkoli E. *Langmuir*. 2008; 24:13518–13524. [PubMed: 18954096]
57. Demirgöz D, Pangburn TO, Davis KP, Lee S, Bates FS, Kokkoli E. *Soft Matter*. 2009; 5:2011–2019.
58. Garg A, Tisdale AW, Haidari E, Kokkoli E. *Int. J. Pharm.* 2009; 366:201–210. [PubMed: 18835580]
59. Garg A, Kokkoli E. *Curr. Pharm. Biotechno.* 2011; 12:1135–1143.
60. Shroff K, Kokkoli E. *Langmuir*. 2012; 28:4729–4736. [PubMed: 22268611]
61. Pangburn TO, Georgiou K, Bates FS, Kokkoli E. *Langmuir*. 2012; 28:12816–12830. [PubMed: 22827285]
62. Rexeisen EL, Fan W, Pangburn TO, Taribagil RR, Bates FS, Lodge TP, Tsapatsis M, Kokkoli E. *Langmuir*. 2010; 26:1953–1959. [PubMed: 19877715]
63. Shroff K, Rexeisen EL, Arunagirinathan MA, Kokkoli E. *Soft Matter*. 2010; 6:5064–5072.
64. Krishnamurthy M, Li J, Al-Masri M, Wang R. *J. Cell Commun. Signal.* 2008; 2:67–79. [PubMed: 19023675]
65. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB. *Endocrinology*. 1992; 130:167–178. [PubMed: 1370150]
66. Cline GW, Pongratz RL, Zhao X, Papas KK. *Biochem. Bioph. Res. Co.* 2011; 415:30–35.
67. Berndt P, Fields GB, Tirrell M. *J. Am. Chem. Soc.* 1995; 117:9515–9522.
68. Mardilovich A, Kokkoli E. *Langmuir*. 2005; 21:7468–7475. [PubMed: 16042481]
69. Fenske, DB.; Maurer, N.; Cullis, PR. *Liposomes: A Practical Approach*. Oxford University Press; Oxford: 2003. p. 167–191.
70. Chen PS, Toribara TY, Warner H. *Anal. Chem.* 1956; 28:1756–1758.
71. Takahashi S, Abe T, Gotoh J, Fukuuchi Y. *Neurochem. Int.* 2002; 40:441–448. [PubMed: 11821152]
72. Wattendorf UTA, Merkle HP. *J. Pharm. Sci.* 2008; 97:4655–4669. [PubMed: 18306270]
73. Atchison N, Fan W, Brewer DD, Arunagirinathan MA, Hering BJ, Kumar S, Papas KK, Kokkoli E, Tsapatsis M. *Angew. Chem.-Int. Edit.* 2011; 50:1617–1621.
74. Petersen MA, Hillmyer MA, Kokkoli E. *Bioconj. Chem.* 2013; 24:533–543.
75. Wileman T, Harding C, Stahl P. *Biochem. J.* 1985; 232:1–14. [PubMed: 2867759]
76. Marchini C, Pozzi D, Montani M, Alfonsi C, Amici A, De Sanctis SC, Digman MA, Sanchez S, Gratton E, Amenitsch H, Fabbretti A, Gualerzi CO, Caracciolo G. *Cancer Gene Ther.* 2011; 18:543–552. [PubMed: 21394110]
77. Resina S, Prevot P, Thierry AR. *Plos One*. 2009; 4:e6058. [PubMed: 19557145]
78. Cheng K, Ho K, Stokes R, Scott C, Lau SM, Hawthorne WJ, Connell PJO, Loudovaris T, Kay TW, Kulkarni RN, Okada T, Wang XL, Yim SH, Shah Y, Grey ST, Biankin AV, Kench JG, Laybutt DR, Gonzalez FJ, Kahn CR, Gunton JE. *J. Clin. Invest.* 2010; 120:2171–2183. [PubMed: 20440072]
79. Nachas N, Pinson A. *FEBS Lett.* 1992; 298:301–305. [PubMed: 1544465]
80. Khaw B-A, Torchilin VP, Vural I, Narula J. *Nat. Med.* 1995; 1:1195–1198. [PubMed: 7584994]
81. Schindler H. *Biochim. Biophys. Acta.* 1979; 555:316–336. [PubMed: 476108]
82. Sandra A, Pagano RE. *J. Bio. Chem.* 1979; 254:2244–2249. [PubMed: 429283]
83. Fretz MM, Koning GA, Mastrobattista E, Jiskoot W, Storm G. *Biochim. Biophys. Acta.* 2004; 1665:48–56. [PubMed: 15471570]
84. Puri A, Jang H, Yavlovich A, Masood MA, Veenstra TD, Luna C, Aranda-Espinoza H, Nussinov R, Blumenthal R. *Langmuir*. 2011; 27:15120–15128. [PubMed: 22053903]
85. Yavlovich A, Singh A, Tarasov S, Capala J, Blumenthal R, Puri A. *J. Therm. Anal. Calorim.* 2009; 98:97–104. [PubMed: 20160877]

