Glucose-Responsive Implantable Polymeric Microdevices for “Smart” Insulin Therapy of Diabetes

by

Michael Kok Loon Chu

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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University of Toronto

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ABSTRACT

Diabetes mellitus is a chronic illness manifested by improper blood glucose management, affecting over 350 million worldwide. As a result, all type 1 patients and roughly 20% of type 2 patients require exogenous insulin therapy to survive. Typically, daily multiple injections are taken to maintain normal glucose levels in response glucose spikes from meals. However, patient compliance and dosing accuracy can fluctuate with variation in meals, exercise, glucose metabolism or stress, leading to poor clinical outcomes. A ‘smart’, closed-loop insulin delivery system providing on-demand release kinetics responding to circulating glucose levels would be a boon for diabetes patients, replacing constant self monitoring and insulin. This thesis focuses on the development of a novel, ‘smart’ insulin microdevice that can provide on-demand insulin release in response to blood glucose levels.

In the early stage, the feasibility of integrating a composite membrane with pH-responsive nanoparticles embedded in ethylcellulose membrane to provide pH-responsive in vitro release was examined and confirmed using a model drug, vitamin B12. In the second microdevice, glucose oxidase for generating pH signals from glucose oxidation, catalase and manganese dioxide nanoparticles, as peroxide scavengers, were used in a bioinorganic, albumin-based membrane cross-linked with a polydimethylsiloxane (PDMS) grid-microdevice system. This
prototype device demonstrated insulin release in response to glucose levels \textit{in vitro} and regulating plasma glucose in type 1 diabetic rats when implanted intraperitoneally.

Advancement allowing for subcutaneous implantation and improved biocompatibility was achieved with surface modification of PDMS microdevices grafted with activated 20 kDa polyethylene glycol (PEG) chains, dramatically reducing immune response and local inflammation. When implanted subcutaneously in diabetic rats, glucose-responsive insulin delivery microdevices showed short and long-term efficacy up to an 18 day period. Finally, to improve insulin stability within microdevice reservoirs, an \textit{in situ} gelling zinc-insulin formulation was designed. High concentration insulin gel complexed with zinc provided physical and chemical stability against thermal denaturation over a 30 day period. Long-term stability of the zinc-insulin gel formulation shows potential for sustained release application, providing low-level, basal insulin release. These combined technologies present significant progress towards the goal of an ‘artificial pancreas’ to combat diabetes through ‘smart’ insulin therapy.
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Firstly, I would like to thank my supervisor, Dr. Xiao Yu Wu, for giving me this opportunity to do research in her lab. With her wisdom, patience, and support, I have learned so much in our time together during my Ph.D studies. She always expected the most from me and I cannot thank her enough for her endless guidance, which has pushed me to improve continuously. I will never forget what she has done for my professional and personal growth, throughout all the future paths I shall take.

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Michael
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<td>Arg</td>
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<tr>
<td>Asn</td>
<td>Asparagine</td>
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<tr>
<td>Asp</td>
<td>Aspartic Acid</td>
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<tr>
<td>BIS</td>
<td>N,N-methylenebisacrylamide</td>
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<td>CAT</td>
<td>Catalase</td>
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<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>CGM</td>
<td>Continuous glucose monitor</td>
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<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
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<tr>
<td>Con A</td>
<td>Concanavalin A</td>
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<tr>
<td>Gln</td>
<td>Glutamine</td>
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<tr>
<td>Glu</td>
<td>Glutamic acid</td>
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<tr>
<td>Gly</td>
<td>Glycine</td>
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<tr>
<td>GOX</td>
<td>Glucose oxidase</td>
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<tr>
<td>HbA1c</td>
<td>Glycated hemoglobin</td>
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<tr>
<td>His</td>
<td>Histidine</td>
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<tr>
<td>HMWP</td>
<td>High molecular weight protein</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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KPS – Potassium persulfate

LCST – Lower critical solution temperature

Lys – Lysine

MAA – Methacrylic acid

MnO₂ – Manganese dioxide

NAD/NADH – Nicotinamide adenine dinucleotide

NIPAM – N-isopropylacrylamide

NPH – Neutral Protamine Hagedorn

PBA – Phenylboronic acid

PBS – Phosphate buffered saline

PDMS – Polydimethylsiloxane

pI – Isoelectric point

pKa – Acid dissociation constant

PMMA – Polymethylmethacrylate

PNIPAM-MMA – Poly(N-isopropylacrylamide-co-methacrylic acid)

Pro – Proline

PVP – Polyvinylpyrrolidone

RP-HPLC – Reversed-Phase High Performance Liquid Chromatography
s.c. – Subcutaneous

SDS – Sodium dodecyl sulfate

UCST – Upper critical solution temperature

VB_{12} – Vitamin B_{12}

WBC – White blood cell

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Chapter 1  Introduction

1.1 Background

The health and socio-economic impact of diabetes mellitus is increasing steadily with the aging population. The number of diabetes patients worldwide has hit 350 million and is predicted to double by the year 2030. Insulin therapy remains a necessary treatment option for patients suffering from type 1 and about 20% of patients with type 2 diabetes. Regardless of classification, the goal of insulin therapy is to manage glycemia within a tight, normal range. Optimal insulin therapy requires frequent glucose detection and regular insulin administration to match up with anticipated peaks in blood glucose level after meals. This treatment method requires strict patient compliance and intervention. Nevertheless, it is difficult to accurately predict blood glucose levels due to variations in meal schedule, glycemic index of food, body metabolism, and exercise intensity. As a result, rigorous insulin therapy is often associated with acute hypoglycemia or low blood glucose, stressing the importance of maintaining a narrow glycemic window. Therefore, many efforts have been made in past few decades to develop glucose-responsive insulin delivery systems with an ultimate goal to mimic the homeostatic capability of the human pancreas, also known as an “artificial pancreas.”

A variety of delivery systems have been investigated to achieve glucose-responsive closed-loop insulin delivery which can be divided into two categories: (1) an electromechanical system; and (2) a chemically-driven system. The first type of system consists of a mechanical delivery system, i.e. an insulin pump, which is controlled by a continuous glucose monitor wired or wirelessly. Through a negative feedback system, continuous glucose monitors are connected via an electrode beneath the skin to determine blood glucose levels, and information is transmitted to
the insulin pump through radio signals to stop or deliver insulin according to low or high glucose levels, respectively \(^{69,70,88}\). Despite this advancement of technology, the glucose sensors require calibration and have a limited lifetime (~3 days) of efficacy, and maintenance of these systems is relatively expensive due to manufacture and upkeep. Recently, insulin pumps integrated with continuous glucose monitors have been explored clinically, providing insulin through a subcutaneous catheter in response to hyperglycemic alarm from the implanted glucose sensor. However, this system requires extensive physician/patient education, susceptible to catheter occlusion and requires replacement after a few days of operation. Clinical trials with electromechanical continuous glucose monitor/pump systems have been carried out with mostly positive results \(^{81,83,87,88}\), but have limited commercial reach, as upkeep is expensive compared with chemically-driven materials or multiple injection strategies.

The second type of system is non-electronic and utilizes chemical signals generated by either binding of glucose with polymer-conjugated glucose-responsive moieties or enzymatic oxidation of glucose (GOX) integrated with stimulus-responsive polymers. In the former, glucose can bind competitively with Concanavalin A (ConA), a plant-based lectin \(^{124-127}\) or reversibly forming a covalent bond with phenylboronic acid (PBA) \(^{135-137}\), causing a drastic change in swelling of the polymer network. This change is utilized to design insulin-loaded materials that release insulin in response to environmental glucose levels, depending on polymer swelling or shrinking. However, application in \textit{in vivo} system is limited due to toxicity from Con A systems \(^{129,130}\) and unfavorable operating pH for PBA which is higher than physiological pH \(^{136,137}\). Alternately, enzyme-driven glucose-responsive materials combined with stimulus-sensitive materials, specifically pH-responsive materials, have shown advantages for use in insulin delivery in physiological conditions. Combined with pH-sensitive materials, glucose
oxidase has been used as a catalyst for gluconic acid production, causing volumetric shrinkage of anionic polymer networks such as carboxylates, or swelling of cationic polymer networks, such as aminoethyl and aminomethyl methacrylates. The rapid response of these polymer materials has been previously utilized by our group to produce pH-responsive composite membranes for modulated drug release.

In this thesis, we have proposed a closed-loop, implantable insulin microdevice. This device is a chemically-driven system, which does not require electronic and mechanical parts and is inexpensive to manufacture. The device consists of a glucose-responsive insulin releasing membrane and a biocompatible insulin reservoir. pH-responsive nanoparticles are embedded in the membrane, which swell or shrink, acting as nanovalves according the microenvironmental pH affected by glucose levels in the presence of GOX. Combining these technologies, I intend to achieve long-term euglycemia in a type 1 diabetic rat model.

To maintain physicochemical stability and bioactivity of insulin in an implanted microdevice at body temperature for a long term (e.g. 30 days), we have proposed a new thermostable insulin gel formulation with high insulin concentration. Currently commercial insulin solutions have low concentration and limited shelf-life, owing to potential fibrillation due to concentration-mediated kinetics and denaturation stresses from liquid formulation. These formulations are unsuited for use in insulin implant for a long-term, as agitation and physiological temperatures would accelerate loss of insulin stability and bioactivity. Zinc-complexed, in situ insulin gel formulation has been designed to combat these pitfalls. The integration of materials science, microfabrication, pharmaceutical formulation, and drug delivery technologies are the crux of this thesis, to provide a novel closed-loop insulin therapy strategy for diabetes patients.
1.2 Hypotheses

We hypothesize that:

1) A polymeric microdevice can be made to provide modulated drug release according to environmental pH by integrating a pH-responsive polymeric composite membrane with a PDMS microreservoir.

2) An implantable glucose-responsive microdevice can be engineered to provide modulated insulin delivery on demand and thereby control blood glycemia in diabetic rats by using a PDMS grid-bioinorganic glucose-sensitive gel membrane integrated with a PDMS reservoir.

3) The biocompatibility and longevity of a subcutaneously implantable glucose-responsive insulin microdevice can be improved by surface modification with PEG of a suitable chain length, which can effectively reduce localized acute and chronic immune response in a diabetic rat model.

4) A high concentration, zinc-complexed insulin in situ gel formulation can provide thermal stability against insulin denaturation, thus maintaining its bioactivity for extended period at body temperature.

1.3 Objectives

The four objectives of this thesis are as follows:

1) To design and test a prototype pH-responsive membrane-based microdevice capable of rapid response to environmental pH change, allowing pulsatile delivery of model drug

2) To design and test an implantable glucose-responsive insulin delivery microdevice based on a PDMS grid-bioinorganic gel for glycemia control in a diabetic rat model.
3) To optimize a subcutaneously implantable glucose-responsive insulin microdevice for extended efficacy and biocompatibility in a diabetic rat model.

4) To design and test a physically and chemically stable, highly concentrate insulin formulation for use in an insulin microdevice for long-term, sustained basal insulin release.

1.4 Rationale and Scope of This Thesis

This thesis focuses on the development of a novel, implantable system for stimulus-responsive drug delivery device, in particular, a glucose-responsive closed-loop insulin delivery device for glycemia control in the diabetic condition. To design an implantable microdevice consisting of a chemically-driven, glucose-sensitive membrane integrated with an insulin reservoir, we started from a simple device with such design concept using pH-responsive composite membrane previously developed in our laboratory. Rapid response of pH-sensitive hydrogel nanoparticles was harnessed to obtain a rapidly responding membrane with desirable mechanical strength used in prototype microdevices. Both hydrophobic and hydrophilic membranes were tested for their responsiveness in a microdevice system, delivering Vitamin B$_{12}$ and insulin, respectively. As this is the first non-degradable glucose-responsive implant system showing long-term in vivo efficacy, a thorough methodology was necessary to confirm glucose-responsive character in both in vitro and in vivo experiments. As well, specifically with the glucose-responsive PDMS microdevices, the biocompatibility and device integrity was critical for maximizing device efficacy and safety tested in a diabetic rat model.

This thesis consists of seven chapters. Chapter 1 provides a brief background of the thesis, hypothesis, objectives, and scope of the thesis. Chapter 2 presents a comprehensive review of
literature in the field pertinent to the thesis topics. Chapter 3 details a prototype pH-responsive ethylcellulose membrane-PDMS microdevice system for modulated Vitamin B₁₂ release. Chapter 4 demonstrates the first implantable, bioinorganic membrane-PDMS insulin microdevice for in vivo glucose-responsive insulin release. Chapter 5 optimizes this glucose-responsive insulin microdevice system for improved biocompatibility through a subcutaneous design and surface PEGylation strategies and improved long-term efficacy. Chapter 6 outlines a novel insulin gel formulation, utilizing zinc complexation and in situ Pluronic polymer to create a high-concentration thermostable insulin formulation for sustained insulin release systems. Chapter 7 provides an overview of findings and further potential in this field.

Chapter 3 concerns the initial integration of hydrophobic ethylcellulose pH-responsive membranes with a PDMS-based microdevice. A reservoir system joined with pH-responsive membranes containing poly(N-isopropylacrylamide-co-methacrylic acid) (PNIPAM-MAA) nanoparticles embedded in ethylcellulose. PNIPAM-MAA nanoparticles were selected because they exhibit a sharp pH-dependent volume transition at body temperature due to its pKa slightly lower than physiological pH and thermostresponsive PNIPAM units which sensitize pH-triggered volume change as body temperature is above its LCST. This property is ideal for in vivo application, providing significant change in release rate of model drug, Vitamin B₁₂, at varying pH levels. As well, membrane characterization of embedded PNIPAM-MAA particles showed cluster morphology when embedded in ethylcellulose, providing physical ‘nano-pore’ formation within the base membrane. This provided the basis of microfabricated reservoir devices, utilizing soft PDMS as a non-reactive biomaterial adequate for potential use in vivo.

Chapter 4 focuses on albumin-based hydrophilic membranes integrated with PDMS grid microdevices. Crosslinked albumin was chosen as a hydrophilic base membrane material over
hydrophobic ethylcellulose previously used, citing its improved permeability to diffusion of glucose and easy incorporation of enzymes, reduced enzyme leakage and maintained bioactivity of enzyme and insulin, compared with hydrophobic materials. With embedded PNIPAM-MAA nanoparticles as before, membranes were also crosslinked with GOX, catalase (CAT) and embedded MnO₂ nanoparticles to create a glucose-sensing and insulin-releasing combined unit, which is then integrated with a PDMS insulin reservoir. For in vivo translation, this GOX-containing chemically-driven system was chosen over other glucose-responsive materials such as PBA and Con A, because the latter exhibit poor responsiveness at physiological pH and toxicity, respectively. To improve the mechanical strength of the composite membrane, we utilized microfabrication to design a PDMS grid as a framework to crosslink the albumin-based membrane. The device was able to regulate insulin release rate according to environmental glucose levels in vitro, with an over two-fold increase in the release rate when glucose level was increased from normal to hyperglycemia. In vivo, we demonstrated glucose-responsive activity of intraperitoneal (i.p.) implanted PDMS grid insulin microdevices in diabetic rats, with robust analysis of short-term blood glucose, insulin and c-peptide analysis. Also, long-term efficacy of implanted microdevices showed maintenance of normal glucose within a diabetic rat model over a one-week period, representing the first prototype implant glucose-responsive insulin reservoir system.

Chapter 5 is an extension of the previous glucose-responsive PDMS microdevice technology, modifying device design to suit subcutaneous (s.c.) implantation with a stronger focus on the biocompatibility and host immune response. Using silicone tubing as the insulin reservoir, surface-modification was performed on the hydrophobic surface, by introducing a hydrophilic polymer, PEG, a commonly used ‘stealthing’ agent, to minimize host immune response and
improve biocompatibility. We showed that longer chain PEG of 20 kDa molecular weight gave improved biocompatibility compared with 2 kDa PEG and untreated microdevices, with respect to local tissue histology and capsule analysis around implanted devices. Furthermore, improved efficacy was seen in diabetic rats when 20 kDa PEG-treated glucose-responsive microdevices were implanted s.c., giving over a 18 day control of normal glucose level with reduced local inflammation and minimal surgical complications.

Chapter 6 describes the development of a novel insulin gel formulation for use in long-term use of implantable insulin delivery devices. Commercial insulin formulations have strict handling requirements, such as refrigeration, and have limited shelf-life once opened to maintain potency. Owing to this, we designed a zinc-complexed human recombinant insulin combined with in situ gel polymer, Pluronic F-127, to maintain complete protein stability when incubated at physiological temperatures over a 30 day period. As well, strong insulin stability coincided with maintenance of bioactivity when reinjected in a diabetic rat model, giving glycemic activity similar to fresh insulin. When tested in a silicone tubing microdevice system, a gradient-based insulin gel formulation showed good linear release kinetics over a two-week period. This optimized insulin gel formulation showed potential for use in implant microdevices to protect against protein denaturation, depletion and improve long-term efficacy.

Chapter 7 discusses the conclusions and original findings of this thesis work and use in the field of ‘smart’ insulin therapy. Potential future work for these integrated glucose-responsive technologies is included as well.
Chapter 2 Literature Review

2.1 Diabetes

2.1.1 History and Global Impact

Diabetes mellitus is a metabolic disorder that has affected over 350 million people worldwide in 2013\(^1\) and the cases are rising, exacerbated by the progressing issues of sedentary lifestyle, population aging and poor nutrition habits. By 2030, this number is expected to double along with an increasing burden on health care resources and services\(^2,4\). Diabetes is a metabolic disorder marked by the inability to control blood glucose within a normal range in both fasted and fed conditions, leading to chronic complications in vascular, neural and renal systems\(^3\). Specifically, diabetes is a major concern in developed populations, affecting over 100 million adults in India, China and the United States combined\(^2\). This can be explained where ample access to food can lead to obesity, which is a major contributing factor for onset of diabetes\(^4,5\). Growing numbers of overweight populations has contributed to both an increase in diabetic and cardiovascular diseases, totaling roughly 12% of total health care expenditures\(^6\). Rapidly developing countries that are becoming more ‘westernized’ have seen an increase in diabetes cases and risk factors, owing to urbanization over the last decade, leading to a growth that vastly outpaces developed countries\(^5,6\). Although preventative measures to halt the prevalence of diabetes have been emphasized, the increasing average lifetimes from improved health care lends itself to an increase in chronic diseases, such as diabetes. Efficient treatment of diabetes is of paramount importance, not only to minimize patient complications, but hospitalization time and expenses.


2.1.2 Pathogenesis

On a general scale, diabetes mellitus, or simply diabetes, is classified as a chronic metabolic disorder of insulin management. Insulin is a hormone that drives uptake of circulating glucose from blood into cells, allowing glucose to be used for energy. Poor physiological insulin management causes poor glycemic management, which can be manifested in different ways, which will be explained in detail. The imbalance in this glycemic homeostasis disturbs downstream physiological processes (Fig. 2.1). Diabetes mellitus is separated into two forms: Type 1 or insulin-dependent diabetes, and Type 2 or insulin-independent diabetes.

Type 1 diabetes is defined by the inability to produce insulin in the body due to autoimmune destruction of beta cells in the islets of Langerhans in the pancreas. Often identified at a young age, hence ‘juvenile’ diabetes, beta-cell specific CD4+ and CD8+ T cells proliferate in type 1 patients, as a result of risk factors and environmental triggers7,8. There is an established genetic influence for developing type 1 diabetes, with direct relatives susceptible to a higher incidence of type 1 diabetes, if another relative is affected8,9. Autoantibodies causing self-destruction of islet cells are present at onset, giving rise to the analogous term autoimmune-mediated diabetes. There have been cases in which viral infection can induce this autoimmune state, in children and pregnant women10. Genetic variation of the immune response has been identified on chromosome 6 and 11, but a cure for type 1 diabetes is unavailable at the moment11,12.

