Glucose-Responsive Materials for Self-Regulated Insulin Delivery

by
Todd Charles Zion

B.S. Chemical Engineering
Cornell University, 1997

Submitted to the Department of Chemical Engineering in Partial
Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Chemical Engineering

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 2004

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Abstract

Achieving normal glycemic control in diabetic patients is nearly impossible given the pharmacokinetics after subcutaneous injection of commercial insulin. Glucose-regulated insulin delivery (GRID) would vastly improve glycemic control while reducing (i) the required frequency of subcutaneous injections and finger-stick glucose tests, (ii) the incidence of hypoglycemia and hyperglycemia, and (iii) the resulting nerve, kidney, retinal and cardiovascular complications associated with the disease. We have chosen to formulate a GRID system into a repeated dosage form capable of being administered through injection, inhalation, or oral routes to eliminate the need for surgery. In our particular system, dextran, a glucose-containing polymer, was crosslinked using the tetrafunctional glucose-binding protein, concanavalin A (Con A). Glucose then directly competed with the polymer for Con A binding sites, causing displacement of the polymer and disruption of the crosslinks.

Through careful manipulation of dextran molecular weight (MW) and Con A/dextran crosslink ratio we have synthesized self-contained gels that erode at rates that depended directly on environmental glucose concentration. Since Con A binding affinity and glucose set-point (GSP) decreased from 25°C to 37°C, covalent mannosylation was required to increase binding affinity and minimize gel dissolution at glucose concentrations below 100 mg/dl. Hybrid gels constructed from varying weight ratios of mannosylated and unmodified dextrans further provided a convenient means for fine-tuning the GSP to closely match the physiological, glucose-dependent pancreatic β-cell response. The rate of gel dissolution increased by as much as 150× from hypo- to hyperglycemia, and decreased rapidly when glucose concentrations returned to hypoglycemic levels. The reversibility was a direct result of the high degree of crosslinking that restricted component dissolution to the gel surface.

By conjugating insulin to mannosylated and non-mannosylated dextran and optimizing the crosslink ratio and dextran MW, we have synthesized the first self-contained GRID system, requiring no external membranes while preserving the precise glucose sensitivity achieved with the membrane-encapsulated soluble system first
developed by Brownlee and Cerami in 1979. Further benefits of insulin-dextran conjugation included (i) purely glucose-sensitive release with no glucose-independent leakage, (ii) controlled dosing by varying non-conjugated dextran to insulin-dextran ratio, (iii) tunable glucose sensitivity based on dextran mannosylation, and (iv) reversible insulin release due to direct coupling with reversible glucose-responsive polymer dissolution. Furthermore, the insulin-dextran gels were active in vivo as demonstrated by their ability to control both fasting and mealtime-simulated blood glucose levels in streptozotocin-induced diabetic rats.

Reverse microemulsions (RM’s) were chosen as a means to formulate our GRID into a nanoparticle dispersion for convenient delivery. The phase behavior of biocompatible RM’s has been determined in the presence of dissolved dextran, insulin, and Con A using a novel rapid screening technique developed in our laboratory. By manipulating the composition of oils and surfactants as well as the relative ratio of surfactant to oil, the size of the RM domains was easily varied to obtain a wide range of GRID particle sizes. Using the well-characterized RM-Cap system, ~500-nm particles were successfully synthesized, isolated, and characterized for their glucose-dependent dissolution properties. As was observed with the macroscopic GRID gel systems, mannosylation of the dextran polymer and optimization of the Con A/dextran crosslink ratio were necessary to control glucose sensitivity in the desired physiological blood glucose range. Since the RM excipients were all biocompatible and FDA-approved, the entire system could be administered orally without isolating the nanoparticles. For inhaled delivery, the nanoparticles might be isolated, dried, and delivered through a dry powder inhaler (DPI). Finally, the isolated nanoparticles could be redispersed in a buffered saline solution for convenient injectable, subcutaneous delivery.

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Acknowledgments

I thank Professor Jackie Ying for providing me with guidance as a member of her research group, for allowing me to pursue new and aggressive research directions and to present my work in a number of different technical forums. I would also like to thank my committee members, Professors William Deen, Paula Hammond, and Gordon Weir for the valuable suggestions and support throughout this project.

Four UROP students also contributed to this thesis, and I gratefully acknowledge the pioneering work of Neville Mehenti in developing our biocompatible reverse microemulsion systems and synthesizing the earliest stimuli-responsive materials. Henry Tsang performed the rapid-screening of reverse microemulsion systems and ternary diagram construction. Monica Sircar provided valuable assistance in developing the insulin-dextran synthesis, and Anita Kris has carried on the insulin-dextran synthesis and in vitro release studies. I also thank Jennifer Lock at the Joslin Diabetes Center for her assistance in training and developing methods for our animal studies, Geoff Moeser and Professor Alan Hatton for the use of the DLS equipment, and Dr. Anthony Garratt-Reed for expert electron microscopy assistance.

I thank the members of the NMRL for their friendship and support over the years. In particular, I would like to thank Thomas Lancaster for many helpful discussions regarding thesis work and graduate student life. Yuhua Hu will continue with this work and has made the transfer of knowledge and methods seamless through her hard work and dedication. I also acknowledge the contributions provided by Dr. Edward Ahn, Dr. Javier Garcia-Martinez, Dr. Dejian Huang, Dr. John Lettow, Dr. Justin McCue, Dr. Suniti Moudgil, Pemakorn Pitukmanorom, Dr. Yee Su, Dr. Jason Sweeney, Steven Weiss, Tseh-Hwan Yong, Noreen Zaman, and Dr. Andrey Zarur.

I thank my mother, father, and sister as well as my mother and father-in-law for their unconditional support. Thank you, Jonathan, my son, for providing me the strength and confidence to fearlessly pursue my dreams. Finally, thank you to my wonderful wife, Karyn, for your unwavering support and love over the years. The extent of my accomplishments is a reflection of your belief in me...even when I had doubts.

This research was supported by the National Science Foundation (Small Grant for Exploratory Research, CTS-0118705) and the Singapore-MIT Alliance.
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Chapter 1 – Background and Objectives

1.1. Limitations of Conventional Insulin Therapy

Insulin-dependent diabetes mellitus (IDDM) is characterized by the autoimmune destruction of pancreatic islet cells leading to the loss of glucose-stimulated insulin secretion and the subsequent disruption of glucose homeostasis. Until the introduction of insulin in the 1920’s, a young diabetic would be fortunate to survive two years after the time of diagnosis. Since then, subcutaneous insulin injection therapy has saved and prolonged millions of patients’ lives, but at the cost of a lifetime of medical complications associated with poor glycemic control.

Achieving normal glycemic control is nearly impossible given the pharmacokinetics after subcutaneous injection of commercial insulin. Therefore, attempts to achieve normoglycemia have involved a multiplicity of insulin preparations, delivery systems, and injection regimens to meet both basal and meal-related insulin requirements. Such painful and cumbersome regimens would be unnecessary if insulin replacement could keep up with the body’s fluctuating blood glucose demands.

1.2. Benefits of Glucose-Regulated Insulin Delivery

Glucose-regulated insulin delivery (GRID) would reduce the required frequency of subcutaneous injections and finger-stick glucose tests. Multiple daily injections are required to supply high doses of short-acting insulin before meals and low doses of intermediate-acting insulin between meals. Glucose tests are the only way to examine how the food and insulin dose immediately affect blood glucose levels. Frequent test readings help patients manage their diabetes day-by-day or even hour-by-hour. A GRID system would provide appropriate insulin delivery at both fasting and mealtime blood glucose levels without requiring mealtime glucose testing and dose adjustment.

Furthermore, GRID would help reduce the incidence of hypoglycemic episodes, hyperglycemia, and the nerve, kidney, retinal and cardiovascular complications associated with the disease. Research studies estimate between 4% and 13% of the insulin-dependent diabetic patients who die each year perish in hypoglycemic-related accidents. Furthermore, people who aggressively manage their diabetes are three times
more likely than other diabetics to suffer severe low-blood-sugar episodes according to the Diabetes Control and Complications Trial (DCCT). However, failure to aggressively manage diabetes results in prolonged hyperglycemia as measured by a rise in glycosylated HbA\textsubscript{1c} levels. Elevated HbA\textsubscript{1c} levels have been directly correlated to an increased risk in developing severe microvascular and premature macrovascular complications. An ideal GRID system would shut off insulin release below a critical blood glucose level to prevent hypoglycemia, thereby allowing more aggressive insulin treatment to reduce hyperglycemia and the associated diabetic complications.

### 1.3. Technical Approaches to Glucose-Regulated Delivery

Thus far, no approach has successfully yielded a safe, convenient, physiologically relevant glucose-regulated insulin delivery system. Transplantation of pancreatic islets containing glucose-responsive, insulin-producing cells would provide the optimal glucose-regulated insulin delivery, but is limited by the supply of viable cells. Implantation of an electromechanical pancreas would provide control without using cells, but is limited by the lack of a long-term, viable glucose sensor capable of frequent and accurate measurements. Finally, artificial materials that biochemically sense glucose and release insulin do not require cells or glucose sensors, but no material has been designed to properly mimic the physiological glucose response.

#### 1.3.1. Islet Transplantation

Pancreatic islets are mainly comprised of \( \beta \)-cells that respond rapidly and with high sensitivity to changes in the extracellular glucose concentration in order to attain an effective feedback control system. A near maximal \( \beta \)-cell insulin secretion rate may be attained within seconds to a few minutes after raising the extracellular glucose concentration. The magnitude of this initial response is proportional to the glucose concentration over a wide range of levels, demonstrating a sigmoidal dose-response curve (Fig. 1.1). If the glucose concentration is maintained, the initial response lasts for only 5–7 min after which the secretion rate falls to near basal levels. This initial spike is then followed by a gradual increase in insulin secretion, thus giving its typical biphasic appearance (Fig. 1.2). Therefore, the \( \beta \)-cell represents the ideal, physiological model for GRID.
Fig. 1.1. Sigmoidal relationship between extracellular glucose concentration and insulin release from collagenase-isolated mouse islets. Note large increase in insulin secretion rate when glucose concentration exceeds normal value for fasting blood sugar.10

Fig. 1.2. Schematic illustration of latency period and the two phases of insulin release. Depending on experimental conditions, the second phase of secretion may be of much longer duration than shown here.10

The first successful islet transplant was performed by Shapiro and coworkers using a glucocorticoid-free immunosuppressive regimen, the so-called Edmonton Protocol.11, 12 The protocol requires invasive surgery, a lifetime dependence on immunosuppressants, and most importantly, two healthy human pancreases to transplant
for each patient, such that only 3,000 of the approximately 1 million eligible recipients can receive the transplants. Xenotransplantation of porcine islets\(^{13-15}\) and β-cell neogenesis from embryonic stem cells or pancreatic progenitor cells\(^{16-18}\) have been proposed to address the shortage of insulin-producing cells. Before xenotransplantation achieves success in the clinical arena, researchers must overcome the immunological response to xenografted islets and the transmission of infectious agents between species.\(^{13}\) Cell replacement therapies based on differentiated stem cells or transdifferentiated adult pancreatic cells will remain fiction rather than fact until stable, fully functional cells can be efficiently and reproducibly generated \textit{in vitro}.\(^{17}\) Even then, safe suppression of autoimmunity must be achieved before this technology can lead to a dramatic shift in clinical practice.\(^{15}\)

\subsection*{1.3.2. Electromechanical Artificial Pancreas}

To overcome the scarcity of functional β-cells, researchers have attempted to design a completely artificial pancreas. The electromechanical pancreas requires three key components: (i) a sensor capable of frequent blood glucose measurements, (ii) an algorithm that translates glucose sensor measurements into an insulin infusion rate, and (iii) a pump that delivers the required insulin from a reservoir to lower blood glucose levels and close the feedback control loop (Figure 1.3). Marliss and coworkers first demonstrated glycemic control in diabetic patients using an artificial endocrine pancreas in 1977.\(^{19}\) Since then, research has aimed at (i) miniaturizing the glucose sensor while preserving the ability to sample blood glucose frequently over long periods of time, (ii) developing a more robust algorithm capable of onboard computer storage backed up with redundancies, and (iii) miniaturizing the pump while providing a stable, refillable insulin reservoir.

Pump miniaturization has successfully led to external wearable pumps, but their use limits physical activity because they can be jostled loose from the body and access through the skin can become infected.\(^{20}\) Miniaturized implantable pumps are designed to confront the limitations of external pumps, but according to the EVADIAC study,\(^{21}\) the benefits come at the cost of frequent catheter blockage, adverse tissue response at the implantation site, and pump mechanical failure.\(^{21,22}\)
In anticipation of a properly designed continuous glucose sensor, control algorithms have been developed to operate the feedback loop. These algorithms have dealt with compensation for glucose sensor reading drift over time, re-calibration based on individual insulin requirements, re-computation of dose-response based on weight and metabolic function, and recognition of sensor malfunction.

![Diagram of blood glucose control system](image)

**Fig. 1.3.** Key elements to restore closed-loop blood glucose control in type 1 diabetic subjects.

The only major missing element is a practical and reliable glucose sensor that is precise, linear, sensitive, selective, and stable *in vivo*. Non-invasive optical sensors that aim to detect blood glucose by shining a beam of light through a fingertip are limited by interference that prevents a sustained, stable, and specific glucose signal. Devices such as GlucoWatch™ (Cygnus, Redwood City, CA) based on reverse iontophoresis have unacceptably slow sensor time lags of 18 min, a problem in sensing low glucose concentrations, and skin reactions at the site of the electrodes. Systems based on the glucose oxidase-catalyzed reaction between glucose and oxygen to produce hydrogen peroxide followed by dissociation to produce electric current must be calibrated against capillary blood glucose at least four times a day due to drift. Perhaps the most reliable continuous glucose sensor design based on intravenous enzymatic oxygen-based sensors must be inconveniently implanted by direct jugular access in the superior vena cava under general anesthesia, and even then has yet to be validated for long-term sensor accuracy.
1.3.3. Biochemically-Regulated Artificial Materials

Artificial biomaterials overcome the limitations of a scarce cell supply and the lack of a viable glucose sensor by combining readily available biocompatible polymers with highly specific glucose-binding proteins. However, these systems all suffer from (i) insulin leakage at hypoglycemic conditions, (ii) slow response to acute glucose concentration rises, (iii) an inability to stop releasing insulin once triggered, and (iv) an inability to controllably dose insulin. Two different mechanisms have been used in the development of glucose-sensitive materials, each with its own limitations. One mechanism utilizes enzyme substrate reactions, while the other uses competitive desorption for rate control.29

1.3.3.1. Enzyme-Substrate Reaction Control

Enzyme-substrate reaction control requires immobilization of glucose oxidase (GOD) in a polymer substrate containing pendant amine groups with a pKₐ at or near the physiological range. As glucose diffuses into the insulin-loaded material, GOD catalyzes its conversion to gluconic acid, thereby lowering the pH in the microenvironment, causing pH-dependent polymer swelling30 and subsequent insulin release (Fig. 1.4). These are not specifically glucose-sensitive materials, but rather pH-sensitive materials triggered to change conformation by the presence of glucose. The indirect coupling leads to unwanted insulin release due to environmental pH changes, the presence of more readily oxidizable molecules, and drift in response over time due to loss of GOD activity. Furthermore, progressive response to glucose concentration over a range of glucose concentrations can be achieved only with a sufficiently low GOD loading; otherwise, depletion of oxygen causes the system to become insensitive to glucose.31 Most importantly, because the system relies on insulin permeation through the material to control release rate, there is always insulin leakage even at low glucose concentrations. Furthermore, these systems require hours instead of minutes to swell, increase permeability and increase insulin release rate to step changes in glucose concentration.31
**Fig. 1.4.** Schematic representation of a glucose-sensitive hydrogel consisting of a poly(amine) and glucose oxidase.\(^{32}\)

### 1.3.3.2. Competitive Desorption Rate Control

Competitive desorption overcomes the limitations of enzyme-substrate rate controlled systems by directly coupling glucose to the material response. Brownlee and Cerami pioneered this concept by preparing insulin covalently attached to sugar molecules, which are complementary to the major binding site of sugar-binding proteins such as concanavalin A (Con A).\(^{33,34}\) Glycosylated insulin (GI) is then displaced by glucose in direct proportion to the concentration of external glucose (Fig. 1.5). In practical applications, the Con A-GI complex is enclosed within a membrane permeable to glucose and GI but not to Con A, relegating the use of this system to an implantable or extracorporeal device. Furthermore, the system is inherently irreversible in that once GI is released to confront a spike in glucose concentration, the system must be reloaded with GI to continue maintaining the same response.