86. Yavlovich A, Singh A, Blumenthal R, Puri A. *Biochim. Biophys. Acta*. 2011; 1808:117–126. [PubMed: 20691151]
87. Ramakers BEI, van den Heuvel M, Tschlis I Spithas N, Brinkhuis RP, van Hest JCM, Löwik DWPM. *Langmuir*. 2012; 28:2049–2055. [PubMed: 22185241]
88. Johnston DS, McLean LR, Whittam MA, Clark AD, Chapman D. *Biochemistry*. 1983; 22:3194–3202. [PubMed: 6309218]
89. Cantley J, Selman C, Shukla D, Abramov AY, Forstreuter F, Esteban MA, Claret M, Lingard SJ, Clements M, Harten SK, Asare-anane H, Batterham RL, Herrera PL, Persaud SJ, Duchon MR, Maxwell PH, Withers DJ. *The J. Clin. Invest.* 2009; 119:125–135.
90. Puri S, Cano DA, Hebrok M. *Hybridoma*. 2009; 58:433–441.

Figure 1.

Binding and internalization of PR_b functionalized liposomes. A) Confocal micrographs of INS-1 cells treated with PEG CAL or PR_b-PEG CAL liposome for 1 hour at 4 or 37 °C. Nuclei are shown as blue and liposomes with encapsulated calcein appear green. The second column shows the image overlain onto the bright field image so cell boundaries can be discerned. Scale bars are 8 μm. B) Fluorescent images of INS-1 cells treated with PEG CAL or PR_b-PEG CAL liposomes for 1 hour at 37 °C. Scale bars are 8 μm. C) Representative flow cytometry data. INS-1 cells were incubated with liposomes for 1 hour prior to analysis. Blocked samples were incubated with 500 μg free PR_b peptide for 30 minutes prior to liposome addition. D) Flow cytometry cumulative analysis. The first column represents the autofluorescence of the INS-1 cells. Data are presented as mean ± SE of n=3-5 experiments. Symbols directly above each bar represent the significance compared to the autofluorescence at the same temperature: † no significance; ** p<0.01; *** p<0.001.

†Electronic Supplementary Information (ESI) available: Liposome characterization, live/dead staining of INS-1 cells exposed to ischemic conditions, flow cytometry histograms of rhodamine labelled lipid transfer, confocal images of lipid transfer to INS-1 cells, and polymerizable liposome data. See DOI: 10.1039/b000000x/

Figure 2.

Live/dead staining of INS-1 cells exposed to ischemic conditions (glucose depleted minimal medium, 37 °C, 95% N₂, 5% CO₂) for 6 hours. Cells were treated with 200 μM free ATP or liposomes at 250 μM lipid concentration and stained with calcein AM (green-live) and propidium iodide (red-dead). Scale bars are 20 μm.

Figure 3.

