Porous Phospholipid Nanoshells as Enzyme Delivery Agents

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Abstract

Diabetes is an epidemic in developed nations. Glucokinase (GK) is vital for glucose sensing, and is directly implicated in particular forms of diabetes. Studying pancreatic cells with altered GK activity would facilitate studies, but current methods for altering proteomes are lacking. Porous phospholipid nanoshells (PPNs) have traditionally been used as platforms for biologically derived nanosensors, though their biocompatibility and protease resistance well suits them as enzyme delivery agents. GK kinetics were investigated with an enzyme coupled reaction to determine the effect of encapsulation. It was determined that encapsulation increased the Hill coefficient by 5.8% and the $S_{0.5}$ by 1.8%. This small deviation may not be significant in physiological conditions. To observe a recovered function in cell lines upon reintroducing GK, constitutively expressed GK must first be knocked down with siRNA. As initial work toward an siRNA knockdown, immunoblotting conditions were optimized resulting in a detection limit below 10 ng of GK. Immunoblotting verified suspected constitutive expression of GK in INS-1 cell lines. While further investigation is necessary to demonstrate the utility of GK-containing PPNs for cell delivery, this thesis outlines the generation and characterization of this novel enzyme delivery platform.
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Introduction

Diabetes is a complex metabolic disorder characterized by high blood glucose. It is traditionally classified into two types: type I diabetes is characterized by a lack of insulin while type II diabetes is caused by factors other than insufficient insulin, such as insulin resistance (1). These simple definitions ignore many facets of the disease. Onset of diabetes increases susceptibility to other disorders including retinopathy, nephropathy and heart disease (2). Unfortunately, diabetes is still poorly understood and managed. Furthermore, type II diabetes is a growing epidemic, likely linked to a growing obese population in developed nations (2, 3). Preventing widespread onset will likely require intervention on many fronts including social structure, environmental influences and lifestyle changes (2). However, the treatment of those already afflicted will require better molecular understanding of the disorder.

In healthy individuals, increased blood glucose following ingestion is offset by the release of insulin from pancreatic β-cells (4). Insulin initiates uptake of glucose by tissues, initiates glycogenesis in the liver, and halts mobilization of fatty acids. In diabetics, when the signal from insulin is lost, glucose remains at high concentrations in the blood and is ultimately excreted in urine. Tissues are then forced to metabolize fatty acids and proteins, generating ketone bodies and ketoacidosis (5).

The glucose transduction pathway of pancreatic β-cells, shown in Figure 1, can suggest targets for treatment of diabetes. While numerous proteins are involved in glucose transduction, glucokinase (GK) has metabolic control of the pathway, ultimately determining the glucose concentration necessary for insulin release. As such, GK has been vaunted as the “glucose sensor” of the body (6). Mutations, knockdowns, and defects in GK are directly implicated in diabetic disease states (7, 8, 9). Patients with certain forms of type II diabetes characterized by chronic hyperglycemia have mutated GK with lower glucose affinity, thereby increasing the threshold of insulin release (6).

The kinetics of GK are well suited for glucose sensing and fixing. Though other hex-
okinases are essentially a dimer of GK (10, 11), their kinetics are drastically different. GK has a much higher $S_{0.5}$ of 8 mM for glucose (compared with $\mu$M for hexokinases (12)) and a sigmoidal binding curve with Hill coefficient ca. 1.7 (6). $S_{0.5}$ is analogous to $K_m$ for enzymes displaying cooperativity. Surprisingly, GK is not a multimeric protein; the observed cooperativity results from transitions between protein conformations of differing glucose affinity (12).

In addition to pancreatic $\beta$-cells, GK is present in pancreatic $\alpha$-cells, endocrine enterocytes and hypothalamic neurons (13) as well as liver hepatocytes, where GK was initially identified (14). Studies based on knockdowns and GK inhibitors in neuron cultures demonstrate GK is necessary for correct neuronal glucose sensing (15). GK is hypothesized to participate in glucose transduction by the same mechanism as in $\beta$-cells, though the specific role of GK in neurons is still unclear (16). In hepatocytes, GK is utilized in a markedly different way (17). Instead of rate-limiting, GK is present at high concentrations; almost 99% of GK in the body is concentrated in the liver (4). There, GK rapidly phosphorylates excess glucose as an initial step in glycogenesis (18). Since GK is not inhibited by its product like other hexokinases, it is uniquely suited to glucose fixing (6). Mice with selective liver knockouts of GK have impaired insulin release due to chronic hyperglycemia from insufficient glucose fixing (19).

Though heavily studied, the role of GK in carbohydrate metabolism is complex. Many questions are still unanswered, including the function of GK in glucose transduction within pancreatic $\alpha$-cells and glucose-sensing neurons. Further investigations of these pathways are limited by current methods of protein modulation within cellular systems. Transfection is usually not possible for primary cell lines and the target protein is not immediately available (20). RNA silencing lowers protein levels without modifying the background genome, but cannot introduce mutated proteins or increase expression (21). Cell penetrating peptides (CPPs) (22) or microinjection (23) introduce proteins immediately into cellular systems, but the internalized peptide is degraded by inherent protein turnover, making these approaches unsuitable for long-term studies. Microinjection has additional disadvantages including dis-
rupting the cell membrane and inability to multiplex. Ideally, a protein delivery system would be minimally invasive, allow for prolonged expression and usable in primary cell lines.

