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Delivery of modified biomimetic nanoparticles and synthetic insulin in a closed loop regulated system for extended glycemic control



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# Delivery of modified biomimetic nanoparticles and synthetic insulin in a closed loop regulated system for extended glycemic control

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in Bioengineering

by

Crystal Yorklum Xiao

2022

ABSTRACT OF THE THESIS

**Delivery of modified biomimetic nanoparticles and synthetic insulin in a closed loop regulated system for extended glycemic control**

by

Crystal Yorklum Xiao

Master of Science in Bioengineering

University of California, Los Angeles, 2022

Professor Song Li, Chair

Traditional self-administered treatment of type 1 diabetes typically requires multiple injections on a daily basis, which is painful and inconvenient and often results in poor compliance and glucose regulation. Closed-loop insulin systems have been proposed such as electronic based glucose-responsive pumps and synthetic glucose-sensitive materials. In this report, erythrocyte membrane-coated nanoparticles with GLUT receptors on the surface of the particles and bonded synthetic glucose-conjugated insulin (Glu-insulin) were created for mounting glucose-sensitive release of insulin. Multiple biomaterial delivery systems were tested *in vitro* to achieve optimal extended and sustained release. In hyperglycemic conditions, high concentrations of glucose in interstitial fluid can displace Glu-Insulin via a competitive interaction with GLUT, leading to a quick release of Glu-Insulin and subsequent regulation of blood glucose (BG) levels.

The thesis of Crystal Yorklum Xiao is approved.

Andrea M. Kasko

Jacob J. Schmidt

Peter Cawood Butler

Song Li, Committee Chair

University of California, Los Angeles

2022

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# Chapter 1. Introduction

Diabetes Mellitus is a chronic disease that is characterized by the loss of ability to regulate or produce insulin, resulting in high blood glucose levels (BG) (Mo et al. 2014). It has affected 537 million people worldwide in 2021 and the prevalence is expected to rise to 1,054 billion by 2045 (Sun et al. 2022). Genetic disposition is suspected to be the main contributing factors to developing type 1 diabetes (T1D) or type 2 diabetes (T2D)(Phlips and Radermecker 2012). Traditional treatment management of type 1 diabetes is daily self-monitoring and administration of insulin, which is typically delivered via subcutaneous injections(Wu et al. 2011; Shah et al. 2016). Insulin is a hormone typically produced in the pancreas by β-islet cells, which can secrete appropriate amounts of insulin that correspond to the level of glucose in the bloodstream (Veiseh et al. 2015). At higher levels, insulin inhibits release of glucose from the liver and promotes glucose uptake from blood into to muscle, fat and the liver. At low blood glucose levels, the drop in insulin no longer suppresses release of glucose from the liver or lipids from adipose tissue. In healthy individuals, β-islet cells sense rising glucose concentrations and respond by releasing insulin, providing basal insulin levels to restrain glycogenolysis and lipolysis. Failure of glucose control can lead hyperglycemia state which may result subsequent consequences, including blindness, limb amputation, coma, and complications affecting eyes, kidneys, nerves, and heart (Nathan 1993).

Excess insulin leads to hypoglycemia, which can lead to cognitive disturbances, brain damage, coma, seizure, or even death (Ohkubo et al. 1995). Insulin treatments are self-administered, and many patients struggle with administering insulin with proper dosage, timing, and location (Trief et al. 2016). There is an urgent need to develop a smart glucose-responsive

system that can mimic the pancreatic beta cell's ability secrete the proper amount of insulin to regulate BG levels and to improve the quality of life for those with diabetes. (Veiseh et al. 2015; Gilroy et al. 2016)

Close-looped systems where continuous glucose monitoring sensors and corresponding insulin dose injection pumps have been in development since 1979 to achieve this objective (Brownlee and Cerami 1979; Veiseh et al. 2015). These systems are commonly called artificial pancreas devices (APD) and mainly consist of a continuous glucose monitor and an insulin infusion pump that is controlled by a computer-controlled algorithm. (Long et al. 2019) The goal of these devices is to minimize user involvement; however, algorithms require daily carbohydrate intake and physical activity information from users (American Diabetes 2020).

Synthetic material-based glucose responsive systems such as a matrix-based insulin delivery device has gained popularity over the past decade in the research field show potential as a low cost alternative to continuous glucose sensor monitoring systems. (Wang et al.). These insulin delivery systems consist of three main aspects: a glucose-sensing motif, a stimuli responsive material, and a system for insulin release (Yang and Cao 2017; Wang et al. 2020). The systems rely on three main glucose-sensitive motifs and can be classified into 3 systems: glucose oxidase (GOx) (Bankar et al. 2009), glucose binding proteins (Sharon and Lis 1972), and phenylboronic acid (PBA) (Chou et al. 2015; VandenBerg and Webber 2019; Yu et al. 2020). GOx is a natural glucose specific enzyme that catalyzes the oxidation of glucose to hydrogen peroxide and gluconic acid. GOx based systems are combined with insulin and GOx in an environmental stimuli sensitive material (Qiu and Park 2001). The reaction between glucose and oxygen catalyzed by GOx generates $H_2O_2$ and D-glucono1,5-lactone with a decline in pH . The protein-

induced volume change of the material expands the size of the polymeric pores and thus releases insulin (Zhang et al. 2018).

Glucose-binding proteins consist of lectins that can reversibly bind to both glucose and mannose such as concanavalin A (ConA) (Pramudya and Chung 2019). In Con-A based glucose responsive delivery systems, Con A is immobilized on polymeric matrixes and upon encountering elevated glucose levels, glucose binds to Con A and competitively replaces saccharide moieties, breaking down the structure and releasing insulin (Wang et al. 2020).

PBA is a synthetic glucose binding compound and structurally reactive upon glucose binding (Antonio et al. 2019). Insulin can be released via swelling as materials with PBA moieties undergo volume expansion and pore matrix enlargement (Huang et al. 2019.) All three synthetic material strategies face drawbacks as most byproducts or the alternative form of the moiety itself cause safety or toxicity issues (Harris et al. 2013; Wu and Zhou 2013; Yang and Cao 2017; Mansoor et al. 2019). A wide variety of formulations and structures such as scaffolds, gels, self-assembled, or emulsion based nanoparticles have been explored to respond to a high glucose level by physical and chemical release mechanisms (Mi et al. 2008; Kim et al. 2012; Gu et al. 2013; Matsumoto et al. 2017).

Another upcoming method is utilizing competitive binding by taking advantage of GLUT variant binding affinities (Uldry et al. 2002; Wang et al. 2017). The GLUT transporter is abundant on the surface of red blood cell membranes (RBCs mem) and can undergo rapid and reversible binding with glucose molecules. RBC structures are highly flexible, which allows the RBCs to pass thorough narrow capillary networks and through organs such as spleen and the liver (Fang et al. 2012). The presence of complex polysaccharides on the cellular surface acts as a hydrophilic coating to achieve spatial stability (Raveendran et al. 2003; Lemarchand et al. 2004). Nanoparticles

with high surface energies can interact more with stabilized polysaccharide membranes to minimize energy compared to RBC nanoparticles surfaces that is not as reactive to membrane interactions. The stabilization ensures monolayer film coating in the presence of excess RBC membrane (Luk et al. 2014). The translocation of bilayer cellular membranes transfers to the associated membrane proteins onto the particle surfaces (Hu et al. 2011). RBCs in both animals and humans have a month-long lifespans and are inherently biocompatible, which makes RBC coated nanoparticles ideal for delivering glucose responsive insulin delivery carriers (Wang et al. 2017). RBC or erythrocyte membranes have been utilized to camouflage particles and extend sustained delivery of drugs through both passive and active mechanisms (Geng et al. 2007; Alexis et al. 2008; Yoo et al. 2010). The small size of particles reslts in accmulate in smaller blood vessels such as capillaries and arteries. By cloaking the nanoparticles in RBC membrane, particles evade immune cell detection and extend circulation times by over 24 hours (Hu et al. 2011). By 72 hours majority the particles are uptake by the reticulated endothelial system (RES) (Luk et al. 2014).

Here, we develop a glucose-responsive insulin delivery system based on the glucose transporter (GLUT) molecules including glucose transporter 4 (GLUT4), the main glucose transporter on mouse red blood cells (RBC) (Mueckler 1994; Montel-Hagen et al. 2008; Montel-Hagen et al. 2009; Vrhovac et al. 2014). Modified glucosamine-modified insulin (Glu-insulin) can bind to the nanoparticles coated with RBC membranes via a specific GLUT- glucosamine interaction (Uldry et al. 2002; Ranganathan et al. 2014). Glu-insulin has been synthesized and proven to not lose its biochemical activity and functions like native insulin by Gu et al (Wang et al. 2017). The interaction between Glu-insulin and GLUT is reversible, and bound Glu-Insulin can be released quickly, owing to the displaced interactions between free glucose and GLUT. (Figure 1). To achieve administration (Figure 2), RBC vesicles bound with Glu-insulin (GINP) were

loaded into thermo-responsive hydrogels composed of hyaluronic acid and Pluronic F127, a well characterized biocompatible biomaterial (Akash et al. 2015). When exposed to interstitial fluids with high glucose concentration, Glu-Insulin can be quickly released from RBC-insulin particles. Additionally, particles were loaded into microarrays to test the feasibility of transcutaneous administration. (Figure 3)