Metabolic activity of INS-1 cells treated with liposomes at various lipid concentrations (μM) containing HBSE buffer (solid bars) or Tris buffer (hatched bars) and exposed to ischemic conditions for 6-9 hours at 37 °C. Data are expressed as a percentage of the metabolic activity of INS-1 cells cultured in a normal environment (complete media, 37 °C, 5% CO_2). Data represents the mean $\pm$ SE (Control: $n=7$, 5 repetitions per n ; HBSE: $n=4$, 3 repetitions per n ; Tris 100, 200 μM : $n=3$, 3 repetitions per n ; Tris 300 μM : $n=5$, 5 repetitions per n). Symbols directly above each bar represent the significance compared to the ischemic control (white bar): * $p<0.05$; ** $p<0.01$; *** $p<0.001$. There is no significant statistical difference between HBSE and Tris samples at each lipid concentration for both PEG and PR_b-PEG, or between the PEG and PR_b-PEG liposomes at each lipid concentration for both buffers.

Figure 4.

Transfer of rhodamine labelled lipids to INS-1 cells. A) Representative flow cytometry histogram for INS-1 cells incubated with PEG HBSE* and PR_b-PEG HBSE* liposomes in an anoxic environment at 37 °C. Both liposome formulations contain 1 mol% lissamine rhodamine B DPPE. Liposomes were incubated for indicated time periods and after washing the fluorescence was monitored via flow cytometry. Cumulative flow cytometry data for anoxic (B) and normoxic (C) environments is displayed as the mean $\pm$ SE for n=3-4 experiments. Symbols directly over bars represent the significance compared to the autofluorescence (AF) of the INS-1 cells at the same temperature: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. There is no significant statistical difference between the PEG HBSE* and PR_b-PEG HBSE* samples at each time point for both conditions.

Figure 5.

Temperature dependence of rhodamine labelled lipids. A) Confocal images of INS-1 cells incubated with PEG HBSE* and PR_b-PEG HBSE* liposomes. Liposomes were incubated for 1 hour at 4 and 37 °C and imaged immediately. Nuclei are shown in blue and rhodamine labelled lipids are red. Scale bars are 8 μm. B) Cumulative flow cytometry data for INS-1 cells incubated for 1 hour with PEG HBSE* and PR_b-PEG HBSE* liposomes. Data are expressed as mean ± SE of n=3-4 experiments. Symbols directly over bars represent the significance compared to the autofluorescence of the INS-1 cells at the same temperature: * p<0.05; ** p<0.01; *** p<0.001. There is no statistical difference between PEG HBSE* and PR_b-PEG HBSE* at either temperature.

Figure 6. INS-1 cells were treated with liposomes containing 1 mol% rhodamine labelled DPPE and encapsulating 2 mM calcein for 1 hour at 37 °C, 5% CO₂. Confocal micrographs show the nucleus in blue, calcein in green, and rhodamine in red. Scale bars are 8 μm.

Figure 7.

Metabolic activity of INS-1 cells treated with 3.6 μM free PR_b peptide, 120 μM free ATP, 3.6 μM free PR_b and 120 μM free ATP combined, or different liposomes (300 μM lipid concentration) and exposed to 6 hours of ischemia at 37 $^{\circ}\text{C}$. PEG ATPL liposomes delivered 110 ± 29 μM ATP and PR_b-PEG ATPL liposomes delivered 122 ± 41 μM ATP. Data are expressed as a percentage of the metabolic activity of INS-1 cells cultured in a normal environment. Data shown as mean $\pm$ SE of $n=3-11$ experiments, 3-5 repetitions per n . * symbols directly over bars represent the significance compared to the ischemic control cells, o symbols directly over bars represent significance compared to free PR_b sample: *, o $p < 0.05$; **, oo $p < 0.01$; ***, ooo $p < 0.001$. If no symbol is displayed, there is no statistical significance for that pair.

Figure 8.

Functional activity of INS-1 cells treated with 150 μ M free ATP or liposomes at a 300 μ M lipid concentration and exposed to 6 hours of ischemia at 37 $^{\circ}$ C followed by 16 or 24 hours of normal culture (white and grey bars respectively). PR_b-PEG ATPL liposomes delivered 162 ± 16 μ M ATP. Data are expressed as the ratio of insulin released at 15 mM glucose to the insulin released at 2.8 mM glucose. Data shown as mean $\pm$ SE of n=3-4 experiments, 2 repetitions per n. Symbols directly over bars represent the significance compared to the ischemic control * p<0.05