Stabilized synthetic vesicles present a possible alternative for protein delivery. Spontaneous vesicle formation, due to the hydrophobic effect, was initially utilized as a scaffold for polymer construction (24). Introduction of polymerizable groups in hydrophobic tail regions allows crosslinking between lipid to generate highly stabilized vesicles. Additional modifications, including lipid chain length (25), head groups (26), locations and types of polymerizable groups (27, 28), and copolymers (29), can tailor synthetic vesicles for specific applications. Hydrophilic molecules encapsulated within the vesicles drastically increase possible uses. Cleverly chosen encapsulants allow stabilized vesicles to be used as nanosensors for membrane permeable substances such as oxygen (30). Within the core of the vesicle, sensing chemistry is concentrated and protected from environmental threats. The main limitation to widespread use is the pristine membrane, which prevents diffusion of hydrophilic substrates into the core. Reconstitution of protein channels into pristine vesicles could selectively allow substrates into the core, but would add another layer of complexity.

In 2006, Cheng, et al. utilized 1,2-bis-[10-(2',4'-hexadienoyloxy)decanoyl]-sn-glycero-3-phosphatidylcholine (bis-SorbPC) to develop stabilized porous vesicles. Bis-SorbPC exhibits an inherent porosity to small, charged molecules and is stabilized by UV-initiated copolymerization with ethylene glycol dimethacrylate (EGDMA). The structure, in Figure 2, shows that unlike most phospholipids, bis-SorbPC has a relatively polar sorbyl group in its tail. It is suspected these groups prevent tight packing of the tails, leading to the observed permeability. The apparent molecular weight cutoff of the membrane is 1.8 kDa (32), allowing many biologically-relevant substrates to pass through but restricting larger proteins. To date, porous phospholipid nanoshells (PPNs) composed of bis-SorbPC have been utilized as protective shells for biologically-derived sensing chemistries including a glucose fluorescent indicator protein (32) and firefly luciferase for the detection of ATP (33). PPNs show distinct advantages compared to other nanoparticles, such as polyacrylamide based PEBBLES (34),
including increased biocompatibility from the lipid exterior and faster diffusion. PPNs also prevent degradation of encapsulated proteins from interaction with proteases (32, 33).

Thus far, PPNs have been utilized solely as nanosensors. However, they are well-suited for delivery of other protein cargo. In particular, enzymes encapsulated within PPNs could catalyze reactions with small molecules and release the products back into solution. The current work will explore PPNs as enzyme delivery agents by demonstrating their utilization for encapsulation of a physiologically relevant enzyme, GK. The synthesis and characterization of GK-containing PPNs for enzyme delivery is described. To date, PPNs have been modified with the HIV-derived TAT cell-penetrating peptide to facilitate cellular uptake. It is vital to demonstrate PPNs, once internalized by a cell, will be available in the cytosol. The mechanism of uptake for PPNs is unknown, but it is possible they adhere to the surface and then are phagocytosed and become entrapped in acidified lysosomes. To confirm cytosolic availability, a novel PPN pH sensor based on ratiometric fluorescence of pHluorin (35) and tdTomato was synthesized to determine local pH of PPNs within a cell. pHluorin is a pH-sensitive mutant of GFP with decreased quantum yield in acidic solutions and a dynamic range between pH 6 and 8. By including a red GFP variant, tdTomato, as a reference dye, the ratiometric response can be used to measure local pH.

From kinetic studies of GK, it was found that PPNs provide a highly-stable, protective shell for encapsulated proteins while altering kinetic parameters by less than 6%. Further efforts are necessary to demonstrate PPN utility with primary cells, including GK knock-downs with siRNA, but initial steps, including detection of GK via immunoblotting, are also described. Fluorometry and confocal microscopy measurements with pHluorin-based pH nanosensors suggest utility for determining cytosolic availability of PPNs, but so far this effort has been exploratory. This is the first reported use of PPNs for the encapsulation of a physiologically relevant enzyme with the intent to modulate cell function. Successful delivery of arbitrary protein cargo protected within nanoshells would provide another route for modifying cell physiology, and may ultimately transform enzyme therapeutics.
Materials and Methods

Materials

General chemicals were purchased from Sigma Aldrich and used as received. All other suppliers are named where pertinent. Aqueous solutions were made with deionized (18 MΩ) water from a Barnstead water purifier. Unless otherwise noted, chemicals were used without further purification.

Cloning

pETDuet-GK expression vector (Figure 3) was generated with standard molecular biology techniques. Briefly, human GK cDNA was subcloned by PCR with forward primer 5′-GGCGGC GGATCC G GACGATGACGATAAGGAT ATG CTG GAC GAC AGA GCC AGG ATG and reverse primer 5′-GGCGGC GAATTC TCA CTG GCC CAG CAT ACA GGC CTT to express the human islet isoform of GK (36). The forward primer includes an enterokinase (EK) cleavage site for removing the N-terminal 6xHis tag from the expressed protein. pHluorin was also subcloned by PCR with forward primer 5′-GGCGGC GGATCC C ATG GTG AGC AAG GCG AG and reverse primer 5′-GGCGGC GAATTC TTA CTT GTA CAG CCG GGC AT. tdTomato was subcloned by direct digestion with BamHI and EcoRI (New England Biolabs). The amplified sequences were purified with a QIAquick PCR purification kit (QIAGEN). Next, amplified DNA and pETDuet vector (Novagen) were digested with EcoRI and BamHI at 37°C for 2 hours. The digest was purified using the QIAquick DNA cleanup kit (QIAGEN). Resulting fragments were ligated with T4-ligase (New England Biolabs) at room temperature for one hour. The ligation product was transformed into XL1-Blue electrocompetent cells, plated onto LB-ampicillin agar plates, and grown at 37°C overnight. Putative clones were screened by PCR with the above primers and sequenced to confirm positive clones (DNA sequencing facility, University of Arizona).
Expression and Purification