Instead of enclosing a soluble competitive binding system within a membrane, other researchers have sought to develop insoluble membranes based on competitive binding that controls the rate of insulin release from a reservoir.\(^{31,32}\) As with the
membrane-enclosed Con A-GI complex, these systems are designed to be devices. The membrane is formed by physically crosslinking water-soluble, glycosylated polymers with tetravalent Con A to form a gel. Free glucose then competes with the glycosylated polymer for Con A and disrupts the crosslinks, causing a gel-to-sol transition.\textsuperscript{35} The system is then sandwiched between two porous support membranes to minimize polymer and Con A leakage.

![Diagram](image)

\textbf{Fig. 1.5.} Schematic representation of glycosylated insulin bound to Con A. Binding site on lower right subunit of Con A is shown to be occupied by a glucose molecule. At the upper right binding site, glucose is shown displacing a glycosylated insulin molecule. Pores in the membrane permit passage of glucose and glycosylated insulin but not Con A.\textsuperscript{34}

The use of support membranes ultimately leads to a complex system with slow diffusion rates. Consequently, excessively high glucose concentrations (> 400 mg/dl) are required to significantly increase insulin diffusion. Furthermore, once glucose is removed from the system, the decrease in insulin release rate lags behind by several hours. Nevertheless, due to the competitive binding mechanism, the gel-to-sol transition itself, unlike the glucose-oxidase induced polymer swelling, is rapid and directly dependent on glucose concentration over a wide range of values. The key to properly utilizing this approach is to develop a self-contained material that does not require the use of rate-limiting membranes.
1.4. Research Goals and Design Criteria

Ideally, a GRID would be surgically implanted only once to provide glycemic control over the patient’s lifetime. However, devices such as those based on the artificial electromechanical pancreas and glucose-responsive membrane-controlled reservoirs will always require maintenance and therefore repeated invasive surgery. Rather than concentrate on developing more reliable devices, we have chosen to formulate GRID into a repeated dosage form capable of being administered through injection, inhalation, or oral routes to eliminate the need for surgery altogether.

Such an approach will lead to drastically improved glycemic control with a minimally invasive system. Glucose-sensitive, artificial materials are best suited for this application, due to their relative simplicity in construction and their ability to be designed from biocompatible and biodegradable polymers. Furthermore, glucose-sensitive materials based on competitive binding are preferred because of the inherently rapid, glucose concentration-dependent material response. To accomplish this goal we must:

1. Develop a novel, self-contained, biodegradable, glucose-responsive material based on competitive binding that rapidly and reversibly responds to fluctuating glucose concentrations in the physiological range. (Chapter 2)

2. Directly couple insulin to the material to obtain precise control over dosing, minimize leakage at glucose concentrations < 100 mg/dl, release insulin rapidly as glucose concentrations increase from 100 to 500 mg/dl, and slow down and stop insulin release as glucose concentrations return to ≤ 100 mg/dl. (Chapter 3)

3. Formulate the material into a dispersion of micro- and nano-particles while preserving the glucose-responsive insulin release properties to enable injectable, pulmonary or oral delivery. (Chapter 4)

Successful completion of these goals will lead to the first and only platform for repeated dose, self-regulated insulin delivery that (i) provides basal and mealtime insulin in a single dose, (ii) prevents insulin leakage at low glucose concentrations to avoid hypoglycemia, and (iii) reduces long-term hyperglycemia and associated microvascular and premature macrovascular diabetic complications.
1.5. References


Chapter 2 – Design of a Novel, Self-Contained, Glucose-Degradable Material

2.1. Introduction

An ideal glucose-responsive material for therapeutic self-regulated insulin delivery must: (i) be self-contained and biodegradable, (ii) respond rapidly in minutes to increasing glucose concentrations from 50 to 500 mg/dl, and (iii) quickly decrease response in minutes as glucose concentrations decrease to normo- and hypo-glycemic concentrations (50–100 mg/dl).

Direct competitive binding between glucose and the glucose-responsive material offers the best approach for a rapid, glucose-dependent response. In this type of system (Fig. 2.1), a glucose-containing polymer is crosslinked using a tetrafunctional glucose-binding protein, such as concanavalin A (Con A). Free glucose directly competes with the polymer for Con A binding sites, causing displacement of the polymer and disruption of the crosslinks.

![Fig. 2.1. Schematic representation of a crosslinked network formed by the interaction between tetravalent Con A and a glucose-containing polymer. In this system, free glucose from the environment displaces the polymeric glucose to disrupt the Con A-crosslinked network.](image)

Previous attempts at synthesizing such materials\(^1\) resulted in extremely viscous liquids (> 200 Pa·s). To prevent components from dissolving in the physiological medium, these materials were enclosed within a membrane that allowed insulin diffusion, but prevented Con A and polymer diffusion. Such a design is limited by the high glucose concentrations (> 500 mg/dl) required to rapidly displace enough crosslinks to significantly lower the viscosity. Furthermore, insulin diffusion through the
encapsulating membrane is substantially hindered and becomes rate-limiting if a sufficiently low molecular weight cut-off (MWCO) membrane is chosen to prevent polymer and Con A leakage.\textsuperscript{7}

Even after overcoming these limitations, insulin diffusion rate will at best increase by only four times when the viscosity changes from 200 Pa-s to 50 Pa-s, which is the most significant, glucose-dependent viscosity change measured by Tanna and coworkers.\textsuperscript{3} This limitation was confirmed by Kim and Park who found that insulin release rates increased by no more than four times when the glucose concentration was increased from 100 to 400 mg/dl.\textsuperscript{6}

The extent of glucose response in these materials was also limited by the relative affinity between Con A and the polymer. No attempt was made to modify the affinity of either the polymer or Con A to tune the glucose sensitivity. Consequently, a system that responded at too low a glucose concentration (< 150 mg/dl) was obtained in the first case,\textsuperscript{5} and a system that responded only to excessively high glucose concentrations (> 400 mg/dl) resulted in the second case.\textsuperscript{1,3}

To address these challenges, we have designed a material based on competitive binding that is insoluble in physiological medium so that no external membranes are required to prevent component leakage. Secondly, instead of volumetrically decreasing viscosity, our material erodes from the surface based on direct competition with glucose. Surface erosion enables rapid, glucose concentration dependent response while preserving the bulk of the material for repeated glucose challenges. To expand the glucose-responsive concentration range, we have modified the polymer with oligosaccharide ligands of higher affinity. As the concentration of higher affinity ligands increased, more glucose would be required to displace the polymer, thereby shifting the material response to higher glucose concentrations.

Con A was chosen as the glucose-binding protein because it is inexpensive, readily available, and one of the most studied proteins in biochemistry. However, as a lectin derived from the jack bean plant, Con A is mitogenic and therefore potentially toxic.\textsuperscript{8,9} Nevertheless, Con A-derived systems represent a tractable model from which we may gain insight to later apply to potentially safer glucose-binding proteins.
Because the end application requires repeated dosage, the polymer must be biocompatible and biodegradable to avoid potentially harmful accumulation. Dextran, poly(α-D-glucose), is attractive because it binds Con A\textsuperscript{10,11} and is already approved for clinical use as a blood plasma volume expander.\textsuperscript{12} It is also a versatile molecule, capable of being covalently modified at the three free –OH groups with a variety of chemicals, drugs, and proteins.\textsuperscript{13} In this study, dextran was covalently modified with (i) fluorophores to measure the extent of surface erosion using standard fluorescence spectroscopy, and (ii) oligosaccharides to increase the binding affinity for Con A. The following study demonstrated how tailoring dextran molecular weight (MW), dextran affinity for Con A, and Con A/dextran crosslink ratio could produce self-contained, glucose-responsive materials that eroded rapidly and reversibly in response to fluctuating glucose concentrations of 50–500 mg/dl.

2.2. Materials and Methods

2.2.1. Materials

Con A (Type VI), BES buffer salts, sodium chloride (NaCl), manganese chloride (MnCl\textsubscript{2}), calcium chloride (CaCl\textsubscript{2}), D-glucose, D-mannose, vinylsulfone, sodium carbonate and glycine were all obtained from Sigma-Aldrich (St. Louis, MO), and used without further purification. Fluorescent-labeled dextrans were obtained as fluorescein-isothiocyanate (FITC) or tetramethylrhodamine-isothiocyanate (TRITC) derivatives with varying MW from Sigma-Aldrich (St. Louis, MO) as follows: TRITC-dextran with MW = 70,000 g/mol (TRITC-Dex70K), TRITC-Dextran with MW = 170,000 g/mol (TRITC-Dex170K), FITC-Dextran with MW = 280,000 g/mol (FITC-Dex280K), and FITC-Dextran with MW = 500,000 g/mol (FITC-Dex500K). Dulbecco’s 1× Phosphate-Buffered Saline containing calcium and magnesium (DPBS) was obtained from Mediatech, Inc. (Herndon, VA). Deionized (DI) water was obtained by ion-exchange chromatography over universal cartridge columns (Cole-Palmer, Vernon Hills, IL).

2.2.2. Dextran Concentration Characterization by Fluorescence Spectroscopy

Solutions containing each of the fluorescent-labeled dextrans were serially diluted by a factor of two from 5.0 mg/ml down to 0.0024 mg/ml in DPBS to establish a fluorescence-concentration calibration curve. 200 μl of each solution were added to each
of the 96 wells of a Microtest™96 polystyrene microplate (Becton Dickinson, Franklin Lakes, NJ). The fluorescence of each solution was determined using a Molecular Devices (Sunnyvale, CA) fmax fluorescence spectrophotometer (TRITC: $\lambda_{ex} = 544$ nm, $\lambda_{em} = 590$ nm; FITC: $\lambda_{ex} = 485$ nm, $\lambda_{em} = 538$ nm), and plotted against concentration. Samples containing unknown quantities of dextran were diluted with DPBS to obtain fluorescence readings within the linear portion of the calibration curve. 200 µl of the diluted solutions were loaded into each of the 96 wells and their fluorescence was determined using an fmax fluorescence spectrophotometer as described above. The concentrations of fluorescently labeled dextran were then calculated using the calibration curve.

2.2.3. Synthesis of Mannosylated Dextran

Mannosylated fluorescent dextran was synthesized by a modified vinylsulfone coupling procedure. Briefly, 500 mg of dextran were dissolved in 28.6 ml of DI water at room temperature. After complete dissolution, 28.6 ml of a 1-M carbonate buffer (pH = 11.4) were added and stirred. 429 µl of vinylsulfone were then added to activate the dextran for 1 hr while stirring. 2.9 g of D-mannose were then added and stirred for another hour, followed by the addition of 28.6 ml of glycine (5 wt% in DI water) to quench the reaction for 1 hr. The resulting solution was then concentrated by ultrafiltration through a polyethersulfone 10K molecular weight cutoff (MWCO) membrane (Millipore, Bedford, MA) to ~ 30 ml, dialyzed exhaustively against DI water using a 6-8K MWCO tube (Spectrapor, Spectrum Labs, CA), and lyophilized (Virtis Sentry™, Gardiner, NY). A separate fluorescence-concentration calibration curve was established, as described above, for the resulting mannosylated-dextran derivatives (TRITC-ManDex70K, TRITC-ManDex170K, FITC-ManDex280K, and FITC-ManDex500K).

2.2.4. Synthesis of Glucose-Responsive Materials

Con A was dissolved at 100 mg/ml in a 20-mM BES buffer (pH = 7) containing 1 M NaCl and 1 mM MnCl₂ and CaCl₂ (BES-20). The resulting solution was passed through a 0.45-µm syringe filter (Acrodisc® 25 mm diameter, Pall Corporation, Ann Arbor, MI) to remove any high molecular aggregates and insoluble impurities. Each
fluorescently labeled dextran or mannosylated dextran solution (TRITC-Dex70, TRITC-Dex170, FITC-Dex280, and FITC-Dex500) was prepared at 50 mg/ml in a 200-mM BES buffer (pH = 7) containing 0.150 M NaCl (BES-200). The dextran solutions were then diluted by a factor of two from 50 mg/ml to 1.6 mg/ml using the same BES-200 buffer.

Each dextran solution was added to a 1.5-ml conical centrifuge tube, after which an equal volume of the Con A solution was added, and the resulting solution was mixed rapidly. The solutions were allowed to stand at room temperature for 1 hr, after which the resulting aggregates were isolated by centrifugation at 360 × g for 10 min (Allegra 21R, Beckman Coulter, Fullerton, CA). The supernatant was removed and the concentration of dextran was determined by fluorescence. The crosslinking efficiency ($X_{CL}$) for each experiment was calculated according to Equation 1 using the supernatant and total dextran concentrations.

$$X_{CL} = \frac{[\text{dextran}]_{\text{total}} - [\text{dextran}]_{\text{supernatant}}}{[\text{dextran}]_{\text{total}}}$$

The formulations resulting in self-contained gels were then washed twice with DI water for 15 min, followed by centrifugation at 360 × g for 10 min. The gels were then washed and centrifuged twice with DPBS. After removing the last wash solution, the gels were kept at 4°C overnight, and used subsequently for set-point, kinetic or cycling studies.

2.2.5. Synthesis of Mannosylated/Unmodified Dextran Hybrid Materials

Two dextran solutions (TRITC-Dex70 and TRITC-ManDex70) were prepared at 8 mg/ml in BES-200. To 0.00, 0.10, 0.50, 0.90 and 1.0 ml of TRITC-Dex70 solution, enough TRITC-ManDex70 solution was added to make 1.0 ml of total solution volume. Each of these solutions was added to a 1.5-ml conical centrifuge tube, after which an equal volume of the Con A solution was added, and the resulting solution was mixed rapidly. The solutions were allowed to stand at room temperature for 1 hr, after which the resulting aggregates were isolated by centrifugation at 360 × g for 10 min. The gels were then washed twice with DI water for 15 min, followed by centrifugation at 360 × g for 10 min. They were then washed and centrifuged twice with DPBS. After removing
the last wash solution, the gels were kept at 4°C overnight, and used subsequently for set-point studies.

2.2.6. Glucose Set-Point Studies

1.0 ml of DPBS solution containing 50 mg/dl of D-glucose was added to each of the 24 3-ml wells of a Multiwell™ plate (Becton Dickinson, Franklin Lakes, NJ). Each gel was then added to the solution and agitated for 1 hr using a microplate incubator/shaker (“Jitterbug,” Boekel Industries, Philadelphia, PA) set at 25°C or 37°C. After 1 hr, 0.5 ml of release medium was removed and the dextran concentration was determined by fluorescence spectroscopy. The release medium was supplemented with 0.5 ml of a 150-mg/dl glucose solution to make a 100-mg/dl solution, and the gels were agitated for another hour. This process was repeated for release medium with glucose concentrations of 200, 400, 800 and 1600 mg/dl for a total of 6 concentrations over 6 hr. The percent dextran dissolution was then calculated by normalizing the cumulative concentration of dextran in the release medium by that released at 100% dissolution. In all cases, 100% dissolution was obtained after the 1600-mg/dl glucose incubation.