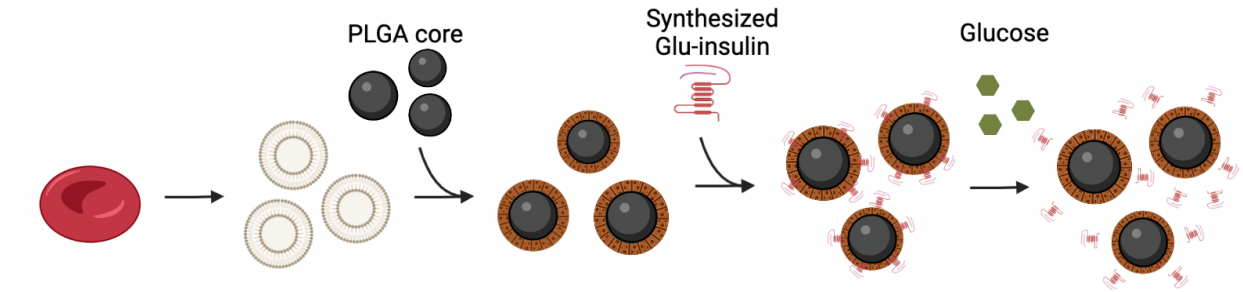



*Figure 1.* ***Schematic of RBC NP formulation and Glu-insulin release.*** *From left to right: Red blood cells are derived of their membrane. PLGA nanoparticles are coated in the RBC membrane and then Glu-insulin is added to the solution of particles. In the presence of glucose, the Glu-insulin should unbind*

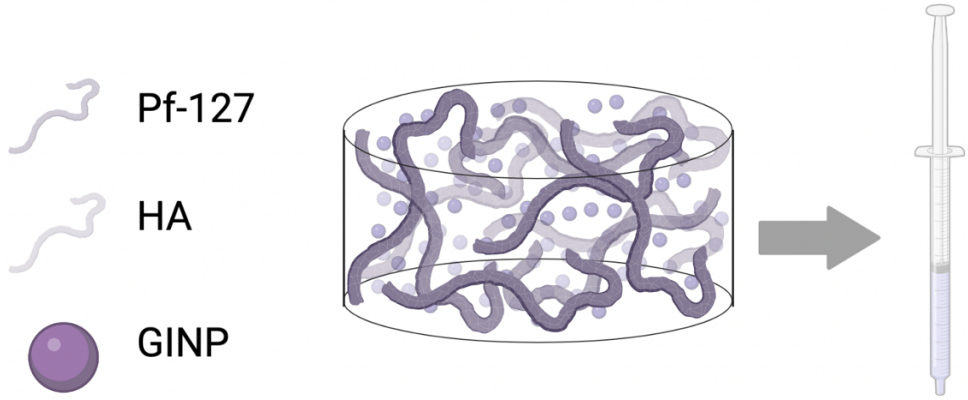



*Figure 2.* ***Schematic of GINPs in injectable thermosensitive hydrogel composed of Pf-127 and HA.*** *Schematic of hydrogel suspended in Pf-127 and HA hydrogel. The thermosensitive hydrogel can be injected.*

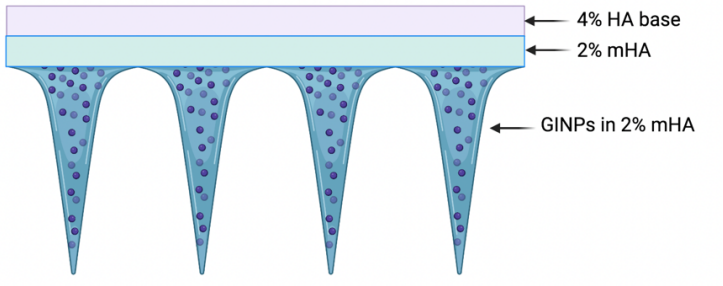



*Figure 3.* ***mHA Microneedle fabrication layering with GINP particles.*** *Layer of microneedle with 2% mHA with GINP loaded as the tip layer, 2% mHA as the base layer, and 4% HA as the final covering layer to prevent shrinkage.*

# Chapter 2. Experimental

## 2.1 RBC nanoparticles

*Erythrocyte membrane deriving*

Fresh whole blood from C57BL/6J mice was collected, centrifuged at 4 °C, and buffy coat and serum was removed to isolate the erythrocytes. The isolated cells were then washed with PBS (pH=7.4) three times to remove the glucose. Then, red blood cells (RBCs) were resuspended in DI water for a minute and then diluted to 1x PBS before centrifuged at 18000 xg for 5 minutes at 4 °C. Supernatant was discarded and above procedures are repeated until supernatant is clear. White membrane was collected and concentrated was measured with a Branford colorimetric assay (BCA). Membrane is collected and stored in DI water at -20°C until usage. Membrane is sonicated before usage.

*RBC coated membrane nanoparticles*

To prepare RBC membrane coated nanoparticles (RBCNP), a stock solution of 10mg/ml Poly(D,L-lactide-co-glycolide)(50:50)-b-poly(ethylene glycol) in acetone was prepared. The solution is stirred for 2 hours or until acetone has evaporated. Nanoparticles are measured using DLS and size is on average 90 d.nm. Membrane is then added to the nanoparticles in a 1:2 ratio in

a 75T Aquasonic bath sonicator for 2 minutes. Resulting particles are spun down and washed three times at 5000g for 10 minutes at room temperature to wash out uncoated membrane. Coated particles are then filtered with a 10k molecule weight cutoff (MWCO) Amicon Ultra-4 centrifugal filters (Millipore).

*RBC membrane nanoparticle characterization*

Particle size was measured via dynamic light scattering (DLS) on Malvern zetasizer. GINPs were heated with 2-mercaptoethanol 10% and Bromophenol blue at 80°C for 1 minute. The samples were then cooled on ice and then pipetted into a gel. The total proteins were separated on 12% sodium dodecyl sulfate (SDS) Bis-Tris-Polyacrylamide gels and then electrically transferred (at 250 mA for 75 min) onto nitrocellulose membranes (BioRad, 0.45 μm). After that, the membranes were blocked in TBST (BioRad) containing 3% milk for 1 h at room temperature, and then incubated in TBST containing 3% milk and primary antibody against GLUT4 (1:500, Novusbio, USA) overnight at 4°C. After being incubated with primary antibody, membranes were washed thrice with TBST and incubated with goat anti-rabbit IgG-HRP secondary antibody for 1 h at room temperature. Finally, membranes were stained by 1-StepTM TMB-Blotting Substrate Solution (Thermo Scientific) after thrice washing with TBST.

## 2.2 Glu-Insulin Synthesis

Glu-Insulin conjugate was synthesized by a two-step production. In brief, human insulin was thiolated by reacting with the Traut's Reagent (2-iminothiolane, Pierce) in PBS (pH=8.0) at a molar ratio of 1:5 for 2 h at 4 °C. After 2 h of reaction, excess Traut's Reagent was removed using a centrifugal filter device (molecular weight cut-off MWCO =3 kDa) to purify the SH-insulin. In the meantime, D-(+)-Glucosamine was mixed with sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (Sulfo-SMCC, Pierce) in PBS (pH=7.6) at a molar ratio of 1:1 for 2h

at RT. At last, the SMCC-activated glucose and insulin-SH were mixed in PBS (pH=7.0) at a molar ratio of insulin: glucosamine=1:100. After 24 h reaction at 4 °C, the excess glucosamine was removed using the centrifugal filter device (MWCO =3 kDa). The obtained Glc-Insulin was stored at 4 °C until use.

*Glu-Insulin FITC*

FITC-NHS was reconstituted in DMSO and mixed with insulin in PBS on ice for 4 hours to obtain FITC-labeled insulin. The fluorescent insulin was used following the same protocol as the synthesis of glu-insulin conjugates to produce FITC-labeled glu-insulin.

*Glu-Insulin analysis*

Matrix-assisted laser desorption/ionization mass spectrometry (MADLI-TOF MS) was utilized to analyze the resulting glu-insulin. Parameters were acetonitrile to water ratio as 70:30 and matrix material was sinapic acid.

## 2.3 Glu-insulin nanoparticles

RBCNPs in DI water were mixed with Glu-insulin solution and mixed at 4 °C overnight. Glu-insulin attached to RBCNP (GINPs) were subsequently centrifuged and washed with DI water at 5000 g for 10 minutes three times to remove the unloaded Glu-insulin.

*Glu-Insulin nanoparticle characterization*

To confirm the attached Glu-insulin on the surface, fluorescent RBCNPs and glu-insulin were imaged. 1,1-Dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine (DiD) was mixed with membrane for 5 minutes before coating. Likewise, Glucose inuslin conjugated with FITC was incubated overnight with fluorescent RBCNPs overnight at 4 °C in the dark. GINPs were subsequently centrifuged and washed with DI water at 5000 g for 10 minutes three times to remove

the unloaded glu-insulin. Resulting GINPs are added to RAW264.7 murine macrophage cell line 3 hours before imaging.

*Glu-insulin nanoparticle in vitro release and loading*

GINPs were suspended in 500ul solutions of either 0 mg/dL, 100 mg/dL, 400 mg/dL of glucose in PBS at 37 °C. GINPs were centrifuged at 5000g for 10 minutes before extracting supernatant. After extracting appropriate amount of supernatant, groups were maintained at 500ul of each respective solution. Samples were taken at 20 minutes, 1 hr, 2hr, and 6hr. At 24 hrs, samples directly from sample to quantify total Glu-insulin loading. Results were analysis by Human Insulin ELISA kit according to the manufacturer's protocol. Glu-insulin and ELISA concentration was verified previously byt Wang et al. (Wang et al. 2017).

## 2.4 GINP in hydrogel

*Hydrogel formulation*

Pluronic f127 (PF127) and hyaluronic acid (HA) were added to deionized water separately at 40% and 4% weight percentage respectively. The resulting solutions were then allowed to dissolve at 4°C for 7 days. After dissolution, PF127 and HA were combined in a 1:1 ratio and allowed to amalgamate overnight at 4°C. The resulting hydrogel of 20% PF127 and 2% HA was stored at 4°C before usage.