BL21 (DE3) E. coli were transformed with pETDuet-GK by heat shock at 42 °C for 45 seconds and screened on LB-ampicillin agar plates. Individual colonies in ∼5 mL of LB-ampicillin precultures were grown overnight at 37 °C. The preculture was back inoculated into a larger culture of LB-ampicillin (0.1-1 L) at a 1:400 dilution to an approximate OD$_{600}$ of 0.05. The large culture was grown at 37 °C for 3 hours, induced with 0.1 mM isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG) and further incubated at room temperature for at least 2 days. Cultures were harvested by centrifugation at 2,800 xg for 10 minutes at 7 °C. Typical yields were greater than 3 g of bacteria per liter of culture. Bacterial pellets were stored at -80 °C for later purification.

For GK purification, pellets were resuspended in 30 mL lysis buffer containing 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethanesulfonylfluoride (PMSF) and 10 mM 2-mercaptoethanol ($\beta$ME), pH 8.0. Suspended cells were disrupted by sonication on ice with a Branson Sonifier 250 (Emmerson). The sonicator was set at the microtip-power level and 50% duty cycle. After ten pulses, sonication was halted and the solution allowed to cool for 1 minute before resuming. This cycle was repeated 24 times for 2 minutes of active sonication.

pHluorin and tdTomato were lysed with CelLytic B (Sigma Aldrich). Following harvesting, pellets were frozen at -80 °C to initiate fracture of the cell wall. Next, cells were suspended in lysis buffer enriched with 0.2 mg/mL lysozyme, 50 U/mL benzonase and 1X CelLytic B and gently rocked at room temperature for 15 minutes. Fifteen mL of lysis buffer were added for each gram of bacteria.

Resulting lysates from each method were clarified in two steps. First, cellular debris and unlysed cells were pelleted at 4,300 xg for 20 minutes at 7 °C. Next, DNA and RNA complexes were removed from the supernatant by addition of 0.7 mg/mL protamine sulfate followed by centrifugation as before.

Protein was purified and concentrated from clarified lysates by Ni$^{2+}$-affinity chromatog-
raphy with Ni-NTA resin (QIAGEN). Prior to use, the column was washed with 2 column volumes of nanopure water and 5 volumes of lysis buffer. The lysate was loaded, the column washed with at least 5 volumes of lysis buffer, and the protein eluted with 3 volumes of lysis buffer enriched with 250 mM imidazole. The column was cleaned with 2 volumes of nanopure water, 5 volumes of 6 M guanidine HCl, 2 volumes of nanopure water, and stored with 3 volumes of 30% ethanol at 4 °C for future use. Protein concentration was determined by a standard Bradford assay and purity confirmed with 10 or 15% SDS-PAGE. One gram of bacteria yielded approximately 10 mg of purified protein. Activity of GK was determined as described below. Fluorescent protein activity is taken as a scaled fluorescence emission.

**Synthesis of PPNs**

Prior to encapsulation, purified protein was concentrated using a 3 kDa size exclusion spin column (Millipore). For GK-PPN synthesis, the protein solution was enriched with 20 mM glucose to prevent degradation or inactivation (14, 37). PPN synthesis was performed by thin film hydration derived from Cheng (2006). Briefly, 10 mg of bis-SorbPC dissolved in benzene was dried with a stream of argon followed by vacuum desiccation for at least 4 hours. The lipid was rehydrated with 1 mL of protein concentrate by incubation at 37 °C and vortexing. Samples were subjected to 10 freeze-thaw-vortex cycles between dry ice/isopropanol (-77 °C) and a 37 °C water bath followed by extrusion 21 times through two stacked 200 nm Nucleopore polycarbonate filters using a stainless steel extruder (Avanti Polar Lipids).

To polymerize, vesicles were UV photo-irradiated with a Hg arc lamp (Newport model 6281) operating at 100 W with a UV band pass filter (Edmund U-330) and a water IR filter. Vesicle solutions were enriched with a 0.125 mole fraction EGDMA and 0.047 mole fraction irgacure 907 in the presence of 1.5 mg/mL ascorbic acid for 15 minutes with stirring. Polymerized vesicles were centrifuged at 16,000 x g for 10 minutes and supernatant discarded to remove the bulk portion of non-encapsulated protein. Vesicles were resuspended and non-
entrapped protein was removed via size-exclusion chromatography (SEC) with Sepharose CL-4B (Sigma Aldrich). Vesicles were finally passed through a Ni-NTA column to ensure removal of extra-vesicular protein.

**Synthesis of TAT-PEG-DOPE Lipid for Loading of pH nanosensors**

PPNs were functionalized with a CPP, TAT from HIV, to facilitate cellular uptake. TAT (American Peptide Co.) was conjugated to 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) with a poly(ethylene glycol) (PEG) linker (38). Briefly, 3.4 g of PEG ($M_n$ 3400) were coevaporated with dichloromethane twice and placed under vacuum for 30 minutes. The resulting solid was dissolved in 5 mL dichloromethane and combined with 4 mmol $p$-nitrophenyl chloroformate and 4 mmol triethylamine (TEA) on ice. After mixing, the ice bath was removed and the reaction incubated at room temperature overnight. The product was precipitated with 200 mL diethyl ether at -20 °C and isolated with vacuum filtration, washing with cold ether. The product, PEG($p$NP)$_2$, was dried under vacuum overnight.