Clinically, type 1 diabetes patients must take exogenous insulin to survive, giving rise to the term ‘insulin-dependent’ diabetes8.

Type 2 diabetes is caused by insufficient insulin from deficient beta cells unable to cope with cellular resistance to insulin. This usually arises later in adulthood, although increasing degrees
of unhealthy or sedentary lifestyle in childhood is causing earlier onset of Type 2 diabetes. Predisposing factors of type 2 diabetes are normal aging, excess body fat or inactivity, which may lead to a deficiency in insulin receptor sensitivity\textsuperscript{5,6,13}. The severity of type 2 diabetes can differ, but progression of the disease worsens and unless treated vigorously at an early stage, rarely enters remission. Compared with type 1 diabetes, there are many more confirmed genes predisposed to type 2 diabetes, either insulin secretory defects or insulin resistance, with progression linked to a combination of mutations \textsuperscript{14,15}. Upon diagnosis, most Type 2 patients will often be required to manage symptoms and treatments for the rest of their lives, with a few able to reverse the glycemic disorder. Roughly 90\% of diabetes cases are Type 2 and this group is growing due to social and economic reasons mentioned previously \textsuperscript{2}.

\textbf{Fig. 2.1} - Scheme of regulation of blood glucose. Glucose input is from food intake via the gastrointestinal tract or during the basal state from hepatic glucose production, which is modulated by basal insulin secretion. The brain and nervous tissue use glucose independent of insulin, while insulin stimulates glucose uptake and utilization by peripheral tissues (here represented by muscle and adipose tissue) \textsuperscript{15}. Diabetes is officially diagnosed based on fasting and 2-hour fed blood glucose values.

According to the World Health Organization, when a patient has fasting glucose levels of 7.0
mmol.L$^{-1}$ (125 mg.dL$^{-1}$) or 2-hour fed (75 g glucose load) levels of 11.1 mol.L$^{-1}$ (200 mg.dL$^{-1}$), they are considered diabetic $^{15,17}$. Alternately, the American Diabetic Association has listed diabetes at glycosylated hemoglobin levels (HbA$_{1C}$) above 6.5% (a long-term average measurement of blood glucose)$^3$. These are all variants of the hyperglycemic condition, although not detrimental for short periods of time, can lead to a number of symptoms from long-term exposure. For Type 1 patients, both situations are present, and insulin replacement therapy is required to control blood sugar. For Type 2 patients, the level of fasting glucose and glucose tolerance can affect the type of therapy required, and insulin may be required in half of cases. As well, Type 2 patients with untreated hyperglycemia can worsen in glucose tolerance and insulin sensitivity, compounding the problem, leading to a loss in beta cell mass and function $^{14,15}$. Unchecked hyperglycemia can lead to a variety of degenerative illnesses which include, but are not limited to: nephropathy, retinopathy, neuropathy, ketoacidosis and related microvascular complications.

Diabetic nephropathy is a renal disease affecting protein filtration through the endothelial walls of the glomerulus inside the kidneys. Damage to these endothelial cells occurs from high blood glucose, driving formation of glycosylated end-products, which can damage glomerular endothelial cells and basement membrane $^{18,19}$. This reduces the kidney’s ability to properly filter large macromolecules, causing excess albumin excretion in the urine. Although albuminuria can be reversed with glycemic control, around 20 to 40% of patients experience some degree of nephropathy after 10 years $^{19}$. Severe macroalbuminuria is identified as a loss of over 300 mg over a 24 h period and is a strong risk factor for future end-stage renal failure and cardiovascular disease, specifically hypertension $^{18,20,21}$.  

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Retinopathy is the most common disorder affecting patients with diabetes, with 90% of patients affected to some degree in their lifetime. Similar to nephropathy, the endothelial cells in the capillaries of the retina are damaged from hyperglycemia, causing leaky vessels, ischemia and abnormal regeneration of disordered capillaries. Early damage is reversible, but continued hyperglycemic exposure can lead to lesions or occlusions across the retinal wall and can result in cataract formation, blurred vision and eventually, complete blindness. As well, the excess fluid buildup within the eye can lead to glaucoma, causing optic nerve damage or vision loss. Macular problems are often a combination of edema, neural and vascular damage and can be exacerbated by hypertension.

As well, neuropathy can develop from multiple pathways from chronic hyperglycemia damaging neural vasculature. Buildup of advanced glycosylated end-products which damage capillary beds feeding nerve systems compromise neural axon integrity and promote apoptosis through compromised blood supply. Triggering protein kinase C initiates a cascade of stress markers, triggering vasoconstriction and downstream generation of reactive oxidative species, further exacerbating nerve injury. Activation of the polyol pathway leads to an imbalance in the NAD:NADH ratio for neural transmission and directly causes neural damage. The result of these physiological changes, which often co-exist with macrovascular damage, can manifest in numbness or pain in the feet or hands, nausea and sexual dysfunction.

From an acute, metabolic standpoint, diabetic ketoacidosis is an acute disorder that results from a gross disturbance in glucose metabolism. Despite high glucose levels in the bloodstream, poor cellular uptake from a lack of insulin or extreme insulin resistance causes the body to redirect towards fat stores for energy. Excessive breakdown of triglycerides leads to a buildup of their oxidation product, ketones, in the body. Buildup of acidic ketones decreases the physiological
pH, causing ketoacidosis, which is often accompanied by dehydration\textsuperscript{28,29}. Symptoms of ketoacidosis include dizziness, sweating and rapid breathing, often to compensate for bicarbonate loss from buffering excess ketones to maintain pH. If unchecked, this can lead to cerebral edema, cardiovascular failure from low blood pressure or kidney disease from low pH, with increased danger in younger children due to undeveloped regulatory physiology\textsuperscript{30}. Ketoacidosis was one of the first recognized symptoms of diabetes in 1886, and an inevitably fatal diagnosis before the discovery of insulin\textsuperscript{28}. Ketoacidosis is typically found in Type 1 patients, and infrequently in severe Type 2 patients\textsuperscript{18}.

Although some of these diabetic complications can be treated on a symptomatic basis, the root of diabetic morbidity is centered on poor glycemic control. This makes proactive treatment of these physiological symptoms of paramount importance. Although there are many accepted antidiabetic drugs used management of Type 2 patients, such as sulfonylureas and metformin\textsuperscript{31,32}, insulin therapy is critical for Type 1 and severe Type 2 patients. Precise and disciplined administration of exogenous insulin to combat high blood sugar is required.
2.2 Open-loop Insulin Therapy

Blood sugar management is directly affected by meals throughout the day. Meals cause a spike in blood glucose, which can easily reach values above 8 mol.L\(^{-1}\), depending on carbohydrate load. The healthy body produces insulin, which counteracts circulating blood glucose and directs excess glucose into cells, to prevent extended hyperglycemia, dropping this spike within 5 to 10 minutes. Insulin is a hormone responsible for cellular uptake of glucose into muscle and fat tissues from circulating blood. Diabetic patients do not have enough insulin or insulin sensitivity to reduce this drastic glucose spike \(^3\). This delayed drop in blood glucose leads to a protracted period of hyperglycemia, at an acute and/or chronic level. As well, during the fasted state, the lack of insulin allows for a higher level of blood glucose circulation above healthy levels, causing chronic hyperglycemia. As a result, the requirement for effective insulin therapy includes basal circulating insulin to suppress fasting blood glucose levels, but periodic administration before meals to counteract glucose spikes. Combining appropriate short and long-term insulin delivery is the core goal of optimizing diabetic patient outcomes. This is defined as ‘open-loop’ insulin therapy, as the patient or physician is required to administer insulin at regular intervals to manage blood glucose. Various open-loop strategies have been attempted at treating diabetic patients with respect to physiological insulin administration and appropriate dosage timing.

2.2.1 Parenteral Injection

Historically, the earliest and most common method of insulin administration has been parenteral injections, typically via syringe in the subcutaneous abdominal fat area. This method has the simplest route of administration, as it directly bypasses external barriers to elicit its effects.
Injections of insulin solutions are self-administered prior to meals and combined with proper blood glucose monitoring and carbohydrate documentation can provide excellent patient outcomes, reducing overall hyperglycemia duration and HbA1c levels. With combinations of rapid and long-acting insulin solutions commercially available, an accurate, patient-specific dosing schedule can be achieved. The caveat for this method involves proper patient education to design and follow an optimal dosing schedule and past this point, the efficacy of insulin therapy is outside the jurisdiction of the physician. As well, syringe discipline is required, to avoid reuse, potential infection and rotating injection sites. Modern injection pens are discreet and more precise, with a pressurized dosage, avoiding filling error and insulin storage concerns with the classic vial and syringe method. This can help maintain a regular insulin injection schedule with an active lifestyle with comparable clinical outcomes. Although there are increased costs initially with pen injection systems, this is offset by lowered overall health care costs from hyper/hypoglycemic episodes causing in-patient hours and hospital visits. With multiple injection regimens, wide variability in meals and daily activity can lead to less than accurate assessments of insulin dosage. Strict compliance is mandatory for positive patient outcomes. However, manual insulin injections are still the traditional ‘gold standard’ for diabetes treatment.

2.2.2 Alternative Forms of Insulin Delivery

Non-injectable, non-invasive forms of insulin delivery have been proposed, to provide simpler methods of administration and improve patient compliance. This includes those with aversion to needles and younger or older patients who cannot regularly provide their own injections or effectively maintain a pump system. Strategies to administer insulin across the more common routes of delivery, such as orally, through the gastrointestinal or respiratory tract, nasal mucosa, and transdermally, have been attempted.
Oral delivery is the most common form of drug administration, with numerous solid dosage forms available. As with most protein-based biologics, insulin has very sensitive characteristics that require care when considering this pathway. Mainly, the insulin protein is readily degraded in acidic stomach pH and proteolytic enzymes present in the GI tract, destroying bioactivity \cite{47,49,50}. Using specific nanoparticle polymer and enteric coating systems, oral delivery of insulin has been achieved, demonstrating efficacy in a number of diabetic rat models, through insulin encapsulation and polymer mucoadhesion, allowing diffusion across the GI tissue \cite{51,52}. Oral insulin tablets may require penetration enhancers to bypass tight junctions across the epithelial layer of stomach or enzyme inhibitors can be applied to the intestinal mucosa, albeit with concerns of toxicity and weakened immune response \cite{47,49}. This also endangers patients to exposing GI barrier to diffusion of unwanted proteins. Oral insulin delivery has the benefit of introducing insulin directly into the hepatic portal circulation, where it can elicit its effects in liver tissue, which is more sensitive to glycemic metabolism than both fat and muscle tissue \cite{49,50}. However, difficulties arise with insulin loading and low bioavailability from protein denaturation and limited diffusion across membranes from absorption kinetics. As well, current oral delivery systems have not reached commercialization outside of laboratory settings, likely due to complications of scale-up and costs.

Insulin delivery through the respiratory tract has seen recent commercialization, with products such as Exubera \cite{53}, AERx \cite{54} and AFREZZA \cite{55} seeing time on the market. The benefit of pulmonary delivery involves the much greater surface area of the respiratory tract and deep alveoli as well as good diffusion of molecules through the thinner mucosa and high vascularization \cite{56,57}. The reduced proteolytic enzyme activity in the respiratory mucosa compared with the GI tract was another noted benefit. Early strategies using sprayed liquid
insulin showed limited efficacy, due to difficulty in reaching the lower respiratory tract and high shear forces which caused insulin denaturation. Commercial products use aerosolized dry powder insulin, with metered dosages that can extend to deep pulmonary walls with \( \approx 10\% \) of the administered insulin reaching the bloodstream, depending on patient inhalation technique and system\textsuperscript{57,58}. Ease of use and patient compliance with dry powder inhalers were obvious benefits for respiratory insulin delivery, compared with constant needle use with injectable methods. Testing in humans showed similar outcomes compared to subcutaneous administration, citing rapid onset of activity (within 5 minutes) and comparable HbA\textsubscript{1c} values \textsuperscript{53,54,58,57}. Some specific complications with inhaled insulin include potential cases of an induction of insulin antibody development, which can dampen dosing through potential binding affinity \textsuperscript{59,60}. Along with this, the prohibitive cost of these respiratory insulin pumps and negligible improvement compared with cheaper, established injectable therapies ultimately will be the largest detractor \textsuperscript{61,62}.

External methods of insulin delivery have been explored, with attempts at rectal, ocular, buccal and transdermal routes. Rationales behind these attractive non-invasive methods include a thinner epithelial layer and high vascularization, obtaining a more direct and rapid diffusive barrier. Studies have shown improved insulin stability at the sites of action, with limited enzymatic inhibition and thinner mucosal barriers, compared to GI and respiratory tissue \textsuperscript{47,48}. This can translate to a greater proportion of active insulin at the diffusion barrier, improving bioavailability. However, since these routes are not naturally absorptive, most strategies require a permeation enhancer for passive diffusion which may lead to localized irritation or compromised immune function. Also, the short duration of action must be compensated with elongated retention of insulin at the delivery site. For ocular, buccal and rectal delivery, \textit{in situ} gels have been proposed, using high viscosity, mucoadhesive polymers to retain insulin for an extended
period of time at the site of diffusion, coupled with permeation enhancers \(^{63-66}\). Alternately, transdermal patches utilize microneedles, absorption enhancers and electric iontophoresis to penetrate the skin barrier \(^{67}\). As with oral and respiratory routes, bioavailability relative to current injectable or pump methods must be competitive for clinical acceptance. With gel-based systems, contact with the barrier surface must be maintained for a long period of time, which precludes the ability to give short, rapid postprandial doses of insulin. Microneedles and iontophoresis allow for rapid, transdermal delivery, but the reduced bioavailability incurs an added cost of ‘overadministering’ to reach therapeutic effect. This is a relatively new area of insulin therapy and more \textit{in vivo} efficacy needs to be demonstrated before reaching clinical practice.
2.3 Closed-loop Insulin Delivery

Beyond the route of administration, periodic insulin administration in response to glycemic fluctuation is the true goal of optimal insulin therapy. The ideal strategy would elicit increased insulin release in response to high blood glucose, and reduction at low blood glucose, as opposed to a single insulin bolus or sustained basal release. This pulsatile insulin response to glycemic levels is akin to the action of the healthy beta cells in the pancreas, giving rise to the idea of an ‘artificial pancreas’ system. Such a system involves multiple mechanisms working in conjunction to deliver insulin at the correct dosage at the correct time, with negative feedback to halt or slow insulin release when it is unnecessary. The basis of a closed-loop insulin delivery system requires three parts: (1) a glucose sensor, to determine real-time blood glucose levels, (2) an insulin delivery module or pump to efficiently deliver correct doses of insulin, often subcutaneously, and (3) an algorithm to trigger controlled insulin release in response to glucose sensing, linking the two systems \(68-71\) (Fig. 2.2). This feedback system provides glucose-responsive insulin release, the basis of ‘hands-off’ insulin therapy that does not require patient or physician intervention. Current research in this field as well as the work demonstrated in this thesis show significant progress towards this goal.
Fig. 2.2 – A closed-loop insulin delivery system, where a glucose sensor detects physiological glucose levels. Through a control algorithm, controlled insulin delivery is activated/deactivated based off sensor values. Hyperglycemia positively triggers insulin release, while hypoglycemia antagonizes insulin release to the diabetic patient. Patient blood glucose is continuously monitored by the glucose sensor to complete the loop.

2.3.1 Continuous Glucose Monitor/Insulin Therapy

Strategies to ‘close’ the loop of glucose sensing linked with smart insulin delivery have been explored with the use of commercial continuous glucose monitors (CGM). The latest generation of commercially available glucose monitors include DexCom’s SEVEN Plus\textsuperscript{72,73}, two from MiniMed: the Continuous Glucose Monitor System (CGMS) Gold\textsuperscript{74,75} and the Guardian Real-Time\textsuperscript{76,77} and Abbott’s Freestyle Navigator\textsuperscript{78,79}, which have all been approved by the FDA (Table 2.1). These CGM systems operate through a minimally invasive catheter attached to the abdominal wall, penetrating deep enough for interstitial fluid, but not vasculature. They all use a similar mechanism of glucose detection, with an enzyme-tipped catheter connected to an electrode to give measurements within minutes, giving warnings when above or below normal
glucose ranges. Sensor augmented pump therapy combines the use of CGMs with continuous insulin pumps, allowing for precise electromechanical control of insulin pump release rate according to blood glucose levels. Steady, low-level release of insulin is provided for basal therapy, while sensor alarms can notify patients of atypical fluctuations. Continuous glucose monitors combined with either insulin pumps or multiple insulin injections gave similar results with respect to self-monitored, postprandial blood glucose management.\textsuperscript{80-88} Moreover, achieving target long-term HbA\textsubscript{1C} goals (<7 %) is more successful with the sensor/pump combination than multiple injections alone.\textsuperscript{84-88} This is due to more blood glucose information to identify acute individual fluctuations, allowing for correct insulin dosing during fed and fasting periods, compared to discontinuous self-measurements via glucose strips. However, these patient outcomes can only be achieved with a strict patient regimen and continuous education on the optimal use of sensor augmented pump systems.

<table>
<thead>
<tr>
<th>CGM System</th>
<th>Manufacturer</th>
<th>Sensor Location</th>
<th>Sensor Lifetime</th>
<th>Warmup Time</th>
<th>Monitoring Interval (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous Glucose Monitoring System Gold</td>
<td>Medtronic</td>
<td>S.C. Abdomen</td>
<td>72 hours</td>
<td>2 hours</td>
<td>5</td>
</tr>
<tr>
<td>Guardian RT</td>
<td>Medtronic</td>
<td>S.C. Arm</td>
<td>72 hours</td>
<td>2 hours</td>
<td>5</td>
</tr>
<tr>
<td>SEVEN Plus</td>
<td>DexCom</td>
<td>S.C. Abdomen</td>
<td>7 days</td>
<td>2 hours</td>
<td>5</td>
</tr>
<tr>
<td>Freestyle Navigator</td>
<td>Abbott</td>
<td>S.C. Arm</td>
<td>72 hours</td>
<td>1 hour</td>
<td>1</td>
</tr>
</tbody>
</table>

\textbf{Table 2.1} – Comparison of current generation continuous glucose monitors (CGM)

Despite this, the system still requires patient intervention, and the glucose sensing algorithm requires precise twice-daily calibration, a sensor ‘warmup’ period and does not activate insulin administration on-demand, so it behooves patients to continuously monitor and self-administer insulin. As well, the current technology in CGM systems allows for continuous monitoring up to 7 days, at which point the sensor must be replaced.\textsuperscript{91,92} This makes the initial and maintenance
costs quite high, averaging over $5000 per year more expensive in direct and indirect costs than normal glucose monitoring for patients, which does not include necessary insulin therapy. Despite this, there is promise in the burgeoning field of CGM technology towards integrating glucose sensing with insulin delivery, especially for patients with ‘brittle’ diabetes, where there are larger than normal swings in blood glucose. A true ‘artificial pancreas’ system requires a cyclic, repeatable response to glycemic levels in the body. Controlled release drug delivery systems stress the importance of rapidly responding materials that give predictable and consistent reaction to physiological triggers. The next section will outline the use of stimuli-responsive polymeric materials to attain pulsatile, hands-off drug release, the basis for ‘smart’ insulin therapy.