2.2.7. Glucose Kinetic Experiments

1.0 ml of DPBS containing 50, 100 or 400 mg/dl of D-glucose was added to each of the 24 3-ml wells of a Multiwell™ plate (Becton Dickinson, Franklin Lakes, NJ). Three of each gel formulation were then added to each of the three glucose solutions, and agitated using a microplate incubator/shaker (“Jitterbug,” Boekel Industries, Philadelphia, PA) set at 25°C or 37°C. 0.5 ml of release medium was removed at specified time intervals, and the dextran concentration was determined by fluorescence spectroscopy. The release medium was supplemented with 0.5 ml of DPBS containing the same concentration of D-glucose as the solution removed, and the process continued for a total of 24 hr. The percent dextran dissolution at glucose concentrations of 50, 100 and 400 mg/dl was calculated by normalizing the cumulative dextran concentration released at a specific time by the total amount released after a 24-hr incubation at 400 mg/dl.

2.2.8. Glucose Cycling Experiments
A flow cell was constructed (Fig. 2.2) using a HPLC pump system equipped with a UV/Visible absorbance detector (Waters Corporation, Milford, MA). The inlet to the flow cell was connected to two reservoirs containing DPBS solutions of low and high glucose concentrations. The outlet of the flow cell was connected to the absorbance detector to continuously measure the amount of dextran dissolved at a wavelength equal to the maximum \( \lambda_{\text{ex}} \) of the dextran fluorophore. The instantaneous amount of dextran dissolved was measured continuously over time and normalized by that originally contained within the gel. In addition, samples were taken at regular intervals and diluted appropriately with DPBS to measure the glucose concentration with a Prestige™ Smart System glucose meter (Home Diagnostics, Ft. Lauderdale, FL). In each experiment, a specific gel was equilibrated at a glucose concentration of 50 mg/dl for 20 min at 25°C or 37°C. Then, the HPLC pumps were programmed to cycle the inlet glucose concentration from a low to high value in 30 min, and then back down again in another 30 min. The cycle was repeated five times for a total of six cycles over 6 hr. In select experiments, the program was changed such that the glucose concentration remained at the low value for 30 min prior to the next cycle.

**Fig. 2.2.** Flow cell constructed to perform glucose cycling studies. Reservoir A and Reservoir B contain pH 7 BES buffered saline solutions of different glucose concentrations. The flow rate is set and the ratio of A to B is varied linearly over time to increase or decrease the glucose concentration in the flow cell. Gel dissolution is monitored continuously through a visible absorbance detector at \( \lambda = \lambda_{\text{ex}} \).

### 2.3. Results and Discussion

#### 2.3.1. Self-Contained Glucose-Responsive Gel Synthesis

The interaction between Con A and dextran was evaluated at a number of dextran MW values and Con A/dextran ratios. Fig. 2.3 shows that \( X_{CL} \) reached a maximum at a
Con A/dextran ratio of ~ 6 for dextran MW of 70K-500K. In addition, $X_{CL}$ increased with increasing dextran MW, approaching nearly 100% for FITC-Dex500K. Similar studies performed on both FITC-Dex10K and FITC-Dex20K yielded less than 10% $X_{CL}$ (data not shown), indicating a minimum MW was required to induce polymer crosslinking.

Fig. 2.3. Gel crosslinking efficiency ($X_{CL}$) vs. Con A/dextran (w/w) ratio at a constant [Con A] of 100 mg/ml, and a dextran molecular weight of (●) 70 K, (■) 170 K, (▲) 280 K and (●) 500 K.

I. Viscous liquid

ii. Insoluble gel

III. Insoluble precipitates

Fig. 2.4. Schematic representation of three distinct regions of behavior in varying Con A/dextran (w/w) ratio at a constant [Con A] of 100 mg/ml. Region I (Con A/dextran < 4): viscous liquid, Region II (4 < Con A/dextran < 24): insoluble gel, and Region III (Con A/dextran > 24): insoluble precipitates.
Three distinct regions of behavior were identified when equal volumes of Dextran (1–50 mg/ml) were mixed with Con A (100 mg/ml) (Figs. 2.3 and 2.4). In Region I, the relative amount of Con A was insufficient to form a crosslinked network. However, the solution viscosity increased appreciably, indicating the formation of higher MW, soluble dextran-Con A complexes. In Region III, the Con A crosslinker was in excess, resulting in insoluble precipitates. However, at an optimal Con A/dextran weight ratio of approximately 2–24 (Region II), the crosslinked network fell out of solution as an insoluble, water-swellable gel. The gels did not require an external membrane to prevent component dissolution and were therefore self-contained.

A Con A/dextran (w/w) ratio equaled a (Con A binding site)/(dextran anhydroglucose subunit) molar ratio of ~ 0.013. Since each anhydroglucose molecule was a potential binding site for Con A, these results indicated a deficiency in Con A binding sites for the number of carbohydrate ligands available. However, Hogg and Winzor found that dextrans of high MW bound to Con A with an average of 80 anhydroglucose residues per site. The maximum capacity of binding was limited by geometrical constraints in that the centers of two ligand molecules could not be closer than the sum of their effective radii. Adjusting for this effect, Con A/dextran (w/w) ratio of 2 would correspond to one Con A binding site per effective dextran carbohydrate ligand, in agreement with the minimum ratio required for sufficient crosslinking (Fig. 2.3). At least two binding sites must be available per dextran chain to ensure network formation. With an average MW of 13K per dextran binding site (80 anhydroglucose residues × 162 g/mol), the average MW of dextran chains must be greater than 26K, thus confirming the existence of a minimum MW requirement for effective Con A/dextran crosslinking. The dextran with an average MW of 70K most likely contained a significant fraction of lower MW dextrans that disrupted crosslinking and decreased the measured $X_{CL}$.

2.3.2. Tailoring Glucose Sensitivity

The gels formed in Region II were investigated for their ability to dissolve when challenged by increasing concentrations of glucose. The intrinsic glucose sensitivity, or glucose set-point (GSP), for various gel formulations was compared. Fig. 2.5 shows the
cumulative dextran dissolution for TRITC-Dex170K gels synthesized at a Con A/dextran (w/w) ratio of 4, 8 and 16 at 25°C and 37°C. At both temperatures, the gels dissolved more rapidly as the glucose concentration in the release medium increased from 50 to 1,600 mg/dl. More importantly, the extent of dissolution at low, physiologically hypoglycemic glucose concentration (< 70 mg/dl) was negligible at room temperature. Only above 100 mg/dl did the gels dissolve rapidly, indicating that there existed a critical glucose concentration above which a sufficient number of Con A-dextran crosslinks would be disrupted to allow rapid gel dissolution. At 37°C, the gels dissolved to a greater extent at low glucose concentrations. Increasing the Con A/dextran ratio minimized the extent of dissolution but did not prevent dissolution even at 50 mg/dl. In general, increasing the Con A/dextran ratio shifted the glucose set-point curve toward higher glucose concentrations. The greater the extent of crosslinking, the higher the glucose concentration required to disrupt the crosslinks.

**Fig. 2.5.** Glucose set-point (GSP) curves at (a) 25°C and (b) 37°C for TRITC-Dex170K gels formed at a Con A/dextran (w/w) ratio of (●) 4, (■) 8 and (▲) 16.

Set-point curves were measured for TRITC-Dex70K, FITC-Dex280K and FITC-Dex500K as well. Rather than comparing the individual curves for each temperature, MW and crosslink ratio, a metric was developed to readily compare the various formulations. The two most relevant glucose concentrations on the GSP curves were (i)
the point at which significant gel dissolution began, and (ii) the point at which gel dissolution proceeded most rapidly. The glucose concentrations corresponding to 10% gel dissolution ($G_{10\%}$) and 50% gel dissolution ($G_{50\%}$) were chosen to characterize these two points, respectively. GSP data were pooled for each dextran MW and plotted against Con A/dextran ratio for both the 25°C and 37°C experiments (Fig. 2.6). As observed for TRITC-Dex170K, both $G_{10\%}$ and $G_{50\%}$ increased with increasing Con A/dextran ratio. In addition, both $G_{10\%}$ and $G_{50\%}$ decreased as the temperature increased from 25°C to 37°C for a given Con A/dextran ratio.

![Graphs showing GSP values as a function of Con A/dextran ratio](image)

**Fig. 2.6.** (a) $G_{10\%}$ and (b) $G_{50\%}$ values pooled for all dextran MW values as a function of Con A/dextran (w/w) ratio measured at (●) 25°C and (■) 37°C.

Using isothermal titration calorimetry (ITC), Sanders and coworkers have evaluated the temperature-dependent binding properties of Con A for various oligosaccharides. The enthalpy ($\Delta H_b$) and entropy ($\Delta S_b$) of binding to glucose were determined to be $-17$ kJ/mol and $-6$ J/mol-K, respectively, indicating that an increase in temperature would decrease the binding affinity for glucose residues and ultimately lead to a decreased GSP. $G_{10\%}$ values for all MW, Con A/dextran ratios and temperatures investigated in this study were less than 100 mg/dl, and $G_{50\%}$ values at 37°C were all less than 150 mg/dl. Such materials would be too sensitive to environmental glucose concentration, and would lead to unacceptably high rates of dissolution at hypoglycemic glucose concentrations.
Mannose binds Con A with ~ 4 times the affinity of glucose. Therefore, covalent attachment of mannose to dextran is expected to shift the GSP curve to higher glucose concentrations. Fig. 2.7 shows that as the polymer weight fraction of TRITC-ManDex70 increased relative to TRITC-Dex70, the $G_{10\%}$ value at 37°C increased from ~ 35 mg/dl to 110 mg/dl, while $G_{50\%}$ increased from 130 mg/dl to 350 mg/dl. More importantly, a simple blend of different weight fractions of mannosylated and unmodified dextrans would allow us to program virtually any glucose sensitivity into our materials. Tuning glucose sensitivity is extremely important to (i) dial in a specific dissolution rate for a given environmental glucose concentration, and (ii) closely match the material response to the local glucose concentration at the site of application. For example, changes in glucose concentration in the subcutaneous fluid (SCF) are well correlated to changes in intravenous (IV) concentration, but the average value in the SCF may be much lower.

![Graphs](image)

**Fig. 2.7.** (a) 37°C GSP curves for TRITC-Dex70K gels (Con A/dextran (w/w) ratio = 12.5) containing (◆) 0.0, (■) 0.1, (▲) 0.5, (●) 0.9, and (*) 1.0 weight fraction of TRITC-ManDex70K. (b) $G_{x\%}$ as a function of the weight fraction of TRITC-ManDex70K, where x = (■) 10 and (◆) 50.

### 2.3.3. Glucose-Dependent Kinetics

GSP curves allowed one to readily compare the difference in glucose sensitivity between specific formulations, but they did not capture the glucose-dependent dissolution kinetics. To determine the relative glucose-dependent dissolution rates, gels were
incubated in DPBS solutions containing different glucose concentrations. The gels
dissolve at markedly different rates depending on the glucose concentration in the release
medium. The TRITC-Dex70K gel (Fig 2.8(a)) and FITC-Dex500K gel (Fig. 2.8(b))
exhibited sustained degradation over several hours at low glucose concentrations, but
completely dissolved in ~ 2 hr at 400 mg/dl. At 50 and 100 mg/dl, the FITC-Dex500K
gels dissolved to a greater extent than the TRITC-Dex70K gels. This was most likely due
to the incorporation of physically entrapped FITC-Dex500K molecules within the Con A-
crosslinked gels. As the gels dissolved, the physically entrapped dextran diffused out of
the network into the release medium. The much smaller TRITC-Dex70K molecules were
less likely to be entrapped within the network during crosslinking, and the subsequent
release kinetics reflected only the direct competition of glucose for the crosslinked
dextran.

![Graphs](Image)

**Fig. 2.8.** Time-dependent (a) TRITC-Dex70K and (b) FITC-Dex500K gel dissolution at
25°C at glucose concentrations of (♦) 50 mg/dl, (■) 100 mg/dl and (▲) 400 mg/dl. Con A/dextran (w/w) ratio = 8.

Assuming linear, first-order kinetics for the first 2 hr of gel dissolution, the rate
increased by ~ 40-fold for TRITC-Dex70K and ~100-fold for FITC-Dex500K when the
glucose concentration increased from 50 to 400 mg/dl. Such a sharp rate increase was
required to properly confront acute post-prandial blood glucose challenges. The kinetic
data were consistent, therefore, with the $G_{10\%}$ and $G_{50\%}$ data obtained for both
formulations (Fig. 2.6). Furthermore, as expected based on GSP data, the FITC-
Dex500K gels dissolved more rapidly at 37°C for each of the three glucose
concentrations (Fig. 2.9(a)). In this case, the dissolution rate increased by only 14-fold when the glucose concentration increased from 50 to 400 mg/dl. In contrast, FITC-ManDex500K exhibited almost negligible release at hypo- and normo-glycemic concentrations, with a 100-fold increase in dissolution rate when the glucose concentration increased from 50 to 400 mg/dl at 37°C (Fig. 2.9(b))

\[ \text{Fig. 2.9. Time-dependent (a) FITC-Dex500K and (b) FITC-ManDex500K gel dissolution at 37°C at glucose concentrations of (\( \blacklozenge \)) 50 mg/dl, (■) 100 mg/dl and (▲) 400 mg/dl. Con A/dextran (w/w) ratio = 8.} \]

2.3.4. Glucose Cycling Studies

The kinetic curves demonstrated the ability of the gels to dissolve rapidly and with high sensitivity to increased glucose concentration. However, they did not capture the reversibility of gel dissolution when, for example, the glucose concentration decreased back to hypo- and normo-glycemic levels. In practice, when blood glucose levels rise, our materials must dissolve to release insulin, which should decrease blood glucose concentration, in turn causing dissolution to slow down to basal levels. Using a flow cell (see Fig. 2.2), glucose concentrations were repeatedly cycled from low to high values over 30 min and back to low values in the next 30 min. Fig. 2.10 shows that the TRITC-Dex70K gels were capable of releasing at a higher rate as the glucose concentration increased. Even more importantly, the gels could slow down their release as the glucose concentration decreased. As expected, the extent of the response was governed by the overall glucose concentration range. When the gels were cycled
between 50 and 500 mg/dl glucose, the extent of the response diminished more rapidly after each cycle as the gel approached complete dissolution.

Unlike the unmodified dextran gels, which dissolved completely after 1 cycle at 37°C (data not shown), FITC-ManDex500K gels repeatedly responded to cycling glucose concentrations at body temperature (Fig. 2.11). In this particular experiment, glucose concentration was cycled between 50 and 500 mg/dl as before, followed by 30 min of constant glucose concentration at 50 mg/dl. This program was repeated over 4 cycles, and the gels not only rapidly and repeatedly responded to increasing and decreasing glucose levels, but also returned to the basal dissolution rates during the constant 50 mg/dl phase and stayed there with minimal baseline drift.

![Graph showing normalized instantaneous dextran dissolution](image)

**Fig. 2.10.** Room-temperature glucose cycling studies of TRITC-Dex70K obtained with the flow cell with [Glucose]ₐ = 50 mg/dl, and [Glucose]ₐ = (a) 500 mg/dl or (b) 250 mg/dl. The glucose concentration was ramped linearly from low to high values in 30 min and back down in the next 30 min. The cycle was repeated 6 times. Gel dissolution was monitored by measuring the absorbance at 544 nm. Con A/dextran (w/w) ratio = 6.1.