For conjugation to the lipid, 5 mg of DOPE was combined with 100 µmol triethylamine in chloroform. To this solution, 0.25 g of PEG($p$NP)$_2$ was added and allowed to react overnight at room temperature in the dark. Next, the chloroform was removed under vacuum for 4 hours. The dried lipid was resuspended in 10 mM HCl and 150 mM NaCl by sonication and the excess PEG($p$NP)$_2$ removed by SEC on a Sepharose CL-4B column monitored at 272 nm. Pooled fractions were freeze-dried and the resulting solid dissolved in chloroform. Shortly before use, the TAT peptide was attached to the lipid by reacting a 1:1.05:1.05 solution of pNP-PEG-DOPE:TAT peptide:TEA in dimethylformamide for 8 hours at room temperature. Solvent was removed and the resulting solid resuspended in chloroform and stored at -20 °C.

To incorporate TAT-PEG-DOPE into vesicle preparations, a 1% (by mole relative to total lipid) solution of TAT lipid was resuspended in desired buffer by sonication for 30 seconds. This solution was added to the purified, polymerized vesicle solution and allowed to
incorporate into the vesicles by incubating at room temperature for 30 minutes with regular vortexing.

**Cell Loading**

MIN6 cells were split onto glass coverslips and allowed to grow a day before loading with vesicles. 0.2 mg of TAT-PEG-DOPE modified bis-Sorb vesicles were then added to the cells in serum free Opti-Mem media (Invitrogen) for 2 hours at 37 °C. Cells were rinsed thoroughly with Opti-Mem before imaging.

**GK Activity Assay**

The GK activity assay was adapted from Pilkis (1975) and Goward (1986) to allow for determination of kinetic constants. NADPH fluorescence (\(\lambda_{ex} 340 \text{ nm}/\lambda_{em} 450 \text{ nm}\)) was utilized as it resulted in less scattering artifacts with vesicle solutions compared with more traditional absorbance monitoring. The assay is based on the enzyme coupled reaction shown below.

\[
\text{Glucose} + \text{ATP} \xrightarrow{\text{Glucokinase}} \text{Glucose 6-Phosphate} + \text{ADP} \\
\text{Glucose 6-Phosphate} + \text{NADP} \xrightarrow{\text{G-6PDH}} 6\text{-phospho-gluconate} + \text{NADPH}
\]

Briefly, concentrations of glucose ranging from 0-80 mM were combined with a master mix yielding a reaction with 10 mM dithiothreitol (DTT), 0.4 mM NADP, 0.8 U/mL glucose 6-phosphate dehydrogenase (G-6PDH) and 5 mM ATP in 100 mM tris, 6 mM MgCl and 150 mM KCl, pH 7.4. Glucokinase concentrations were limited to low \(\mu\)g/mL to prevent saturating G-6PDH. Samples were monitored at \(\lambda_{ex} 340 \text{ nm}/\lambda_{em} 450 \text{ nm}\) for 2 minutes at 1 Hz. The maximal slope was determined in Microsoft Excel for each concentration. A plot of initial velocity, \(v_o\), as a function of glucose concentration was fit with the Hill equation,

\[
v_o = \frac{V_{max}[L]^n}{S_{0.5}^n + [L]^n}
\]
using MATLAB. When necessary, the $V_{\text{max}}$ in AFU/s was converted to mM/s for comparison with literature values based on a standard curve of NADPH in the reaction mixture. Once encapsulated, the concentration of protein is difficult to determine, so it was necessary to scale $v_0$ by $V_{\text{max}}$ to compare the kinetics of free and encapsulated GK.

**Immunoblotting**

Immunoblotting was performed to detect GK in cell lysates as an initial step of siRNA silencing. Samples were separated with 10% SDS-PAGE and then transferred to nitrocellulose at 90 V for 45 minutes. The blot was visualized with ponceau S (Santa Cruz Biotechnology) to confirm transfer and then blocked with 5% (w/v) dry milk with 0.1% (v/v) tween-20 in phosphate buffered saline (PBS) for 1 hour at room temperature. Next, the blot was incubated with 0.4 µg/mL of GK primary antibody H-88 (Santa Cruz Biotechnology) in wash buffer (0.1% dry milk, 0.1% tween-20 in PBS) overnight at 4 °C. The blot was washed 3 times for 10 minutes each and exposed to 20 ng/mL of anti-rabbit IgG-HRP (Santa Cruz Biotechnology) in wash buffer for 45 minutes at room temperature. The secondary antibody was removed and the blot washed as before followed by rinsing with tris-buffered saline. Western Blotting Luminol Reagent (Santa Cruz Biotechnology) was pipetted over the blot and allowed to react for 1 minute at room temperature before exposure to autoradiography film.

**Standardization of pHluorin/tdTomato pH sensor**

The response of the pH nanosensors was monitored by fluorescence of pHluorin ($\lambda_{\text{ex}}$ 470 nm/$\lambda_{\text{em}}$ 508 nm) and tdTomato ($\lambda_{\text{ex}}$ 561 nm/$\lambda_{\text{em}}$ 585 nm) with a Fluorolog 3 Fluorometer (Horiba Inc) as the solution pH was adjusted. For the free proteins, pHluorin and tdTomato were added to 3 mL of 10 mM phosphate buffer with 120 mM NaCl (pH 6.0) at equal emission intensities in a pH 8 solution. During PPN synthesis, the same ratio of pHluorin and tdTomato was used to resuspend bis-Sorb. For each measurement, 10 µL of ~100 mM NaOH
was added to the cuvette, the sample pH measured with a pH meter, and the fluorescence monitored. This was repeated until the pH was above 8, at which point 10 µL of ∼100 mM HCl was added and the process repeated until the pH dropped below 6 to estimate the reversibility. This was performed in triplicate for the free and encapsulated samples.