2.3.2 Responsive Polymeric Hydrogels

Polymer hydrogel systems are a class of long-chain polymer that exhibit unique physical and chemical properties. Inherently hydrophilic, hydrogels swell in the presence of water and as a result, can easily interact with diffused molecules in a physiological system. These soft and naturally high-water content polymers are ideal for use in an aqueous physiological system. The linear chains in hydrogels are flexible and can entangle, creating a matrix for drug loading and release which can be chemically or physically modified with crosslinkers and functionalized with reactive moieties to retain their 3-D conformation. Without crosslinking, polymer chains are soluble and can dissolve in water without a physical structure. Hydrogel matrices can take on bulk, membrane or micro/nanoparticle shapes, depending on release profile or drug delivery system desired. It is through synthesizing these functional moieties onto a polymer backbone that give hydrogels the ability to physically respond to environmental stimuli, through a change in polymer conformation. This volume phase change can be combined with integrated drug to
give a pulsatile or temporally-regulated drug delivery system for \textit{in vitro} or \textit{in vivo} efficacy\textsuperscript{94-99}. That is, an elevated drug release when drug is necessary with a down-regulation of drug release when it is not, giving appropriate therapeutic kinetics. The following section addresses stimuli-responsive hydrogels currently used in the field of drug delivery.

\textbf{2.3.2.1 Thermosensitive Polymers}

Thermosensitive polymers are classified as polymers that respond directly to a change in temperature, which affects intermolecular forces and thus, overall solubility. The balance of inter- and intramolecular forces determines the overall solubility of the polymer network, which is governed by free energy. When the solubility of these polymers undergoes a sudden increase with increasing temperature, this temperature is called lower critical solution temperature (LCST), denoting a limit at which the polymer remains in solution below a certain temperature but precipitate from the solution above this temperature. Poly(N-isopropylacrylamide) (PNIPAM) is an extensively studied polymer exhibiting LCST. It consists of hydrophilic amide group and hydrophobic isopropyl group. At temperatures lower than the LCST, water molecules form a weak hydration shell (cage) surrounding the isopropyl group, making the polymer soluble in water. At a temperature above the LCST, the hydration shell is disturbed due to broken hydrogen bonds among water molecules in the shell by increasing environmental temperature. As a result, the hydrophobic groups are exposed to aqueous medium, which is unfavored thermodynamically. Thus they aggregate by hydrophobic interations and precipitate from the aqueous solution. This thermal transition is driven by a significant entropy increase owing to the change of ordered ice-like water structure in the hydration shell at lower temperatures to more disordered arrangement of water molecules at higher temperatures, although the polymer chains are less flexible due to aggregation\textsuperscript{99-104}. Other examples of polymer types exhibiting LCST
include, but are not limited to, other N-alkyl substituted acrylamides, polyethers and pyrrolidones \(^{99-104}\).

Some polymers show sharp increase in solubility with an increase in temperature i.e., exhibiting an upper critical solution temperature (UCST), where solvation increases above this point. UCST polymers are less common. Normally they comprise certain types of polymer-polymer interactions, such as hydrogen bonding, long fatty chain crystallization, and helical complexation. These interactions or structures are weakened or melted bring the polymer into the solution.\(^{100,103-106}\). That is, polymer-polymer interactions become less favorable compared to polymer-water interactions at higher temperatures. Temperature-sensitive polymers have been used for responsive drug carrier matrices, nanoparticles, micelles, gated release, and protective surface grafts for biomaterials, to respond selectively at physiological temperatures or activation through localized heating \(^{99-106}\).

Alternately, a class of amphiphilic block copolymers exhibit temperature-mediated sol-gel transition. Combining both hydrophobic and hydrophilic polymers, such as polypropylene oxide and polyethylene oxide, these non-crosslinked polymer networks are able to transition between solvated and gel states. At low concentrations, they do not exhibit a useful temperature-sensitive phase transition, although they may form micelles. However, when dissolved at a high concentration, they exhibit a sol-gel transition at higher temperatures. This occurs from self-association of hydrophobic blocks, initially micellar, which become more favorable at higher temperatures creating a viscous, lattice-micelle structure \(^{100,107}\). Increasing concentration of gel reduces the sol-gel transition temperature and allows for tailoring to increase viscosity for drug delivery systems at desired temperatures. They are more difficult to use \textit{in vivo}, as inevitable dilution in an open, physiological environment damages their sol-gel transition capability from
polymer dissolution after injection. However, their non-crosslinked nature gives them potential for use in degradable, sustained drug release systems and allows for relatively rapid clearance post-treatment\textsuperscript{107-109}.

2.3.2.2 pH-sensitive Polymers

Similar to thermosensitive polymers, pH-sensitive polymers exhibit a volume phase transition in response to environmental stimuli, changing the polymer 3-D conformation through intramolecular interactions. This mechanism occurs through electrostatically mediated forces that arise from ionization of pH-responsive functional moieties. Common pH-sensitive polymers include carboxyl or amine groups, which affect polymer swelling according to degree of ionization at a given pH. For ionizable polymers, such as polyacrylic acid and poly(N,N-dimethylaminoethyl) methacrylate, ionization occurs at a pH above or below the pKa, inducing a charged state (COO$^-$ or NH$_3^+$, respectively), which induces repulsive forces between charged moieties and increases the overall hydrodynamic radius. Ionization occurs according to the Henderson-Hasselbach equation, which observes a sinusoidal shift near the pKa\textsuperscript{96,98}. This gives pH-sensitive polymers a drastic change in volume transition across a very tight pH range, ideal for responsive drug delivery. An obvious use for pH-responsive polymers comes from pH shift across the GI tract, going as low as 1 in the stomach to 8 in some sections of the small intestine. Anionic polymers can remain insoluble in the highly acidic stomach region, while swelling or dissolving in the small intestine or colon, reducing gastric irritation and improving bioavailability at the site of action\textsuperscript{110-112}. As well, they have been utilized with some success in pH-controlled delivery of anticancer drugs to tumors, which have slightly acidic microenvironment\textsuperscript{99,113,114}. In the scope of this thesis, utilizing pH-sensitive polymers in
conjunction with glucose oxidase enzyme is a major field of research that has been explored as a glucose-responsive system for closed-loop insulin delivery, which will be discussed later.

2.3.2.3 Light-sensitive Polymers

Light-sensitive polymers utilize a combination of double or triple-bonded moieties that react to electromagnetic irradiation, typically UV or visible light. These polymers undergo a change in isoform through ionization, cis-trans change or ring-opening, which in turn changes the structural character of the polymer\(^{115-117}\). Light is captured by cyclic carbon ring moieties, such as azobenzene, to capture light and change overall polymer conformation. Polymers can undergo micellar degradation or solubility change, which can trigger loaded drug release upon irradiation\(^{118-120}\). Alternately, by controlling polarization of light, azobenzene-based polymers can be mechanically manipulated, giving rise to potential light-responsive micro- or nanoscale materials for drug delivery systems\(^{121,122}\). The limitations of light-sensitive polymers include a relatively slow reaction of the hydrogel to incoming irradiation, compared with pH and temperature-sensitive moieties. As well, practical in vivo application is difficult, requiring an externally focused light source to modulate drug release.

2.3.3 Glucose-Responsive Hydrogel Polymers

As demonstrated, the ability of responsive polymeric materials to reversibly change conformation can make them fundamentally suited for pulsatile release kinetics for drug release. Having a controlled ‘on-off’ switch in response to external stimuli gives treatment profiles geared towards controlling appropriate therapeutic efficacy. Specifically, with diabetes, effective insulin therapy is a function of glucose levels in the blood. An adaptable system that can increase insulin release in response to hyperglycemia and decrease insulin at low blood glucose levels
would provide the best outcomes for maintaining glycemic homeostasis. Utilizing polymers that
directly or indirectly respond to environmental glucose levels through phase transition or
shrinking/swelling dynamics would provide the engine for the basis of ‘glucose-responsive’
insulin delivery. There are three major fields of glucose-responsive polymers, which include
concanavalin A (Con A), phenylboronic acids, and glucose oxidase enzyme-coupled systems.

2.3.3.1 Concanavalin A-conjugated Systems

Extracted from plant sources, Con A is a unique carbohydrate binding lectin protein that has a
sol-gel transition upon interaction with six chain sugars, specifically D-mannose and D-glucose
\(^\text{123-125} \). Typically found in a collapsed, native tetramer formation, one Con A protein has four
binding sites for glucose molecules and this binding induces an increase in solvation, which can
be used as a mechanism for glucose-mediated release of drug, specifically insulin. Con A has
been used through incorporation into polymer network systems to create glucose-responsive
membranes or matrices for insulin release. This is achieved by increasing Con A-bound polymer
swelling, allowing permeation of loaded insulin through increased diffusion rates. Utilizing
glycosylated products allows Con A to act at the crosslinker point, generating sol-gel transition
through competitive binding of glucose, freeing bound glycosylated polymer derivatives, thus,
releasing loaded insulin within Con A crosslinked matrix, although this competitive binding
cannot be cyclic by nature \(^\text{124,125} \). As well, Con A has been previously used as an optical glucose
sensor, utilizing fluorescein isothiocyanate (FITC)-dextran binding as a reagent for fluorescence
detection of physiological glucose, through quenching \(^\text{127,128} \). Con A has demonstrated in vitro
efficacy in glucose-mediated insulin release, although there are some specific toxicity concerns
with the plant protein itself. Con A induces mitogenicity in T cells, causing unwanted
upregulation of cytokine expression and secretion in vivo. With this, increased levels of
cytokines such as tumor necrosis factor alpha and interleukin-2 induce hepatic damage through apoptotic mechanisms, which can lead to organ failure\textsuperscript{129-131}. Moreover, Con A strongly binds to membrane proteins glycoproteins and glycolipids, causing non-specific agglutination and cell binding in interstitial fluid or blood\textsuperscript{128,129}. Use of Con A as a glucose-responsive agent is difficult without effective immobilization of the protein, although many systems utilize Con A as a non-covalently bound moiety to achieve sol-gel transition, lending the danger leakage into physiological systems. As a result, \textit{in vivo} application has been limited.

2.3.3.2 \textit{Phenylboronic Acid-based Systems}

Utilizing a borate moiety, phenylboronic acid (PBA) exhibits strong affinity to non-covalently bind diol sugars such as D-glucose. It can also bind other polyols, but will preferentially bind to sugars, making it attractive for glucose selectivity\textsuperscript{132,133}. Similar to Con A, this competitive binding of the hydroxyl groups of D-glucose to the borate ion causes a phase transition, increasing swelling of the glucose-PBA bound complex. This occurs in the charged protonated form of the borate moiety, which can then bind to D-glucose. As more glucose binds, complexed PBA-glucose gives rise to more protonated borate groups, creating positive feedback for rapid glucose binding and sharp transition. To utilize this glucose-sensitive phase transition, PBA can be functionalized with a hydrophilic polymer, such as chitosan or polyethylene glycol. Upon administration of glucose, the borate moieties in the PBA-polymer complex bind circulating glucose, decreasing viscosity of the system, increasing polymer network solubility and permeability to trigger loaded insulin diffusion, through micellular dissociation or increased matrix permeability\textsuperscript{138-136}. One major concern for practical application is the pH-dependence of PBA ionization. At pH 7.4, PBA is primarily unionized, and the glucose complex formed is readily hydrolyzed, limiting efficacy of glucose-mediated PBA systems in physiological systems.
Steps to decrease the pKa of the borate ion have been attempted, such as adding amine group to shift electron affinity, and promote the charged PBA form at physiological pH \(^{137,138}\). Similar glucose-mediated PBA matrices have been explored, with rapid swelling and insulin release at clinically relevant glucose concentrations. It is important to note, as with Con A and mannose, that specificity to glucose is not secured, as PBA does have higher affinity to fructose, which may pose potential sensitivity error \(\textit{in vivo}\) \(^{139,140}\). However, PBA is a glucose-sensitive component with established history that has potential for non-immunogenic, biologic-free insulin release systems, although ionization in physiological conditions is not guaranteed.

2.3.3.3 \textit{Glucose Oxidase Enzyme-Coupled Systems}

Contrary to Con A and PBA-based systems, glucose oxidase (GOX)-coupled systems do not directly bind or complex with glucose molecules to exhibit sensitivity. Utilizing GOX enzyme, circulating glucose and oxygen can be initially oxidized into gluconic acid and hydrogen peroxide end products. The gluconic acid is used as a trigger for responsiveness when coupled with pH-responsive materials in the second step of this process. Thusly, entrapping or cross-linking GOX with pH-responsive polymers can cause rapid phase swelling/deswelling in response to environmental glucose \(^{123,141}\). GOX activity is reduced slightly upon covalent crosslinking process, but the stability of the enzyme is increased greatly compared with free enzyme, making it ideal for a potential long-term glucose sensor. Strategies have been explored to combine GOX-coupled pH-sensitive materials to achieve glucose-sensitive insulin release. Utilizing cationic hydrogel polymers, swelling and hydrophilicity is increased from acidic gluconic acid challenge, allowing for insulin release through polymer membranes or nano-shells with increased permeability through larger volume and reduced polymer network density \(^{143,142}\). With the opposite phase transition, GOX-coupled, anionic hydrogels collapse in response to
glucose challenge, with application in gating or ‘nano-valve’ membrane systems. Collapse of methacrylic acid or acrylic acid polymer chains increase porosity through increasing void space, allowing for greater permeability and diffusion of insulin\(^ {147,148}\). The presence of oxygen as a reactant in the glucose oxidase reaction must be acknowledged, as some systems required an oxygen regenerating catalyst to prevent the process from becoming oxygen starved, stunting its efficacy as a glucose sensor. Catalase and more recently, manganese dioxide, has been used to both rapidly regenerate oxygen and remove cytotoxic hydrogen peroxide product in a cyclic fashion\(^ {144-151}\). Compared with the previous two systems, glucose-oxidase coupled systems have shown the most promise \textit{in vivo}, with a number of studies showing glucose-sensitive insulin release in animal models, with duration of efficacy ranging from 24h to 14 days\(^ {141,145,152,169}\). As a forerunner of glucose-responsive technology, strategies to integrate GOX-coupled, responsive polymers with insulin delivery systems have a unified goal of achieving not only glucose-responsive insulin delivery, but high biocompatibility and long-term \textit{in vivo} application.
2.4 Continuous Insulin Delivery Systems

The need for a continuous delivery of insulin arises from the physiological nature of insulin release. Basal levels of insulin are provided by the pancreas throughout the day (~0.5 u per kg per day), to maintain blood glucose within a healthy level during non-mealtimes and sleep. Although fast-acting insulin injections provide insulin during mealtimes, this void during fasted times exposes patients to potential uncontrolled hyperglycemia. Patients may compensate by increasing pre-meal injection volume, which can lead to the danger of a hypoglycemic event.

2.4.1 Continuous Insulin Pumps

Continuous insulin pumps provide insulin through a pump-catheter system controlled by an external electromechanical device. An insulin reservoir is housed outside of the body and delivers insulin through a catheter, which is inserted into the abdominal fat tissue. Electronic or even wireless signals can set basal rates of insulin delivery and on-demand bolus delivery as well. This replaces the need for multiple daily injections and provides a steady rate of fast or intermediate-acting insulin delivery that can be programmed based on daily activities. Pump infusion rate can be increased to compensate for meals, decreased to compensate for exercise, and maintained at a steady rate during fasted or sleep periods. This allows for a very flat blood-insulin profile that can mirror physiological insulin. Some examples of commercial insulin pumps include the MiniMed Paradigm and Omnipod systems, which allow for insulin reservoirs up to 200 units and precise basal/bolus insulin control. With rigorous patient compliance, improvement in HbA1C levels for continuous pump system users can be almost identical or better than multiple daily injection regimens. A reduction in Hb A1c also correlates well with reduced risk factors from diabetes, a welcome result. As well, there is a much lower incidence of
hypoglycemic events compared with multiple injections, due to fully regulated insulin delivery, avoiding the potential for ‘overshooting’\textsuperscript{38-43}. It is also good for patients with an aversion to needles, as catheters can be replaced infrequently (~1 week).

More recently, insulin pump systems have been used in conjunction with CGM systems, to provide an external pseudo-‘closed-loop’ insulin delivery system. Linking the CGM with the insulin pump device, the pump can be set to respond to fluctuations in patient glucose levels, with sensor that can give real time or predictive, ‘rate-of-change’ glucose information. Carbohydrate loads and ratios can be set, creating a sophisticated regimen to account for external glucose ingestion and metabolism. As expected, corrective adjustments and calibration are needed in the initial phase of this marriage to account for patient variability in insulin sensitivity. However, due to the limited lifespan of current commercial CGM systems, this strategy can prove costly when compared to potential benefits in patient outcomes. As well, even with CGM/insulin pump integration, the onus is on the patient to set up their insulin pump to deliver the correct basal and bolus amounts according to their specific needs.

Improvement in patient glycemia control with continuous pumps comes with practical tradeoffs. Discounting CGMs, pump systems themselves can cost much more than insulin vials for injection, which makes it unrealistic for use in developing countries without health care. Complications with premature device malfunction, catheter blockage and insulin solution stability within pump systems are all potential quality of life concerns\textsuperscript{38,39,46}. It is important to acknowledge the pump costs may be offset by a reduction in hospitalization and health care costs from acute hypoglycemic episodes from the improved basal/bolus insulin regimen, but this is inconclusive. Again, as is the case with multiple daily injections, patient education and compliance is critical for optimal insulin therapy.
When optimizing therapeutic efficacy with drug delivery systems, looking at a route of administration with minimal barriers can improve bioavailability. Oral, respiratory and other external methods are inherently inferior to intravenous administration when considering onset of activity, bypassing dermal layers and mucosal membranes. However, even with parenteral administration of drug, there is still potential danger of toxicity from high concentration of drug in plasma or low, ineffective plasma levels. Also, considering the aforementioned methods, periodic dosing is still required to maintain therapeutic levels, which may have a narrow window of efficacy.

2.4.2 Implantable Drug Delivery Systems

The rise of implantable drug delivery systems has birthed an opportunity for novel dosing profiles that cannot be effectively achieved through traditional methods. Unique aspects of subcutaneous or intraperitoneal implant systems include, but are not limited to, long-term dosing regimens, minimal patient intervention, ease of achieving sustained plasma drug concentrations and patient-specific profiles. As well, implantable micro- or nano-electromechanical systems (MEMS/NEMS) can provide precise dose control, and in conjunction with a sensor or electromechanical component, responsive, triggered release for pulsatile drug kinetics. Implantable micro or nanodevices can be designed with specific reservoirs, valves or pumps to give the appropriate drug release profile specific to the treatment disease\textsuperscript{149-155}. While commercial CGM/insulin pump systems can operate subcutaneously, most of the equipment is electromechanical, so the externally housed devices have to be maintained to ensure proper implantation.
As implantable systems bypass barriers that may have high enzymatic activity, variable pH, or poor macromolecule diffusion, an ideal choice for drugs of interest include high molecular weight biologics. Large proteins, hormones, nucleic acids and antibodies are often blocked from traditional methods of administration, due to enzymatic degradation, pH and limited diffusion across lipophilic barriers \(^{156,158}\). As well, metabolism from first-pass effect after parenteral injection can drastically reduce bioavailability despite direct administration into the circulation. Circumventing these barriers with an implantable system near the site of action can be an ideal solution. The final advantage of implantable systems arises from its direct contact with interstitial fluid, providing potential for triggered response to environmental stimuli, such as pH, temperature, or, in the case of insulin delivery, glucose \(^{149-155}\).