*In vitro release*

GINPs were suspended in hydrogel in a ratio of 2:3 respectively. Then, 100ul of the combined GINP and hydrogel (GINP-H) were added to 2K MWCO Slide-A-Lyzer MINI Dialysis Devices. The dialysis devices were then submerged in solutions of either 0 mg/dL, 100 mg/dL, 400 mg/dL of glucose in PBS at 37 °C. Extracted solution were centrifuged at 5000g for 10 minutes before collecting supernatant. Groups were maintained at 2 ml of each respective solution. Samples

were taken at 20 minutes, 1 hr, 2hr, and 10hr. Results were analysis by Human Insulin ELISA kit according to the manufacturer's protocol.

## 2.5 Glu-insulin nanoparticle Microneedle

*Fabrication*

Methacrylate hyaluronic acid (mHA) and 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone was mixed in DI water at 70°C so the final concentration is 2% and 1% respectively. 100ul of 2% Methacrylate HA (mHA) polymer solution and GINPs were loaded into tips of a mold with 200x200x600 μm pyramid tips and 500 μm spacing through centrifugation mold and spun down at 2150g for 3 and another layer of just mHA was utilized as a base layer to form a GINP loaded HA MN (GINP-MN). After the mold was crosslinked via UV light for 5 minutes. A layer of 4% HA was then spread on top of the mold and allowed for drying overnight. The purpose of depositing HA as the final layer is to prevent the slight base layer shrinkage as observed when microneedles were fabricated from just mHA.

*In vitro release*

mHA were submerged in 500ul solutions of either 0 mg/dL, 100 mg/dL, 400 mg/dL of glucose in PBS at 37 °C. Extracted solution were centrifuged at 5000g for 10 minutes before collecting supernatant. Groups were maintained at 500ul of each respective solution. Samples were taken at 20 minutes, 1 hr, 2hr, and 10hr. Results were analysis by Human Insulin ELISA kit according to the manufacturer's protocol.

## 2.6 Statistical analysis

One-way ANOVA with post-hoc Turkey analysis was performed on experiments with 3 or more groups, homogeneity of variances. The threshold for statistical significance was $P < 0.05$ and Geisser-Greenhouse correction was utilized.

# Chapter 3. Results and Discussion

## 3.1 RBC nanoparticle

RBC cell membranes were derived from blood from adult C57BL/6J containing GLUT4 using a hypotonic treatment (Figure 1). Dynamic light scattering verified that the coated particles were 167 d.nm. (Figure 5) The negatively charged nanoparticles and the presence of a silica moiety produces a strong electrostatic repulsion that ensures nanoparticle and intracellular fusion. This ensures that outer membrane is orientated outward and surface protein morphology is preserved. (Xia et al. 2019) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) together with Western blot analysis was carried out to verify the presence of GLUTs on RBCNPs. Membrane stripped from RBC NPs by boiling the particles in 80°C and tested in a WB comparing unprocessed membrane and unbound membrane from particle coating process. In Figure 4, signal was detected at ~55kda which confirms that PLGA particle is coated in RBC membrane containing glucose receptors. In this, a 1:2 ratio of particle to membrane was utilized but a significant amount of membrane did not coat the particle as seen by the presence of a significant blot in the RBC NP flow-through lane. The sample was taken from after particle sonication with RBC membrane. Size of particle is crucial as diameter of particle dictates circulation patterns and potential release from biomaterial systems (Hoshyar et al. 2016). DLS analysis showed that GINPs were around 167 d.nm, an adequate size to entrap into biomaterials and avoid detection from the RES. The particles were uniform in size with a polydispersity index of 0.03.

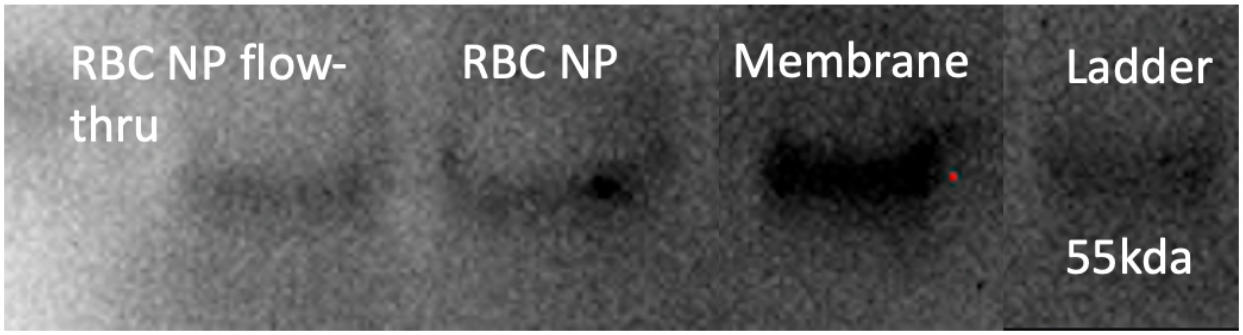



*Figure 4.* ***Western blot of RBC NP flow through, RBC NP, and RBC membrane to confirm presence of GLUT receptors****. Samples were heated with 2-mercaptoethanol 10% and Bromophenol blue at 80°C for 1 minute. After procedure followed manufacture's protocol. Samples from left to right are: RBC NP Flow-through, RBC NP, and membrane.*

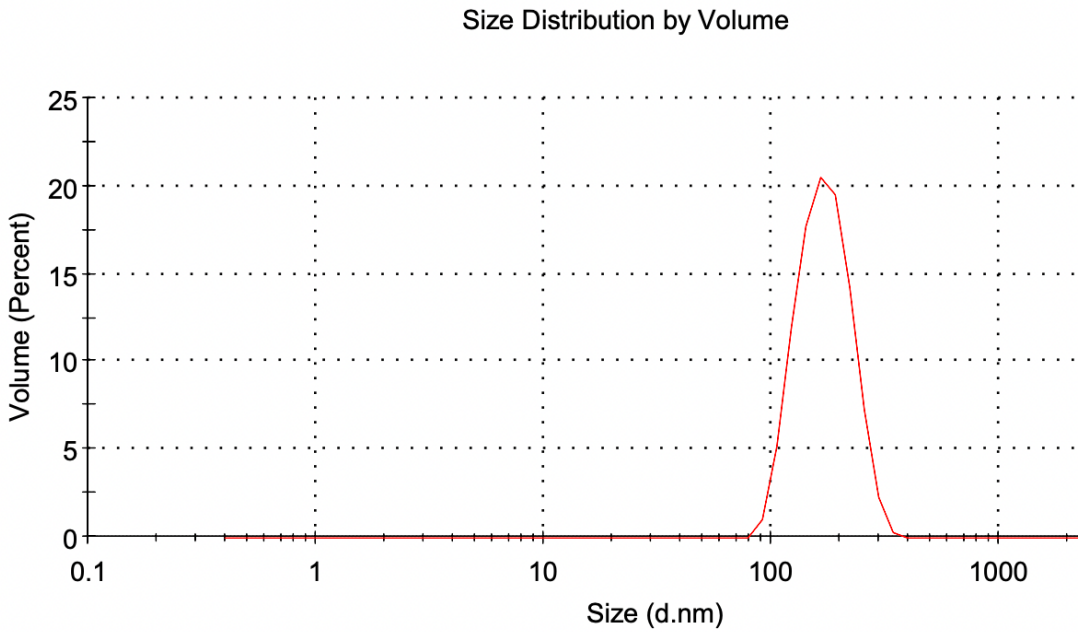



*Figure 5.* ***DLS of 167 d.nm RBC NPs.*** *RBC particles were suspended in DI water after sonication coating and washing. Size was measured via a Malvern Zetasizer.*

## 3.2 Glu-Insulin

Poly(ethylene glycol) (PEG)-protein conjugates exhibit increased plasma half life, reduced immunogenicity and antigenicity, improved resistance to proteolysis, and increased aqueous/organic solubility compared to native proteins (Nucci et al. 1991; Delgado et al. 1992) Insulin has three bioconjugate sites (primary amine groups): GlyA1, PheB1, and LysB29. The

conjugation attachment to PheB1 or LyseB29 does not negatively impact protein's bioactivity and attachment to these residues helps improve insulin's physicochemical and pharmacological activity. (Gliemann and Gammeltoft 1974; Hashimoto et al. 1989; Murrayrust et al. 1992) Glucosamine was conjugated to insulin via a bifunctional NHS-maleimide linker (i.e., sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate), forming Glu-insulin in a two-step reaction (Figure 6). The formation was verified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) as seen in Figure 7. Glu-insulin has been previously synthesized and the effectiveness has been verified by Wang et al (Wang et al. 2017). In Figure 7, Peak at 6200 nm indicates a single insulin with a single GLUT, other peaks indicate either unconjugated insulin (5.8 Kda) or GLUT conjugated with more than one insulin. It has been found by Wang et al. that the circular dichroism (CD) spectra of native insulin and Glu-Insulin were virtually superimposable (Want et al. 2017).. Importantly, the Glu-Insulin conjugate and native insulin did not display any significant difference in their bioactivity profiles upon administration in STZ-induced type 1 diabetic mice in Wang et al.'s previous experiment with Glu-Insulin (Want et al. 2017). The modification of insulin with glucosamine had a negligible impact on bioactivity relative to the native insulin.