To analyze the data, the ratio of pHluorin emission to tdTomato emission for a given pH ($r_{p/t}(pH)$) was normalized as

$$r_{\text{norm}}(pH) = \frac{r_{p/t}(pH) - \min\{r_{p/t}\}}{\max\{r_{p/t}\} - \min\{r_{p/t}\}}$$

where the minimum and maximum are evaluated over the pooled trials. This value was taken as the saturation of the sensing system. The sigmoidal response of $r_{\text{norm}}(pH)$ was then fit to

$$r_{\text{norm}}(pH) = \frac{1}{1 + \exp[-a(pH) + b]}$$

(3)

where $a$ and $b$ are arbitrary constants. The pH of half saturation can be solved as

$$pH_{1/2} = \frac{b}{a}$$

(4)

so the sensitivity is described as

$$\frac{\partial r_{\text{norm}}}{\partial pH} = \left. \frac{ae^{a(pH)+b}}{(e^{a(pH)} + e^b)^2} \right|_{pH=pH_{1/2}} = \frac{a}{4}$$

(5)

showing the linear relationship between sensitivity and fitting parameter $a$. The sensitivity and pH$_{1/2}$ were used to compare pHluorin/tdTomato when in free solution and encapsulated in PPNs.

Confocal Imaging of pHluorin/tdTomato pH sensor-loaded cells

Laser scanning confocal microscopy was performed using a LEICA TCS SP2 (Leica Lasertechnik GmbH) with the following settings: Leica HCX PL APO 63x/1.20/0.17 UV
objective lens, excitation wavelength 488 nm (Ar Laser) and 543 nm (HeNe laser), and a 488/543 double dichroic mirror. Detection of pHluorin was monitored at 505-525 nm and tdTomato at 605-670 nm. To analyze, the two channels were merged to confirm colocalization of the pHluorin and tdTomato signal. Regions of interest were selected by hand and the average intensity measured for at least three regions within and outside the cell for each fluorophore. The ratio of these signals were then averaged to obtain a pooled measure.
Results

Optimization of Protein Purification

Recombinant GK, pHluorin and tdTomato were expressed from pETDUET. Expression of pHluorin and tdTomato were monitored qualitatively based on their color at room temperature or 37 °C and 0.1, 0.5 or 1 mM IPTG over the course of several days. From this optimization, it was determined that expression at 0.1 mM IPTG and room temperature gave the greatest yield after 2-4 days. The same conditions were examined with GK, but the specific activity was the pertinent metric. Not only did the long expression with 0.1 mM IPTG at room temperature increase the yield of GK, the enzyme was more active.

Sonication and CelLytic B were investigated for optimal yield of GK and EGFP (a model for pHluorin and tdTomato). Figure 4 shows an SDS-PAGE used to qualitatively monitor protein within the pellet and supernatant of various lysis conditions for GK. Panel a is the progression of extraction using sonication. Additional sonication releases more protein into the supernatant. Panel b shows lysis using CelLytic B at various concentrations and storage conditions. Many CelLytic B conditions provided near complete solubilization of the pellet. For further investigation, the full 2 minutes of sonication or storage at -80 °C followed by lysis with 1X CelLytic B were used to lyse pellets and the specific activity of GK and EGFP were measured. For GK, a ratio of the maximum velocity to protein concentration was used as the metric for specific activity. EGFP specific activity was taken as the ratio of fluorescence emission to protein concentration. As summarized in Table 1, while CelLytic B provided a 30% higher specific activity for EGFP, it reduced the activity of GK by nearly 70%.

Figure 5 shows a representative SDS-PAGE of the purification scheme for tdTomato. Equal amounts of protein were loaded into each lane to facilitate rough quantification. The first lane contains the whole cell lysate, providing a baseline for total protein within the sample. The next four lanes show the lysis (with CelLytic B) and clarification leading up to affinity chromatography. Lanes 2 and 3 contain the pellet and supernatant following lysis,
respectively, and demonstrate near complete solubilization. DNA and RNA complexes were removed from the lysate by mixing with protamine sulfate; lanes 4 and 5 contain the pellet and supernatant from the precipitation. Most protein is retained in the supernatant, indicating little product is lost in this step. The next four lanes contain samples during affinity chromatography. Lane 6 contains flow-through of the Ni-NTA column. The lack of a band for tdTomato in the flow-through demonstrates high affinity of the 6xHis-tagged protein for the Ni$^{2+}$ column matrix. The next two lanes are samples of the washing steps. Some protein is initially washed through and the last portion of wash contains relatively little protein. Finally, the elution shows high concentration and purity.

**GK Kinetics**

Using the enzyme coupled reaction in Equation 1, GK activity could be indirectly monitored by the increase in fluorescence of NADPH. Sample raw data from a kinetic curve experiment is shown in Figure 6. The maximal velocity of the reactions increases with increasing glucose concentrations, as expected. Comparison of free and encapsulated GK kinetic curves are shown in Figure 7. The Hill coefficient increased from $1.7 \pm 0.25$ to $1.8 \pm 0.25$ upon encapsulation, as did the $S_{0.5}$ from $5.5 \pm 0.56$ to $5.6 \pm 0.53$ mM.