Currently, systems made of biocompatible materials are used to create an implant system to achieve desired drug release profile with minimal host response. Biodegradable systems are one type of drug implant that can provide sustained release, utilizing polymer matrices or gels. Copolymers of polylactic acid, polyglycolic acid and polycaprolactone have been used for biodegradable systems in conjunction with drug, such as nucleic acids, antibodies or hormones \(^{159-163}\). Primarily hydrophilic, these polymers also confer the ability to provide mucoadhesion, retaining their depot near the site of injection or implantation. Biodegradable implants have the advantage of clearing after administration and degradation, limiting chronic toxicity and need for surgical removal \(^{164,165}\). However, there may be concerns with the latent toxicity of degraded material not fully clearing from the system. Also, for degradable matrices, the 3-D shape of the material changes during the duration, potentially affecting release kinetics. Insulin therapy requires a pulsatile release profile for postprandial periods, along with sustained basal release during fasting. Sustained insulin release systems have been developed utilizing an injectable
depot system to provide long-term basal insulin, although pulsatile release for postprandial
treatment has limited long-term capability\textsuperscript{166-169}. Nonetheless, focus on biodegradable implants
has been on short-term sustained or localized zero-order release systems for drug delivery, as the
vehicle dissipates after duration of release, preventing potential refill or reimplantation.

Alternately, non-degradable implant systems have modified long-term designs with different
application. Implantable non-degradable materials are used through either a solid matrix that
diffuses loaded drug within the material or a reservoir system, which holds loaded drug, allowing
controlled diffusion across a membrane barrier. This allows for surgical implantation, providing
a long-term release profile beyond degradable systems. As well, as release is not mediated by
physical degradation of the biomaterial, cyclic, repeatable drug release can be achieved without
compromising the implant structure. A number of non-degradable materials have been used for
implant systems, with goals of long-term structural integrity with minimal immunogenicity.

Poly dimethylsiloxane (PDMS) or silicone rubber has been one of the most common
biomaterials used in non-degradable implant systems, lending itself to its low immunogenicity
and non-reactive hydrophobic surface\textsuperscript{169}. Silicone rubbers are cured through crosslinking via
thermal, chemical (peroxide) or addition chemistry. As it is soft and flexible, even at high
temperatures, PDMS is a viable option for implantable material that can machined in a variety of
shapes through microfabrication\textsuperscript{171,172}. Silicone rubbers confer permeability to low molecular
weight and lipophilic drugs, allowing for potential diffusion of loaded or entrapped drug. Also,
high molecular weight drugs can be loaded, combining intrapenetrating networks in PDMS with
hydrophilic polymers to control porosity, with release driven by concentration gradients\textsuperscript{173,174}.

One example, Norplant, is a commercial subdermal implant system that allows sustained
administration of the matrix-embedded birth control drug, levonorgestel, from a PDMS matrix.
After Norplant implantation, released levonorgestrel can reach therapeutic levels in the bloodstream after 24 hours and provide duration of efficacy over 5 years\textsuperscript{175,176}. PDMS has also been used with integrated copolymers and as a coating for reservoir matrices, providing high resistance to biofouling, while maintaining sustained drug release\textsuperscript{177,178}. Utilized in a PDMS reservoir system, macromolecule release has been demonstrated with degradable polymer membranes. As a soft biomaterial, PDMS has the ability to be easily modified and machined to any number of desired implant designs.

Ceramic implant systems have seen use as non-degradable implant systems, with specificity in bone tissue. One type, poly methylmethacrylate (PMMA), has been used extensively in implantable drug delivery application. Upon crosslinking, the PMMA hardens at the site of injection and after curing, provides sustained release of loaded drug. Relatively rigid and inert, PMMA has a functional methyl ester group that can be used to modify the implant material to improve biocompatibility, drug loading or bioadhesion\textsuperscript{179-181}. Typically used as bone cement, gentamycin-loaded PMMA has been injected and crosslinked at the site of action, with increased release rate when prepared in microbeads, due to increased surface area. Also intratumoral injection in bone lesions has been explored, with observed necrosis in localized tissue, upon sustained delivery of anticancer drugs\textsuperscript{182-184}. Implant systems based off mesoporous silica are another group of ceramic, nondegradable material that utilize porosity, rather than diffusion through the biomaterial. Pores are created by solvent evaporation, etching or surfactant and can be formed in the range of 2 to 50 nm, making pore interactions with loaded drug much more pronounced due to relatively large surface area\textsuperscript{185-187}. Modifications of the silanol moieties can change the surface properties, thus regulating the release of drug to the desired profile and potentially providing stimuli-responsive release with appropriate polymers\textsuperscript{185-190}. Mesoporous
biomaterials loaded with ibuprofen, gentamicin, bovine serum albumin and captopril have been demonstrated, with drug loading values under 50%\textsuperscript{186,187}. Insulin loaded into mesoporous structures has been combined with functionalized PBA or GOX enzyme systems for glucose-responsive release \textit{in vitro}, but long-term \textit{in vivo} efficacy has not yet been demonstrated\textsuperscript{192-194}. The rigidity of ceramic materials has good biocompatibility and high strength, but this makes application difficult for acceptable monolithic implant design. As well, due to loading constraints based on the nanopore volume, total macromolecular drug loading is limited, which may be difficult when trying to achieve clinical efficacy. When selecting an implant biomaterial, it is important to determine desired release kinetics, and both compatibility and loading capacity of the model drug for therapeutic efficacy.
2.5 Insulin Formulation and Design

2.5.1 Background and Structure

Discovered by Fredrick Banting and Charles Best in a small laboratory under the supervision and support of Professor John Macleod in the Department of Physiology at the University of Toronto in 1921, insulin is a hormone produced from the beta cells in the Islets of Langerhans in the pancreas responsible for glycemic homeostasis. Initially, it was identified crudely as an ‘islet extract’ when it was shown that salvaged islet cells when ground and purified before reinjection, counteract diabetic symptoms in diabetic dogs after pancreatic ligation (removal). Upon further purification and analysis, by J.B. Collip, the extract identified as isletin is now known as insulin suggested by John Macleod. The discovery of insulin was a major milestone towards the treatment of diabetes.

Historically, common extracts of insulin include those isolated from porcine and bovine sources; however, the rest of this section will refer to human recombinant insulin, which is the most common native form of insulin used clinically. Both A and B-subunits of insulin are primarily alpha helical in nature, with the A unit consisting of two antiparallel chains (A2-8, A13-20) and B subunit as one chain (B9-19) with a beta-strand tail (B21-30). Before materializing as an active, dual-subunit hormone, insulin is initially produced as the single-chain form of proinsulin in the endoplasmic reticulum, which is cleaved by peptidases at a 1:1 ratio into insulin and c-peptide. This is an important physiological mechanism, as the cleavage product c-peptide is commonly used as an indicator of endogenous beta-cell insulin production when assayed from blood plasma. The distinction between endogenous and exogenous insulin is especially important, with respect to the diabetic animal model work described in this thesis and elucidating
efficacy of exogenous insulin delivery systems. The active insulin hormone travels to the liver to rapidly elicit its effects, triggering uptake of blood glucose into cells.

Structurally, insulin can be found in three major conformations: monomer, dimer or hexamer, which are comprised of one, two or six insulin proteins, respectively (Fig. 2.3). Insulin can be found circulating in blood plasma in primarily monomeric form, where it readily elicits its glycemic activity and can diffuse across endothelia towards insulin receptors \(^{197,198}\). Multiple insulin molecules can coordinate into alternate less active forms, dimer and hexamer, while maintaining equilibrium among all three forms based on insulin concentration and presence of inorganic ions or complexation agents. Dimers are formed from planar contact of hydrophobic monomer surfaces, mainly across the B-chain, burying them from the external aqueous environment to create a more favorable energy state \(^{198-200}\). Hexamers are then formed from multiple dimers, which experience short term conformation as tetramers before reaching the highly stable hexamer form. Hexamers are a spherical conformation of three dimers, with strong polarity in the centre. As mentioned before, the hexamer conformation can be promoted with presence of inorganic multivalent ions, such as calcium or zinc, to neutralize the net negative charge from the amino acid surfaces in the hexamer core. Without metal ions, insulin concentrations above 2 mM promote a hexameric self-association state to reduce the monomeric and dimeric population \(^{197,200}\). Unlike zinc-free hexamers, zinc complexed hexamers have a noted effect on both chemical and physical stability of the bound insulin protein, which will be discussed in depth later. Physiologically, insoluble zinc-insulin hexamers are the inactive storage form of insulin in pancreatic islet cells, and dissociation of zinc from hexamers allows monomer release and acute activity, which is a reversible change \(^{198,201}\). This close relationship between
insulin structure, solubility and activity are major parameters in the design of insulin formulations for different onsets of activity and pharmacokinetic profiles.

**Fig. 2.3** – Diagram of insulin monomer, dimer and hexamer conformations and biological membrane permeability *in vivo*.

### 2.5.2 Commercial Insulin Formulations

With insulin therapy, the end goal of treatment requires maintaining blood glucose for diabetic patients within a healthy range for the longest period of time. As blood glucose fluctuates with meals and fasting periods, the need for insulin fluctuates as well. During meals, the onset of insulin activity must be fast, to quickly metabolize the carbohydrate loads. During fasting periods, blood glucose is relatively stable, and low-level, slow-acting insulin is often enough to maintain euglycemia. Careful balancing of these two types of insulin gives the best clinical outcomes compared to single types alone and can be specifically selected to meet patient requirements. Current commercial formulations are designed for subcutaneous administration.
The different insulin formulations available are categorized by onset of activity, and can be tailored to give the best blood glucose profiles for diabetes treatment.

### 2.5.2.1 Rapid-acting Insulin Analogs

Insulins with the fastest onset of activity are classified under rapid-acting insulin analogs. They typically have an onset of activity of 10 to 20 minutes, roughly twice as fast as native insulin, with peak concentrations at 30 to 45 minutes\(^{201-203}\). Due to this, they are used in acute insulin therapy, to counteract the sharp glycemic peak that occurs after meals. To achieve this, chemical modification of the insulin is required, which usually promotes the active monomer form of insulin, discouraging hexamer formation. As a result, these insulin derivatives are referred to as analogs, with modification of specific amino acids of native human insulin.

Conversion of the amino acid at the B28 position from proline to aspartic acid gives rise to insulin aspart (Novo Nordisk). This elicits a negative charge at the B28 position at physiological pH, creating negative repulsion between insulin molecules. This change makes the monomer-monomer interaction unfavorable, retarding dimer formation\(^{203-206}\). A high insulin monomer population within the formulation allows for immediate diffusion across subcutaneous barriers. With a continuous subcutaneous administration regimen, improved postprandial blood glucose control was achieved with insulin aspart compared with human insulin, and injections could be taken immediately before meals, as opposed to 30 minutes prior, as is necessary with native human insulin\(^{204,206}\).

Another analog, insulin lispro (Eli Lilly), is designed with a change at both the B28 (lysine) and B29 (proline) positions, having a similar retardation effect on monomer-monomer interactions. This is achieved by a disruption in the non-polar surfaces typically found in native insulin, which
are used for conformational binding\textsuperscript{201,207,207}. It has shown improvement in blood glucose profiles compared with human insulin in both continuous and multiple injection therapies\textsuperscript{208}. As expected, considering their near identical chemistry, aspart and lispro have clinically equivalent pharmacokinetics, making them ideal for use in acute insulin therapy with reduced hypoglycemic risks due to rapid clearance\textsuperscript{209,210}.

More recently, a novel analog, insulin glulisine (Sanofi-Aventis), has seen acceptance as a viable rapid-acting insulin derivative. With a lysine and glutamic acid at the B3 and B29 position, respectively, insulin glulisine does not share the same strong monomer dissociation characteristics from B28 position modification, readily forming dimers in solution with polysorbate 20 as a stabilizer, while still maintaining fast dissociation \textit{in situ}\textsuperscript{211,212}. The addition of lysine and glutamic acid impart a negative overall charge to the insulin analog, decreasing pI from 5.5 to 5.1, improving solubility at physiological pH. Upon administration, insulin glulisine readily dissociates, giving it a theoretical onset of activity that is faster than both aspart and lispro. In continuous pump systems, improvement in efficacy compared with human insulins has been well documented\textsuperscript{212-215}, but inconclusive compared with either insulin lispro or aspart\textsuperscript{216-218}. In practice, all of the rapid insulin analogs have shown either clinical improvement or no change when compared directly with normal insulins\textsuperscript{211,218}. As well, the chemical amino acid modifications did not have a significant effect on insulin receptor activity, making dosing equivalence possible\textsuperscript{203,211}. Overall chemical and physical protein stability concerns for rapid-acting insulins is an unavoidable disadvantage of these modified insulins\textsuperscript{221}, and will be discussed further in a later section.
2.5.2.2 Short-acting Insulin

Normal endogenous human insulin falls into the category of short-acting insulin, with onset of activity around 30 minutes and peak concentrations from 2.5 to 5 hours \(^{219,220}\). According to this classification, Humulin R (Eli Lilly) and Novolin (Novo Nordisk) are two commercial human recombinant insulins derived from E.coli bacteria and are identical to native human insulin. Previously, porcine and bovine insulins were used as their extraction was the only source of insulin but human recombinant insulins are now primarily used for treatment \(^{204,218}\). Although this presumably reduces potential immunogenicity compared with modified insulin analogs, the slower onset of efficacy compared with rapid-acting analogs has led short-acting insulins towards limited use in insulin pump strategies with treatment typically confined to multiple injection regimens, from vial/syringe or pen method \(^{36}\). This is the classic treatment regimen for short-acting or regular insulin, with injections of human recombinant insulin usually within 30 minutes of meal ingestion. Alternately, combining these ‘normal’ insulins with rapid acting insulin analogs has been explored, to mimic postprandial insulin management with insulin mixtures, but more recently, studies have shown improved outcomes with rapid-acting insulins mixed with intermediate or long-acting insulins \(^{222-228}\). Careful combination of rapid or short-acting insulin can be administered in conjunction to optimize for specific glycemic profiles and individual metabolism with patient feedback. With established recombinant technology, this is the least expensive form of insulin therapy available and often the choice for diabetic people in developing countries, without access to advanced analogs or health care insurance.
2.5.2.3 Intermediate-acting Insulin

Intermediate-acting insulins typically require twice daily injections, with an onset of activity within 1 to 2 hours and peak from 4 to 8 hours but can have effects up to 24 hours. The only commercial product currently on the market is neutral protamine insulin, called NPH (neutral protamine Hagedorn) insulin. NPH is a cloudy, crystalline-based insulin suspension that achieves a slightly extended release profile from delayed insulin crystal dissolution in the subcutaneous environment. Along with zinc, a sperm protein extract, protamine, is used in tandem to trigger crystallization of insulin monomers, which are native in structure. As it is a suspension formulation, shaking of pens or vials is required to resuspend precipitates before administration to give accurate dosage. In practice, clinical benefits come from mixing crystalline NPH insulin with regular or rapid-acting insulin analogs to create a dual acting regimen, providing postprandial and basal plasma insulin. Combining an injectable ratio of 50/50 or 70/30 of fast acting insulin to NPH insulin or protamine-hexamerized analogs, better postprandial glucose control can be achieved than either insulin alone. Currently used in injectable pen systems, NPH/regular (Novolin) or NPH/rapid (Novolog, Humalog) insulin mixes are used for a twice-daily administration, for rapid mealtime glucose response and reduction in nocturnal hypoglycemic episodes from intermediate release kinetics. Some reports show IgE-mediated immunogenicity from protamine extract, but it is not a widespread concern for most NPH insulin users. However, NPH does not have a flat, peakless profile as the newer long-acting insulin analogs, which will be discussed in the next section, so care must be taken to avoid under/overdosing with respect to individual patient variability. As well, storage longevity of NPH mixes is lower than single species formulations, owing to potential interactions of protamine and zinc with the rapid-acting species within the combination therapy.
2.5.2.4 Long-acting Insulin

Daily use of rapid to intermediate-acting insulins is specific to their capacity to maintain euglycemia during meal ingestion. Speed of bioactivity is important to coincide with glucose metabolism, suppressing sharp glycemic peaks to minimize hyperglycemic periods. During periods of fasting, where there is very little fluctuation in blood glucose, an equally stable, small of insulin is required for glycemic homeostasis. Healthy B-cells in the pancreas secrete a low, basal level of insulin during fasting periods, to counteract endogenous glucose production. As native insulin is cleared relatively quickly, extending activity is only achieved by slow, steady release from the pancreas. Contrary to rapid-acting analogs, long-acting insulins contain modifications that slow the rate of absorption and decrease diffusion across membranes, to give a delayed onset of activity and extended glycemic profile, mimicking the fasted state. This creates a pharmacokinetic profile similar to slow-releasing normal insulin from the healthy pancreas, through a single bioequivalent administration of long-acting insulin.

The earliest long-acting insulins include the lente and ultralente insulins (Eli Lilly). In the past, both bovine and porcine insulins were used, mainly due to the unavailability of recombinant human insulin and the lower solubility of animal insulins, which promoted their long-acting effects. Time to reach systemic circulation for lente and ultralente insulins is 2 and 4 hours, respectively, with clearance from the bloodstream by 24 h for both, used typically as a once-daily injection. Extended release is achieved by zinc chloride addition and subsequent crystallization of zinc-insulin hexamers to create a slow dissolving insulin suspension. Lente and ultralente are distinguished by the amount of amorphous and crystalline insulin present in suspension, with lente having a 3:7 ratio and ultralente being fully crystalline. They are cloudy solutions and similarly to NPH insulin, shaking of vials is necessary before injection, to
resuspend precipitates before administration. The ability for lente and ultralente insulin to exhibit a basal insulin profile was maintained when switched over from animal to human insulin, despite the increased solubility, due to excess zinc in solution strongly binding insulin molecules. This was a boon for their use, as immunogenicity of human insulin is negligible compared with insulins from animal sources. However, despite slow dissolution of lente and ultralente crystals and reduced diffusion across membranes, variability of total insulin and presence of late insulin peaks is more pronounced compared with NPH and newer insulin analogs. This potentially endangers patients to increased risk of hypoglycemic episodes, without negligible physiological improvement as a basal insulin formulation compared with alternative long-acting insulins. As a result, a push towards established NPH insulin and the rise of alternative long-acting analogs has prompted the discontinuation of lente and ultralente insulins in 2005.

Insulin glargine is one such long-acting insulin analog that uses modified solubility at physiological conditions to control insulin diffusion across membranes, giving improved pharmacodynamics. The A21 asparagine residue is replaced with glycine and addition of two arginine groups at the C-terminus of the insulin B-chain shifts the isoelectric point of the insulin molecule from 5.4 to 6.7. This mechanism contrasts with glulisine, which decreases the pI to speed up insulin action by improving solubility. When administered at pH 4, insulin glargine is fully soluble and upon injection, immediately precipitates in the physiological environment. As expect, this delays diffusion of insulin across subcutaneous membranes, and can be further protracted with varying amounts of zinc for hexamer complexation (typically 30 mg.L⁻¹ for commercial product). This makes the use of insulin glargine attractive for overnight insulin therapy, as the extremely delayed insulin profile does not endanger patients to potential peaks which can lead to nocturnal hypoglycemic episodes seen with NPH insulin. Blood insulin
profile for glargine showed less variability compared with both NPH and lente insulins, likely due to the flatter release kinetics. Some concerns with insulin glargine include slightly higher injection site irritation and inability to be mixed with faster acting insulins, both owing to the acidic character of injection solution.