Reversible dissolution was only possible if the gels eroded from the surface inward rather than volumetrically. Surface erosion was not likely due to restricted glucose diffusion into the gels, as the gels were highly porous materials with the capacity to absorb large volumes of water. A more likely scenario was that competitive displacement occurred throughout the volume, but displaced components were unable to escape the surrounding network due to restricted diffusion. Only the dextran molecules
displaced from the surface were capable of being dissolved into the release medium, giving rise to glucose-dependent surface erosion.

![Graph](image)

**Fig. 2.11.** 37°C glucose cycling study of FITC-ManDex500K obtained with the flow cell with $[\text{Glucose}]_A = 50 \text{ mg/dl}$ and $[\text{Glucose}]_B = 500 \text{ mg/dl}$. The glucose concentration was ramped linearly from low to high values in 30 min and back down in the next 30 min, followed by constant inlet glucose concentration at the low value for 30 min. The cycle was repeated 4 times. Gel dissolution was monitored by measuring the absorbance at 485 nm. Con A/dextran (w/w) ratio = 4.

### 2.4. Summary

Through careful manipulation of dextran MW and Con A/dextran ratio, we have synthesized self-contained gels that eroded at rates that depended directly on the environmental glucose concentration. Since Con A binding affinity and GSP decreased from 25°C to 37°C, covalent mannosylation was developed to improve the binding affinity between Con A and dextran, and to minimize gel dissolution at glucose concentrations below 100 mg/dl. Hybrid gels were also constructed from varying weight ratios of mannosylated and unmodified dextrans to provide a convenient means for fine-tuning the GSP to closely match the physiological, glucose-dependent pancreatic β-cell response.

The rate of gel dissolution increased by as much as 150 times from hypo- to hyper-glycemic conditions, and decreased rapidly when glucose concentrations returned
to hypoglycemic levels. The reversibility was a direct result of the high degree of
crosslinking that restricted component dissolution to the gel surface. Surface erosion,
combined with dextran mannosylation, resulted in gels that repeatedly responded at 37°C
to cycling glucose concentrations between 50 and 500 mg/dl. For use as a glucose-
responsive insulin delivery (GRID) system, insulin must be directly coupled to these
materials such that insulin release would match the glucose-dependent gel dissolution
(Chapter 3).

2.5. References

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Chapter 3 – Covalent Coupling of Insulin to Glucose-Degradable Polymers for Self-Regulated Delivery

3.1. Introduction

We have developed and optimized a self-contained, glucose-responsive material based on the physical crosslinking between dextran and tetravalent concanavalin A (Con A). The material erodes rapidly and reversibly in response to fluctuating glucose concentrations with a tunable set-point between 50 and 500 mg/dl (see Chapter 2). To perform as an ideal glucose-regulated insulin delivery (GRID) system, the material must (i) contain a high insulin concentration, (ii) prevent insulin leakage at normo- and hypoglycemic glucose concentrations (< 100 mg/dl), (iii) respond rapidly with a controlled dose of insulin as glucose concentrations increase from 100 mg/dl to 500 mg/dl, and (iv) slow insulin release as glucose concentrations decrease towards 100 mg/dl.

Physical encapsulation of native insulin molecules is preferred to preserve the biological activity, and maintain the absorption, circulation time and elimination rate of pharmaceutical-grade insulin. However, to efficiently immobilize insulin within the glucose-responsive material, the gel pore size must be less than or equal to the size of the insulin molecule. Meeting this criterion is difficult given that the average pore size of the glucose-responsive gels would not likely be smaller than the size of the crosslinking Con A tetramers (~ 11 nm). The hydrodynamic diameter of insulin monomers is only 1.6 nm, but insulin monomers can bind zinc ions to form larger ~ 5-nm hexamers, which exist as trimers of insulin dimers. In the presence of a large excess of zinc ions (>> 6 zinc ions/hexamer), insulin hexamers would further associate to form crystalline or amorphous precipitates ranging in size from 1 μm to 50 μm. In this study, encapsulation of soluble, zinc-free insulin monomers and larger zinc-insulin complexes was compared, and the resulting glucose-dependent release profiles were determined.

When insulin molecules cannot be restricted from diffusing out of the gel, insulin release is uncoupled from the glucose-responsive gel dissolution, posing a risk of sustained insulin release even at hypoglycemic glucose concentrations. To achieve a direct correlation between glucose-responsive gel dissolution and insulin release, insulin must be closely associated with the dissolving dextran polymer. In this way, an exact
number of insulin molecules would be released with every dissolving polymer chain (Fig. 3.1). In this study, covalent conjugation of insulin to dextran was evaluated for its ability to minimize insulin leakage and control dosing, while preserving the pharmacological activity of insulin.

![Diagram of insulin and dextran conjugation]

**Fig. 3.1.** Schematic representation of the proposed GRID system. Glucose competes at the surface with the insulin-dextran therapeutic, causing glucose-dependent gel degradation and insulin release.

Polymer-drug and polymer-protein conjugates are being actively investigated as a research area for improving drug solubility, extending circulation time, reducing protein immunogenicity, and targeting drugs to specific sites of action.\(^6\)-\(^9\) Insulin-dextran conjugates, specifically, were first synthesized using cyanogen bromide activation by a number of research groups interested in determining the mechanism of insulin action. By covalently attaching the large dextran chain to insulin, they found that insulin activity was preserved with respect to glucose metabolism in adipose tissue,\(^10\)-\(^12\) amino acid and glucose transport in muscle tissue,\(^10\) and lowering of blood glucose levels in rodent models,\(^10\)-\(^13\) indicating that insulin must have signaled cells without being able to pass through the cell membrane. These results were later confirmed after the cell-surface transmembrane insulin receptor complex was isolated, purified to homogeneity, cloned, sequenced, and modeled.\(^14\),\(^15\)

To optimize a GRID system based on insulin-dextran, the Con A/insulin-dextran ratio, dextran MW and degree of dextran mannosylation were tailored based on the
results of glucose set point (GSP) studies, glucose- and time-dependent insulin-dextran release profiles, and dynamic glucose cycling studies. In addition, the in vivo performance of the optimized system was evaluated using streptozotocin-induced diabetic rat models. Specifically, GRID system control over blood glucose levels during both fasting and meal-time conditions (simulated by an intraperitoneal (i.p.) glucose tolerance test) was evaluated. Transplantation in the i.p. cavity was chosen because preliminary studies involving subcutaneous (s.c.) transplantation resulted in significant inflammation and walling off of the gel with fibrotic and necrotic tissue. In addition, the i.p. insulin absorption rate was fast and targeted to the liver, which would be the preferred site of insulin action. As such, insulin transport and subsequent biological action would not likely represent the rate-limiting process in the GRID feedback-control system.

3.2. Materials and Methods

3.2.1. Animals Studies

Male Sprague-Dawley (SD) rats weighing 200-250 g were purchased from Taconic (Germantown, NY). All procedures in handling animals adhered to the Principles of Laboratory Animal Care (National Institute of Health Publication 85-23, revised in 1985). Diabetes was induced with an i.p. injection of streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO) (80 mg/kg) using a syringe (1-ml capacity) equipped with a 27-gauge 1-inch needle. Blood glucose levels were measured after a 6-hr fast on day 5 and day 6 after STZ injection. Only those rats with fasting glucose levels of > 350 mg/dl for 2 consecutive days were used for the subsequent studies. Blood glucose levels were measured using Precision QID\textsuperscript{®} test strips and a MediSense\textsuperscript{®} glucometer (Abbott Laboratories, Abbott Park, IL) from ~ 20 µl of blood extracted from the tail vein.

3.2.2. Materials

Con A (Type VI), bovine insulin, human recombinant insulin, BES buffer salts, NaCl, MnCl\textsubscript{2}, CaCl\textsubscript{2}, ZnCl\textsubscript{2}, D-glucose, D-mannose, vinylsulfone, sodium carbonate, glycine, acetic acid, HCl, NaOH, (ethylenedinitrilo)-tetraacetic acid disodium salt (EDTA), ethanol, diethyl ether, acetonitrile, sodium sulfate, and phosphoric acid were all obtained from Sigma-Aldrich (St. Louis, MO), and used without further purification.
Tetramethylrhodamine-isothiocyanate-labeled dextran with MW = 70,000 g/mol (TRITC-Dex70K) and fluorescein-isothiocyanate-labeled dextran with MW = 70,000 g/mol and 280,000 g/mol (FITC-Dex70K and FITC-Dex280K, respectively) were obtained from Sigma-Aldrich (St. Louis, MO). Unlabeled dextran with MW = 280,000 g/mol (Dex280K) was obtained from Polysciences, Inc. (Warrington, PA). Dulbecco’s 1× Phosphate-Buffered Saline (DPBS) containing calcium and magnesium was obtained from Mediatech, Inc. (Herndon, VA). Deionized (DI) water was obtained by ion-exchange chromatography over universal cartridge columns (Cole-Palmer, Vernon Hills, IL). A 45% w/v sterile, endotoxin-free D-glucose solution was obtained from Sigma-Aldrich (St. Louis, MO) for use in i.p. glucose tolerance tests.

3.2.3. Preparation of Zinc-free Bovine Insulin

Samples of insulin were made zinc-free according to the procedure described by Holladay and coworkers.16 1.0 g of bovine insulin was suspended in 100 ml of 2% acetic acid solution, and readily dissolved on addition of one drop of concentrated HCl. 75 mg of EDTA was added to the solution and the pH was adjusted to 5.4 using 5-N NaOH. After standing at room temperature overnight, the precipitate was centrifuged, washed twice with DI water, then twice with a 50:50 (v/v) solution of ethanol and diethyl ether, and dried at room temperature under vacuum.

3.2.4. Synthesis of Mannosylated Dextran

Mannosylated dextran was synthesized by a modified vinylsulfone coupling procedure.17 Briefly, 500 mg of dextran (TRITC-Dex70K or Dex280K) were dissolved in 28.6 ml of DI water at room temperature. After complete dissolution, 28.6 ml of a 1-M pH 11.4 carbonate buffer was added and stirred. 429 μl of vinylsulfone were then added to activate the dextran for 1 hr while stirring. 2.9 g of D-mannose was then added and stirred for another hour, followed by the addition of 28.6 ml of glycine (5 wt% in DI water) to quench the reaction for 1 hr. The resulting solution was then concentrated by ultrafiltration through a 10K molecular weight cutoff (MWCO) polyethersulfone membrane (Millipore, Bedford, MA) to ~ 30 ml, dialyzed exhaustively against DI water using a 6–8K MWCO tube (Spectrapor, Spectrum Labs, CA), and lyophilized (Virtis
Sentry™, Gardiner, NY) to obtain the pure powders, TRITC-ManDex70K or ManDex280K.

3.2.5. Insulin-Dextran Conjugation

Insulin-dextran was synthesized using a modified cyanogen bromide (CNBr) coupling reaction. Briefly, 500 mg of dextran (FITC-Dex70K or TRITC-ManDex70K) were dissolved in 50 ml of deionized water. 56 mg of solid CNBr were added to the resulting solution and the pH was maintained at 10.7 ± 0.2 using 5-N NaOH solution. After stirring for 15 min, another 56 mg of solid CNBr were added and the pH was maintained at 10.7 ± 0.2 while stirring for 45 min. 50–250 mg of human recombinant insulin were then added to the solution, and the pH was adjusted to 9.15 using solid sodium bicarbonate. The solution was stirred overnight, ultrafiltered exhaustively against DI water using a 10K MWCO polyethersulfone disc membrane filter (Millipore, Bedford, MA), and lyophilized. The resulting powder was then purified from unconjugated insulin by high-performance liquid chromatography (Waters, Milford, MA) using a 1-M acetic acid mobile phase over a Superdex™ 75 packed column (Amersham Biosciences, Piscataway, NJ). The insulin-dextran fraction was then lyophilized to obtain the conjugate as a pure powder (InsFITC-Dex70K or InsTRITC-ManDex70K). The degree of insulin conjugation was determined by amino acid analysis (Brigham and Women's Hospital, Boston, MA) based on aspartic acid (ASX), glutamic acid (GLX), isoleucine (ILE), leucine (LEU), and phenylalanine (PHE) content.

3.2.6. Characterization of Dextran and Insulin-Dextran Concentration

Solutions containing each of the fluorescent-labeled dextrans and insulin-dextran conjugates were serially diluted by a factor of two from 5.0 mg/ml down to 0.0024 mg/ml in DPBS to establish a fluorescence-concentration calibration curve. 200 µl of each solution were added to each of the 96 wells of a Microtest™96 polystyrene microplate (Becton Dickinson, Franklin Lakes, NJ). The fluorescence reading of each solution was determined using a Molecular Devices fmax fluorescence spectrophotometer (Sunnyvale, CA) (TRITC: λ<sub>ex</sub> = 544 nm, λ<sub>em</sub> = 590 nm; FITC: λ<sub>ex</sub> = 485 nm, λ<sub>em</sub> = 538 nm) and plotted against concentration. Samples containing unknown quantities of dextran or insulin-dextran were diluted with DPBS to obtain fluorescence readings within the linear
portion of the calibration curve. 200 μl of the diluted solutions were loaded into each of the 96 wells, and their fluorescence reading was determined using a \( f_{\text{max}} \) fluorescence spectrophotometer as described above.

3.2.7. Gel Synthesis

Con A was dissolved at 100 mg/ml in a 20-mM pH 7 BES buffer containing 1 M NaCl and 1 mM MnCl₂ and CaCl₂ (BES-20). The resulting solution was passed through a 0.45-μm syringe filter (Acrodisc® 25 mm diameter, Pall Corporation, Ann Arbor, MI) to remove any high molecular aggregates and insoluble impurities. Each dextran solution (InsFITC-Dex70K, InsTRITC-ManDex70K and ManDex280K) was prepared at 50 mg/ml in a 200-mM pH 7 BES buffer containing 0.150 M NaCl (BES-200). The dextran solutions were then diluted to the desired concentration using the same BES-200 buffer. Each dextran solution was added to a 1.5-ml conical centrifuge tube, after which an equal volume of the Con A solution was introduced with rapid mixing. The solutions were allowed to stand at room temperature for 1 hr, after which the resulting gels were isolated by centrifugation at 360 × g for 10 min (Allegra 21R, Beckman Coulter, Fullerton, CA). The supernatant was removed, and the concentration of insulin-dextran was determined by fluorescence spectroscopy. The crosslinking efficiency \( X_{\text{CL}} \) for each experiment was calculated according to Equation 1 using the supernatant and total dextran concentrations.

\[
X_{\text{CL}} = \frac{[\text{dextran}]_{\text{total}} - [\text{dextran}]_{\text{supernatant}}}{[\text{dextran}]_{\text{total}}}
\]  

The gels were then washed twice with DI water and twice with DPBS. After removing the last wash solution, the gels were kept at 4°C overnight and used subsequently for the following studies.

3.2.8. Physical Insulin Encapsulation and Release Studies

FITC-Dex280K and FITC-ManDex280K gels with a Con A/dextran (w/w) ratio of 6.25 were used to encapsulate zinc-free insulin and zinc-insulin precipitates, respectively. To load the gels with insulin, the synthesis medium was modified to contain a solution of bovine insulin (either zinc-free or as a zinc-complexed precipitate)
at 1 mg/ml. After synthesis and washing, the gels were frozen in liquid nitrogen and freeze-dried to obtain their dry weight. To determine insulin loading, the gels were dissolved in 1.0 ml of 0.1 N HCl, and the resulting solution was analyzed by a high-performance liquid chromatography (HPLC) column (Waters, Symmetry 5-μm C18, 250 mm × 4.6 mm) using a mobile phase of 1.4 ml/min containing 37% v/v acetonitrile in a pH 2.4 sodium sulphate/phosphoric acid buffer. The insulin peak area of the dissolved solution was calculated and compared to a standard linear curve obtained under the same conditions.