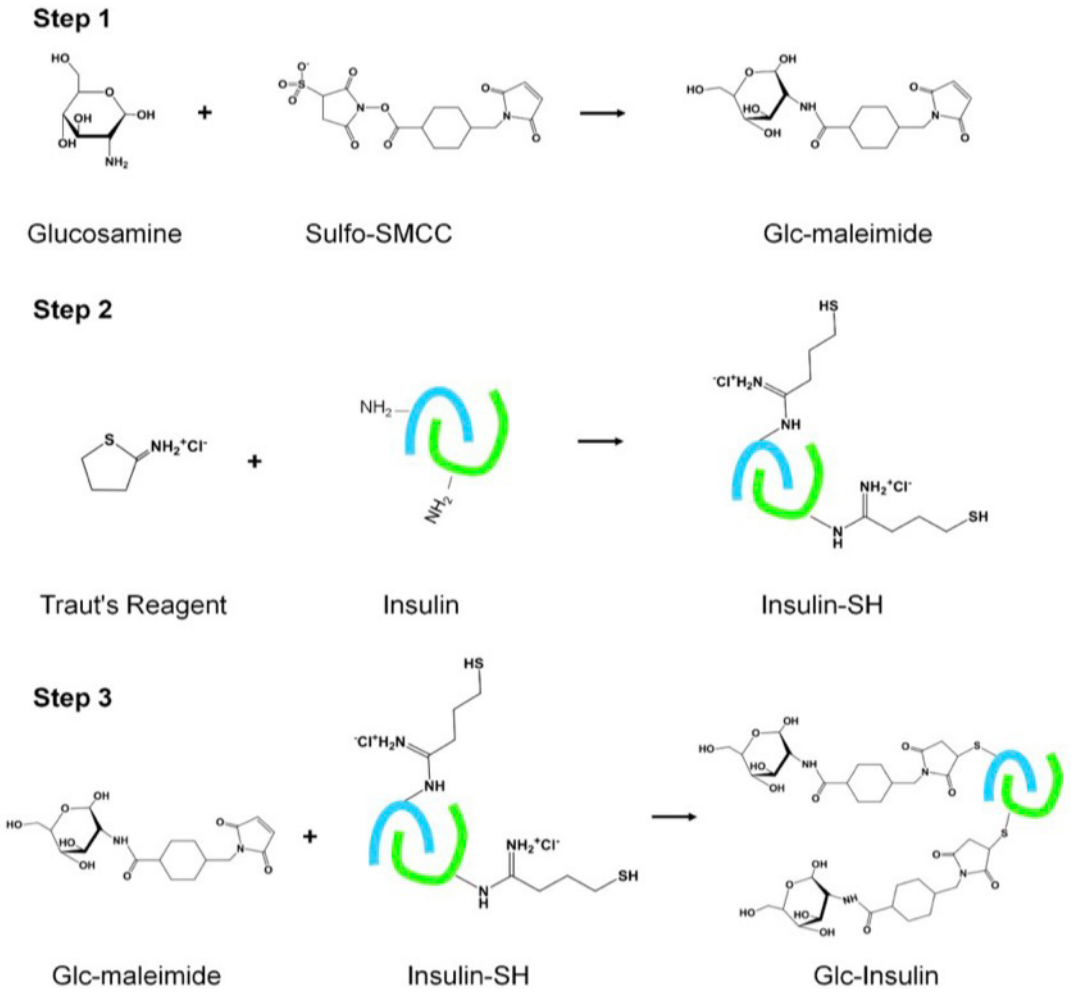



*Figure 6.* ***Glu-Insulin's two- step reactions*** *Step 1. D-(+)-Glucosamine was mixed with sulfosuccinimidyl-4-(N-maleimidomethyl)- cyclohexane-1-carboxylate at a molar ratio of 1:1 for 2h at RT. Step 2. Insulin was thiolated by reacting with the Traut's Reagent at a molar ratio of 1:5 for 2 h at 4 °C. Step 3. the SMCC-activated glucose and insulin-SH were mixed in PBS at a molar ratio of insulin: glucosamine=1:100. The obtained Glc-Insulin was stored at 4 °C until use.*

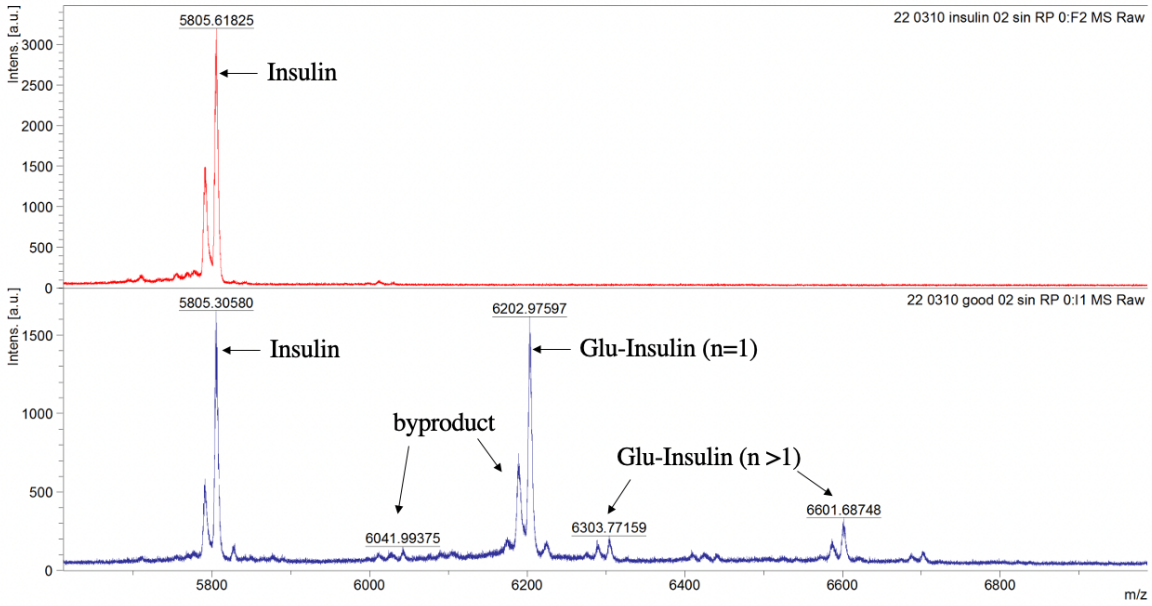
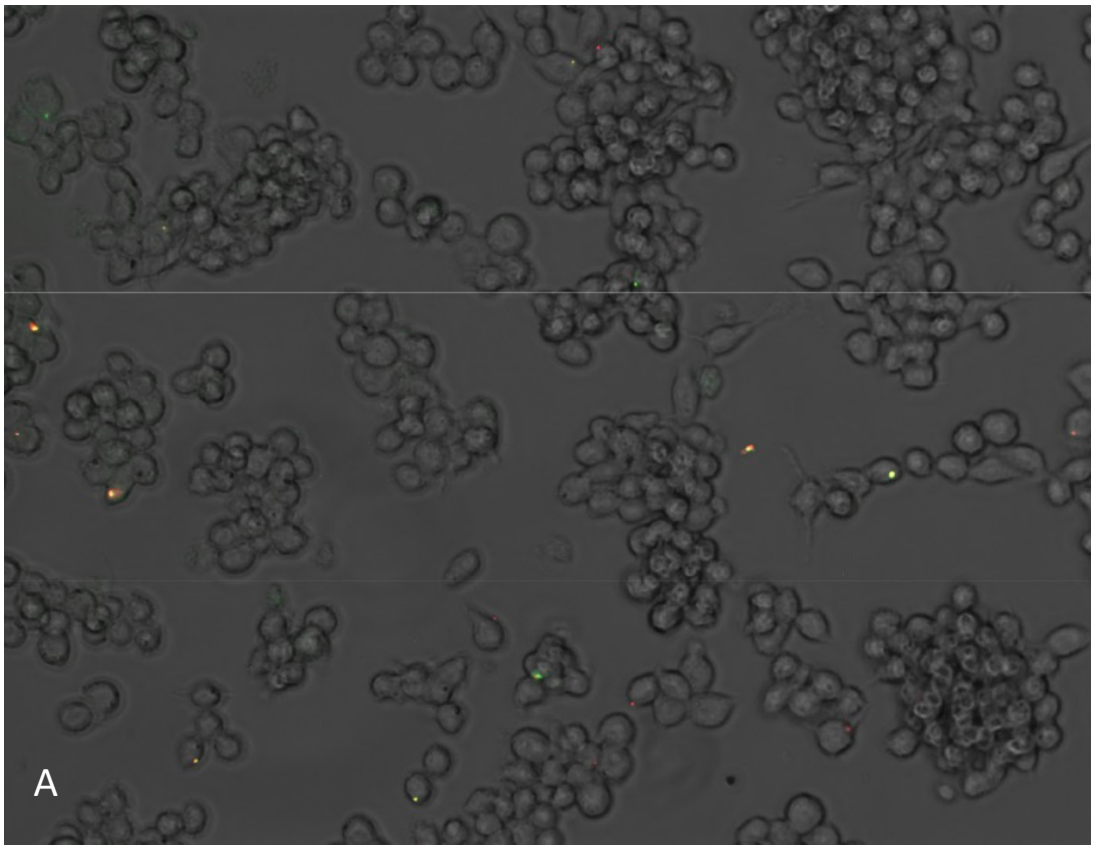



*Figure 7.* ***Mass-Spectrometry of Insulin (top) and Glu-insulin(bottom)****. Glu-Insulin was measured via mass spectrometry and results show that roughly 50% of the insulin was unreacted but the majority of the reacted insulin is glucose conjugated with a single insulin. Other peaks indicate glucose with multiple insulins.*