**Detection of GK in Cell Lines**

Immunoblotting was utilized to monitor GK expression in cell lysates as shown in Figure 8. Specifically, MIN6 and INS-1 cells were screened to confirm GK expression as previously reported (40, 41). HEK293 was included as a negative control as it should not express GK. A range of purified 6xHis-EK-GK masses was also loaded as a positive control and to estimate a limit of detection. Since 10 ng sample is clearly visible, this is a high estimate of the limit of detection. Aliquots of lysates were spiked with 20 ng of 6xHis-EK-GK to better estimate the migration within those lanes. The recombinant protein is approximately 4 kDa larger than wild-type GK, making resolution possible. Based on the log(MW)/$R_f$
plot for the blot, the difference between 54 kDa and 50 kDa is predicted to be a difference of 1.4 cm, approximately the thickness of the 10 ng band. Since the recombinant GK migrated slower than predicted, this should provide a better estimate than determining the R_f of a 50 kDa protein. The expected position of wild-type GK corresponds to the band immediately below 6xHis-EK-GK in INS-1; this band is absent in HEK293 and MIN6 lysates.

*pHluorin/tdTomato pH Nanosensors*

To investigate pHluorin/tdTomato nanosensors, the fluorescence of pHluorin and td-Tomato solutions were monitored by fluorimetry while adjusting the pH. The normalized fluorescence ratio was fit to the sigmoidal function presented in Equation 3. The overlay of free and encapsulated pHluorin/tdTomato saturation curves are given in Figure 9 and the fitting parameters are summarized in Table 2. In general, free protein solutions lost saturation upon decreasing pH from 8 back to 6. The resulting scatter contributed to the greater summed square error of the model (0.887 compared to 0.071 when encapsulated) and larger confidence intervals of parameters. Comparison of the acquired quantities show encapsulation had minor effects on the system. Most notably the sensitivity increased almost 50% from 0.508 ± 0.065 to 0.742 ± 0.030 pH\(^{-1}\) upon encapsulation. Additionally the pH at half saturation, determined by Equation 4, was not statistically different when encapsulated, 7.15 ± 0.40 compared with the free protein value of 7.1 ± 1.3.

pHluorin/tdTomato pH nanosensors were loaded into MIN6 cells using TAT-PEG-DOPE lipid to facilitate uptake. The relative pH of PPNs within and outside of cells were compared qualitatively using two-channel confocal microscopy. A sample image and the fluorescence ratios are presented in Figure 10. Laser backscatter, in panel a, established the cell boundary. The pHluorin and tdTomato signals are in panels b and c, respectively. The ratio of pHluorin to tdTomato is significantly higher outside of the cell, suggesting the pH of intercellular PPNs is lower than the extracellular pH. Since these nanosensors were not standardized with the confocal microscope, the magnitude of the pH difference cannot be established.
Discussion

Purification

Several conditions were tested to optimize the expression temperature, time, and IPTG concentration. Lysis conditions were also examined, specifically between sonication and CelLytic B. Use of CelLytic B for purification of GK reduced the specific activity by nearly 70%. Despite the increased yield in mass of GK, the loss of activity was deemed unacceptable. For EGFP, more protein released and it was more active. Since CelLytic B is a proprietary surfactant, it is possible the conditions were harsh enough to denature GK while the more robust EGFP was unaffected. Based on this observation, purification of GK was performed with sonication while pHluorin and tdTomato were lysed with CelLytic B. It was found that lysis with CelLytic B can be performed in half the time of sonication on multiple cultures simultaneously and more thoroughly lyses cells. If there was no effect on activity, it would be favored over sonication. However, as demonstrated with GK, the CelLytic B can drastically reduce enzyme activity so care and preliminary experiments are necessary.

Following lysis, supernatants were treated with protamine sulfate to precipitate DNA and RNA. As shown in Figure 5, the protamine sulfate pellet contained little protein, indicating tdTomato did not precipitate with the nucleic acids. Since the negatively charged backbone of nucleic acid polymers can interfere with Ni$^{2+}$-affinity resin, it is important to remove DNA and RNA prior to chromatography. A rapid, one-step purification by Ni$^{2+}$-affinity chromatography was performed on the clarified lysate. In the flow-through, little protein passes through the column, demonstrating good capture by Ni-NTA. Washing removed some protein from the column and the eluted product contains a significant amount of pure protein. Though a step gradient of imidazole would provide a purer product, this protocol was optimized for speed and yield of the target protein. PPN synthesis requires a considerable amount of protein so a greater yield was more important than a slightly purer product.

Glucokinase Encapsulation and Immunoblotting

GK activity was determined from an enzyme coupled reaction where the production of
NADPH was monitored by fluorimetry. Figure 6 shows the raw data from a kinetic curve experiment with increasing glucose concentrations. While the maximal velocity a trial does increase as expected, the initial rate is much lower than this value. This behavior may result from either GK kinetics or insufficient mixing within the small-volume cuvette. Since many components of the assay are expensive, the small-volume cuvette is necessary though additional mixing should be attempted in future experiments. A plot of maximal velocity as a function of glucose concentration is shown in Figure 7 for free and encapsulated GK. The kinetics are expected to display positive cooperativity with glucose, as modeled by the Hill equation. The Hill coefficient increased from 1.7±0.25 to 1.8±0.25 upon encapsulation and is close to the literature value of 1.7. The Hill coefficient measures cooperativity and gives rise to the sigmoidal shape of the curve. The $S_{0.5}$ values changed from 5.5±0.56 to 5.6±0.53 mM upon encapsulation and are significantly lower than the literature value of 7.5 mM. $S_{0.5}$ ultimately dictates the threshold of glucose signalling (4). Since the effect is present in each sample, it appears the disagreement with literature is characteristic of the recombinant protein. These differences will hopefully be inconsequential for initial experiments. Overall, the kinetics of this reaction are not drastically altered by encapsulation and suggest PPNs can protect their cargo without affecting its function, a vital characteristic for enzyme delivery.