Each gel was incubated in 1.0 ml of glucose-free DPBS for a predetermined length of time (~ 1 day for zinc-free gels and ~ 2 hr for zinc-complex gels). The gels were then incubated in DPBS containing 100 mg/dl of glucose, followed by incubation in DPBS containing 1,000 mg/dl of glucose. Throughout the course of the experiment, 0.5-ml samples of the release medium were removed and replaced with 0.5 ml of fresh medium to measure insulin concentration by HPLC and dextran concentration by fluorescence spectroscopy.

3.2.9. Environmental Scanning Electron Microscopy (ESEM)

Select gel formulations were washed repeatedly with DI water, frozen in liquid nitrogen, and freeze dried overnight (Virtis Sentry™, Gardiner, NY). The dried gels were sectioned and placed on a ½-inch aluminum specimen mount with a 12 mm-diameter carbon conductive tab (Ted Pella, Inc., Redding, CA). ESEM was performed on the mounted, sectioned, dry gel with a FEI/Philips XL30 FEG ESEM (FEI Company, Peabody, MA) using a 20-kV beam and 1.9 torr of water vapor partial pressure.

3.2.10. Insulin-Dextran Glucose Set-Point Studies

1.0 ml of DPBS solution containing 50 mg/dl of D-glucose was added to each of the 24 3-ml wells of a Multiwell™ plate (Becton Dickinson, Franklin Lakes, NJ). Each gel constructed from InsFITC-Dex70 and InsTRITC-ManDex70 was then added to the solution and agitated for 1 hr using a microplate incubator/shaker (“Jitterbug,” Boekel Industries, Philadelphia, PA) set at either 25°C or 37°C. 0.5 ml of the release medium was then removed, and the insulin-dextran concentration was determined by fluorescence
spectroscopy. The release medium was supplemented with 0.5 ml of a 150-mg/dl glucose solution to make a 100-mg/dl solution, and the gels were agitated for another hour. This process was repeated with release medium glucose concentrations of 200, 400, 800 and 1600 mg/dl for a total of 6 concentrations over 6 hr. The percent insulin-dextran dissolution was then calculated by normalizing the cumulative insulin-dextran released by that released at 100% dissolution. In all cases, 100% dissolution was obtained after the 1600 mg/dl glucose incubation.

3.2.11. Insulin-Dextran Dosing Studies

To adjust the amount of insulin-dextran loaded into each gel formulation, unlabeled, unconjugated ManDex280K was used as a diluent. The total dextran (InsDex70K + ManDex280K) concentration was held constant at 8 mg/ml, while the weight ratio of InsDex70K to ManDex280K was adjusted from 1 to 9. After gel synthesis and centrifugation, the supernatant was removed and measured for insulin-dextran concentration by fluorescence spectroscopy. The total loading of the insulin-dextran conjugate was determined from the calculated \( X_{CL} \) and the effective insulin concentration was determined by multiplying the calculated conjugate concentration by the degree of insulin substitution as determined by amino acid (AA) analysis.

3.2.12. Insulin-Dextran Release Studies

1.0 ml of DPBS containing 50, 100 or 400 mg/dl of D-glucose was added to each of the 24 3-ml wells of a Multiwell\textsuperscript{TM} plate (Becton Dickinson, Franklin Lakes, NJ). Three of each gel formulation were then added to each of the three glucose solutions and agitated using a microplate incubator/shaker ("Jitterbug," Boekel Industries, Philadelphia, PA) set at 37\(^\circ\)C. 0.5 ml of release medium was removed at specified time intervals, and the dextran concentration was determined by fluorescence spectroscopy. The release medium was supplemented with 0.5 ml DPBS containing the same concentration of D-glucose as the solution removed, and the process was continued for a total of 5 hr.

3.2.13. Insulin-Dextran Cycling Studies

A flow cell was constructed using a HPLC pump system equipped with a UV/Visible absorbance detector (Waters Corporation, Milford, MA) (see Fig. 2.2). The
inlet to the flow cell was connected to two reservoirs containing DPBS solutions having glucose concentrations of 50 and 500 mg/dl, respectively. The outlet of the flow cell was connected to the absorbance detector to continuously measure the amount of insulin-dextran dissolved at a wavelength equal to the maximum $\lambda_{ex}$ of the insulin-dextran fluorophore. The instantaneous amount of dissolved insulin-dextran was measured continuously over time and normalized by that originally contained within the gel. The gel was equilibrated at a glucose concentration of 50 mg/dl for 20 min at 37°C. Then, the HPLC pumps were programmed to cycle the inlet glucose concentration from 50 to 500 mg/dl in 30 min and then back down in another 30 min. The glucose concentration was then held at 50 mg/dl for 30 min before the next cycle. The cycle was repeated three more times for a total of four cycles over 6 hr.

### 3.2.14. In Vivo Glucose Control Experiments

Transplant recipients ($n = 6$) were anesthetized by i.p. injection of ketamine (50 mg/kg) and xylazine (10 mg/kg), and the ventral midline area was shaved. A small (5–10 mm) ventral incision was made with aseptic technique, and the glucose-responsive gels were inserted into the peritoneum using a sterile scupula. The incision was closed using sutures and surgical staples. Banamine (0.5 mg/kg s.c.) was used as analgesic during the post-operative recovery. Controls ($n = 6$) were subjected to the same surgical procedure, but a saline solution was injected into the peritoneum in place of the glucose-responsive gels.

Rats were fasted each day after transplantation for 6 hr prior to measuring blood glucose levels by tail vein bleeding. After a 6-hr fast, i.p. glucose tolerance tests (IPGTT) were performed on gel-transplanted rats ($n = 4$) by i.p. injection of a 45% w/v D-glucose solution at a dose of 1 g/kg. Over the course of the next 3 hr, blood samples (25 µL) were taken frequently from the tail vein to determine blood glucose levels. Normal, non-diabetic rats ($n = 4$) were used as controls.

### 3.3. Results and Discussion

#### 3.3.1. Physical Insulin Encapsulation and Release
After synthesis and washing, the zinc-free insulin-loaded FITC-Dex280K gels contained ~1% insulin on a dry weight basis as determined by HPLC. Both FITC-Dex280K dissolution and insulin release were measured in response to increasing glucose concentrations using fluorescence spectroscopy and HPLC, respectively. As shown in Fig. 3.2, after ~18 hr of room temperature incubation in glucose-free DPBS, less than 5% of the FITC-Dex280K dissolved. However, at the same time under the same conditions, almost 80% of the insulin was released. As the glucose concentration was ramped to 100 mg/dl for the next 22 hr and to 1,000 mg/dl over the last 8 hr, the gel dissolved by another 45% and another 50%, respectively, while the remaining insulin was released in a [glucose]-independent manner.

**Fig. 3.2.** Stepwise release profile of (■) physically entrapped zinc-free bovine insulin and (♦) FITC-Dex280K from a glucose-responsive gel formulation (Con A/FITC-Dex280K (w/w) ratio = 12.5). Gels were incubated in DPBS at room temperature for (a) 18 hr at 0 mg/dl of glucose, followed by (b) 22 hr at 100 mg/dl of glucose, and followed by (c) 8 hr at 1,000 mg/dl of glucose.
The release profiles indicated that insulin was able to diffuse out of the pores of the gel, regardless of whether the encapsulating gel has dissolved. To confirm this hypothesis, the pore sizes of a liquid nitrogen-frozen, hydrated gel were characterized by ESEM. They were determined to be ~ 5–10 μm in size (Fig. 3.3), which were too large to entrap the 1.6-nm insulin molecules.

![Fig. 3.3. ESEM micrographs of freeze-dried gels constructed from FITC-Dex280K. Con A/FITC-Dex280K (w/w) ratio = 6.1.](image)

To overcome the size disparity between the pores and insulin molecules, ZnCl₂ (0.1 mM) was added to the gel synthesis medium along with bovine insulin (1 mg/ml). Physical encapsulation of the zinc-insulin complexes resulted in 6.5% (w/w) loading on a dry mass basis, an increase of ~ 6.5 times over the zinc-free insulin loading. However, when subjected to a similar release study (Fig. 3.4), only 30% of the encapsulated insulin was released, despite a rapid and complete gel dissolution during the 1,000-mg/dl glucose incubation period. These results indicated that insulin release was limited by the solubility of the zinc-insulin complex, which was released from the gel but dissolved much more slowly over time. In fact, dissolution was one of the rate-limiting factors for the in vivo absorption of long-acting subcutaneously injected zinc-insulin suspensions.¹⁸ Despite the benefits of increased loading and minimal insulin leakage, zinc-insulin complexation was undesirable because insulin solubility rather than glucose concentration governed the insulin release rate.
Fig. 3.4. Stepwise release profile of (■) insulin from physically entrapped zinc-insulin precipitates and (◆) FITC-ManDex280K from a glucose-responsive gel formulation (Con A/FITC-ManDex280K (w/w) ratio = 12.5. Gels were incubated in DPBS at room temperature for (a) 120 min at 0 mg/dl of glucose, followed by (b) 120 min at 100 mg/dl of glucose, and followed by (c) 160 min at 1,000 mg/dl of glucose.

3.3.2. Insulin-Dextran Conjugation

Covalent conjugation of human recombinant insulin to FITC-Dex70K and TRITC-ManDex70K was achieved through CNBr coupling. After reaction using a CNBr/dextran weight ratio of 0.26 and an insulin/dextran weight ratio of 0.25, the conjugate was purified from unreacted insulin by preparative gel permeation chromatography over a Superdex™ G75 column (Fig. 3.5). Purified InsFITC-Dex70K and InsTRITC-ManDex70K conjugates were obtained with insulin/dextran weight ratios of 0.113 ± 0.010 and 0.073 ± 0.006, respectively (Table 3.1). The degree of insulin conjugation may be increased by increasing the insulin/dextran stoichiometry of the coupling reaction. However, because insulin attaches to a cell-surface receptor to activate intracellular signaling,¹⁴ it is doubtful whether a multiply substituted insulin-dextran conjugate can act on two separate receptors unless the size of the conjugate and the spacing between substituted insulin molecules are sufficiently large.
Fig. 3.5. Preparative HPLC chromatogram for the purification of (a) insulin-dextran (MW = 70K) from (b) free insulin using a Superdex™ G75 column and 1 M acetic acid eluent at a flow rate of 1.0 ml/min. Dashed line represents the isolated fraction.

Table 3.1. Amino acid analysis results for (a) purified InsFITC-Dex70K and (b) purified InsTRITC-ManDex70K.

(a)  

<table>
<thead>
<tr>
<th>Amino Acid (AA)</th>
<th>nmol/mg conjugate</th>
<th>nmol insulin/mg conjugate</th>
<th>mg insulin/mg conjugate</th>
<th>mol insulin/mol chains</th>
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<tbody>
<tr>
<td>ASX</td>
<td>59.1</td>
<td>19.7</td>
<td>0.114</td>
<td>1.38</td>
</tr>
<tr>
<td>GLX</td>
<td>153.5</td>
<td>21.9</td>
<td>0.127</td>
<td>1.54</td>
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<td>LEU</td>
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<td>19.7</td>
<td>0.114</td>
<td>1.36</td>
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<tr>
<td>PHE</td>
<td>56.2</td>
<td>18.7</td>
<td>0.108</td>
<td>1.31</td>
</tr>
</tbody>
</table>

Average over all AA values 0.113 ± 0.010 1.36 ± 0.12

(b)  

<table>
<thead>
<tr>
<th>Amino Acid (AA)</th>
<th>nmol/mg conjugate</th>
<th>nmol insulin/mg conjugate</th>
<th>mg insulin/mg conjugate</th>
<th>mol insulin/mol chains</th>
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</thead>
<tbody>
<tr>
<td>ASX</td>
<td>36.8</td>
<td>12.3</td>
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<td>0.359</td>
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<tr>
<td>GLX</td>
<td>99.1</td>
<td>14.2</td>
<td>0.0822</td>
<td>0.991</td>
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<tr>
<td>ILE</td>
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<tr>
<td>LEU</td>
<td>75.8</td>
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<td>0.885</td>
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<tr>
<td>PHE</td>
<td>36.1</td>
<td>12.0</td>
<td>0.0699</td>
<td>0.842</td>
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</tbody>
</table>

Average over all AA values 0.0729 ± 0.0056 0.879 ± 0.067
3.3.3. Insulin-Dextran Gel Synthesis, Glucose Set Point, and Release Studies

The ability of insulin-dextran conjugates to crosslink with Con A to form insoluble, self-contained gels was investigated as a function of the Con A/dextran ratio and the covalent attachment of D-mannose. Fig. 3.6 illustrates that as the Con A/dextran ratio was increased above 4 mg/mg, an appreciable amount of both the mannosylated and unmodified insulin-dextran conjugates would form precipitated complexes with Con A. Furthermore, as was observed with the non-conjugated dextrans, covalent attachment of the higher affinity mannose molecules prior to insulin conjugation resulted in a molecule that crosslinked ~ 50% more efficiently than the non-mannosylated insulin-dextran conjugate.

![Graph showing crosslinking efficiency (XCL) vs. Con A/dextran (w/w) ratio.]

**Fig. 3.6.** Crosslinking efficiency ($X_{CL}$) of (♦) InsFITC-Dex70K and (■) InsTRITC-ManDex70K vs. Con A/dextran (w/w) ratio at a constant [Con A] of 100 mg/ml.

Furthermore, when the crosslinked gels were subjected to a glucose set-point (GSP) study at 37°C, the InsFITC-Dex70K dissolved by over 20% even at 50 mg/dl of glucose, while the higher affinity InsTRITC-ManDex70K (Con A/dextran = 8 and 16 mg/mg) only released appreciably above 100 mg/dl of glucose (Fig. 3.7). The $X_{CL}$ and GSP data together demonstrated the versatility of systems derived from insulin-dextran in that (i) the InsFITC-Dex70K gels have a low relative loading of insulin-dextran and low GSP to closely match basal insulin requirements, while (ii) the InsTRITC-ManDex70K gels contained a relatively higher concentration of insulin-dextran and only significantly
responded to glucose concentrations above 100 mg/dl, as would be required during mealtime glucose challenges.

**Fig. 3.7.** Glucose set-point (GSP) curves at 37°C for (a) InsFITC-Dex70K and (b) InsTRITC-ManDex70K gels formed at a Con A/dextran (w/w) ratio of (◆) 4, (■) 8 and (▲) 16.

In addition, the insulin content could be independently varied within a given gel composition without significantly altering the encapsulation efficiency. In these experiments, non-conjugated ManDex280K was used as a diluent while the amount of insulin-dextran used in the crosslinking reaction was varied. As shown in Table 3.2, the amount of insulin-dextran encapsulated was directly correlated with the amount loaded during the crosslinking reaction. Therefore, adjusting the relative amount of insulin-dextran to non-conjugated insulin allowed for programmable and precise insulin loading up to a maximum loading corresponding to the degree of insulin-dextran conjugation.

**Table 3.2.** Comparison of control over insulin-dextran loading for InsFITC-Dex70K and InsTRITC-ManDex70K gels by diluting the reaction medium with non-conjugated ManDex280K (Con A/total dextran ratio = 100 mg/mg).