## 3.3 Glu-insulin nanoparticles

Glu-Insulin was bound to RBCNPs through the specific Glut-glucosamine interaction, which is observed through confocal imaging in Figure 8. GINPs were incubated with and uptake by RAW264.7 macrophage cells for 2 hours to ensure that the particles were within the same plane for confocal imaging. The DiD labeled particles are represented as the red and FITC conjugated Glu-insulin are the green dots in Figure 8. Majority of the GINPs had weak FITC signal and overall less Glu-insulin detected (not shown). This signal weakening was confirmed to be a loading limitation as explained in the following section.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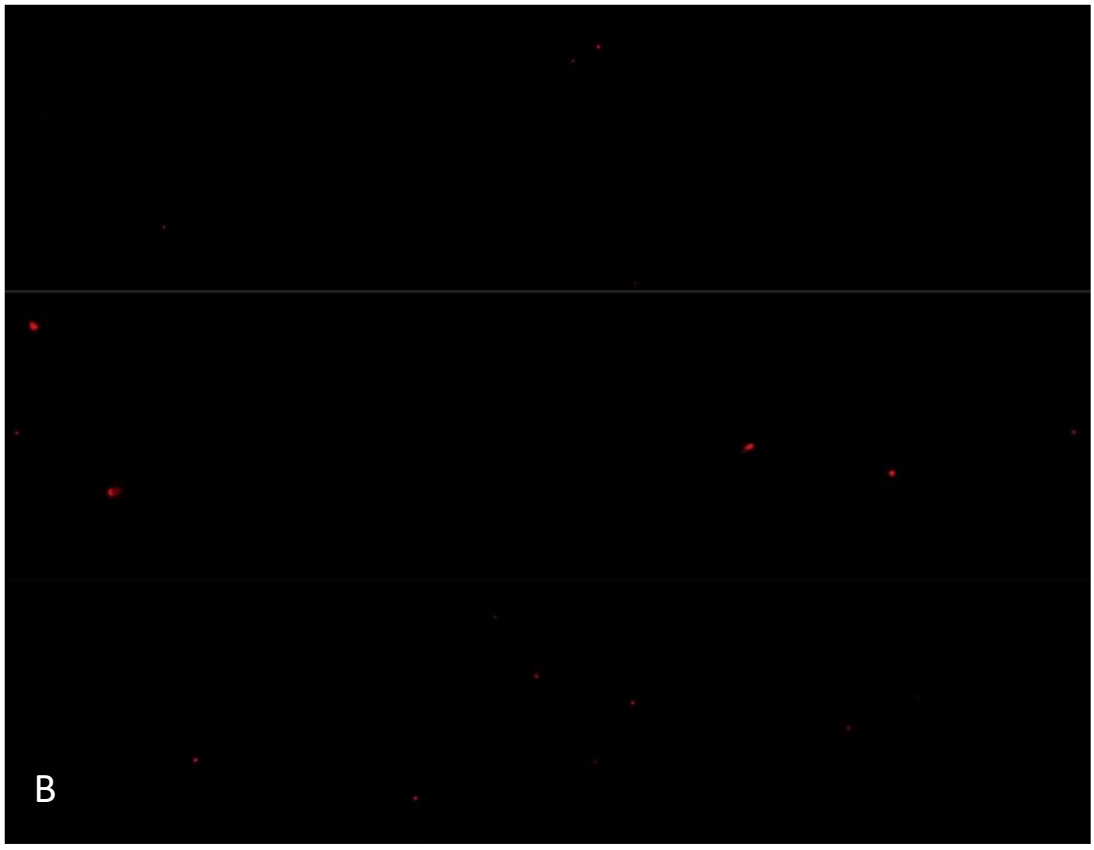
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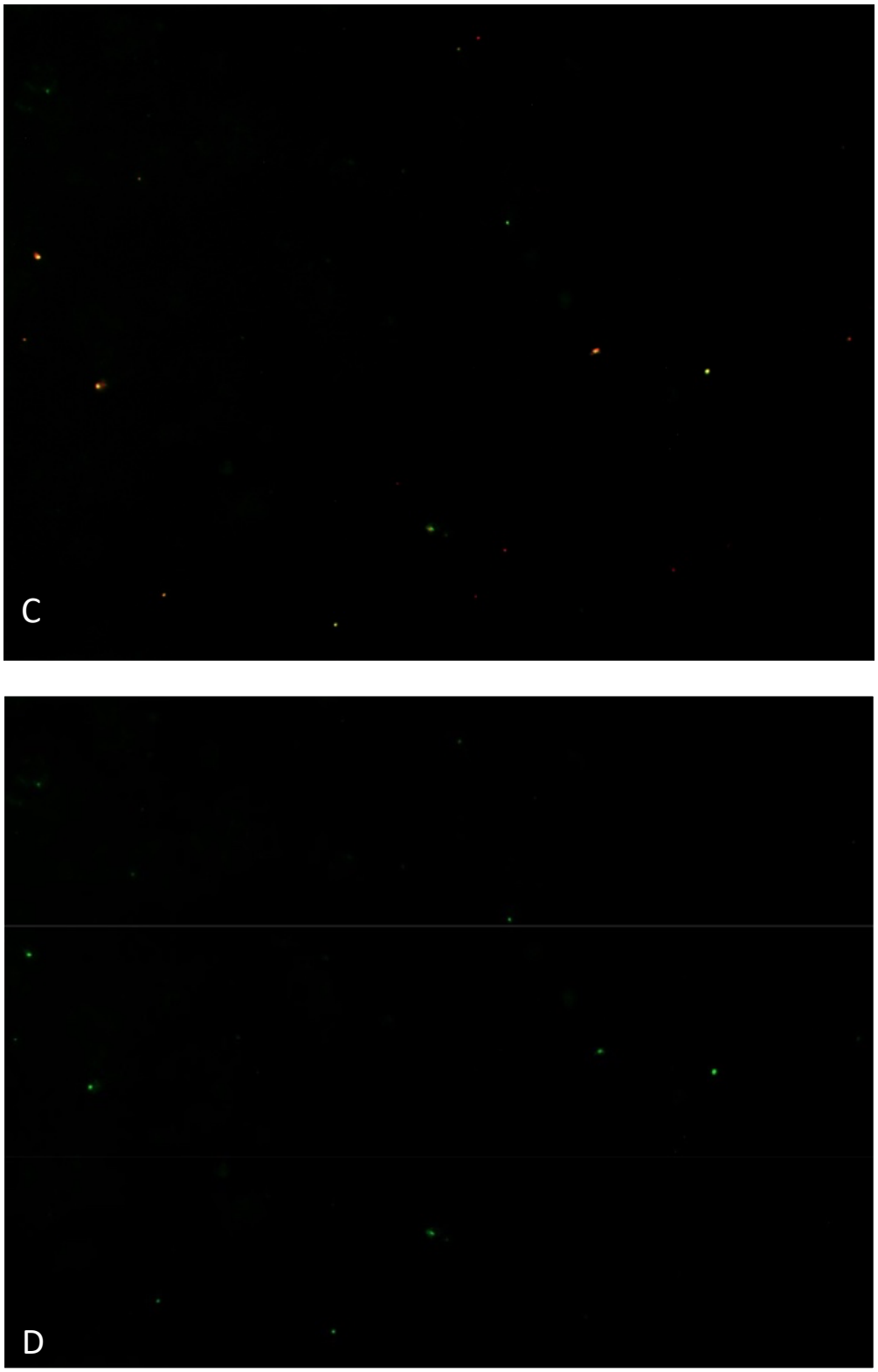


*Figure 8.* ***a) Image of GINPs incubated with RAW264.7 to show the co-localization of the Glu-inuslin (green) and RBC NPs(red). B) RBCNP with DiD dye. C) overlay of RBCNP and Glu-insulin D) Yellow/orange signals indicate co-location*** *RBC NPs were tagged with DiD dye and Glu-insulins were conjugated with FITC. The*

*resulting fluorescent GINPs were incubated with RAW264.7 cells to ensure that the particles are all within the same plane.*

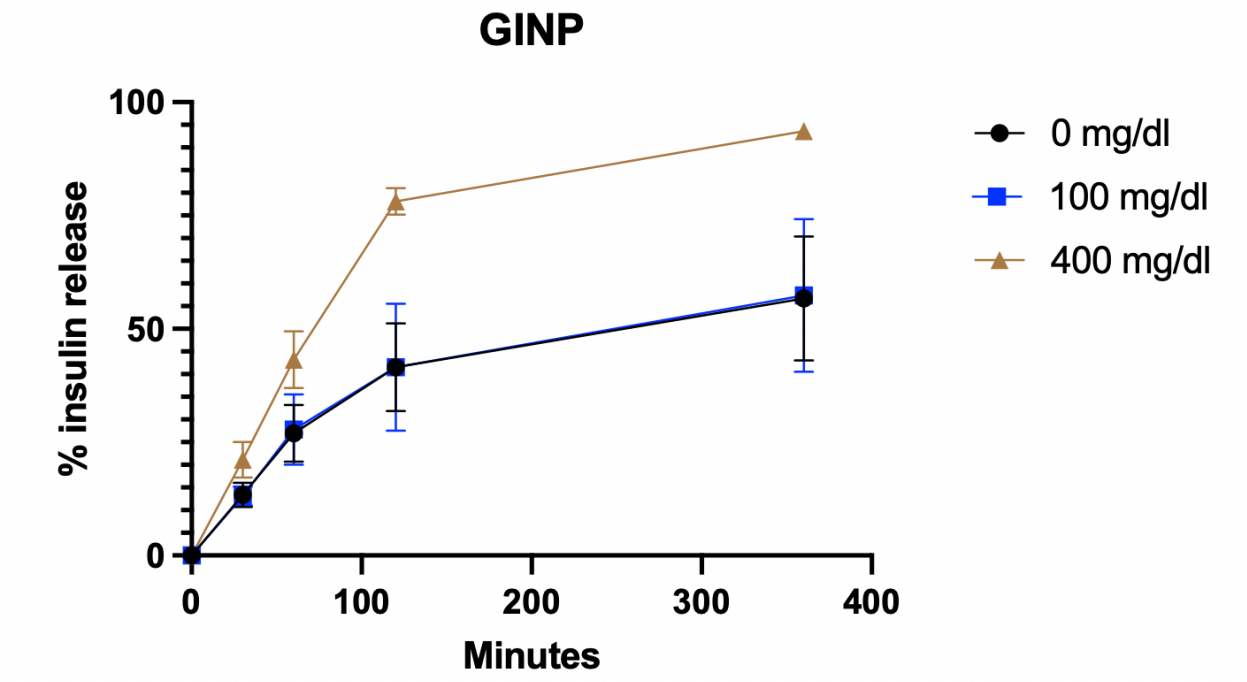


*Figure 9.* ***Release profile of GINP in different levels of glucose over the course of 6 hours.*** *Groups were placed in PBS, 100mg/dl, or 400 mg/dl of glucose.*