Insulin detemir is a long-acting insulin analog that exhibits delayed onset of activity, over 3 to 4 hours, with duration of activity up to 24 hours. As expected, this makes it unsuited for use against acute glycemic peaks but attractive for long-term daily injections for basal background insulin. Insulin detemir is derived from a modification of the B29 lysine to a fatty, long-chain myristic acid and removal of the B30 threonine. Upon injection, a depot is formed, and a combination of zinc, phenol and m-cresol within the injection bolus allow individual insulin detemir molecules to form a hexamer, and with self-association between surface fatty acids, a dihexamer. The dihexamer conformation slows dissociation of insulin detemir monomers across subcutaneous membranes, drastically delaying onset of activity. As well, upon dissociation, the myristic acid monomers are highly albumin bound in circulation, further reducing activity. This reversible binding coupled with the dihexamer complexed depot site gives a two stage extension of insulin detemir bioactivity. In theory, the amount of albumin binding should exhibit patient specific variability, but in practice, the slow protraction of insulin detemir activity renders this moot, as glycemic profile is flat and spread over a 24 hour period. This steady and non-acute profile reduces the risk of hypoglycemic episodes and allows for more aggressive therapy and blood glucose targets for patients. Studies have shown improvement in the reduction of variability compared with NPH insulin, but effect on overall HbA1c values was negligible. As well, insulin receptor activity for insulin detemir is slightly lower with the modified fatty chain, giving a decreased level of activity on a per mole
basis\textsuperscript{243,245}. This requires slight augmentation of treatment to compensate, but is not a major clinical hazard, with its peakless, flat plasma profile.

With the numerous insulin formulations and analogs available, it is easier than ever to achieve optimal glycemic control. Short and long-acting analogs can be combined to mimic postprandial and basal insulin release from a healthy pancreas. As these formulations are primarily used for injectable therapies, there are no external barriers towards complete bioavailability of insulin after administration, although patients still require strict compliance and supervise their diets accordingly. Owing to these improvements and advanced manufacturing processes, costs of insulin analog systems are often several times more than regular insulin, which usually require almost full health insurance coverage to be affordable and thus, analogs are not often utilized by uninsured patients in lower income brackets or developing countries\textsuperscript{251}. However, this initial upfront cost of insulin analogs has shown to be offset by reduced downstream costs from potential hospital visits due to improved patient quality of life outcomes\textsuperscript{252,253}. It is up to the patient to decide the best cost-benefit ratio for their own lifestyle.
Fig. 2.4 – Modified insulin analogs and their amino acid substitutions. Rapid-acting analogs include modifications that resist hexamer formation (aspart, lispro) or increase physiological solubility (glulisine) for faster diffusion and onset of activity. Long-acting analogs have reduced physiological solubility (glargine) or increased hexamerization (detemir) to delay onset of activity for protracted plasma insulin profiles.
<table>
<thead>
<tr>
<th>Insulin Type</th>
<th>Commercial Name</th>
<th>Chemical/Physical Modification</th>
<th>Onset of Activity</th>
<th>Duration of Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rapid</strong></td>
<td>Insulin Aspart</td>
<td>B28 Aspartamine</td>
<td>12-18 minutes</td>
<td>3-5 hours</td>
</tr>
<tr>
<td></td>
<td>Insulin Lispro</td>
<td>B28 Lysine, B29 Proline</td>
<td>15-30 minutes</td>
<td>2-4 hours</td>
</tr>
<tr>
<td></td>
<td>Insulin Glulisine</td>
<td>B3 Lysine, B29 Glutamic Acid, Polysorbate 20</td>
<td>12-20 minutes</td>
<td>3-4 hours</td>
</tr>
<tr>
<td><strong>Short</strong></td>
<td>Human Insulin</td>
<td>None</td>
<td>30 minutes</td>
<td>6-8 hours</td>
</tr>
<tr>
<td><strong>Intermediate</strong></td>
<td>NPH</td>
<td>None, Protamine, crystalline</td>
<td>1-2 hours</td>
<td>12-24 hours</td>
</tr>
<tr>
<td></td>
<td>Lente (disc.)</td>
<td>None, zinc 70% crystalline</td>
<td>1-2 hours</td>
<td>12-24 hours</td>
</tr>
<tr>
<td><strong>Long</strong></td>
<td>Ultralente (disc.)</td>
<td>None, zinc 100% crystalline</td>
<td>4-8 hours</td>
<td>16-24 hours</td>
</tr>
<tr>
<td></td>
<td>Insulin Glargine</td>
<td>A21 Glycine, two Arginines on B-chain, acidic solution</td>
<td>3-4 hours</td>
<td>24 hours</td>
</tr>
<tr>
<td></td>
<td>Insulin Detemir</td>
<td>B29 myristic acid, no B30 residue</td>
<td>3-4 hours</td>
<td>24 hours</td>
</tr>
</tbody>
</table>

**Table 2.2** – List of commercial insulin formulations, properties and physiological parameters.
2.6 Physical and Chemical Stability of Insulin

With the myriad of insulin formulations on the market, there are many options for diabetic patients to select the correct insulin regimen. With varying speeds of activity, balancing fast and slow insulins to best achieve euglycemia is completely viable with patient compliance. However, concerns with insulin formulation stability must be acknowledged when assessing clinical efficacy in real world situations. Potency of insulin formulations is related to individual insulin protein structure, which is directly affected by a number of environmental factors. Even partial unfolding of insulin protein, when unchecked, can lead to large-scale, irreversible batch denaturation. This can cause inaccurate dosing, poor glycemic management and raise costs of insulin therapy from a formulation and health care standpoint. All the commercial formulations discussed earlier have expiration dates within a one month period after opening, longer if kept unopened and refrigerated \(^{202-204,237,254}\). Subjecting insulin formulations to heat, additives, shaking and other various denaturation stresses can irreversibly and drastically reduce potency, rendering them inadequate and potentially dangerous for clinical use.

Insulin stability is a measure of the ability of the protein structure to remain unchanged, from a physical protein folding and chemical structure standpoint. The effects on disturbing protein structure in response to environmental stresses are not unique to insulin, with similar aggregation and unfolding processes, but the end results are quite different. In its final stages of denaturation, protein formulations undergo a two-step process of nucleation and fibrillation, leading to high molecular weight products from covalent bonding of unfolded protein molecules \(^{254,256}\). Insulin denaturation primarily affects the monomeric form of insulin, as exposed hydrophobic surface groups are readily accessible compared with the dimer and hexamer forms, which exhibit
conformation across these planar hydrophobic surfaces, protecting them. Nucleation is the first step in this reaction, where partially unfolded insulin monomers associate and form ‘seeds’ \(^{257-259}\). At this point, insulin nuclei do not aggregate and precipitate out of solution and may be undetectable within a formulation, however, overall activity and thus, potency of the formulation is affected. A long lag phase of nucleation occurs before relatively rapid fibrillation occurs. Fibrillation is defined by the fast, chain polymerization of insulin, initially from monomers attaching to the partially folded nuclei ‘seeds’ and then from extension of these fibril chains from continued monomer binding. Further extension of fibril chains cause aggregation and precipitation of high molecular weight insulin fibrils out of solution, which is an irreversible process \(^{257,259-261}\). The environmental parameters that affect both insulin nucleation and fibrillation of insulin in formulation will be detailed in the next section.

### 2.6.1 Physical Agitation and Surface Adsorption

Physical agitation of pharmaceutical proteins in solution has been established to be a major factor in native protein stability. Specifically, when comparing undisturbed insulin with agitated insulin, there is a clear direct relationship between fibril formation and degree of agitation, through stirring or rocking of insulin formulations. In acidic and neutral solutions, agitation or shaking increased fibril formation dramatically, compared with unshaken samples \(^{257,262,263}\). Shear forces caused agitation via transport of insulin solutions can disturb the folding structure of insulin molecules, triggering potential nucleation from partially unfolded monomer-monomer interactions. As well, the kinetics of insulin-insulin interactions can be augmented by molecular collisions. By itself, this process can be detrimental to insulin formulation storage and potency, but this denaturation stress is further aggravated by hydrophobic surface interactions \(^{263,264}\). Practically speaking, within insulin vials, pens or pumps, insulin solutions are susceptible to
interactions from both air-water interfaces and container wall material. This involves orienting hydrophobic moieties towards air or hydrophobic surfaces and hydrophilic moieties towards aqueous solvent, to reduce overall free energy. Exposure of the hydrophobic surfaces of non-polar insulin monomer and dimer moieties occurs more readily with agitation, increasing contact with hydrophobic surfaces. Insulin proteins interacting with liquid surfaces, bubbles or rough, hydrophobic surfaces (Teflon, rubbers) can induce a small population of denatured insulin across these boundaries, and increase their probability to form nuclei and potentially, long-chain fibrils, rather than refolding into the correct native structure 256,263,265.

2.6.2 Temperature

As with most proteins, insulin structure is negatively affected by increasing temperature. Raising free energy of a system can affect conversion of proteins towards denatured end products, due to amplified kinetics of degradation pathways 254,256. One such pathway is the deamidation at one of six Asn or Gln residues in the insulin molecule, specifically the A21 and B3 sites that are externally situated. Individually, these desamino insulins are non-immunogenic and retain native activity, however increasing populations of hydrolyzed deamidated insulin can affect overall formulation stability. Equilibration of monomeric insulin with the more stable dimer and hexamer forms is disturbed by the increase in free energy, which promotes a looser folding structure and retards the ordered conformation, exposing peripheral Asn and Gln residues 264,266. A major temperature byproduct of higher temperatures is the A8-A9 split product, which occurs from peptide bond breakage between these two residues. These split products lose almost all insulin activity and are readily hydrophobic due to conformation change, which promotes both nucleation and fibrillation pathways 266,267. This peptide bond split product is much more temperature-sensitive than the deamidation pathways, which are also jointly affected by pH.
These degradation pathways follow Arrhenius kinetics at lower temperatures, although they increase exponentially with increasing temperature beyond 30°C, regardless of insulin species, potentially due to increased insulin-insulin interactions 264,268. Effect of crystallization on these chemical denaturation processes is inconclusive, with studies reporting both increased and reduced stability 266,269. When kept refrigerated at 4°C, most insulin formulations exhibit minimal desamido insulin and split product formation, below 5%, well within clinical tolerance 264,266.

2.6.3 pH

Insulin exhibits a pH-solubility curve that has its lowest solubility peak near its pKa of 5.5, as expected with proteins. At its isoelectric point, the net charge of insulin becomes neutral, reducing solubility greatly, but typically, insulin formulations are prepared by dissolving insulin in low pH before titrating towards a neutral pH, which keeps insulin in solution 258,260. Special concern must be taken when handling insulin in both acidic and basic conditions, relative to pKa. As mentioned before, the deamidation reaction occurring at the A21 asparagine is promoted by increasing temperature, but it also is aggravated in acidic conditions. Hydrolytic cleavage of the amide of the asparagine to a carboxyl aspartic acid occurs more readily in a highly protonated environment. Increasing pH to neutral decreases this hydrolytic reaction by 10-fold, maintaining native structure more readily, as water is not a strong nucleophile without protonation 257,258,267,271. The only current acidic insulin formulation is the long-acting glargine analog, which circumvents this A21 Asn deamidation by replacing asparagine with the non-reactive glycine. All other native insulin and insulin analog formulations are susceptible to this acidic deamidation process, and thusly, are stored at neutral pH. This deamidation is an irreversible chemical reaction, and although there is little change in insulin receptor activity, the conversion of amide to a carboxyl group disturbs the overall charge of the insulin molecule, rendering it susceptible to
nucleation and fibrillation in solution\textsuperscript{267,270,271}. Alternately, in basic pH (>7.4) a two-step reaction occurs primarily at the B3 Asn residue, which can also be classified as chemical deamidation. The asparagine residue amide at B3 can be attacked by the adjacent deprotonated peptide bond secondary amine at B4, creating a cyclic succinimide intermediate. This intermediate can hydrolyze rapidly, leaving a B3 aspartic acid, another chemically deamidated insulin product\textsuperscript{257,267,269,271}. This process is much slower than the acid labile A21 Asn, as the succinimide reaction requires the amino acid chain to accommodate the cyclic conformation for this process to occur, requiring the molecule to overcome steric hindrance. This does not readily occur in dimerized or hexamerized forms, due to greater rigidity of insulin within these conformations. In both deamidation processes, a carboxyl is produced from an amide, which directly affects overall protein polarity and charge and as a result, protein-protein interactions. Interestingly, in alkaline solutions, renaturation of acid-fibrillated insulin can be achieved, returning insulin to its native structure\textsuperscript{257,260}. As discussed earlier, the insulin glulisine analog has a modification at the B3 residue, giving it specific resistance to this pathway. The A8-A9 split product is also exacerbated with increasing acidity and temperature, yielding a non-active, highly hydrophobic endproduct\textsuperscript{264,269,271}. As insulin exhibits degradation at both ends of pH and lower solubility nearer pKa (5.5), it is critical in formulation design to maintain pH within these boundaries, opting to keep solution pH near neutral.

\textbf{2.6.4 Insulin Concentration}

Increasing insulin concentration leads to increasing hexamer formation, at the expense of monomer population. In an ideal formulation, this confers increased stability from hexamer isoform, given the appropriate solution. Against shear forces, increased hexamer population minimizes the amount of potential monomer exposed to hydrophobic surfaces or air-water
interfaces, which gives improved stability against initial fibril formation from partially unfolded intermediates \cite{257,258,263}. However, in formulations where conditions are not ideal, the presence of partially unfolded intermediates can more readily trigger nucleation and long-chain fibrillation due to greater intermolecular interactions at higher insulin concentrations. As well, in protein formulations, a low concentration can extend the lag phase of initial nucleation, if the kinetics are not air-water interface mediated \cite{263,272}. For insulin, where larger doses are required for clinical effect, formulation design must be focused on stabilizing relatively high concentration of protein. For practical purposes, at high insulin concentrations, visible aggregates are a simple identifier for denatured formulations, while low concentrations may not create visible high molecular weight products even though the formulation may be completely inactive.

2.6.5 Formulation Additives

As with other proteins, insulin has interaction with additives that can stabilize the protein structure, through structural binding to prevent exposure of deamidation sites. Antimicrobial excipients such as phenol, m-cresol, and methylparaben have shown to improve stability of insulin formulations. Phenol and the phenolic derivative, m-cresol stabilize insulin by inducing alpha helical shape across the early B-chain, rather than its extended native profile, protecting Asn residues on the B-chain \cite{258,260,273}. They are commonly used in low concentrations (~ 1 mg.mL$^{-1}$) as a dual purpose additive for their bacteriostatic and protein stabilizing effects for commercial insulin formulations, specifically the rapid-acting and long-acting analogs \cite{220}. Chemical stability is greatest when combining phenolic derivatives with zinc in solution, promoting hexamer and/or crystalline structure \cite{257,273,275}. Also, methylparaben is used as a stabilizer for crystalline formulations, allowing for protracted crystal dissolution by tightening hexamer interactions between insulin molecules \cite{258,276}. However, there is a concern with the use
of phenolic additives on sensitivity, with a small subset of patients exhibiting allergic reaction to trace levels of phenol or m-cresol upon subcutaneous administration\textsuperscript{[277,278]}.

From a physical stability standpoint, strategies to reduce agitation-induced denaturation include addition of block copolymer hydrophobic-hydrophilic surfactants, which can preferentially associate with hydrophobic surfaces (PVP, polaxomer) and increase solubility of insulin molecules in solution. Small concentrations of non-ionic surfactant below CMC in solution can reduce detrimental interactions of insulin with hydrophobic surfaces, such as pump tubing\textsuperscript{[258,260,263]}. Also, modifying vials and pump tubes to reduce hydrophobic character and minimizing total surface area in contact with insulin molecules can help retain native structure through preventing physical adsorption\textsuperscript{[263,265]}. The reversibility of surface adsorption is still debated, but it cannot be ignored that hydrophobic-hydrophobic surface interactions retain insulin molecules in a partially unfolded conformation, which can result in irreversible aggregate formation. This is commonly seen on ‘frosted’ insulin vials, where the insulin precipitates cannot be resuspended, rendering the entire formulation unsuitable for use.

\textbf{2.6.6 Role of Zinc in Insulin Formulation}

Despite the number of additives used for formulation stability, the largest impact on insulin stability and pharmacokinetics comes from the use of zinc. Most non-rapid formulations utilize zinc to promote insulin hexamerization, reducing solubility and increasing stability of formulations. Zinc ions interact electrostatically with the B10 His residue of the insulin molecule, binding the planar side with three insulin molecules. Conformational surfaces between dimers in a hexamer are not as tight as monomer surfaces in dimer form, so the electrostatic binding of zinc provides much greater physical and chemical stability. Zinc-free insulin
hexamers without this strong binding affinity can readily dissociate between dimer-hexamer forms, and thusly have increased exposure of denaturing stresses.

The typical native ratio of two zinc ions per insulin hexamer is classified as T₆ hexamer, comprised of six T isoform monomers. Alternate zinc-insulin hexamers such as the T₃R₃ and R₆ hexamer are defined by a coiled early B-chain (B1-8) in the bound insulin R isoform monomers, and tighter binding, which is facilitated by the addition of chloride ions and addition of phenol or phenolic derivatives such as m-cresol, respectively. T₆, T₃R₃ and R₆ all require zinc complexation to achieve this more stable ordered state and, in the presence of chloride and phenol, interconvert in solution. These isoforms have greater overall stability in solution, and thusly are not readily bioactive, until dissociated into dimers or monomers. The sensitive denaturation kinetics of the insulin monomer and dimer to environmental stresses makes proper zinc hexamerization critical for long-term stability in an insulin formulation.
Chapter 3 A monolithic polymeric microdevice for pH-responsive drug delivery

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Developed and tested the integrated ethylcellulose membrane-PDMS microdevice prototype. Formulated composite membrane and polymerized hydrogel nanoparticles. Performed in vitro testing of Vitamin B_{12} release and SEM analysis of nanoparticle transition.
3.1 Abstract

A drug-delivery microdevice integrating pH-responsive nano-hydrogel particles functioning as intelligent nano valves is described. The polymeric microdevices are monolithic without requiring peripheral control hardware or additional components for controlling drug-release rates. pH-responsive nanoparticles were synthesized and embedded into a composite membrane. The resulting pH-responsive composite membranes were integrated with PDMS micro reservoirs via a room-temperature transfer bonding technique to form the proof-of-concept microdevices. In vitro release characterization of the microdevices was conducted in which the release rate of Vitamin B\textsubscript{12} (VB\textsubscript{12}) as a model drug increased dramatically when the local pH value was decreased from 7.4 to 4. This device concept can serve as a platform technology for intelligent drug delivery in response to various \textit{in vivo} environmental signals.