To demonstrate GK PPNs internalized by a cell will alter its physiology, a glucose sensitive cell line will be treated with siRNA to knockdown GK in a future experiment. To monitor GK knockdowns with immortal cell lines, GK had to be detected in small quantities from lysates. This required performing an immunoblot to identify which cell lines had detectible amounts of GK constitutively expressed. Figure 8 shows the results of an immunoblot with recombinant 6xHis-EK-GK and several cell lysates. Low amounts of GK can be detected using the described conditions; 10 ng of GK is clearly present on the blot. The primary antibody is sensitive, but not specific as seen from the other bands. Based on the log(MW)/$R_f$ plot of the blot, wild-type GK is detectible in INS-1, but not MIN6. This is not consistent with (40), but may be a limitation on cell growth and GK induction rather
than detection. While good evidence for the expression of GK in INS-1, further verification could include running the recombinant protein with the His tag removed. This would ensure the recombinant GK would migrate with wild-type GK. Perhaps the best evidence would be a successful knockdown with siRNA, where the amount GK in the cells should decrease.

\textit{pHluorin/tdTomato pH nanosensors}

\textit{pHluorin/tdTomato} pH nanosensors performed better than free protein since saturation as a function of pH had less scatter and was more sensitive. However, this may be an artifact from adsorption of the free protein to the pH electrode or due to variations in protein concentrations between the free and encapsulated samples. The experiment should be repeated with a biologically compatible pH electrode to verify this finding. Regardless, \textit{pHluorin/tdTomato} encapsulated in a PPN has a predictable fluorescence as a function of pH. As with GK, the encapsulation has not affected protein properties indicating protons can freely pass through the vesicle membrane. Encapsulated \textit{pHluorin/tdTomato} functions as a protein based pH nanosensor and will be able to quantify intracellular pH in future work.

While PPNs may be successfully internalized by cells, if they are entrapped in a cellular membrane, the encapsulated protein will not affect cellular physiology. The \textit{pHluorin/tdTomato} nanosensor was loaded into cells to determine if PPNs are freely interacting with the cytosol. A confocal microscopy image from this experiment is presented in Figure 10. These nanosensors were not standardized prior to loading, so quantitative measures of pH are not possible. Still, pH-insensitive tdTomato has a fairly constant fluorescence throughout the image, while pHluorin fluorescence drops inside the cell. This implies the pH is lower for PPNs within the cell, but without proper standardization, the magnitude of this difference cannot be determined. As such, the drop in ratio could be from a slight pH differential across the cellular membrane or the PPNs could be entrapped within an acidified compartment. Furthermore, the encapsulation of multiple proteins in PPNs is still in early stages of investigation. It is unknown if the presence of one protein would preclude other
proteins from the core. When measuring few vesicles, as in confocal microscopy, this may drastically alter the measured ratios. These possibilities could be further investigated by performing analytical measurements of the pH based on the pHluorin/tdTomato emission ratio. Alternatively, the cellular membrane could be permeated with a proton-selective channel and the extracellular pH modified. If the PPNs are not surrounded by another membrane, the response would fluctuate with changing pH, otherwise the fluorescence ratio would be roughly constant. These experiments are fairly easy to perform and should give definitive evidence of the cytosolic availability of internalized PPNs.

**Future Directions and Challenges**

This thesis demonstrated significant progress towards a novel use of PPNs, but additional work must be completed to confirm the utility of PPNs for delivering physiologically relevant enzymes. pHluorin/tdTomato pH nanosensors could determine whether PPNs are entrapped in secondary vesicles upon cellular internalization. Once PPNs are shown to be available in the cytosol, work specific to GK can commence. While an *in vivo* assay specific to GK would be ideal, it has not yet been described in the literature and its development would likely be another project in itself. The best prospect for detecting altered cellular proteomes is monitoring physiological responses, be it calcium signaling, electrophysiology, or insulin release. However, since only downstream effects can be monitored, significant effort is required to verify an altered response from GK PPNs. Perhaps GK is not well-suited for a proof of concept experiment, but the ability to modulate GK within cells would immediately assist diabetes research and is worth extra effort.

PPNs show promise as next generation enzyme delivery agents due to their ability to protect biological cargo from proteases while not affecting kinetics. Combined with high stability by polymerization, PPNs should maintain enzyme activity within cells significantly longer than direct injection or transfection with CPP-tagged proteins. PPNs would give researchers a unique tool for manipulating cell proteomes and may ultimately revolutionize direct enzyme therapeutics.
Table 1: Relative specific activity comparison between sonication and CelLytic B lysis methods for GK and EGFP. In general, CelLytic B released more protein into the supernatant, but the specific activity varied based on protein. For GK, the relative activity was based on the response of the kinetic assay. For EGFP, the activity was simply the fluorescence per unit volume. Relative specific activity is the activity scaled by the concentration and normalized to the higher value.