To evaluate the glucose-responsive release of insulin, GINPs were dispersed in phosphate buffer saline (PBS) with different levels of glucose. Interstitial fluid has been previously reported to have about 100 mg/dl of glucose and 400 mg/dl of glucose in normoglycemic and hyperglycemic levels respectively. For those reasons, we chose to test the GINP and further systems with PBS as the control, 100 mg/dl glucose to represent normoglycemic conditions, and 400 mg/dl to represent hypoglycemic conditions. The released Glu-insulin from GINPs were measured by ELISA. In Figure 9 , compared to control or normoglycemic conditions (0 or 100 mg/dl glucose), the release of Glu-insulin significantly increased in the hyperglycemic condition (400 mg/dl glucose), suggesting glucose responsive release of insulin from GINPs. The binding can be competitively

replaced by D-glucose, as seen in Figure 9 as more Glu-insulin was release in the 400 mg/dl solution compared to the 100 mg/dl solution. Insulin release from GINP vesicles incubated in 400 mg/dl glucose solution were higher, indicating rapid release of Glu-Insulin under hyperglycemic conditions. The release of Glu-insulin in normoglycemic or 100 mg/dl group is not significantly different than that of the PBS or control group. The notable change of release rate of Glu-insulin in 400 mg/dl compared to 100mg/dl or PBS indicates that the glucose sensitive release portion of the system is functional and responsive to different levels of glucose.

However, there is some Glu-insulin release without any presence of glucose or with low levels of glucose. This indicates that the binding affinity between GLUT and Glu-insulin is relatively low, and could potentially contribute to hypoglycemia in patients, as 40% of Glu-insulin is passively released by 2 hours. This problematic insulin release without the presence of glucose needs to be improved as the therapeutic range for insulin release is narrow and can be lethal. One potential solution to this is to pre-incubate the particle solution in PBS or ionized solution instead of deionized solution for at least 2 hours and washing thoroughly before usage in order to avoid the burst release. Another solution is to re-formulate the Glu-insulin to optimize the binding affinity. However, this was not accomplished in this project as the aim of the project was to observe the inherent release rates of Glu-insulin different delivery system.

The glu-insulin loading content of GINP was measured to be 0.5% wt by ELISA. The total amount of insulin delivered per particle is relatively low due to the fact that human erythrocyte plasma membrane are composed of 10% GLUT1 receptors (Gorga and Lienhard 1982). In order to deliver, the daily insulin dose for a 70kg person, the system would have to deliver 38.5 U or 80 mg of GINPs to the patient (patient insulin estimates from (Center 2022)). The amount of particle

required is very high and labor intensive, which would not be feasible for translational application unless further modifications to increase loading on particles are achieved.

A burst release is observed in these particles with the rate of release greatly decreasing after 2 hours. Additionally, by 2 hours, 80% and 40% of the Glu-insulin is released in a hyperglycemic state and normoglycemic state respectively. As of the current formulation, GINP is suitable for a singular glucose responsive treatment of insulin at low doses. At higher dosages, this system would not be ideal for once-daily insulin regulation, as the burst release and the passive Glu-insulin release may contribute to rapid hypoglycemia. However, the GINP platform shows promise if the loading and passive release are optimized as RBC NPs have days long retention in circulation (Fang et al. 2012).

## 3.4 Glu-Insulin in Hydrogel

To extend the release profile, GINPs were loaded into a 20% PF127 2% HA thermosensitive hydrogel. The lower critical solution temperature of PF127 and thus the hydrogel can be varied from 25 to 37 °C depending on concentration of PF127 in the formulation.The resulting particle loaded hydrogel (GINP-H) was then tested in vitro with varying levels of glucose that represents normoglycemic and hypoglycemic states in PBS to observe the release profile (Figure 10).

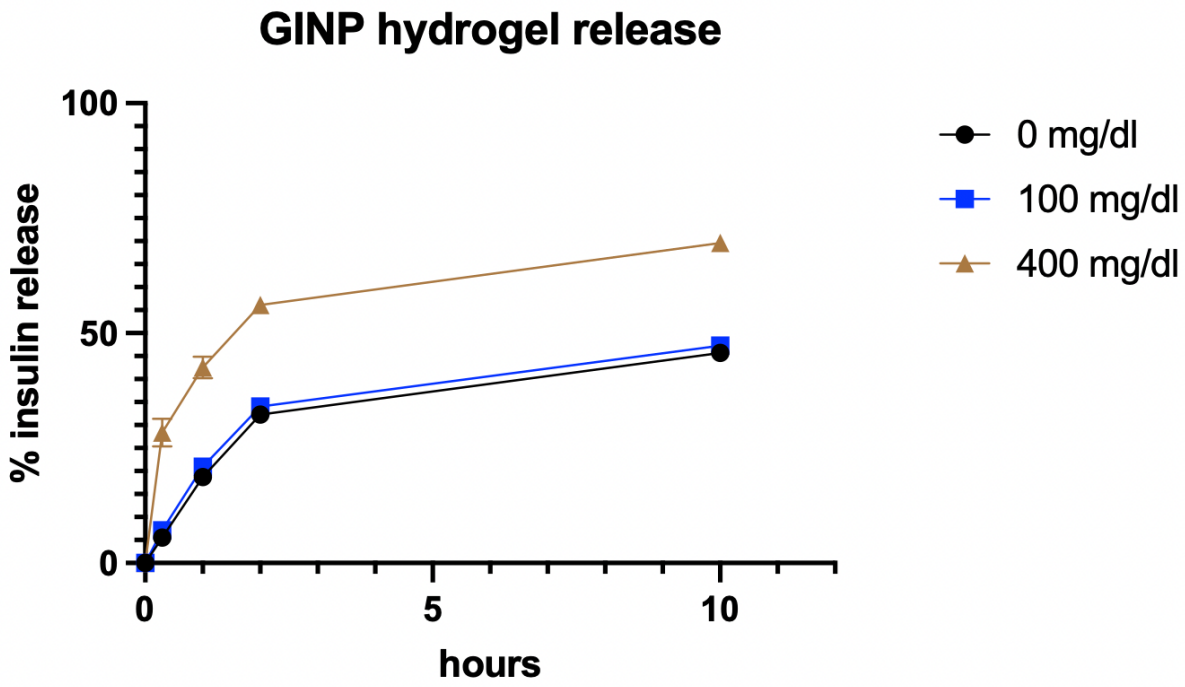


*Figure 10.* ***Release profile of GINP-H in different levels of glucose over the course of 10 hours in different glucose concentrations. .*** *Groups were placed in PBS, 100mg/dl, or 400 mg/dl of glucose.*

As expected, the release of Glu-insulin in GINP-His is slower in a normoglycemic state compared to the hyperglycemic state, similar to the results of just GINP. The burst release has been attenuated by the incorporating the particles into a hydrogel delivery system. However, the passive release of Glu-insulin has been slowed but not fully eliminated, as the overall release rate of the system has significantly decreased. In this system, it takes 10 hours for the system to release 40% Glu-insulin in normoglycemic conditions compared to 2 hours for just particles. These results suggest that the hydrogel hinders the diffusion of Glu-insulin or glucose, thus reducing the overall release rate.

The attenuated release rate shows promise for a larger volume of GINP delivery as the passive release of Glu-insulin has overall decreased. These release trends indicate that PF127+HA hydrogel serves as an effective delivery method for prolonging the release rates while also allowing for diffusion of Glu-insulin sand other fluids to permeate across the gel.

## 3.5 Glu-Insulin nanoparticles in microneedles

With the promising results of a material release system, we then incorporated the particles into microneedles to facilitate transdermal delivery for a painless and convenient administration. Methacrylate hyaluronic acid was utilized for its material properties including its biostability and biocompatibility, and ability to provide a sustained release.

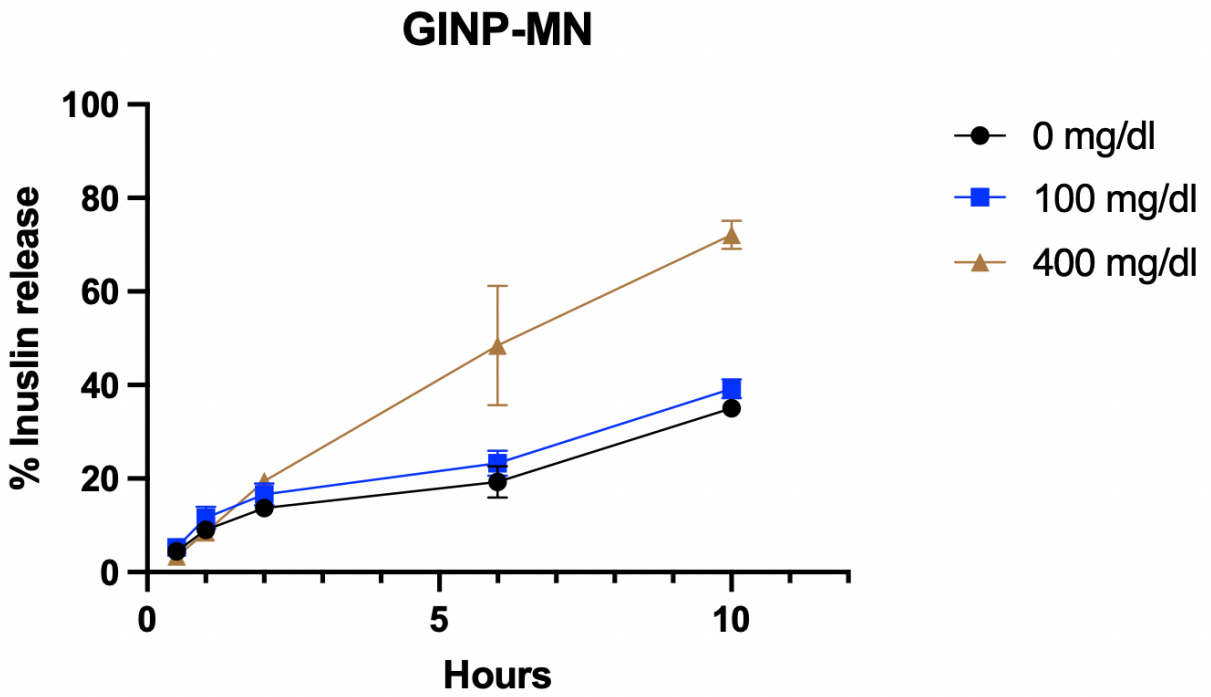


*Figure 11.* ***Release profile of GINP-MN in different levels of glucose over the course of 10 hours in different glucose concentrations .*** *Groups were placed in PBS, 100mg/dl, or 400 mg/dl of glucose.*

Similarly, the GINP-MN's release profile was studied in the same set up as the GINP and GINP-H in vitro release profiles. in Figure 11, data shows that the release profile follows the same trend following the GINP and GINP-H where in higher levels of glucose, more Glu-insulin is released. However, the burst release in GINP-MNs is greatly attenuated compared to GINP particles but the

diffusion of Glu-insulin in the absence of glucose is still observed. Both the GINP-H and the GINP-MN system release roughly 40% of the Glu-insulin levels by 10 hours in a normoglycemic state and are responsive to a change of glucose levels.