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Amount loaded (mg)</th>
<th>Amount encapsulated (mg)</th>
<th>Insulin-Dextran ( X_{CL} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>InsFITC-Dex70K</td>
<td>0.80</td>
<td>0.33</td>
<td>41%</td>
</tr>
<tr>
<td>InsFITC-Dex70K</td>
<td>1.44</td>
<td>0.53</td>
<td>37%</td>
</tr>
<tr>
<td>InsTRITC-ManDex70K</td>
<td>0.80</td>
<td>0.49</td>
<td>61%</td>
</tr>
<tr>
<td>InsTRITC-ManDex70K</td>
<td>1.44</td>
<td>0.95</td>
<td>66%</td>
</tr>
</tbody>
</table>
To illustrate the power of this technique, glucose-dependent insulin-dextran release was measured over time at 37°C for each of the four gels described in Table 3. Figs. 3.8(a) and (b) show that the InsFITC-Dex70K gels and the InsTRITC-ManDex70K gels released low doses of insulin-dextran at 50 and 100 mg/dl of glucose. However, the increase in the extent and rate of release was much more dramatic for the InsTRITC-ManDex70K gels at 400 mg/dl of glucose (Fig. 3.8(b)). In other words, insulin-dextran materials that were mannosylated have the capacity to release basal amounts of insulin at hypo- and normo-glycemic glucose concentrations, but rapidly responded to glucose concentrations above 100 mg/dl. The InsFITC-Dex70K gels, on the other hand, have a lower capacity to respond with large doses of insulin-dextran when confronted with high glucose concentrations.

Fig. 3.8. Time-dependent release of (a,c) InsFITC-Dex70K and (b,d) InsTRITC-ManDex70K at 37°C at glucose concentrations of (•) 50 mg/dl, (■) 100 mg/dl, and (▲) 400 mg/dl. Con A/total dextran (w/w) ratio = 8; insulin-dextran/ManDex280K (w/w) ratio = (a,b) 1 and (c,d) 9.
Furthermore, the relative glucose sensitivity between 50 and 400 mg/dl did not significantly decrease when the ratio of InsTRITC-ManDex70K to ManDex280K was increased from 1 to 9 (Figs. 3.8(b) and (d)). However, the InsFITC-Dex70K gels became much more sensitive to lower glucose concentrations when the relative amount of ManDex280K was decreased (Figs. 3.8(a) and (c)). As was observed for non-conjugated dextrans, the GSP of physical mixtures of mannosylated and unmodified dextrans was generally higher than homogeneous unmodified dextran gels. This technique provided a convenient means to adjust glucose sensitivity without mannosylating the insulin-dextran therapeutic directly.

Finally, the InsFITC-Dex70K/ManDex280K (50:50 w/w) gel dissolution was found to be reversible at 37°C when the glucose concentration was cycled (Fig. 3.9). In this particular experiment, the inlet [glucose] was ramped linearly from 50 mg/dl to 500 mg/dl in 30 min, and then back down again in 30 min, followed by holding the inlet [glucose] at 50 mg/dl for 30 min. This program was repeated over four cycles, and the insulin-dextran not only rapidly and repeatedly responded to increasing and decreasing glucose levels, but returned to basal dissolution rates during the constant [glucose] hold at 50 mg/dl.

![Graph](image)

**Fig. 3.9.** Gel dissolution of InsFITC-Dex70K during glucose cycling studies, as monitored by measuring the absorbance at 485 nm. Con A/total dextran (w/w) ratio = 8; InsFITC-Dex70K/ManDex280K = 1 mg/mg.
3.3.4. *In Vivo* Glucose Control

To determine whether the insulin-dextran/Con A gels were capable of controlling blood glucose levels *in vivo*, the gels were surgically implanted into the i.p. cavity of STZ-induced diabetic rats. The i.p. cavity was chosen because no adverse immune response was observed in pilot studies, and the site was well-vascularized to ensure rapid systemic uptake of the released insulin-dextran. In the first study, fasting blood glucose levels were measured in two populations of rats (n = 6) that were either implanted with a glucose-responsive gel (Con A/InsTRITC-ManDex70K = 4, 0.400 ml total gel synthesis volume) or a saline solution control in day 0. The particular gel formulation was capable of reducing fasting blood glucose to physiologically normal values for over 2 days (see Fig. 3.10(a)). The study also indicated that (i) the insulin-dextran conjugate was indeed bioactive, and (ii) the gels were capable of preventing insulin release during the 6-hr fast on both day 1 and day 2 of the experiment due to their glucose-responsive insulin release mechanism.

![Graphs](image)

**Fig. 3.10.** (a) Fasting blood glucose levels over time for STZ-induced diabetic rats containing (●) i.p. implants of InsTRITC-ManDex70K gels (n = 6), and (■) saline solution controls (n = 6). (b) IPGTT in (●) STZ-induced diabetic rats containing i.p. implants of InsTRITC-ManDex70K gels (n = 4), and (■) normal non-diabetic rat controls (n = 4).

Furthermore, two sets of 4 rats (non-diabetic and diabetic with i.p.-implanted glucose-responsive gels) were subjected to an i.p. glucose tolerance test (IPGTT) at 1 g/kg, and their blood glucose levels were measured frequently over the course of two
hours. Fig. 3.10(b) shows that the diabetic rats implanted with glucose-responsive gels have a peak glucose concentration that was actually lower than the controls and occurred only 15 min later. Furthermore, the blood glucose returned to fasting levels in less than 2 hr as was observed in the non-diabetic rats. The findings presented in Fig. 3.10 illustrated that a single i.p.-implanted gel formulation was capable of controlling blood glucose levels during both fasting and mealtime-simulated conditions.

3.4. Summary

The first competitive binding, glucose-responsive system was based on the interaction between maltose, maltotriose, manntriose, and mannotetrose-functionalized insulin conjugates with Con A. Covalent modification of insulin was necessary to achieve a direct correlation between glucose concentration and insulin release from Con A. However, the system was completely soluble, and required a glucose-permeable and glycosylated insulin-permeable, Con A-impermeable membrane to hold the system together, relegating its use to a device rather than a therapeutic. By conjugating insulin to mannosylated and non-mannosylated dextran and optimizing the crosslink ratio and dextran MW, we have synthesized the first self-contained GRID system, requiring no external membranes while preserving the precise glucose sensitivity achieved with the membrane-encapsulated soluble system.

Further benefits of insulin-dextran conjugation included (i) purely glucose-sensitive release with no glucose-independent leakage, (ii) controlled dosing by varying the ratio of non-conjugated dextran to insulin-dextran, (iii) tunable glucose sensitivity based on dextran mannosylation, and (iv) reversible insulin release (due to direct coupling of insulin and dextran) with reversible glucose-responsive polymer dissolution. Furthermore, the glucose-responsive insulin-dextran gels were active in vivo as demonstrated by their ability to control both fasting and mealtime-simulated blood glucose levels in STZ-induced diabetic rats. Surgical i.p. transplantation, however, would not be a practical means for the frequent dosing of our GRID system. Therefore, the gels must be developed into a formulation suitable for injectable, inhalable or oral delivery for convenient, repeated GRID administration (see Chapter 4).
3.5. References


Chapter 4 – Reverse Microemulsion-Mediated Synthesis of Nanoparticles for Glucose-Responsive Insulin Delivery

4.1. Introduction

We have designed a self-contained glucose-responsive insulin delivery (GRID) system comprised of a gel crosslinked by the physical interaction between tetravalent concanavalin A (Con A) and dextran-insulin covalent conjugates (Chapters 2 and 3).\(^1,2\) While the system released insulin rapidly and reversibly in a glucose-dependent manner from 50 mg/dl to 500 mg/dl with minimal leakage at hypo- and normo-glycemic glucose concentrations, the GRID had to be surgically transplanted intraperitoneally to function \textit{in vivo} (Chapter 3).\(^2\) To enable less invasive, repeated dosing of insulin, the GRID materials must be formulated into a more convenient delivery vehicle.

Development of alternative or more convenient methods of administering proteins is already an intense area of research.\(^3\) In the case of insulin treatment, efforts have focused on either (i) reducing the number of injections, or (ii) facilitating administration through non-invasive routes such as oral or pulmonary delivery. Polymer nanoparticles and microparticles (Fig. 4.1) have shown promise in addressing these challenges by first providing a means to encapsulate the protein and release it over extended periods of time, thereby potentially reducing the dosing frequency. In addition, it has been demonstrated that protein encapsulation within certain polymers provides protection from proteolytic enzymes to facilitate oral delivery.\(^4-6\) Furthermore, it has been shown that small (submicron) colloidal particles are absorbed and transported through the intestinal mucosa,\(^7-9\) thereby enhancing the oral bioavailability of the protein. It is known that particles delivered to the lung are preferentially within the size range of 500 nm to 3.3 \(\mu\)m for deposition in the alveoli.\(^10,11\) Systemically absorbed nanoparticles may also avoid rapid elimination due to macrophage digestion, provided that the particle size is less than \(\sim 300\) nm.\(^12\) Despite the attractive features of nanoparticles in enhancing insulin delivery, the usual mechanism of sustained release is independent of physiological blood glucose concentration. We are therefore interested in constructing our GRID materials in the
form of nanoparticles to not only improve the means of insulin delivery as outlined above, but also provide the desired glucose-responsive release profile once administered.

Fig. 4.1. Examples of particles used for injectable, inhalable and oral protein delivery: (a) injectable poly-lactide-coglycolide (PLGA) particles (1–500 μm) from Macromed, Inc. (Sandy, UT), (b) inhalable insulin microspheres (1–3 μm) from Nektar, Inc. (San Carlos, CA), and (c) PLGA particles (< 2 μm) from Spheric, Inc (Lincoln, RI).

Our laboratory has developed a reverse microemulsion (RM) synthesis of inorganic nanoparticles for catalytic applications. In this research, we sought to apply this synthesis technique to derive polymer nanoparticles for controlled drug delivery. RM’s are spontaneously forming, thermodynamically stable, water-in-oil (w/o) systems having a uniform aqueous particle size in the range of 5–200 nm (Fig. 4.2). The nanometer-sized aqueous domains are used as a medium for the controlled synthesis of nanoparticles. RM’s are different from reverse macroemulsions, which are optically cloudy instead of transparent/translucent in appearance, thermodynamically unstable, and require a large input of energy to prepare.

Fig. 4.2. Schematic representation of a reverse microemulsion comprised of a continuous oil phase and dispersed aqueous droplets of 5–200 nm, stabilized with the use of nonionic surfactants and co-surfactants.
Although the RM synthesis of polymer particles has been extensively investigated in the past decade, the surfactants that were typically used, such as sodium di-2-ethylhexylsulfosuccinate (AOT) and polyethoxylated alcohols, were highly toxic and could not be separated completely from the resulting polymer particles without severely deteriorating the chemical structure of the polymers. Therefore, conventional RM systems would not be suitable for the preparation of materials for drug delivery applications.

We have examined a variety of RM formulations containing only surfactants and oils that were non-toxic and FDA-approved for food and drug use. The surfactants were generally glycerides and sorbitan-derived oleates. The non-polar phases consisted of naturally derived oils, such as soybean and coconut oils. RM's based on such materials represented a particularly attractive environment for the nanoencapsulation of labile proteins because (i) they would not require high-intensity shear to form stable nanometer-sized droplets, and (ii) they could be formulated at room temperature with nonionic surfactants, thus avoiding shear-, temperature- and/or surfactant-induced protein denaturation. Furthermore, lipid-based RM's have been proposed to enhance the oral bioavailability of peptides and proteins. In fact, one group has actually synthesized polymer nanocapsules of insulin from biocompatible RM's to combine the advantages of polymeric protection from acidic and proteolytic gastro-intestinal degradation with the intestinal absorption enhancing effects of the RM components.

The success of such a synthesis technique depends on the ability to form stable RM's even in the presence of high molecular weight and potentially surface-active materials, such as the dextran, insulin and Con A used to construct our GRID system. Indeed, in spite of the growing number of applications involving RM-assisted drug solubilization and delivery, surprisingly few studies have examined the effect of drug on phase behavior, this was despite the fact that a large number of drug molecules were themselves surface-active, and as such would be expected to influence the RM phase behavior.

The behavior of RM's is usually characterized by a pseudo-ternary phase diagram, such as the one depicted in Fig. 4.3. Each corner represents a binary mixture of two of
the three components, the surfactant/cosurfactant, water/drug, and oil/drug. In general, a RM boundary is identified by the cloud point, or a particular composition beyond which an isotropically clear dispersion would become cloudy. Constructing phase diagrams is both time- and material-intensive, particularly when the aim is to accurately delineate a phase boundary. The procedure most often employed is to prepare a series of (pseudo) binary compositions and titrate with the third component, evaluating the mixture after each addition. Detection of phase boundary is usually subjective, depending on the observer’s qualitative analysis of solution turbidity.

![Phase Diagram](image)

Fig. 4.3. Schematic representation of a pseudo-ternary phase diagram of an oil/surfactant/water system with emphasis on microemulsion and emulsion phases.

One of the goals of this study was to develop the first quantitative, rapid screening approach to establish RM pseudo-ternary diagrams for a particular surfactant/oil system. Emphasis was placed on evaluating the effects of temperature, pH, as well as the concentrations of Con A, insulin and dextran in the aqueous phase. Secondly, we investigated how the stability of RM’s, constructed from different oils and surfactants, was affected by the concentrations of dissolved proteins and polymers. Finally, GRID particles were synthesized using an optimal RM composition, and tested for glucose-responsive dissolution and insulin-dextran release.
4.2. Materials and Methods

4.2.1. Materials

Fluorescein-isothiocyanate-labeled dextran with MW = 280,000 g/mol (FITC-Dex280K), dextrans (MW = 10,000 and 70,000 g/mol), Con A (Type VI), bovine insulin, polyoxyethylene (20) sorbitan monooleate (Tween 80), sorbitan monooleate (Span 80), soybean oil, BES buffer salts, NaCl, MnCl₂, CaCl₂, D-glucose, D-mannose, vinylsulfone, sodium carbonate, glycine, HCl, and NaOH were obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco’s 1× Phosphate-Buffered Saline containing calcium and magnesium (DPBS) was obtained from Mediatech, Inc. (Herndon, VA). Deionized (DI) water was obtained by ion-exchange chromatography over universal cartridge columns (Cole-Palmer, Vernon Hills, IL).

Captex 355 (C₈/C₁₀ triglycerides) and Capmul MCM (C₈/C₁₀ mono-/di-glycerides) were generously donated by Abitec Corporation (Janesville, WI). Arlacel 186 (monoolein:propylene glycol 90:10) was generously donated by ICI Americas (Wilminton, DE). Cremophor EL (polyoxyethylene glycerol tricinoleate 35 DAC) was generously donated by BASF (Parsippany, NJ). Myvacet (distilled acetylated monoglycerides) and Myverol 18-92 (distilled sunflower oil monoglyceride, 90% glycerol linoleate) were generously donated by Eastman Chemicals (Kingsport, TN).

Zn²⁺-free insulin was prepared according to a previously published procedure.²² Mannosylated FITC-Dex280K (FITC-ManDex280K) was prepared using the vinylsulfone procedure described previously.²³

4.2.2. Dynamic Light Scattering Measurements

A Brookhaven Instruments Photon Correlation Spectrometer model BI-9000 AT (Holtsville, NY) equipped with an argon ion laser model Stabilite 2017 from Spectra-Physics (Mountain View, CA) was used for dynamic light scattering (DLS). DLS was monitored at 90° angle and 25°C with polystyrene beads as a standard. The particle size and size distribution were obtained by non-negatively constrained least squares fitting of the autocorrelation function. Kinematic viscosity measurements were obtained using a
falling ball viscometer (VWR, Boston, MA) with a constant of 0.04231 cSt/s. Multiplying this value by the density gave the viscosity in cP. An Abbe refractometer (American Optical, Buffalo, NY) was used to determine the refractive index. Both viscosity and refractive index values were required to determine the hydrodynamic diameter from the measured diffusion coefficient according to the Stokes-Einstein equation.