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Table 2: Summary of fitting parameters for pHluorin/tdTomato pH sensor platform. The summed square error (SSE) is a measure of the goodness of fit for the data. It is generally defined as $\sum(y_i - f(x_i))^2$ where $y_i$ is the empirical data and $f(x_i)$ is the value predicted from the model with optimized parameters. The larger value of SSE for the free protein is a result of the larger scatter in saturation for the trials. Parameters $a$ and $b$ correspond to Equation 3 and the pH$_{1/2}$ is from Equation 4. Sensitivity is taken as the predicted slope at pH$_{1/2}$ as presented in Equation 5. Encapsulation does not affect the pH at half saturation, but does increase the sensitivity at pH$_{1/2}$. 
Figure 1: Simplified glucose transduction pathway in pancreatic β cells, adapted from (42). Extracellular glucose is freely transported into the cell by GLUT2. GK then phosphorylates glucose, metabolically controlling the rest of the pathway. Excess ATP from the citric acid cycle and electron transport chain causes K\(_{\text{ATP}}\) channels to close, depolarizing the cell and opening voltage-gated calcium channels. This initiates insulin granule migration and fusion, releasing insulin into the blood.
Figure 2: Structure of 1,2-bis-[10-(2',4'-hexadienoyloxy)decanoyl]-{sn}-glycero-3-phosphatidylcholine (bis-SorbPC). Polymerization occurs at the sorbyl groups near the end of the tail. The slight polarity of these ester moieties likely gives rise to the porosity of bis-SorbPC bilayers.
Figure 3: Vector map of pETDuet containing 6xHis-EK-GK. Islet isoform GK cDNA (36) was subcloned into pETDUET with PCR followed by restriction digests with EcoRI and BamHI and finally ligation. The EK cleavage site was added in the forward primer to facilitate removal of the 6xHis tag. pHluorin and tdTomato were subcloned between the same restriction sites, but the EK site was not added.
Figure 4: Optimization of lysis for GK. Every pair of lanes contain a single lysis condition, the left containing the pellet while the right is the supernatant. The same proportion of pellet and supernatant were loaded into each lane. The band for GK is boxed for each set of gels. Lanes marked L contain the ladder (Precision Plus Protein Standard, Bio-Rad Laboratories). For sonication (a), the suspension was sampled every three cycles, approximately 15 seconds of sonication. For CelLytic B (b), three concentrations of CelLytic B were investigated at three different incubation temperatures. Pellets of bacteria were incubated at these temperatures for 20 minutes prior to lysing with CelLytic B at room temperature for 15 minutes.
Figure 5: 15% SDS-PAGE of intermediate steps during purification of tdTomato (boxed). An equal amount of supernatant and pellet was loaded into each lane except the elution, which underwent significant concentration on the Ni-NTA column.
Figure 6: Glucokinase activity assay with glucose concentrations increasing from 0-80 mM. Reactions contained 10 mM DTT, 0.4 mM NADP, 0.8 U/mL G-6PDH and 5 mM ATP in 100 mM tris, 6 mM MgCl and 150 mM KCl pH 7.4. The fluorescence ($\lambda_{\text{ex}}$ 340 nm/$\lambda_{\text{em}}$ 450 nm) was monitored at 1 Hz for 2 minutes. Initial work determined 2 minutes was sufficient to obtain the maximal reaction rate. From this raw data, 60 points were used to estimate the initial activity from 60-120 s. Note the reactions do not achieve their maximum velocity until later in the recording. Fluorescence is relative to the maximal value within this figure.
Figure 7: Kinetic curves of free and encapsulated glucokinase. The initial velocities of each sample are scaled by their $V_{\text{max}}$ for easier comparison. 95% confidence intervals for each kinetic parameter are given in the inset table.
Figure 8: Immunoblot of cell lysates suspected to constitutively express GK. The first four lanes contain decreasing masses of 6xHis-EK-GK as positive controls for each antibody and for semi-quantification. The Cruz-Marker ladder (Santa Cruz Biotechnology) is a positive control for the secondary antibody and the HEK293 cell lysate was used as a negative control as it should not express detectible amounts of GK. INS-1 and MIN6 cell lysates, suspected to contain GK, were run with 20 ng of added 6xHis-EK-GK to estimate the migration of wild-type GK. There are several other bands present in the cell lysates due to non-specific binding of the primary antibody. Based on the difference in mass between wild-type and 6xHis-EK-GK, the wild-type band is suspected to be immediately below the 6xHis-EK-GK band.
Figure 9: Standardization curve of encapsulated and free pFluorin/tdTomato pH sensor fit to Equation 3. The free protein has much larger scatter in saturation, leading to an order of magnitude higher summed square error and much larger confidence intervals. The larger sensitivity of the encapsulated sample is evident from the slope of the fits. The encapsulated data seems to be well modeled by Equation 3.
Figure 10: Two-channel confocal microscopy of a MIN6 cell loaded with pHluorin/tdTomato nanosensors. pHluorin/tdTomato containing PPNs were functionalized with TAT-PEG-DOPE lipids and incubated with MIN6 cells for 2 hours at 37 °C. The backscatter, in panel a, was used to determine the cell boundary. Panels b and c contain the fluorescence of pHluorin and tdTomato, respectively. The average ratio of pHluorin/tdTomato for vesicles outside of the cell was $1.77 \pm 0.16$ while inside the cell the ratio dropped to $0.455 \pm 0.035$. 
References