# Chapter 4. Conclusion

The need for a smart glucose responsive delivery system that effectively delivers insulin without interrupting a patient's daily life is currently being investigated. Modern solutions face not only technical and biological challenges but also cost barriers as current smart insulin delivery systems are unaffordable to most. Therefore, an effective self-contained glucose responsive insulin delivery system must present a low barrier of access for patients. The microarray patch fabricated in this project is low cost due to the usage of common materials.

Challenges to glucose-responsive insulin delivery include a rapid response, biocompatibility, and long-term retention/release. This strategy based on delivering RBC nanoparticles containing surface bound GLUT with glucosamine-modified insulin in a sustained release microneedle was effectively developed for a glucose-responsive insulin delivery. By taking advantage of competitive binding between glucose insulin and glucose, glu-insulin can be quickly released. Unlike most existing smart insulin delivery systems, this system avoids the need for multiple components such as a continuous glucose monitor and an insulin pump, which eliminates potential errors by reducing the complexity of the system. The reversible binding between glu-insulin and GLUT actives rapidly, which enables a quick real time response without interference. By validating the GLUT and glu-insulin release abilities in two different sustained release platforms, this project has shown the promise for future developments of a more convenient diabetes treatment. However, the passive release of inuslin in low concentrations of glucose must be eliminated as the therapeutic range of inuslin is narrow. The hydrogel system allows for a single insulin injection that could potentially be tuned to a release course of multiple days as the hydrogel formulation has been proven in other papers to be stable for 3 to 5 days in vivo. The integration into a microneedle array allows for a long term sustained release, and potentially paves the way

for an over-the-counter alternative to daily glucose monitoring and insulin injection for patients. GINP-MN system allows for a sustained release of Glu-insulin while also attenuating a burst release and is potentially removable in case the patient develops hypoglycemia.

In order to improve the insulin delivery system, the amount of Glu-insulin loaded on nanoparticles must be significantly increased. The amount of resources dedicated to creating one daily dosage of 38.5 U insulin with 0.5% wt loading of nanoparticles would not be feasible from a cost perspective to make 80 mg of GINPs (Trief et al. 2016). Another solution is to re-formulate the Glu-insulin to optimize the binding affinity and increase the overall total of Glu-insulin per particle. Additionally, the microneedle platform cannot load enough particles without major adjustments to the particle loading capacity as the workable solution is relatively low, less than 500ul. Utilizing a hydrogel is a good compromise to ensure a sustained release of Glu-insulin as a scalable hydrogel system can load significantly more particles without compromising the innate structure and ability. However, hydrogel cannot be extracted once injected and the diffusion of Glu-insulin without any glucose present may contribute to hypoglycemia.

Overall, the results from this project highlight a promising method of circumventing complex and patient involved insulin delivery method by utilizing GLUT transporter and GLUT variants in two delivery systems - ultimately enabling glucose-responsive insulin release. Major improvements to get this to a more translational stage would be to eliminate passive insulin release, improve loading of Glu-insulin, and test the system out in vivo models.

# Chapter 5. References


Alexis, F., Pridgen, E., Molnar, L.K., and Farokhzad, O.C. (2008). Factors affecting the clearance and biodistribution of polymeric nanoparticles. *Mol Pharm* 5(4), 505-515. doi: 10.1021/mp800051m.

American Diabetes, A. (2020). 7. Diabetes Technology: Standards of Medical Care in Diabetes-2020. *Diabetes Care* 43(Suppl 1), S77-S88. doi: 10.2337/dc20-S007.

Antonio, J.P.M., Russo, R., Carvalho, C.P., Cal, P., and Gois, P.M.P. (2019). Boronic acids as building blocks for the construction of therapeutically useful bioconjugates. *Chem Soc Rev* 48(13), 3513-3536. doi: 10.1039/c9cs00184k.

Bankar, S.B., Bule, M.V., Singhal, R.S., and Ananthanarayan, L. (2009). Glucose oxidase--an overview. *Biotechnol Adv* 27(4), 489-501. doi: 10.1016/j.biotechadv.2009.04.003.

Brownlee, M., and Cerami, A. (1979). A glucose-controlled insulin-delivery system: semisynthetic insulin bound to lectin. *Science* 206(4423), 1190-1191. doi: 10.1126/science.505005.

Center, D.T. (2022). "Calculating Insulin Dose". (UCSF: UCSF).

Chou, D.H., Webber, M.J., Tang, B.C., Lin, A.B., Thapa, L.S., Deng, D., et al. (2015). Glucose-responsive insulin activity by covalent modification with aliphatic phenylboronic acid conjugates. *Proc Natl Acad Sci U S A* 112(8), 2401-2406. doi: 10.1073/pnas.1424684112.

Delgado, C., Francis, G.E., and Fisher, D. (1992). The Uses and Properties of Peg-Linked Proteins. *Critical Reviews in Therapeutic Drug Carrier Systems* 9(3-4), 249-304.

Fang, R.H., Hu, C.M.J., and Zhang, L.F. (2012). Nanoparticles disguised as red blood cells to evade the immune system. *Expert Opinion on Biological Therapy* 12(4), 385-389. doi: 10.1517/14712598.2012.661710.

Geng, Y., Dalhaimer, P., Cai, S., Tsai, R., Tewari, M., Minko, T., et al. (2007). Shape effects of filaments versus spherical particles in flow and drug delivery. *Nat Nanotechnol* 2(4)**,** 249-255. doi: 10.1038/nnano.2007.70.

Gilroy, C.A., Luginbuhl, K.M., and Chilkoti, A. (2016). Controlled release of biologics for the treatment of type 2 diabetes. *J Control Release* 240**,** 151-164. doi: 10.1016/j.jconrel.2015.12.002.

Gliemann, J., and Gammeltoft, S. (1974). Biological-Activity and Binding Affinity of Modified Insulins Determined on Isolated Rat Fat-Cells. *Diabetologia* 10(2)**,** 105-113. doi: Doi 10.1007/Bf01219665.

Gorga, F.R., and Lienhard, G.E. (1982). Changes in the intrinsic fluorescence of the human erythrocyte monosaccharide transporter upon ligand binding. *Biochemistry* 21(8)**,** 1905-1908. doi: 10.1021/bi00537a031.

Gu, Z., Dang, T.T., Ma, M., Tang, B.C., Cheng, H., Jiang, S., et al. (2013). Glucose-responsive microgels integrated with enzyme nanocapsules for closed-loop insulin delivery. *ACS Nano* 7(8)**,** 6758-6766. doi: 10.1021/nn401617u.

Harris, J.M., Reyes, C., and Lopez, G.P. (2013). Common causes of glucose oxidase instability in in vivo biosensing: a brief review. *J Diabetes Sci Technol* 7(4)**,** 1030-1038. doi: 10.1177/193229681300700428.

Hashimoto, M., Takada, K., Kiso, Y., and Muranishi, S. (1989). Synthesis of Palmitoyl Derivatives of Insulin and Their Biological-Activities. *Pharmaceutical Research* 6(2)**,** 171-176. doi: Doi 10.1023/A:1015992828666.

Hoshyar, N., Gray, S., Han, H., and Bao, G. (2016). The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction. *Nanomedicine (Lond)* 11(6)**,** 673-692. doi: 10.2217/nnm.16.5.

Hu, C.M.J., Zhang, L., Aryal, S., Cheung, C., Fang, R.H., and Zhang, L.F. (2011). Erythrocyte membrane-camouflaged polymeric nanoparticles as a biomimetic delivery platform. *Proceedings of the National Academy of Sciences of the United States of America* 108(27)**,** 10980-10985. doi: 10.1073/pnas.1106634108.

Huang, Q., Wang, L., Yu, H., and Ur-Rahman, K. (2019). Advances in phenylboronic acid-based closed-loop smart drug delivery system for diabetic therapy. *J Control Release* 305**,** 50-64. doi: 10.1016/j.jconrel.2019.05.029.

Kim, H., Kang, Y.J., Kang, S., and Kim, K.T. (2012). Monosaccharide-responsive release of insulin from polymersomes of polyboroxole block copolymers at neutral pH. *J Am Chem Soc* 134(9)**,** 4030-4033. doi: 10.1021/ja211728x.

Akash, M. and Rehman, K. (2022). Recent progress in biomedical applications of Pluronic (PF127): Pharmaceutical perspectives. *J Control Release* 209,120-138. doi.org/10.1016/j.jconrel.2015.04.032

Lemarchand, C., Gref, R., and Couvreur, P. (2004). Polysaccharide-decorated nanoparticles. *Eur J Pharm Biopharm* 58(2)**,** 327-341. doi: 10.1016/j.ejpb.2004.02.016.