3.2 Introduction

Stimuli-responsive hydrogels change their behavior according to environmental conditions\textsuperscript{285-287}. These properties can be utilized for the construction of smart drug-delivery systems to be responsive to temperature\textsuperscript{288-291}, pH\textsuperscript{292,293}, electric field\textsuperscript{294}, light\textsuperscript{295,296}, or magnetic field\textsuperscript{297}. Taking pH as an example, normal organs, tissues and cellular compartments have different pH levels. Additionally, certain cancers as well as inflamed or damaged tissues exhibit a pH deviation from 7.4. Hence, pH can be a suitable target stimulus for responsive drug delivery, and pH-responsive hydrogels have been developed for controlled drug delivery\textsuperscript{298,299}. pH-responsive hydrogels are composed of polymeric backbones with ionic pendant groups. Commonly studied ionic polymers for pH-responsive behavior include poly(acrylamide) (PAAm), poly(acrylic acid) (PAA), poly(methacrylic acid) (PMAA), poly(diethylaminoethyl
methacrylate) (PDEAEMA), and poly(dimethyl-amoethy methacrylate) (PDMAEMA). In aqueous media of appropriate pH and ionic strength, the pendant groups ionize and develop fixed charges on the polymer network, generating internal electrostatic repulsive or attractive forces responsible for pH-dependent swelling or deswelling of the hydrogel, thereby controlling drug release. The pH-responsive swelling and deswelling behavior has been used to induce controlled release of model compounds (e.g., caffeine and indomethacin).

Since transient swelling-deswelling behavior of the stimuli-responsive hydrogel is limited by the diffusion of stimuli into the hydrogel matrix and by the absorption and expulsion of the solvent (usually water), stimuli-responsive micro and nano hydrogels have been developed for controlled drug delivery with shorter response time. For example, a closed-loop insulin delivery system utilizing micro-hydrogel via microfabrication was developed. In this design, a thin hydrogel poly(3-methacrylamido phenylboronic acid-co-acrylamide)(PMPBA-AAm) was confined in a cavity that allowed an external glucose solution to diffuse through a stiff porous membrane. The hydrogels volume change produced deflections of a diaphragm that opened and closed an intake orifice of a valve to deliver insulin. In order to obtain fast response and enhanced mechanical strength/integrity of stimulus-responsive polymeric hydrogels, prototypes of nano-hydrogel composite membranes containing poly(N-isopropylacrylamide-co-MAA) (PNIPAm-MAA) nanoparticles were developed, which responded to temperature and pH changes. The much smaller sizes of hydrogel particles (hundreds of nanometers) led to faster responses than bulk hydrogels. The membranes also possessed significantly enhanced mechanical strength/integrity.

An alternative method for achieving pulsatile release of drugs is based on microfabrication technologies. In the first implantable drug-delivery device, electrical pulses were
employed to dissolve a gold membrane via electrochemical dissolution, allowing the diffusion of drugs out of silicon-made reservoirs on demand. In contrast to the electrochemical dissolution approach, electrothermal activation to open reservoirs for controlled drug release has also been demonstrated. For the construction of implantable drug-delivery microdevices, biodegradable membranes have also been proposed for multi-dose drug delivery. The first biodegradable polymeric microchip was formed by compression-molding polylactic acid (PLA). Individual membrane recipes were prepared by using various ratios of lactic acid/glycolic acid and different molecular-weight polymers for controlled drug release. An advantage of biodegradable polymeric microchips is the elimination of a second surgery for device removal. In addition, the lack of electronics reduces size and other manufacturing restrictions.

In these implantable drug-delivery microdevices, although drugs can be released on demand, the microdevices are not capable of regulating drug-delivery rate according to local micro environmental signals such as pH and glucose changes. In this study, we have developed the first pH-responsive drug-delivery microdevice targeting short-term implantation use in rodents. The proof-of-concept devices integrate pH-sensitive composite membranes embedded with responsive hydrogel nanoparticles with microfabricated reservoirs. The polymeric microdevices are monolithic without requiring peripheral control hardware or additional components for controlling drug-release rates.

Compared to normal physiological pH (7.4), the embedded pH-responsive nanoparticles control the drug-delivery rate to provide higher release rates at lower environmental pH values. This has been explored with pH-responsive nanoparticle injected into circulation to induce drug release in tumor tissue. As shown in Fig. 3.1, the patterned PDMS structure forms a drug reservoir and provides physical support for the thin nano-hydrogel embedded composite membrane. The
embedded hydrogel nanoparticles in the composite membrane detect environmental pH changes as intelligent nano valves. Corresponding volumetric swelling and shrinking response of the nanoparticles controls drug-release rates.

**Fig. 3.1** – Illustration of the mechanism for pH-responsive drug release out of the microdevice. *Top:* nanoparticles are in the swollen state when the surrounding pH value is higher than pKa (acid dissociation constant) of the nanoparticles. *Bottom:* Nanoparticles are in the shrunk state when the surrounding pH value is lower than pKa. Resulting volumetric swelling and shrinking of the nanoparticles control drug-release rates. *(Reproduced by permission of Biomedical Microdevices)*
3.3 Materials and Methods

*pH*-responsive nanoparticle synthesis and characterization

For nanoparticle synthesis, N-Isopropylacrylamide (NIPAm, 99%), methacrylic acid (MAA), N,N-bisacrylamide (BIS), and potassium persulfate (KPS) were from Sigma. Sodium Lauryl Sulfate (SDS) was from Fisher. Ethylcellulose powder (viscosity 45) for composite membrane fabrication was from Dow.

The pH-responsive poly(N-isopropylacrylamide-co-methacrylic acid) (PNIPAm-MAA) nanoparticles with 1:1 molar ratio of NIPAm to MAA were prepared by an aqueous dispersion polymerization process using N’N’-methylenebisacrylamide (BIS) as the crosslinking agent, potassium persulfate as the initiator (KPS), and sodium dodecyl sulfate (SDS) as the stabilizer. Monomer mixtures (763.83 mg of NIPAm and 0.57 ml of MAA) were dissolved in 100 ml distilled water. After the incorporation of 133.22 mg of BIS, 11.54 mg of SDS was added to the reaction mixture. The reaction mixture was purged with nitrogen for 0.5 h and then polymerization was initiated by the addition of 56.77 mg of KPS. The polymerization process was conducted under a nitrogen blanket at 70°C for 4 hours at 200 rpm. Typically, nanoparticles in aqueous solution with a polymer concentration of ~1.5 wt% were prepared. More concentrated samples were obtained through centrifugation.

Synthesized nanoparticles were dispersed in 10 mg.ml⁻¹ concentration with distilled water. A volume of 50 μl of solution was taken and placed into glass light scattering test tube with 700 μl of phosphate buffer solution (PBS) at different pH values. Particle sizer measurements were performed with a NICOMP 380 particle sizer.
Synthesis and Characterization of Composite Membrane

To form a useful material for device construction, the nanoparticles were embedded into a composite membrane. Casting of composite membranes started with dissolving 0.65 g of ethylcellulose powder in 15 ml of anhydrous alcohol at room temperature (22°C). Ethylcellulose solution was mixed with dispersed nanoparticle solution for another 2 h.

Ethylcellulose/nanoparticle solution was then poured into a 10 cm Teflon plate and placed in a vacuum tank for 15 minutes for degassing. Solution was allowed to dry for 24 hours in a sealed desiccator to slow evaporation and prevent contamination. SEM analysis of the membrane was performed to obtain visual characterization of the nanoparticle and membrane.

Permeability testing of nano-hydrogel composite membranes was conducted in side-by-side diffusion cells using Vitamin B<sub>12</sub> as a model drug (M<sub>w</sub> = 1355). Multiple 2 cm diameter circular samples were soaked in pH 7.4 phosphate buffer solution for 24 h before testing. Side-by-side water jacketed diffusion cells housed both the receptor and donor cells for permeability testing shown in Fig. 3.2. The volume of each cell was 3 ml and the area for permeation was 0.63 cm<sup>2</sup>. Polyethylene tubes connected to a peristaltic pump allowed for continuous flow from the receptor cell to a cuvette. The cuvette was placed in a UV spectrometer for kinetic measurements of VB<sub>12</sub> diffusion. Donor cell was filled with 1 mg.ml<sup>−1</sup> VB<sub>12</sub> solution. Data were collected over 4 h periods at 10 min intervals at different pH values.
Solute permeability $P = \frac{DK}{h}$ was calculated according to the following equation $Mt = PSC\, d\,(t-t_L)$ based on Fick’s first law of diffusion with assumptions including: (1) steady state is reached in the membrane after a lag time, $t_L$; (2) the area for permeation, $S$, and solute concentration in the donor cell, $C_d$, are constant; (3) sink condition is maintained at the receptor side; where $M_t$ is the mass of a drug permeated till time $t$; $D$ and $K$ are, respectively, the diffusion coefficient and partition coefficient of the drug; $h$ is the thickness of the membrane. The $P$ value was calculated from the slope of the curve of $Mt$ vs. $t$ at the steady state.

**Microdevice fabrication and characterization**

The integration of nano-hydrogel composite membranes and PDMS drug reservoirs must ensure the physical and functional integrity of the composite membranes without exposing the hydrogel nanoparticles to harsh processing conditions, such as UV, plasma, high temperatures or wet chemical etchant.

The PDMS reservoir was formed via standard soft lithography using SU-8 as the mould master. The thickness of the SU-8 master was approximately 1 mm, obtained by spinning two layers of SU-8 2100. After the PDMS drug reservoir was peeled off from the substrate, room-temperature transfer bonding was used to integrate nano-hydrogel embedded composite membranes. A thin
layer of PDMS was spun on a substrate as a bonding adhesive layer (Fig. 3.3a). Through micro-
contact printing, the adhesive layer was transferred to the PDMS drug reservoir (Fig. 3.3b and c).
The drug reservoir was then bonded to a nano-hydrogel composite membrane and left to cure at
room temperature (Fig. 3.3d).

[Diagram of microfabrication steps]

**Fig 3.3** – Microfabrication steps for the integration of nano-hydrogel composite membranes with
patterned PDMS structures. *(Reproduced by permission of Biomedical Microdevices)*

*In vitro* testing was conducted on the micro devices filled with 5 mg.ml$^{-1}$ VB$_{12}$ solution. Device-
containing chambers were soaked in a water bath at 37°C and solutions around micro devices
were measured by a UV spectrometer. Data was collected over 8 h periods with a change in pH
from 7.4 to 4 triggered by adding dilute acetic acid. After each change in pH, the devices were
washed several times with distilled water before re-testing.

To verify the suitability of the microdevices for short-term implantation use in animals, *in vivo*
bio compatibility testing was conducted in rats. The rats were randomly divided into 2
groups and underwent implant surgery under isoflurane anesthesia. The sham group (n = 6)
underwent surgery without an implant while in the device group (n = 6), rats were implanted
subcutaneously in the interscapular tissue with ethylene oxide sterilized empty micro devices for two weeks.

Statistical Analysis

Student’s t-test was utilized to determine statistical significance between two groups, respectively. A $p$ value $< 0.05$ was considered to be statistically significant.

3.4 Results and discussion

Nanoparticle and membrane characterization

Figure 3.4a shows that the diameters of the nanoparticles decrease dramatically from 490 ± 42 nm (pH 7.4) to 327 ± 29 nm (pH 4), showing a sharp transition below physiological pH. This makes PNIPAm-MAA nanoparticles appropriate for an acute ‘on-off’ pH response for drug delivery ($\mu = 0.15$M). Figure 3.4b shows a scanning electron microscopy picture of a composite membrane after freezing and cross-sectional fractioning. The membranes have sufficient mechanical strength and are suitable for physical handling.

![Figure 3.4](image)

**Fig. 3.4** – (a) Size transition of poly(NIPAm-MAA) nanoparticles resulting from varied pH values at 37°C. (b) SEM picture of a cross section of 40% nanoparticle-loaded ethylcellulose membrane. Circled are nanoparticles into membrane channels in ethylcellulose matrix. *(Reproduced by permission of Biomedical Microdevices)*
Permeability testing of composite membranes

Figure 3.5 shows a representative set of permeability testing data, which proves the pH responsiveness of the nano-hydrogel composite membranes. After the addition of dilute acid to decrease the pH of the side-by-side diffusion cell from 7.4 to 4 (μ = 0.15M), VB12 amount diffused into the receptor cell increased steadily with a calculated permeability of $1.17 \times 10^{-5} \pm 1.46 \times 10^{-6}$ cm/s.

Fig. 3.5 – pH-dependent permeation of VB12 through ethylcellulose membranes (n = 3) that contain 40% w/w nanoparticles. Dilute acetic acid was added at 80 min to decrease pH from 7.4 to 4. Error bars represent standard deviations. (Reproduced by permission of Biomedical Microdevices)

Nanoparticle percentages ranging from 10% to 40% were embedded into the ethylcellulose membranes. At 30% and lower, there was no obvious response to pH, possibly due to the insufficient amount of nanoparticles to create nano-channels for drug diffusion. At 40%, pH response was strong (Fig. 3.5). Even higher percentages (>40%) resulted in uneven ethylcellulose membranes.
Alcohol-based ethylcellulose was chosen as the base polymer since it showed highly satisfactory film formation at room temperature, and membrane synthesis was relatively fast. Furthermore, the membranes remained mechanically strong after the addition of nanoparticles. Cellulose acetate, despite being a similar base polymer, required a much longer synthesis time (24 hours vs. ~3.5 days), and the resulting membrane was poor in quality (e.g., surface uniformity).

Aquacoat, an aqueous suspension of ethylcellulose, was also tested for membrane synthesis. The resulting membranes were weak in mechanical strength and remained weak with the addition of plasticizers, making physical handling of the membranes difficult.

**Microdevice characterization**

Fig. 3.6 shows prototypes of the pH responsive drug-delivery microdevices. The drug reservoirs were constructed with PDMS due to its mechanical stability, the feasibility of precise patterning using microfabrication, and its biocompatibility and insusceptibility to protein adsorption and fouling. Other biocompatible materials such as PEVA, poly(ethylene-co-vinyl acetate) can also be valid options. VB$_{12}$ filling and refilling were conducted through the backside of the PDMS reservoirs with a small-gauge syringe needle, made possible by the self-sealing property of PDMS.
Figure 3.6 – Prototype microdevices for pH-responsive drug release (Reproduced by permission of Biomedical Microdevices)

Figure 3.7 shows VB₁₂ release profiles of two-cycle tests of two microdevices. After the addition of dilute acid to decrease the pH of the surrounding medium from 7.4 to 4, VB₁₂ amount in the surrounding medium increased steadily. The results proved the release concept and the capability of the microdevices for pH-responsive drug delivery.
Fig. 3.7 – *In vitro* testing results of pH-responsive release. Data show two cycles of *in vitro* testing of VB$_{12}$ permeation on two microdevices. Dilute acetic acid added at 2 h. *(Reproduced by permission of Biomedical Microdevices)*

The lag time for diffusion could be due to the chain of chemical events. The hydrogen ions from acid need to come in contact with the embedded nanoparticles, which requires diffusion into the membrane. Although the nanoparticle shrinkage time is short, drug diffusion takes time to permeate the composite membrane. By adjusting nanoparticle percentages and the thickness of the composite membranes, the response time could be further shortened. The slight drop of VB$_{12}$ after the surrounding pH decreased from 7.4 to 4 could be attributed to osmotic pressures and hydrogen ion gradients across the membrane which could have brought a small amount of VB$_{12}$ together with water into the microdevices, as nanoparticles began to shrink. As well, an increase in environmental ionic strength from acid addition would draw water out from the microdevices in an osmotically driven process.
Biocompatibility testing of microdevices

Microdevices after implantation were retrieved. They demonstrated satisfactory mechanical strength for implantation. The total white blood cell (WBC) at the end of the experiment for the “device implantation” ($5.35 \times 10^9 \pm 1.78 \times 10^9$) group was slightly but not significantly increased compared to the “sham” group ($3.78 \times 10^9 \pm 1.32 \times 10^9$) (Fig. 3.8). This may indicate the presence of some inflammation due to the foreign implant. The count of specific inflammatory cells shows mostly an increase in the number of lymphocytes, which may be an indication of chronic inflammation. This, however, was not significant ($p > 0.05$).

![Fig. 3.8](image)

**Fig. 3.8** – Total white blood cell (WBC) numbers for the control group (no device implanted, $n = 6$) and the device implantation group ($n = 6$). *(Reproduced by permission of Biomedical Microdevices)*

3.5 Conclusion

In summary, this paper demonstrated the first monolithic pH-responsive drug-delivery microdevices. The microdevices integrated pH-responsive nanoparticles as intelligent nano-valves that were embedded into composite membranes. *In vitro* release characterization proved the concept and the capability of the microdevices for pH-responsive drug delivery. *In vivo* biocompatibility testing verified the suitability of the devices for short-term implantation in rodents.
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Chapter 4  *In vitro and In vivo* Testing of Glucose-Responsive Insulin-Delivery Microdevices in Diabetic Rats

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Designed and tested PDMS reservoir-bioinorganic membrane microdevice prototype. Tested preliminary *in vitro* glucose-responsive insulin release. Performed animal surgery for implantation and *in vivo* assays for glucose, insulin and C-peptide.
4.1 Abstract

We have developed glucose-responsive implantable microdevices for closed-loop delivery of insulin and conducted in vivo testing of these devices in diabetic rats. The microdevices consist of an albumin-based bioinorganic membrane that utilizes glucose oxidase (GOX), catalase (CAT) and manganese dioxide (MnO$_2$) nanoparticles to convert a change in the environmental glucose level to a pH stimulus, which regulates the volume of pH-sensitive hydrogel nanoparticles and thereby the permeability of the membrane. The membrane is integrated with microfabricated PDMS (polydimethylsiloxane) structures to form compact, stand-alone microdevices, which do not require tethering wires or tubes. During in vitro testing, the microdevices showed glucose-responsive insulin release over multiple cycles at clinically relevant glucose concentrations. In vivo, the microdevices were able to counter hyperglycemia in diabetic rats over a one-week period. The in vitro and in vivo testing results demonstrated the efficacy of closed-loop biosensing and rapid response of the ‘smart’ insulin delivery devices.

4.2 Introduction

The global effect of diabetes has been an issue that is increasingly weighing on our population. With around 285 million people affected in 2010, the number of diabetic patients is expected to drastically increase to 440 million by 2030$^{321}$. Diabetes is marked by the destruction of islet β-cells and the inability to produce endogenous insulin, known as Type 1 diabetes, or by the development of insulin resistance and inability of the pancreas to provide adequate insulin, known as Type 2 diabetes$^{322,323}$. In all diabetic patients, the body is unable to control blood glucose levels, leading to a state of hyperglycemia, a precursor for further complications, such as
micro/macrovacular, renal and neural damage. The necessary treatment of diabetes complications is an increasing financial burden\textsuperscript{321-323}.

Insulin replacement therapy is the current standard treatment for all Type 1 diabetic patients and more than one third of Type 2 diabetic patients. Directly providing exogenous insulin to lower blood glucose is required to maintain normoglycemia for these patients and is often necessary for survival\textsuperscript{324}. Although effective in short periods, insulin therapy cannot reproduce normal physiological insulin secretion patterns. Even when using combinations of short and long-acting insulin types, complications can arise when insulin dosage amount and timing are not precise, leading to intermittent periods of hypoglycemia due to an excess of insulin and chronic complications from periods of hyperglycemia due to lack of insulin.