4.2.3. Effect of Aqueous pH on RM Stability in the Presence of Con A and Insulin

Solutions containing varying concentrations of Con A (0–50 mg/ml) and insulin (0–20 mg/ml) were mixed with a RM comprising 65% (v/v) oil (3:1 w/w Captex 355/Capmul MCM), 25% (v/v) surfactant (3:2 w/w Tween 80/Span 80), and 10% (v/v) aqueous phase (designated RM-Cap). In the neutral pH experiment, Con A was dissolved in a 20-mM pH 7 BES buffer containing 1 M NaCl and 1 mM MnCl₂ and CaCl₂ (BES-20), and zinc-free bovine insulin was dissolved in a 200-mM pH 7 BES buffer containing 0.150 M NaCl (BES-200). In the acidic pH experiment, both Con A and zinc-free bovine insulin were dissolved in 0.1 N of HCl. The particle size and size distribution of the resulting blends of oil, surfactant and aqueous phase were then measured by DLS.

4.2.4. Effect of Composition on RM Stability in the Presence of Con A and Insulin

Solutions containing varying concentrations of Con A (0–50 mg/ml) and insulin (0–20 mg/ml) in BES-20 and BES-200, respectively, were mixed with RM’s comprising 87% oil, 10% surfactant and 3% aqueous phase by volume. The particle size and size distribution of the resulting mixtures were determined by DLS for each of three different RM’s: RM-Cap, RM-Myv and RM-Soy (see Table 4.1).

<table>
<thead>
<tr>
<th>RM</th>
<th>Oil</th>
<th>Surfactant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cap</td>
<td>Captex 355/Capmul MCM (3:1 w/w)</td>
<td>Tween 80/Span 80 (3:2 w/w)</td>
</tr>
<tr>
<td>Myv</td>
<td>Myvacet/Capmul MCM (8:5:1 w/w)</td>
<td>Cremophore EL/Myverol 18-92 (10:1 w/w)</td>
</tr>
<tr>
<td>Soy</td>
<td>Soybean Oil/Arlacel 186 (3:1 w/w)</td>
<td>Tween 80</td>
</tr>
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</table>

4.2.5. Ternary Diagram Construction for RM’s

Various pseudoternary compositions were prepared and evaluated for optical
clarity. Clear, transparent formulations were indicative of stable RM's as the size of the aqueous domains would have been much smaller than the wavelength of light. We have developed a rapid screening approach to quantify the optical clarity of a particular system at a number of compositions and temperatures. This approach minimizes the time and materials required for mapping out the ternary diagram. For example, for RM-Cap, varying amounts of surfactants (3:2 w/w Tween 80/Span 80) and oils (3:1 w/w Captex 355/Capmul MCM) were micropipetted into a glass, flat-bottomed, 96-well microplate (500-μl well capacity, Alltech, Deerfield, IL) and mixed thoroughly. The appropriate amount of aqueous solution was then added to make a total volume of 350 μl, and mixed again in such a way that each well contained a specific ternary composition. Each composition was run in duplicate, and the absorbance values for all 96 wells were collected at a wavelength of 450 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA) to quantify the turbidity of various mixtures. The internal plate-reader thermal incubator was then adjusted to take absorbance readings of plates equilibrated at elevated temperatures. The data resulting from such experiments were plotted on a pseudoternary contour diagram, yielding a pseudoternary surface; while pseudoternary surfaces plotted as a function of temperature produced a prism.

To evaluate the effect of dissolved proteins and polymers on the RM phase behavior, varying concentrations of each protein and polymer were dissolved in the aqueous phase before addition to the surfactant/oil mixture. Then, new pseudoternary contour diagrams were constructed to illustrate the region of RM in the presence of different types and concentrations of aqueous dissolved species.

4.2.6. Characterization of Dextran Concentrations by Fluorescence Spectroscopy

Solutions containing each of the fluorescent-labeled dextrans (FITC-Dex280K and FITC-ManDex280K) were serially diluted by a factor of two from 5.0 mg/ml down to 0.0024 mg/ml in DPBS to establish a fluorescence-concentration calibration curve. 200 μl of each solution were added to each of the 96 wells of a Microtest™96 polystyrene microplate (Becton Dickinson, Franklin Lakes, NJ). The fluorescence of each solution was determined using a Molecular Devices (Sunnyvale, CA) fmax fluorescence
spectrophotometer (FITC: $\lambda_{ex} = 485$ nm, $\lambda_{em} = 538$ nm), and plotted against concentration. Samples containing unknown quantities of dextran were diluted with DPBS to obtain fluorescence readings within the linear portion of the calibration curve. 200 µl of the diluted solutions were loaded into each of the 96 wells, and their fluorescence was determined using a $f_{max}$ fluorescence spectrophotometer as described above.

4.2.7. Synthesis of Nanoparticles

Glucose-sensitive nanoparticles were prepared using a RM formulation comprising 65% v/v oil (3:1 w/w Captex 355/Capmul MCM), 25% v/v surfactant (3:2 w/w Tween 80/Span 80), and 10% v/v aqueous phase. 160 µl of a 0.1-N HCl solution containing Con A and FITC-Dex280K or FITC-ManDex280K of predetermined concentrations were added to 1.8 ml of premixed oil and surfactant. Next, 20 µl of a pH 7.0 200 mM BES buffered saline solution containing 1 M NaCl, 20 mM MnCl$_2$ and CaCl$_2$, and 20 µl of a 1-N NaOH solution were added to neutralize the pH and activate Con A/dextran crosslinking. The resulting mixtures were allowed to stand overnight at room temperature prior to particle isolation and characterization.

4.2.8. Particle Isolation and Purification

The particle-containing RM’s were diluted 1:10 (v/v) with ethanol, and centrifuged for 30 min to separate the particles from the ethanol, oil and surfactant. The centrifuge cake was redispersed by sonication, washed twice in ethanol to remove residual oil and surfactant, followed by washing with DPBS to remove any unbound dextran. The resulting dispersion was kept at 4°C overnight, and used for the following studies.

The supernatant of the first DPBS wash was removed, and the concentration of dextran was determined by fluorescence spectroscopy. The crosslinking efficiency ($X_{CL}$) for each experiment was calculated according to Equation 1 using the supernatant and total dextran concentrations.
\[ X_{CL} = \frac{[dextran]_{\text{total}} - [dextran]_{\text{supernatant}}}{[dextran]_{\text{total}}} \]  

(1)

**4.2.9. Environmental Scanning Electron Microscopy**

In select cases, the washed particles were redispersed in ethanol by sonication and dried under vacuum at room temperature on a ½-inch aluminum specimen mount containing a 12 mm-diameter carbon conductive tab (Ted Pella, Inc., Redding, CA). Environmental scanning electron microscopy (ESEM) was performed on the mounted, dry particles with a FEI/Philips XL30 FEG ESEM (FEI Company, Peabody, MA) using a 20-kV beam and 1.9 torr of water vapor partial pressure.

**4.2.10. Glucose Set-Point Studies**

The particles isolated from each experiment according to the procedure above were redispersed in a known volume of DPBS containing 50 mg/dl of glucose, and mixed for 30 min at room temperature, followed by centrifugation at 360 \( \times g \) for 30 min (Allegra 21R, Beckman Coulter, Fullerton, CA) to separate the particles from the release medium. Half the volume was collected to determine the amount of dissolved dextran by fluorescence spectroscopy, and replaced with a 150-mg/dl glucose buffer to increase the glucose concentration in the release medium to 100 mg/dl. This process was repeated with release medium glucose concentrations of 1,000 and 10,000 mg/dl for a total of 4 progressively higher concentrations over 4 hr. The percent of dextran dissolution was then calculated by normalizing the cumulative dextran released by that released at 100% dissolution. In all cases, 100% dissolution was obtained after the incubation at 10,000 mg/dl of glucose.

**4.2.11. Dextran Release Kinetics**

The particles were redispersed in known volumes of DPBS containing 50, 100 or 400 mg/dl of D-glucose, and mixed at room temperature for the entire experiment. At each time point, the solutions were ultracentrifuged at 3600 \( \times g \) for 5 min (Allegra 21R, Beckman Coulter, Fullerton, CA) to separate the particles from the release medium. Half the volume was collected to determine the amount of dissolved dextran by fluorescence
spectroscopy. The solution was then replaced with a DPBS solution containing the same D-glucose concentration and the particles were mixed with it until the next time point. This process was repeated for each time point over the course of the 24-hr experiment. The percent of dextran dissolution at glucose concentrations of 50, 100 and 400 mg/dl was calculated by normalizing the cumulative dextran released at a specific time by the total amount released after the 24-hr incubation at 400 mg/dl of glucose.

4.3. Results and Discussion

4.3.1. Effect of Aqueous pH on RM Stability in the Presence of Con A and Insulin

In 0.1 N of HCl, both Con A-containing and insulin-containing RM-Cap systems were transparent and stable over the entire concentration range investigated in this study, with particle sizes of < 10 nm (Fig. 4.4). However, in neutral pH buffered saline solutions, Con A and insulin induced a transition from transparency to turbidity as observed by the sharp increase in particle size.

![Graph](image1)

**Fig. 4.4.** Particle size of the RM-Cap system as a function of aqueous phase concentration for (a) Con A and (b) insulin dissolved in (▲) 0.1 N HCl and (■) BES-20.

Possible explanations for this observation included pH-dependent differences in protein charge, state of aggregation, and tertiary structure. For example, both Con A and insulin would form tetramers and hexamers, respectively, at neutral pH, which might result in decreased solubility in the aqueous domains and increased droplet aggregation,
similar to the effect noted for dextran. In addition, other researchers have found large structural changes when proteins were added to reversed micelles as was the case, for instance, with cytochrome c, which enhanced percolation due to increase in attractive interactions between reversed micelles.\textsuperscript{24} In our system, these attractive interactions were minimized at low pH, but increased upon pH neutralization.

4.3.2. Effect of Composition on RM Stability in the Presence of Con A and Insulin

To investigate whether stable RM’s could be obtained using neutral pH buffered saline solutions of Con A and insulin, the surfactant and oil components were varied from RM-Cap to RM-Myv and RM-Soy. Fig. 4.5 shows that RM-Soy compositions were not only stable over the entire concentration range for both Con A and insulin, but also gave rise to extremely small aqueous domain sizes (~10 nm). The RM-Myv system, on the other hand, exhibited a decrease in particle size from ~1 µm to ~15 nm as the aqueous phase concentrations of both Con A and insulin were decreased. Different RM systems could therefore be used subsequently to control the size of our GRID particles.

![Graphs showing particle size](image)

**Fig. 4.5.** Particle size of the (■) RM-Cap, (◆) RM-Myv and (○) RM-Soy systems as a function of aqueous phase concentration for (a) Con A and (b) insulin dissolved in BES-20.

The general reduction in particle size could be correlated with the decrease in the hydrophilic-lipophilic balance (HLB) value of the low-HLB excipients used in each mixture (i.e. Capmul MCM (5.5) for RM-Cap, Myvacet 18-92 (3.7) for RM-Myv, and
Arlacel 186 (2.8) for RM-Soy). The lower the HLB value, the greater is the tendency to form w/o rather than o/w emulsion systems. Therefore, there was most likely a greater tendency toward aqueous droplet interactions in the RM-Cap system than the RM-Soy system, leading to a higher probability of droplet collision-induced aggregation. The hypothesis of enhanced droplet interaction in the RM-Cap system was supported by the fact that only this system spontaneously formed o/w fine emulsions when diluted with an excess of water.

4.3.3. Phase Behavior of Reverse Microemulsions

To explore the RM-Cap phase behavior further, the RM stability was evaluated as a function of surfactant, oil, and aqueous phase composition. Fig. 4.6 depicts the ternary diagram obtained from our rapid screening approach, which agreed remarkably well with the one in the literature obtained by multiple cloud point titrations.

Fig. 4.6. Pseudo-ternary diagram of the RM-Cap system at 25°C constructed using the rapid screening approach for a 0.1-N HCl aqueous phase. Absorbance at $\lambda = 450$ nm: \begin{itemize} \item \[ \leq 0.25 \] \item \[ \leq 0.50 \] \item \[ \leq 0.75 \] \item \[ \leq 1.00 \] \item \[ \leq 1.25 \] \item \[ \leq 1.50 \] \item \[ > 1.50 \] \end{itemize}

DLS results (Fig. 4.7) obtained at select ternary compositions confirmed the existence of RM in the region of optical transparency shown in Fig. 4.6. As the aqueous concentration increased at constant concentrations of surfactant (Fig. 4.7(a)) and oil (Fig. 4.7(b)), the dispersed phase progressed from reverse micelles to larger water-swollen reverse micelles, until a turbid multiphase region was obtained. As the surfactant concentration increased at a constant aqueous concentration (Fig. 4.7(c)), the system
underwent transition from a kinetically stabilized reverse emulsion toward a RM containing droplets of decreasing particle size. Thus, the size of the GRID nanoparticles could be subsequently tailored by varying the ratio of surfactant to oil at a constant aqueous concentration in this RM system.

Fig. 4.7. DLS results for select pseudo-ternary compositions in the RM-Cap system at 25°C as a function of aqueous volume fraction at (a) a constant surfactant volume fraction of 0.25 and (b) a constant oil volume fraction of 0.65, and (c) as a function of surfactant volume fraction at a constant aqueous volume fraction of 0.10.

The phase prism of Fig. 4.8 illustrates that as temperature was increased, the region of RM was expanded to contain up to 30% water by volume. The increased water volume fraction would translate to a higher yield of particles for a given RM system.
Fig. 4.8. Temperature-dependent pseudo-ternary prism for the RM-Cap system. Absorbance at $\lambda = 450$ nm: $\square \leq 0.25$, $\square \leq 0.50$, $\square \leq 0.75$, $\square \leq 1.00$, $\square \leq 1.25$, $\square \leq 1.50$, $\square > 1.50$.

Fig. 4.9 shows that the region of RM diminished upon the addition of dextran in a concentration-dependent manner. As the dextran MW increased from 10,000 to 70,000 g/mol, the region of RM was diminished further. Other researchers have studied the effects of the water-soluble polymer, polyoxyethylene (POE), on RM phase behavior using ionic AOT/H$_2$O/paraffin$^{26}$ and nonionic pentaethylene glycol monododecyl ether (C$_{12}$E$_5$)/H$_2$O/paraffin$^{27}$ systems and found that the polymer might induce droplet cluster formation, leading to macroscopic phase separation in some cases. They also found that this effect was more prevalent at higher polymer concentrations and higher polymer MW. Possible explanations for this behavior included a polymer-induced increase in droplet attractive interactions and/or a tendency to maximize chain entropy by forming larger-sized water pools.
Fig. 4.9. Dependence of the RM-Cap system on the concentration of dextran of MW = (a) 10,000 and (b) 70,000 g/mol dissolved in 0.1 N HCl aqueous phase at 25°C. Absorbance at \( \lambda = 450 \) nm: \( \square \leq 0.25 \), \( \square \leq 0.50 \), \( \square \leq 0.75 \), \( \square \leq 1.00 \), \( \square \leq 2.50 \), \( \square \leq 1.50 \), \( \square \geq 1.50 \).