Long, M.T., Coursin, D.B., and Rice, M.J. (2019). Perioperative Considerations for Evolving Artificial Pancreas Devices. *Anesth Analg* 128(5)**,** 902-906. doi: 10.1213/ANE.0000000000003779.

Luk, B.T., Hu, C.M.J., Fang, R.N.H., Dehaini, D., Carpenter, C., Gao, W.W., et al. (2014). Interfacial interactions between natural RBC membranes and synthetic polymeric nanoparticles. *Nanoscale* 6(5), 2730-2737. doi: 10.1039/c3nr06371b.

Mansoor, S., Kondiah, P.P.D., Choonara, Y.E., and Pillay, V. (2019). Polymer-Based Nanoparticle Strategies for Insulin Delivery. *Polymers (Basel)* 11(9). doi: 10.3390/polym11091380.

Matsumoto, A., Tanaka, M., Matsumoto, H., Ochi, K., Moro-Oka, Y., Kuwata, H., et al. (2017). Synthetic "smart gel" provides glucose-responsive insulin delivery in diabetic mice. *Sci Adv* 3(11), eaaq0723. doi: 10.1126/sciadv.aaq0723.

Mi, F.L., Wu, Y.Y., Lin, Y.H., Sonaje, K., Ho, Y.C., Chen, C.T., et al. (2008). Oral delivery of peptide drugs using nanoparticles self-assembled by poly(gamma-glutamic acid) and a chitosan derivative functionalized by trimethylation. *Bioconjug Chem* 19(6), 1248-1255. doi: 10.1021/bc800076n.

Mo, R., Jiang, T., Di, J., Tai, W., and Gu, Z. (2014). Emerging micro- and nanotechnology based synthetic approaches for insulin delivery. *Chem Soc Rev* 43(10), 3595-3629. doi: 10.1039/c3cs60436e.

Montel-Hagen, A., Blanc, L., Boyer-Clavel, M., Jacquet, C., Vidal, M., Sitbon, M., et al. (2008). The Glut1 and Glut4 glucose transporters are differentially expressed during perinatal and postnatal erythropoiesis. *Blood* 112(12), 4729-4738. doi: 10.1182/blood-2008-05-159269.

Montel-Hagen, A., Sitbon, M., and Taylor, N. (2009). Erythroid glucose transporters. *Current Opinion in Hematology* 16(3), 165-172. doi: 10.1097/MOH.0b013e328329905c.

Mueckler, M. (1994). Facilitative Glucose Transporters. *European Journal of Biochemistry* 219(3), 713-725. doi: DOI 10.1111/j.1432-1033.1994.tb18550.x.

Murrayrust, J., Mcleod, A.N., Blundell, T.L., and Wood, S.P. (1992). Structure and Evolution of Insulins - Implications for Receptor-Binding. *Bioessays* 14(5)**,** 325-331. doi: DOI 10.1002/bies.950140507.

Nathan, D.M. (1993). Long-term complications of diabetes mellitus. *N Engl J Med* 328(23)**,** 1676-1685. doi: 10.1056/NEJM199306103282306.

Nucci, M.L., Shorr, R., and Abuchowski, A. (1991). The Therapeutic Value of Poly(Ethylene Glycol)-Modified Proteins. *Advanced Drug Delivery Reviews* 6(2)**,** 133-151. doi: Doi 10.1016/0169-409x(91)90037-D.

Ohkubo, Y., Kishikawa, H., Araki, E., Miyata, T., Isami, S., Motoyoshi, S., et al. (1995). Intensive insulin therapy prevents the progression of diabetic microvascular complications in Japanese patients with non-insulin-dependent diabetes mellitus: a randomized prospective 6-year study. *Diabetes Res Clin Pract* 28(2)**,** 103-117. doi: 10.1016/0168-8227(95)01064-k.

Phlips, J.C., and Radermecker, R.P. (2012). [Type 1 diabetes: from genetic predisposition to hypothetical environmental triggers]. *Rev Med Liege* 67(5-6)**,** 319-325.

Pramudya, I., and Chung, H. (2019). Recent progress of glycopolymer synthesis for biomedical applications. *Biomater Sci* 7(12)**,** 4848-4872. doi: 10.1039/c9bm01385g.

Qiu, Y., and Park, K. (2001). Environment-sensitive hydrogels for drug delivery. *Adv Drug Deliv Rev* 53(3)**,** 321-339. doi: 10.1016/s0169-409x(01)00203-4.

Ranganathan, D., Thamake, S., Tworowska, I., and Delpassand, E. (2014). Chelator based glucosamine derivative for Ga-68 based diagnostic PET imaging of malignant tumors. *Journal of Nuclear Medicine* 55.

Raveendran, P., Fu, J., and Wallen, S.L. (2003). Completely "green" synthesis and stabilization of metal nanoparticles. *Journal of the American Chemical Society* 125(46)**,** 13940-13941. doi: 10.1021/ja029267j.

Shah, R.B., Patel, M., Maahs, D.M., and Shah, V.N. (2016). Insulin delivery methods: Past, present and future. *Int J Pharm Investig* 6(1)**,** 1-9. doi: 10.4103/2230-973X.176456.

Sharon, N., and Lis, H. (1972). Lectins: cell-agglutinating and sugar-specific proteins. *Science* 177(4053)**,** 949-959. doi: 10.1126/science.177.4053.949.

Sun, H., Saeedi, P., Karuranga, S., Pinkepank, M., Ogurtsova, K., Duncan, B.B., et al. (2022). IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes Res Clin Pract* 183**,** 109119. doi: 10.1016/j.diabres.2021.109119.

Trief, P.M., Cibula, D., Rodriguez, E., Akel, B., and Weinstock, R.S. (2016). Incorrect Insulin Administration: A Problem That Warrants Attention. *Clin Diabetes* 34(1)**,** 25-33. doi: 10.2337/diaclin.34.1.25.

Uldry, M., Ibberson, M., Hosokawa, M., and Thorens, B. (2002). GLUT2 is a high affinity glucosamine transporter. *Febs Letters* 524(1-3)**,** 199-203. doi: Pii S0014-5793(02)03058-2

Doi 10.1016/S0014-5793(02)03058-2.

VandenBerg, M.A., and Webber, M.J. (2019). Biologically Inspired and Chemically Derived Methods for Glucose-Responsive Insulin Therapy. *Adv Healthc Mater* 8(12)**,** e1801466. doi: 10.1002/adhm.201801466.

Veiseh, O., Tang, B.C., Whitehead, K.A., Anderson, D.G., and Langer, R. (2015). Managing diabetes with nanomedicine: challenges and opportunities. *Nature Reviews Drug Discovery* 14(1)**,** 45-57. doi: 10.1038/nrd4477.

Vrhovac, I., Breljak, D., and Sabolic, I. (2014). Glucose transporters in the mammalian blood cells. *Periodicum Biologorum* 116(2)**,** 131-138.

Wang, C., Ye, Y.Q., Sun, W.J., Yu, J.C., Wang, J.Q., Lawrence, D.S., et al. (2017). Red Blood Cells for Glucose-Responsive Insulin Delivery. *Advanced Materials* 29(18). doi: ARTN 1606617

10.1002/adma.201606617.

Wang, J., Wang, Z., Yu, J., Kahkoska, A.R., Buse, J.B., and Gu, Z. (2020). Glucose-Responsive Insulin and Delivery Systems: Innovation and Translation. *Adv Mater* 32(13)**,** e1902004. doi: 10.1002/adma.201902004.

Wang, Z., Wang, J., Kahkoska, A.R., Buse, J.B., and Gu, Z. (2021). Developing Insulin Delivery Devices with Glucose Responsiveness. *Trends Pharmacol Sci* 42(1)**,** 31-44. doi: 10.1016/j.tips.2020.11.002.

Wu, Q., Wang, L., Yu, H.J., Wang, J.J., and Chen, Z.F. (2011). Organization of Glucose-Responsive Systems and Their Properties. *Chemical Reviews* 111(12)**,** 7855-7875. doi: 10.1021/cr200027j.

Wu, W., and Zhou, S. (2013). Responsive materials for self-regulated insulin delivery. *Macromol Biosci* 13(11)**,** 1464-1477. doi: 10.1002/mabi.201300120.

Xia, Q., Zhang, Y.T., Li, Z., Hou, X.F., and Feng, N.P. (2019). Red blood cell membrane-camouflaged nanoparticles: a novel drug delivery system for antitumor application. *Acta Pharmaceutica Sinica B* 9(4)**,** 675-689. doi: 10.1016/j.apsb.2019.01.011.

Yang, J., and Cao, Z. (2017). Glucose-responsive insulin release: Analysis of mechanisms, formulations, and evaluation criteria. *J Control Release* 263**,** 231-239. doi: 10.1016/j.jconrel.2017.01.043.

Yoo, J.W., Chambers, E., and Mitragotri, S. (2010). Factors that control the circulation time of nanoparticles in blood: challenges, solutions and future prospects. *Curr Pharm Des* 16(21)**,** 2298-2307. doi: 10.2174/138161210791920496.

Yu, J., Wang, J., Zhang, Y., Chen, G., Mao, W., Ye, Y., et al. (2020). Glucose-responsive insulin patch for the regulation of blood glucose in mice and minipigs. *Nat Biomed Eng* 4(5)**,** 499-506. doi: 10.1038/s41551-019-0508-y.

Zhang, Y., Wang, J., Yu, J., Wen, D., Kahkoska, A.R., Lu, Y., et al. (2018). Bioresponsive Microneedles with a Sheath Structure for H2 O2 and pH Cascade-Triggered Insulin Delivery. *Small* 14(14)**,** e1704181. doi: 10.1002/smll.201704181.