Ideally, insulin therapy should provide the proper amounts of insulin in response to blood glucose concentration. Current attempts towards this goal involve the use of continuous insulin delivery via pumps\textsuperscript{325-327}. With a needle inserted in the abdominal fat, insulin is delivered subcutaneously from an insulin reservoir, providing a constant basal release of insulin, more closely mimicking the physiological insulin profile than subcutaneous injections\textsuperscript{325-329}. However, this treatment approach still requires regular glucose monitoring, that is, it is an open-loop approach. Combination of insulin pumps with real-time glucose sensors have been investigated for closed-loop insulin delivery by collecting blood glucose data and converting it into a release profile for responsive insulin delivery. Compared with standard pumps, the pump and sensor combination resulted in shorter durations of hypoglycemia. However, there were no significant differences in hemoglobin A1c, a measurement of long-term glycemic index\textsuperscript{326,330,331}.
Another glucose-responsive closed-loop insulin delivery option is the use of glucose-responsive hydrogel-based insulin delivery systems. The systems require the synergy of a real-time glucose sensor and a responsive insulin release element. Glucose oxidase has been successfully used as a glucose sensing component in combination with pH-responsive materials, such as films or hydrogel particles. The enzymatic oxidation of glucose into gluconic acid is harnessed to produce an acidic ‘trigger’ for a pH-responsive delivery system. The pH signal is utilized to modulate shrinking/swelling response or disintegration of pH-responsive hydrogels, releasing loaded insulin. The in vivo application of insulin-loaded hydrogel systems is limited by slow response times, clinically irrelevant glucose-responsive range, low insulin loading capacity, bioavailability, stability and non-cyclic release profiles.

To overcome the limitations of hydrogel systems, Yam and Wu designed hydrogel nanoparticle-containing composite membrane systems for stimulus-responsive drug delivery. Utilizing environment-sensitive hydrogels as physical ‘nano-valves’, drug delivery across the membrane is modulated by shrinking or swelling of embedded responsive nanoparticles. The nano-size allows for rapid response, within seconds, while the hydrogel polymer structure maintains three-dimensional geometry and repeatable volumetric responses. Typical hydrogel nanoparticles used in the composite membranes are comprised of crosslinked copolymers, specifically poly(N-isopropylacrylamide) (PNIPAM) and poly(methacrylic acid) (PMAA). PMAA acts as a pH-sensor that changes ionization degree according to pH, affecting nanoparticle hydration and volume. At pH levels above pKa, the MAA groups are ionized and the charged carboxyl groups generate repulsive forces, causing the nano-hydrogel to swell. At low pH levels, the MAA groups take unionized form, causing collapse of the nano-hydrogel particles. pH-responsive membranes with embedded PNIPAM-MAA nanohydrogels have been previously studied for pH-
responsive protein/peptide drug delivery\textsuperscript{334,341,351,352,354,355} and incorporated into a microdevice system that provided a 2-fold increase in vitamin B\textsubscript{12} release when environmental pH drops from neutral to acidic pH\textsuperscript{356}.

A new generation of glucose-responsive composite membrane systems was devised by Gordijo et al. to address the problem of oxygen-limited glucose sensing encountered by glucose oxidase-based biosensors\textsuperscript{341}. Utilizing the capability of MnO\textsubscript{2} nanoparticles to fully recover oxygen consumed by glucose oxidation, an albumin-based bioinorganic membrane system was developed with a glucose sensing component that possesses a self-renewable oxygen supply\textsuperscript{341}. The bioinorganic composite membrane was integrated with an insulin reservoir to form an implantable tube-shaped microdevice made of biocompatible silicone. The device, when implanted, reduced hyperglycemia in streptozotocin (STZ) induced-diabetic rats for up to 5 days\textsuperscript{342}.

The previous tube-shaped microdevices, although proven effective in lowering hyperglycemia, exhibited certain technical restraints. Due to small surface areas for permeation limited by the construction method, the implantation of a number of these devices (vs. a single device) was required to achieve normoglycemic state in STZ-diabetic rats. In addition, the smaller reservoir volume limited the total amount of insulin that can be stored. Hence, in this work we intended to develop a microdevice with larger permeation surface area and reservoir volume. However, an increased membrane surface area faces the higher risk of membrane rupture \textit{in vivo}, which can cause device failure leading to severe hypoglycemia and even host death. To solve this problem, we designed PDMS grid sheet-covered microdevices with glucose-responsive bioinorganic membranes crosslinked to the PDMS grid, which is adhered to a PDMS reservoir. We chose to use PDMS to form microdevice structures because of its ease of microfabrication, relatively
accepted biocompatibility for implantable devices particularly for animal studies, its resealing property which is important for drug refilling, and mechanical strength\textsuperscript{356}. Compared with the soft bioinorganic membranes with a Young’s modulus value of approximately 10 kPa\textsuperscript{332}, PDMS has a 25 to 50-fold higher Young’s modulus value, which is critical for maintaining membrane constitution\textsuperscript{359,360}. These new integrated microdevices increase net insulin permeation and minimize device numbers required for implantation. The integrated PDMS grid also reinforces the albumin-based glucose-responsive permeable membrane, maintaining membrane integrity, with a larger, more efficient area for insulin permeation.

Following \textit{in vitro} testing of the glucose-responsive insulin release from the grid sheet and the microdevices, we conducted \textit{in vivo} testing of the capability of the microdevices to control hyperglycemia in STZ rats. This Type 1 diabetic rat model is an important vehicle for the \textit{in vivo} analysis of prototype insulin microdevices. Utilizing both glucose and insulin assays can quickly provide the information needed to discern the efficacy of microdevices \textit{in vivo}, which cannot ethically be performed in humans. Our microdevices reported in this paper were developed for the diabetic rat model as a proof-of-concept glucose-responsive insulin-delivery system. Achieving glucose-responsive delivery of insulin in a fashion that mimics the way of normal body controlling glycemic profile is the crux of the implantable microdevice system. Furthermore, our implantable insulin-delivery microdevices allow for maintenance of blood glucose under true, untethered \textit{in vivo} conditions, avoiding cannulated attachment and wiring seen with alternate intravenous systems.
4.3 Methods

Materials

All materials were analytical grade and used without further purification unless noted. Bovine serum albumin (99%), catalase (solution 13mg ml⁻¹), 3-aminopropyltrimethoxysilane (97%), glutaraldehyde (25%, Grade I), n-octyl-β-glucopyranoside and poly(ethylene glycol) methyl ether (Mₚ 2 000) were purchased from Sigma-Aldrich (USA). Glucose oxidase (230u mg⁻¹) was purchased from Wisent (Canada). HEPPS (4-(2-hydroxyethyl)⁻¹-piperazinethanesulfonic acid) was purchased from MP Biomedicals (USA). PDMS, 184 silicone elastomer was obtained from Ellsworth Adhesives Canada (Burlington, ON, Canada). PNIPAM-co-PMAA nanoparticles (250 ± 50nm in pH 7.4 and 160 ± 35 at pH 5.0 PBS) and nano-MnO₂ nanoparticles(80 ± 30nm) were prepared as previously described 341,342.

Preparation of activated PDMS grid microdevices

To fabricate glucose-responsive membranes, standard soft lithography was first used for forming a PDMS grid sheet. The insulin reservoir compartment was constructed with PDMS due to its mechanical stability, the feasibility of precise patterning using microfabrication, and its short-term biocompatibility. SU-8 pillars were constructed via standard photolithography and used as a mold master (see supplemental material). PDMS was spin-coated on the SU-8 mold master. The cured PDMS membrane with a thickness of 150 μm was peeled off from the substrate, producing a PDMS grid sheet. PDMS grid sheets were bonded with the insulin reservoir via a thin layer of pre-cured PDMS as an adhesive layer. PDMS grid microdevices were modified by oxygen plasma treatment to introduce reactive hydroxyls and then soaked in 0.1 M aminopropyl trimethoxysilane solution for 24 hours at room temperature to produce primary amine groups,
which provide active sites for covalently crosslinking bioinorganic membrane to the PDMS grid, improving membrane integrity\textsuperscript{342}.

\textit{Preparation of Glucose-responsive Bioinorganic PDMS Grid-gel Microdevices}

\(\text{MnO}_2\) nanoparticles (6mg) were dispersed in 143\,\mu l of phosphate buffered saline (pH 4) using a UP100H Hielscher ultrasonicator. Albumin (28\,mg), glucose oxidase (3\,mg) and catalase (0.86\,mg) were added to the suspension and dissolved under light agitation in a 37\degree C water bath. To the \(\text{MnO}_2\)-protein mixture, 65\,\mu l of 200\,mg.m\,l\textsuperscript{-1} NIPAM:MAA hydrogel nanoparticle suspension was added in slowly under stirring with a stir bar. To initiate crosslinking, 25\% glutaraldehyde solution (15\,\mu l) was introduced and stirred rapidly for 5s, and then 60\,\mu l of the mixture was pipetted onto an activated grid device surfaces and spread evenly. Each batch provided enough volume for three grid device surfaces. Grid-gel membrane devices were allowed to crosslink for 10 minutes, and then placed in PBS 7.4 solution at 4\degree C for 24h.

To reduce the hydrophobicity of PDMS and improve insulin compatibility with the device, we introduced activated polyethylene glycol (PEG) on the PDMS surface. After completion of crosslinking, grid-gel devices were soaked in an activated-PEG solution for 24h at 4\degree C to initiate hydrophilic surface modification of the devices. The devices were washed several times in PBS 7.4 to remove soluble unreacted activated-PEG and then incubated in fresh PBS 7.4 at 4\degree C before use.

\textit{ESEM Analysis of PDMS Grid-Bioinorganic Gel}

Environmental scanning electron microscopy (ESEM) imaging was conducted to analyze the morphology and integrity of crosslinked PDMS grid sheets with bioinorganic gels and the surface of the microdevices. ESEM images were taken at x160 magnification on membrane grid
holes. To determine grid-gel membrane integrity after in vivo testing, grid-gel microdevice samples were retrieved 0, 5 and 15 days after in vivo implantation and incubated in formalin (1% Paraformaldehyde, 4% glutaraldehyde) at 4°C for 24h to prepare samples and fix residual cellular adhesion. Retrieved PDMS grid-gel bioinorganic membranes were also analyzed to investigate degradation and cell adhesion for biocompatibility analysis at x200 magnification.

**Reservoir Insulin Formulation and Stability Analysis**

Insulin formulation was prepared in a buffered solution of 3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid (HEPPS) (pH 7.4). To make 2ml of 25mg.ml⁻¹ insulin reservoir formulation, 50mg of human recombinant insulin (M₇, 5808, Wisent) was dissolved in 1.2ml 0.1M NaOH solution. Octyl β-d-glucopyranoside (8mg) and Pluronic F68 (8mg) were added as surfactant to stabilize insulin and dissolved slowly. Then 0.252g of HEPPS was added and 800ul of 0.1M HCl was added to adjust pH to approximately 7. Insulin solution was prepared one day before injection into microdevices.

Insulin solution (100μl) was injected into the PDMS reservoir immediately before implantation into rats using a 27 gauge syringe needle. The self-sealing property of PDMS maintains the integrity of the device after injection. Insulin filled devices were kept in sterile saline solution before implantation. After implantation period, microdevices were removed and insulin was retrieved from microdevice reservoirs.

Retrieved insulin and freshly prepared samples were analyzed by reversed-phase HPLC using a Waters HPLC equipped with a Waters NovaPak C18 column, 3.9mm x150mm (4.0 μm pore size). The column was eluted with two mobile phase solutions in two-gradient modes at a flow
rate of 1ml/min. Solvent A was water (0.1% trifluoroacetic acid) and solvent B was acetonitrile (0.1% trifluoroacetic acid). The column was initially run in an 85/15 ratio of A to B, with a linear gradient to 35/65 over 10 minutes, then returned to 85/15 over 10 minutes. Insulin samples were detected by a UV detector (Waters PDA 2899) at 215nm.

**In vitro Testing of Insulin Release from Microdevices**

At the start of experiment, devices were incubated in PBS 7.4 at 37ºC with 5mmol.L^{-1} d-glucose (corresponding to normal glucose levels) and increased to 20mmol.L^{-1} glucose (corresponding to hyperglycemic glucose levels) after 2.5h, while slowly rotating on a hematological blot mixer. UV measurements of insulin were taken by Agilent UV Spectrometer at 276nm every 30 min. Three normal glucose-high glucose cycles were tested, with multiple washing of microdevices in between to remove residual glucose and insulin from prior tests. A calibration curve was determined with serial dilution of human recombinant insulin stock solution.

The *in vitro* insulin release in response to glucose concentration was measured at 20mmol.L^{-1} glucose and 5mmol.L^{-1} glucose in the release medium. From the permeation-time profiles, insulin permeability was determined according to: \[ P = \frac{s \cdot h}{a \cdot c} \], where \( P \) = Permeability (cm².s⁻¹), \( s \) = Slope of the permeation vs. time plot (mg.s⁻¹), \( h \) = Membrane thickness (cm), \( a \) = Membrane surface area for permeation (cm²), and \( c \) = Insulin concentration in the device (mg.cm⁻³). Permeability ratio was calculated by dividing the permeability at high glucose by the permeability at low glucose (\( P_{20}/P_{5} \)). Three cycles were plotted to examine microdevice repeatability.
In vivo Testing with Microdevice-Implantated STZ-Diabetic Rats

Male Sprague-Dawley rats were injected with 65mg.ml\(^{-1}\) streptozotocin (STZ) to induce diabetes 3 days before implantation. STZ destroys pancreatic beta cells, thus removing endogenous insulin production and glycemic control, leading to hyperglycemia. The rats were kept under 12/12 reverse light cycle. Blood glucose was taken before STZ-injection and after to ensure beta cell deficiency. All rats were implanted with a single microdevice in two groups: one group was implanted with a microdevice filled with 25mg.ml\(^{-1}\) insulin formulation and the other group was implanted with a sham microdevice filled with saline as control. Surgery was performed on the abdominal wall of the rats, and the microdevice was implanted intraperitoneally. Blood glucose was measured on Day 0, 1 and 2 and then measured three times a day with a LifeScan OneTouch meter. Blood samples from the tail vein were also taken for insulin and C-peptide measurements by radioimmunoassay which was performed with commercial kits (Linco).

In vivo Glucose Challenge Testing with Microdevice-implantated STZ-Diabetic Rats

In the glucose challenge tests, STZ-diabetic rats treated with insulin microdevices were given a 1g.kg\(^{-1}\) injection of glucose via a previously cannulated jugular vein to induce hyperglycemia, while blood for glucose, C-peptide and insulin measurements were taken as described before via a previously cannulated carotid artery.

Statistical Analysis

Student’s t-test was utilized to determine statistical significance between two groups, respectively. A \(p\) value < 0.05 was considered to be statistically significant.
4.4 Results and Discussion

Microdevice Working Mechanism

As shown in Figs. 4.1a and 4.1b, a PDMS drug reservoir and a layer of PDMS grid provides physical support for the bioinorganic composite membrane. In the composite membrane, the enzymes GOx and CAT are directly crosslinked with the albumin macromolecules, as well as suspended nano-MnO$_2$ particles, forming the base membrane. Albumin was chosen as a base material, as it is hydrophilic, allowing for unrestricted diffusion of small molecule solutes while providing sufficient membrane integrity when crosslinked within microdevices. As well, through non-specific crosslinking with glutaraldehyde, this hydrophilic composite membrane can easily covalently bind with glucose oxidase and catalase, preventing enzyme leakage. The hydrogel nanoparticles embedded in the base membrane detect and respond to local pH changes caused by gluconic acid produced from the oxidation of environmental glucose by GOx, acting as intelligent ‘nano-valves’. The MnO$_2$ nanoparticles and catalase act as catalysts to remove harmful hydrogen peroxide produced by the glucose oxidase reaction and recover consumed oxygen. Volumetric swelling and shrinking of the hydrogel nanoparticles in response to the fluctuation of glucose levels control the porosity of the membrane, resulting in regulated insulin release from the microdevice reservoir, powered by a concentration gradient (Fig. 4.1c).

With our microdevice system, intraperitoneal implantation allows for rapid response to glucose and delivery of insulin. Fabrication of the devices requires careful integration of PDMS grid to PDMS reservoir, and bioinorganic gel to PDMS grid. Compared with tube-shaped devices developed by Gordijo et. al$^{341,342}$, improved device integrity is achieved in the present microdevices. The reservoir volume of the present microdevices is twice as large as the previous tube-shaped devices (100μl vs. 50μl). Upon incubation in buffer on a rotating mixer for 24h,
none of the fully prepared microdevices showed any signs of rupture or damage that could cause dose dumping *in vivo*.

**Fig. 4.1** – a) Schematic for PDMS grid-gel microdevice with integrated bioinorganic membrane (with inset for c)). b) Size comparison of completed PDMS grid-bioinorganic gel membrane microdevices. c) Cross-sectional diagram showing triggered insulin release in a glucose-rich environment to form open ‘nano-pores’. (*Reproduced by permission of The Royal Society of Chemistry*)

**In vitro Glucose-Responsive Insulin Release from Microdevices**

Using three-cycle testing protocol, amount of insulin permeated vs. time was measured using a clinically relevant glucose concentration change in the buffer solution (5 to 20 mmol.L⁻¹). As seen in Fig. 4.2a, the slope of the curves (rate of insulin release) increased significantly at 2.5h when glucose concentration was increased from 5 mmol.L⁻¹ to 20 mmol.L⁻¹. All three cycles had a permeability ratio above 2 (P₂₀/P₅ > 2) (Fig. 4.2b and Table 4.1) showing consistent response to
glucose levels over multiple cycles. This cyclic response is important for in vivo applications under the diabetic condition, as meals can cause large shifts in glucose levels, to which the microdevice has shown a rapid, repeatable response. The amount of insulin released over 4 hours was determined to be $50 \pm 11\mu g$, which is clinically relevant as it equates to roughly 5 units per day, within the tolerance range for in vivo testing on rats. Based on this finding, only one PDMS grid-gel microdevice per rat was necessary for controlling hyperglycemia, compared with our previous studies that required the implantation of five tube-shaped devices to achieve efficacy $^{341,342}$. The change in normal to hyperglycemic release rates over the three cycles was not significant (p>0.05).

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**Fig. 4.2** – a) Three cycle testing of in vitro insulin permeability testing for PDMS grid-gel glucose-responsive microdevices over 5mmol.L$^{-1}$ (shaded area) and 20mmol.L$^{-1}$ (open area). Error bars represent standard deviation (n = 5). b) Three cycle in vitro insulin permeability of PDMS grid-gel microdevices. Error bars represent standard deviation (n = 5). (*Reproduced by permission of The Royal Society of Chemistry*)
<table>
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<tr>
<th>Cycle</th>
<th>$P_{20}$</th>
<th>$P_{5}$</th>
<th>$P_{20}/P_{5}$</th>
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</tr>
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<tr>
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<td>$1.13 \times 10^{-4}$ cm$^2$/s</td>
<td>$5.60 \times 10^{-5}$ cm$^2$/s</td>
<td>2.01</td>
</tr>
</tbody>
</table>

Table 4.1 - Three-Cycle *In vitro* Insulin Permeability Data for Glucose-Responsive Microdevices.

**Morphology and Integrity of PDMS Grid-Gel Membrane**

As seen in Fig. 4.3a, the bioinorganic membrane evenly covered the grid holes. Examination of membrane surface showed no gaps in grid-gel membrane, indicating complete crosslinking of bioinorganic gel membrane to surface-modified PDMS grid. This confirms that the grid-gel membrane interface between the reservoir and the external environment was fully sealed, avoiding issues such as dose dumping. This integrated grid-gel membrane serves as a scaffold for the PDMS reservoir, providing the membrane with strong physical support. Also, this allows for a surface area approximately 4 times larger than previous tube-shaped devices (8 mm$^2$ vs 2 mm$^2$), allowing a higher rate of insulin delivery per device. During incubation in PBS pH 7.4, none of the grid holes showed gel membrane damage after agitation in a scintillation vial for 24 h on a rotating stirrer.