The effect of dissolved proteins on RM phase behavior was determined using a 0.1-N HCl aqueous phase. Fig. 4.10(a) showed that insulin has minimal effect on the RM region compared to the control (Fig. 4.6). In contrast, addition of \( \geq 25 \) mg/ml of Con A led to the appearance of two distinct regions of optical clarity, separated by a turbid region at high surfactant and moderate oil concentrations (Fig. 4.10(b)). The ternary volume composition that consisted of 10% aqueous phase, 25% surfactant and 65% oil was chosen for the mediated synthesis of nanoparticles at 25°C since it was found to yield the most stable RM for all three components (i.e. dextran, insulin and Con A) over the concentration ranges of interest. In addition, based on the pH-dependent protein stability, the RM was formulated at low pH, followed by neutralization to induce Con A crosslinking and nanoparticle formation.
Fig. 4.10. Dependence of the RM-Cap system on the concentrations of (a) insulin and (b) Con A dissolved in 0.1 N HCl aqueous phase at 25°C. Absorbance at λ = 450 nm: \[ \begin{array}{c} \square \leq 0.25, \quad \square \leq 0.50, \quad \square \leq 0.75, \\
\square \leq 1.00, \quad \square \leq 1.25, \quad \square \leq 1.50, \quad \square > 1.50. \end{array} \]

4.3.4. Particle Characterization

Fig. 4.11 shows an ESEM micrograph of particles obtained from a formulation comprising FITC-Dex280K (8 mg/ml) and Con A (50 mg/ml). In this RM system, the particles obtained were much larger than the original aqueous domains, most probably due to the pH-dependent change in the RM phase behavior. In addition, the interaction between Con A and dextran might have increased the droplet attraction, leading to particle growth. Nevertheless, the entire population of particles was present in the submicron range of < 200 nm, demonstrating our ability to restrict the reaction size domain and form physically crosslinked nanoparticles via RM-mediated synthesis.
Fig. 4.11. ESEM micrograph of particles obtained via RM-Cap mediated synthesis at 25°C. Aqueous concentrations of FITC-Dex280K and Con A = 8 mg/ml and 50 mg/ml, respectively.

4.3.5. Nanoparticle Crosslinking Efficiency, Glucose Set-Point and Release Kinetics

Fig. 4.12 shows that the extent of crosslinking ($X_{\text{CL}}$) for both FITC-Dex280K and FITC-ManDex280K was highly dependent on the Con A concentration in the aqueous phase. It further illustrates that $X_{\text{CL}}$ steadily increased with the Con A/dextran weight ratio until a maximum value was reached, as observed also in the macroscopic gel crosslinking experiments (Chapter 2).

Fig. 4.12. Crosslinking efficiency ($X_{\text{CL}}$) for (a) FITC-Dex280K and (b) FITC-ManDex280K in RM-Cap with 10% (v/v) aqueous phase, 25% (v/v) surfactant and 65% (v/v) oil. Concentration of Con A in aqueous phase = (■) 25 mg/ml and (◆) 50 mg/ml.
The amount of crosslinked polymer might be improved by a factor of 2 through covalent modification of the dextran polymer. The higher affinity of Con A for mannose \(K_{d,\text{mannose}} = 3.8 \ K_{d,\text{glucose}}\)\(^{28}\) ensured a higher proportion of bound polymer at a given concentration of Con A and dextran. In addition, mannosylation of the dextran backbone effectively increased the degree of branching, which was known to facilitate the precipitation reaction between Con A and \(\alpha\)-1,6-linked polysaccharides.\(^{29}\)

The GSP of the RM-synthesized nanoparticles was also measured for both FITC-Dex280K and FITC-ManDex280K as a function of Con A/dextran ratio. Fig. 4.13 shows that all nanoparticle formulations preserved the glucose-sensitive dissolution properties observed in their macroscopic gel counterparts. The GSP could be controlled by the Con A/dextran weight ratio; increasing this ratio from 1.6 to 6.3 resulted in particles that were more sensitive to higher glucose concentrations. In addition, as expected from the GSP studies on macroscopic gels, mannosylated dextran nanoparticles were much more sensitive to higher glucose concentrations (> 100 mg/dl) than the unmodified dextran nanoparticles.

Fig. 4.13. Glucose set-point (GSP) curves at 25°C for (a) FITC-Dex280K and (b) FITC-ManDex280K nanoparticles synthesized in RM-Cap with 10% (v/v) aqueous phase, 25% (v/v) surfactant and 65% (v/v) oil. Concentration of Con A in aqueous phase = 50 mg/ml. Con A/dextran (w/w) ratio = (▲) 1.6, (■) 3.1, and (◆) 6.2.
Fig. 4.14 depicts the time-dependent nanoparticle dissolution as a function of glucose concentration. FITC-Dex280K nanoparticles were shown to dissolve too rapidly at 50 and 100 mg/dl of glucose. In contrast, FITC-ManDex280K nanoparticles exhibited < 40% dissolution over 24 hours at 50 mg/dl of glucose. When the glucose concentration was increased to 400 mg/dl, over 60% of the FITC-ManDex280K nanoparticles would dissolve in less than 30 min. Together with the gel crosslinking and GSP studies, these results indicated that our RM-synthesized GRID systems preserved their glucose-dependent dissolution profiles, and were suitable for in vivo testing of their ability to control blood glucose levels.

Fig. 4.14. Room-temperature time-dependent dissolution of dextran nanoparticles at glucose concentrations of (◆) 50 mg/dl, (■) 100 mg/dl and (▲) 400 mg/dl. (a) FITC-Dex280K and (b) FITC-ManDex280K nanoparticles were derived from RM-Cap mediated synthesis with Con A/dextran (w/w) ratios of 3.1 and 6.2, respectively.

4.4. Summary

The phase behavior of biocompatible RM’s has been determined in the presence of dissolved polymers and proteins using a novel rapid screening technique developed in our laboratory. By manipulating the composition of oils and surfactants, as well as the relative ratio of surfactant to oil, the size of the RM domains could be easily varied to derive a wide range of GRID particle sizes. Using the well-characterized RM-Cap system, particles of < 200 nm were successfully synthesized, isolated and characterized
for their glucose-dependent dissolution properties. As was found for the macroscopic
GRID gel systems, mannosylation of the dextran polymer and optimization of the Con
A/dextran crosslink ratio were necessary to control glucose sensitivity in the desired
physiological blood glucose range.

Since the RM excipients are all biocompatible and FDA-approved, the entire
system may be administered orally without isolating the nanoparticles. For delivery via
inhalation, the nanoparticles may be isolated, dried, and delivered through a dry powder
inhaler (DPI). The isolated nanoparticles may also be redispersed in a buffered saline
solution for convenient injectable, subcutaneous delivery.

4.5. **References**

[1] Zion, T. C.; Hu, Y.; Ying, J. Y., Design of a novel, self-contained, glucose-
degradable material. *To be submitted 2004*.

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oral bioactivity of insulin in diabetic rats using nanocapsules dispersed in


Chapter 5 – Conclusions and Recommendations for Future Work

5.1. Conclusions

Achieving normal glycemic control in diabetic patients is nearly impossible given the pharmacokinetics after subcutaneous injection of commercial insulin. Glucose-regulated insulin delivery (GRID) would vastly improve glycemic control while reducing (i) the required frequency of subcutaneous injections and finger-stick glucose tests, (ii) the incidence of hypoglycemia and hyperglycemia, and (iii) the resulting nerve, kidney, retinal and cardiovascular complications associated with diabetes. We have chosen to formulate a GRID system into a repeated dosage form capable of being administered through injection, inhalation, or oral routes to eliminate the need for surgery. In our particular system, dextran, a glucose-containing polymer, was crosslinked using the tetrafunctional glucose-binding protein, concanavalin A (Con A). Free glucose would then directly compete with the polymer for Con A binding sites, causing displacement of the polymer and disruption of the crosslinks.

5.1.1. Development of A Self-Contained, Glucose-Degradable Material

Through careful manipulation of dextran molecular weight (MW) and Con A/dextran ratio, we have synthesized self-contained gels that could be diluted in an infinite volume of biological medium without requiring enclosure within a size-selective membrane to prevent component leakage. The resulting insoluble materials would erode from the surface due to the competitive binding of glucose. This direct interaction with glucose at the surface enabled a rapid, glucose-dependent erosion rate. Furthermore, surface erosion, as opposed to volumetric dissolution, provided the system with the ability to turn on and off in response to repeated glucose challenges. Lastly, by manipulating the dextran affinity for Con A and combining varying ratios of low and high affinity constructs, the glucose sensitivity was tailored to closely match the physiological, glucose-dependent pancreatic β-cell response. Eliminating the need for membranes, auxiliary pumps and machinery enabled the use of these materials for repeated doses of self-regulated insulin rather than restricting their use to extra- or intra-corporeal devices.
5.1.2. Covalent Coupling of Insulin to Glucose-Responsive Materials

By covalently conjugating insulin to affinity-modified dextrans and optimizing the crosslink ratio and dextran MW, we have synthesized the first self-contained GRID system while preserving the precise glucose sensitivity achieved with a membrane-encapsulated soluble system. This system combined the specificity of previously designed soluble glycosylated insulin/Con A complexes with the ability of polymeric systems to form insoluble constructs to eliminate the need for membrane enclosure. Despite covalent insulin modification, the glucose-responsive insulin-dextran gels were active in vivo as demonstrated by their ability to control both fasting and mealtime-simulated blood glucose levels in STZ-induced diabetic rats following surgical intraperitoneal (i.p.) transplantation. Further benefits of insulin-dextran conjugation included (i) purely glucose-sensitive release with no glucose-independent leakage, (ii) controlled dosing by varying the ratio of non-conjugated dextran to insulin-dextran, (iii) tunable glucose sensitivity based on dextran mannosylation, and (iv) reversible insulin release (due to direct coupling of insulin and dextran) with reversible glucose-responsive polymer dissolution.

5.1.3. Synthesis of Nanoparticles for Convenient Delivery

Reverse microemulsions (RM) constructed from biocompatible oils and surfactants were used to synthesize GRID nanoparticles for more convenient delivery. Since the RM excipients were all biocompatible and FDA-approved, the entire system might be administered orally without isolating the nanoparticles. For delivery via inhalation, the nanoparticles could be isolated, dried, and delivered through a dry powder inhaler (DPI). The isolated nanoparticles might also be redispersed in a buffered saline solution for convenient injectable, subcutaneous delivery.

Particles of < 200 nm were successfully synthesized, isolated and characterized for their glucose-dependent dissolution properties after exploring the RM phase behavior using a novel rapid screening technique developed in our laboratory. By manipulating the composition of oils and surfactants, as well as the relative ratio of surfactant to oil, the size of the RM domains was easily varied to derive a wide range of GRID particle sizes.
The nanoparticles were found to preserve the relative glucose-sensitive dissolution of their macroscopic counterparts, but the overall response rate tended to increase with the increase in surface area. However, the rate of GRID nanoparticle dissolution at a given glucose concentration was easily adjusted by modifying dextran with the higher affinity mannose.

5.2. Recommendations for Future Work

5.2.1. Optimization of the Biocompatibility of Glucose-Binding Molecule

The glucose-binding protein, Con A, like all proteins is fully biodegradable, but the biocompatibility needs to be evaluated independently and in conjunction with the GRID system. Con A, a plant lectin, is known to be both mitogenic and antigenic,\(^1\)\(^3\) and as such may yield both acute and long-term immunological responses. However, Ueno and coworkers\(^1\) found that covalent modification of Con A with poly(ethylene glycol) (PEG), which is commonly referred to as pegylation, significantly reduced the mitogenicity, immunoreactivity and immunogenicity to elicit anti-Con A antibody production. Furthermore, Kim and Park found that pegylated Con A still retained its glucose-binding properties \textit{in vitro}.\(^4\) Both the length of PEG groups and the extent of pegylation influenced the \textit{in vivo} immune response as well as the \textit{in vitro} glucose-binding properties. Therefore, these parameters should be evaluated in future studies to improve the biocompatibility of the GRID system.

In addition, there exist a number of human sugar-binding proteins, or collectins, with properties similar to Con A. For example, human mannan-binding protein (MBP) contains a hexamer of trimeric sugar-binding sites with specificity for both mannose and glucose oligosaccharide residues.\(^5\)\(^-\)\(^8\) Studies have shown that the concentration of glucose required for 50% inhibition of MBP-mannan binding was only 13% greater than that required to inhibit Con A-mannan binding.\(^7\) Surfactant proteins A and D were also well-studied human proteins containing multimeric sugar-binding moieties.\(^9\)\(^,\)\(^10\) SP-D, in particular, has a specificity for \(\alpha\)-glucosyl residues,\(^10\) and has been used to precipitate viruses, bacteria, and fungi expressing specific surface oligosaccharides containing these residues.\(^9\) Human Con A analogues along with pegylated Con A constructs should be
evaluated in parallel to quantify the improvement in safety, while preserving the glucose-dep-
dendent functionality of our system.

5.2.2. In Vivo Studies and Performance Optimization

While preliminary studies with i.p.-implanted glucose-responsive gels demonstrated blood glucose control under both fasting and glucose tolerance test conditions, the corresponding insulin-dextran concentration profile was not measured. Future studies should develop a means to reliably quantify serum insulin-dextran concentration using an adapted ELISA protocol. After establishing a reproducible standard calibration curve, the dynamics of insulin-dextran release must be measured in vivo to quantify and minimize the delay time between an acute glucose challenge and the peak serum insulin-dextran levels.

Furthermore, the first practical application of a repeated dose of glucose-regulated insulin would be via subcutaneous (s.c.) injection, because it has been the most widely used and generally accepted method of administering insulin. However, a transition from i.p. to s.c. administration requires our system to overcome the s.c.-associated molecular transport and immunological barriers. Since our therapeutic is constructed from dextran, a biocompatible and biodegradable polymer that is already approved by the FDA for blood plasma volume expansion,\textsuperscript{11} we expect minimal adverse immunological and inflammatory responses in vivo. However, less is known about s.c. administered dextran and dextran-therapeutic conjugates. Furthermore, most clinical experience involved dextran with a molecular weight of 70K. Much less is known about the absorption and elimination profiles of lower and higher MW dextrans. Therefore, a major focus of future studies should include evaluation of the safety, pharmacokinetics and pharmacodynamics associated with s.c.-injected insulin-dextran conjugates of varying MW.

5.2.3. Optimization of Nanoparticle Synthesis, Stabilization and In Vivo Properties

The same studies used to evaluate the RM-Cap system should be applied to the RM-Soy and RM-Miyv systems to generate a wide range of particle sizes. Isolated nanoparticles may then be redispersed in physiological buffered saline solution and
administered via s.c. injection. Varying particle sizes not only will influence the rate of insulin delivery at a given glucose concentration, but may also affect the biocompatibility of the injection with regard to macrophage uptake and response.

The injectable particle dispersion must be as concentrated as possible to minimize the volume required per injection and maximize the duration of glycemic control. However, because the GRID nanoparticles are uncharged and have exposed complementary Con A and dextran surfaces, they tend to aggregate significantly as the aqueous concentration increases. Therefore, future studies should focus on stabilizing the aqueous particle dispersion by adding biocompatible surfactants and co-surfactants to minimize the extent of aggregation.

Finally, to evaluate non-injection-based delivery, the biocompatible RM-derived nanoparticles may be administered intraduodenally without purification to diabetic rats to determine particle uptake, blood glucose depression profiles, and the corresponding oral bioavailability. The effect of RM oil and surfactant composition in addition to particle size should be studied to determine the ability of the biocompatible RM carrier vehicle to facilitate oral delivery of self-regulated insulin. Successful oral delivery of nanoparticles has the distinct advantage of absorption into the portal circulation and accumulation in the liver, the site of physiological insulin secretion in non-diabetic individuals.

5.3. References