Membrane samples were taken from grid-gel microdevices retrieved at the end of experiments and treated with formalin. Comparing the ESEM image of control bioinorganic microdevice surface (Fig. 4.3b) with the insulin-loaded bioinorganic microdevice surface (Fig. 4.3c) after 5 days, there was little change in structure and morphology. Little cellular adhesion and
accumulation were seen on the implanted insulin-filled device. However, on the membrane of the microdevice loaded with saline after 5 day implantation, there was heavy buildup of cells, likely resulting from immune response, and a completely different morphology was observed (Fig. 4.3d). The porous structure of the membrane seemed to have become collapsed or covered with cells.

**Fig. 4.3** – a) ESEM image of PDMS grid hole crosslinked with bioinorganic membrane (Magnification x160). Grid holes were on average 420µm wide. ESEM analysis of bioinorganic membrane surface after *in vivo* testing. Comparison of control (day 0) (b), implantation with insulin (day 5) (c) and implantation with saline (day 5) (d) (Magnification x200). *(Reproduced by permission of The Royal Society of Chemistry)*
Implanted Microdevice Controlled Hyperglycemia and Provided Sustained Release of Insulin

Fig. 4.4a shows that implanted insulin delivery microdevices can maintain glucose levels in diabetic rats at normoglycemia for at least 7 days, while in the control rats with sham devices, glucose concentrations reached above 20mmol.L$^{-1}$. In the insulin microdevice-treated group, the blood glucose concentration dropped dramatically after implantation. The blood glucose levels in the microdevice-treated group gradually increased after day 10, possibly due to the depletion of reservoir insulin or insulin inactivation over time. The insulin microdevice-treated rats were healthy with no mortality seen in any of the subjects.

Insulin profiles for the treated group and control group are presented in Fig. 4.4b. The insulin levels in the control group were very low as expected, much lower than normal physiological levels due to destruction of the pancreatic beta cells. Insulin microdevice-treated rats showed a rapid increase in insulin levels after implantation, with the insulin relatively stable over ten days. Insulin levels at day 10 were not significantly different from initial implantation at day 0 (p > 0.05). The plasma insulin levels in the microdevice implanted rats were high; however, the rats did not show associated hypoglycemic conditions, which suggests a possibility of compensation for reduced insulin bioactivity or insulin insensitivity (Fig. 4.4b).
Fig. 4.4 – a) Long-term plasma blood glucose measurements in STZ-Diabetic rats. Shaded area indicates normoglycemic range. Implantation of microdevices occurred at day 2. Error bars represent standard error (n=5). b) Long-term plasma insulin measurements in STZ-diabetic rats. Implantation of microdevices occurred at day 2. Error bars represent standard error (n=5).

(Reproduced by permission of The Royal Society of Chemistry)

**Implanted Insulin Microdevice Response to Glucose Challenge**

To examine if the glycemia-control effect of the insulin microdevices was due to sustained insulin release or glucose-responsive insulin release, a short-term glucose challenge test was conducted on diabetic rats with implanted insulin microdevices. Fig. 4.5a shows that after the injection of a 1g.kg\(^{-1}\) bolus of glucose, the blood glucose peaks at 2 min followed by a drastic decrease. Over the next 30 minutes, blood glucose returned to normal baseline levels (Fig. 4.5a). Fig. 4.5b shows that insulin release was regulated by glucose concentration. After glucose challenge, plasma insulin concentrations increased immediately and significantly by 300pM at 10 min. Once blood glucose returned to normal, insulin concentrations leveled off at 30 minutes and onwards (Fig. 4.5b).
Absolute C-peptide, an indicator of endogenous insulin production, was assayed to investigate if the insulin was from the microdevices or from the endogenous source. Fig. 4.5c shows no change in C-peptide concentration with glucose level, indicating that the rise in insulin level was not from residual β-cell activity. These results demonstrate the capability of the microdevices to release insulin in response to glucose levels. Furthermore, these results agree with the previous findings with tube-shaped devices \(^{341,342}\), demonstrating that the implantable microdevice can provide glucose-responsive insulin release on demand.

**Fig. 4.5** – a) Plasma blood glucose in STZ-diabetic rats with implanted microdevices after glucose challenge at t=0. Error bars represent standard deviation (n=3) b) Change in plasma insulin measurements in STZ-diabetic rats with implanted microdevices after glucose challenge at t=0. Error bars represent standard deviation (n=3). c) Plasma C-peptide measurements in STZ-diabetic rats with implanted microdevices after glucose challenge at t=0. Error bars represent standard deviation (n=3). Dotted lines represent normal physiological levels of C-peptide. *(Reproduced by permission of The Royal Society of Chemistry)*
Stability of Insulin Retrieved from Ex Vivo Microdevices

Remaining insulin retrieved from the microdevice reservoirs after in vivo implantation was examined by RP-HPLC for studying structural integrity and formulation stability. The chromatograms of insulin retrieved one day after the 14 day implantation was compared with fresh human recombinant insulin and intraperitoneal (IP) fluid (Supplemental Fig. 4.7). The insulin peak at 8.7 min elution time was still present in the retrieved samples, suggesting insulin did not degrade into denatured products, because aggregated insulin samples usually produce a much wider, erratic peak at a broad range of retention times, as reported in literature. However, a second peak was found in the retrieved insulin samples, which seems to match with the peak from the IP fluid samples. It is possible that some protein in the IP fluid infiltrated the membrane and mixed with the insulin in the microdevice reservoir. The in vivo data (Fig. 4.4b) showed much higher insulin levels than normal physiological levels in normal rats, suggesting that insulin bioactivity was possibly compromised in part. Although the high insulin levels did not adversely affect the rat survival rates or glucose profiles, this phenomenon needs to be better understood for improving insulin formulations in our future studies.

The goal for implantable microdevices is to provide a self-regulated approach to insulin therapy. The current proof-of-concept system was designed to allow potential future refilling for long-term efficacy, utilizing the resealing property of the PDMS material. The vicinity of the sensor (glucose oxidase) to the release mechanism (hydrogel nanoparticles) is necessary for the rapid response and insulin release for our microdevice. As well, a backup external glucose sensor can be utilized with this system for patients to monitor blood glucose levels.
One concern for this microdevice to translate into human testing versus a rat model is the variability of insulin dosing. The Sprague-Dawley rat model is a controlled group of identical species and similar body weights. Human dosing schedule is based upon insulin sensitivity, glucose metabolism and weight (measured via unit/kg), and must be finely tuned before a safe and efficacious implantable microdevice can be achieved. Furthermore, insulin dosing must be controlled with respect to carbohydrate levels in meals. The results reported in this paper demonstrate our recent progress in the controlled STZ-diabetic rat model. Devices will undergo critical optimization before clinical trials can be considered.

4.5 Conclusions

This paper presented new PDMS grid-gel integrated glucose-responsive microdevices and \textit{in vitro} and \textit{in vivo} testing results. Glucose-responsiveness was realized through a glucose-triggered acidic byproduct causing a reversible volumetric shrink/swell dynamic of embedded nanohydrogels and alteration of membrane permeability, without the use of electronic input. Device performance was maintained over multiple cycles \textit{in vitro}, with insulin released at clinically relevant glucose levels. \textit{In vivo} efficacy of the microdevices for hyperglycemia control was maintained for a period of 7 days. Under glucose challenge, these microdevices responded with an acute increase in insulin delivery within minutes, attenuating blood glucose levels. Like all implantable devices, avoiding complications of surgical administration is not possible. However, these proof-of-concept microdevices demonstrated their feasibility for use in studying diabetes in animal models.
4.6 Supplemental Content

Fig. 4.6 – Fabrication process of PDMS grid from casting on SU-8 pillars from mold master.

Fig. 4.7 – RP-HPLC chromatogram of soluble insulin from retrieved microdevices after 15 days of *in vivo* implantation compared with fresh human recombinant insulin and intraperitoneal (IP) fluid with endogenous proteins.
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Chapter 5 \textit{In vivo} Performance and Biocompatibility of a Subcutaneous Implant for Real-Time Glucose-Responsive Insulin Delivery

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Submitted to Diabetes Technology and Therapeutics (2014).

Designed and tested surface-modified insulin microdevices. Completed \textit{in vitro} testing of glucose-responsive insulin release and performed animal surgery for \textit{in vivo} efficacy studies.
5.1 Abstract

An implantable, glucose-responsive insulin delivery microdevice was reported previously by our group, providing rapid insulin release in response to hyperglycemic events and efficacy in vivo over one week when implanted intraperitoneally (i.p.) in diabetic rats. Herein, we focused on the improvement of the microdevice prototype for long-term glycemic control by subcutaneous (s.c.) implantation which allows for easy retrieval and replacement as needed. To surmount the strong immune response to the s.c. implant system, the microdevice was treated by surface modification with high molecular weight polyethylene glycol (PEG). In vitro glucose-responsive insulin release, in vivo efficacy and biocompatibility of the microdevice were studied. Modification with 20 kDa PEG chains greatly reduced immune response without a significant change in glucose-responsive insulin release in vitro. The fibrous capsule thickness was reduced from ~1000 µm for the untreated devices to 30 – 300 µm for 2 kDa PEG-treated and to 30-50 µm for 20 kDa PEG-treated devices after 30 day implantation. The integrity of the glucose responsive bioinorganic membrane and resistance to acute and chronic immune response were improved with the long-chain 20 kDa PEG brush layer. The 20 kDa PEG-treated microdevice provided long-term maintenance of euglycemia in a diabetic rat model for up to 18 days. Moreover, a consistent rapid response to short-term glucose challenge was demonstrated in multiple-day tests for the first time on diabetic rats with the implanted devices. The improvement of the microdevice is a promising step towards a long-acting insulin implant system for a true, closed-loop treatment of diabetes.

Keywords: Closed-loop insulin delivery, microdevice, subcutaneous implant, insulin formulation, glucose challenge test, biocompatibility, in vivo glycemia control
5.2 Introduction

Diabetes mellitus is a chronic metabolic disease that is characterized by the inability of the body to control blood glucose. Diabetes affected 366 million people worldwide in 2011 and projected to 552 million in 2030, with numbers increasing from the prevalence of obesity due to poor diet and lifestyle\textsuperscript{361,362}. While oral therapies are available for early stage treatment of Type 2 diabetes, which is caused by insulin resistance, at later stages of the disease patients will eventually become insulin dependent, resulting in approximately 20\% of Type 2 patients requiring insulin therapy\textsuperscript{363}. All Type 1 diabetes patients critically depend on exogenous insulin due to autoimmune destruction of insulin-producing beta cells in the pancreas.

The goal of insulin therapy is to provide insulin at defined time points to achieve euglycemia and minimize periods of hyperglycemia. Currently, the established ways of insulin administration are limited to use of syringes, pens or sensor-augmented pumps to provide compensatory insulin based on individual patient requirements. These therapeutic regimes may lead to periods of hyper- or hypoglycemia due to variability in meal schedule, metabolism, exercise, inaccurate caloric evaluation, poor compliance, or specific insulin resistance of patients, compromising patient quality of life\textsuperscript{364-366}. To minimize the incidence of hypoglycemia, sensor augmented insulin pump therapy has been developed. This approach utilizes the signal transmitted from a continuous glucose monitor (CGM) to an insulin pump to suspend insulin infusion at a preset low glucose level (e.g. 60 mg.dL\textsuperscript{-1}). Thereafter, more advanced closed-loop insulin delivery systems using controlled algorithms with combination of CGMs and insulin pumps have been investigated in multicenter clinical trials. These systems have shown promising results towards the goal to control glucose levels within the target range. A lower combined rate of severe and moderate hypoglycemia in type 1 diabetes patients has been reported\textsuperscript{367-373}. Nevertheless, this
therapeutic approach requires substantial patient education and resources to achieve these goals, compared with standard injectable regimens.

An ideal insulin delivery system should provide sustained basal insulin at low glycemic levels, along with responsive, pulsatile release during periods of high glucose levels. In addition, a ‘smart’, on-demand delivery system that can provide a ‘hands-off’ means to minimize hyper- and hypoglycemic episodes without patient intervention would improve quality of life and clinical outcomes. Up to date, the existing technologies based on integration of electronic (CGMs) and mechanical parts (pumps) and algorithms are still far from achieving this goal. In addition, the overall cost of the electromechanical devices and accessories could limit the number of uninsured patients who can afford the therapy or even have access to it at all 375-377.

Other systems, such as oral and intranasal formulations, transdermal patches and implant pumps have been explored as alternative means for insulin delivery, yet there remain concerns about clinical efficacy, low bioavailability due to limited penetration, absorption, or enzymatic degradation before reaching the site of action 378-387. Furthermore, most of these systems can only provide patient-mediated, open-loop insulin delivery.

Sustained insulin implant systems have shown promise through the use of polymeric materials and microdevices with long periods of efficacy, which can provide basal insulin 388-390. However, they must be used in conjunction with fast-acting insulin injections. Still, rapid, pulsatile release in response to glycemic challenge cannot be achieved without strict patient intervention and compliance. To this end, chemically driven closed-loop insulin systems utilizing stimulus-responsive polymer hydrogels could overcome the aforementioned limitations. They do not require complex electronic and mechanical parts, reducing the cost and complexity of potential implant systems, while providing real-time glucose monitoring and insulin delivery as needed. In
these ‘closed-loop’ insulin therapy systems, seamlessly integrating the glucose sensing component with the insulin delivery mechanism is paramount.

Responsive polymer hydrogels have been studied extensively to provide cyclic, pulsatile insulin delivery. Specifically, pH-responsive hydrogel moieties integrated with nano- or microparticles, film, or bulk hydrogels have been used to create a reversible, modulated insulin release system. From delivery systems, insulin release is controlled by the ionization of the anionic or cationic pH-responsive polymer moieties which causes shrinking or swelling of the hydrogels depending on the environmental pH and pKa of the functional groups. Glucose-specific enzymes for detection of glucose, e.g. glucose oxidase, have been well-studied in glucose-responsive insulin delivery systems. Glucose oxidase converts environmental glucose into gluconic acid, creating an acidic pH trigger to elicit a response from pH-sensitive polymeric materials. As a result, the glucose-responsive systems undergo cyclic response to glucose levels, providing faster or slower insulin release through polymer membranes, microspheres or bulk hydrogels via a diffusion controlled mechanism.

Based on a bioinorganic nanocomposite membrane, our group has developed implantable microdevices that deliver insulin at physiological and clinical glucose levels. Rapid, pulsatile insulin delivery and in vivo efficacy for 5 to 10 days were obtained with these devices when implanted i.p. Within these devices, a glucose-sensing, enzyme-based bioinorganic membrane is integrated with an insulin reservoir made of a silicone tube. Insulin is released from a reservoir through the porous, hydrophilic membrane that modulates permeability through rapid-acting pH-responsive nanoparticles in response to the environmental glucose concentration. The increase in glucose concentration results in a faster reaction catalyzed by glucose oxidase, producing more gluconic acid. This acidic product causes the shrinkage of
poly(N-isopropylacrylamide-co-methacrylic acid) (PNIPAM-co-MAA) hydrogel nanoparticles embedded in the bioinorganic, albumin-based membrane. The porosity across the membrane is thus increased due to vacated ‘nano-pores’ created by the collapse of nanoparticles which leads to an increase in insulin diffusion (Fig. 5.1). Owing to rapid response of pH-sensitive nanohydrogels, the device enabled rapid attenuation of plasma glucose levels \textit{in vivo} after a glucose bolus injection, mimicking a physiological response to the post-meal hyperglycemia in a healthy subject\textsuperscript{404}.

For long-term application and patient safety, easy retrieval and replacement/refill of the implants are important. To this end, s.c. implantation is preferred over i.p. implantation. Moreover, the stability and longevity of the device, in particular the glucose-responsive membrane are crucial. Our preliminary study suggested that the previous version of the device was susceptible to heavy fibrous capsule formation when implanted either i.p. or s.c\textsuperscript{342}. Degradation of the bioinorganic membrane also occurred after two weeks, due to cellular adhesion and immune response from destructive cell-mediated oxidization, which compromised long-term application of the microdevice.
Fig. 5.1 – Schematic for glucose-responsive insulin microdevices. PDMS (silicone) tubing microdevice with bioinorganic plug and cross-section of device with embedded hydrogel nanoparticles (top). Cross-sectional diagram of membrane response to glucose at high glucose concentrations (bottom).

Therefore, this work was aimed to optimize the microdevice for long-term application. We improved the system design for s.c. implantation, modified the surface of the microdevices to reduce immune response from the host to the implants, and investigated the effect of polyethylene glycol (PEG) chain length on the inflammation and foreign body response in diabetic rats.

5.3 Materials and Methods

*Materials:* All chemicals were analytical grade and used without further purification. Bovine serum albumin (99%), catalase (solution 13 mg.mL\(^{-1}\)), 3-aminopropyltrimethoxysilane (97%),
glutaraldehyde (25%, grade I), poly(ethylene glycol) methyl ether (\(M_w\) 2 000 and 20 000), 4-nitrophenyl chloroformate (NPC) and tri-pentylamine (TPA) were purchased from Sigma-Aldrich. Glucose oxidase (230 U.mg\(^{-1}\)) was purchased from Calzyme (USA). Recombinant human insulin (r-DNA origin, 27 U.mg\(^{-1}\)) was purchased from Wisent (Canada). Pluronic F-68 and F-127 were provided by BASF Corporation (Germany). HEPPS (4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid) was from MP Biomedicals (USA). Silicone tubing (Nalgene 50 silicone tubing, ID = 1.6 mm, OD = 3.2 mm, wall = 0.8 mm) was purchased by Nalgene (USA). Ethylene-vinyl acetate copolymer resin (EVAC, ELVAX-40W) was provided by E. I. Du Pont. Poly(N-Isopropyl acrylamide-co-methacrylic acid) (NIPAM/MAA) nanoparticles (200 ± 110 nm in pH 7.4 phosphate buffered saline (PBS) and 60 ± 50 nm in pH 5.0 PBS, volume distribution) and powder nano-MnO\(_2\) (80 ± 30 nm) were prepared as previously described by our group without further modifications\(^{403,404}\). Distilled and deionized (DDI) water were obtained from a Milli-Q water purifier (Millipore Inc.). PBS pH 5.0 or pH 7.4 was prepared with 0.01 M phosphate and 0.15 M NaCl in DDI water.

**Activation of PEG chains:** mPEG with 2 kDa or 20 kDa was activated with NPC as described elsewhere 403. Briefly, NPC and TPA were mixed in the molar ratio 1:5:5 (PEG: TPA: NPC). mPEG (2 g) was dissolved with toluene (20 mL) under nitrogen atmosphere. The temperature of the resulting solution was increased to 60°C and 0.15 mL of TPA was added followed by 100 mg of NPC. After 24 hrs under stirring, the temperature was reduced to 35°C and 20 mL of methyl-t-butyl ether was added and solution was allowed to stir for 5 min. The reaction flask was then placed in an ice bath and the resulting thick slurry was stirred for another 30 min. The obtained solid was isolated by centrifugation (3000 rpm), dried under vacuum and re-dissolved with methanol (10 mL) at 40°C. The methanol solution was added dropwise in ice cold isopropanol.
(30 mL) under stirring, and the white solid obtained was collected by centrifugation (3000 rpm). The washing step was repeated two more times to remove unreacted molecules, and finally dried under vacuum for 24 hrs at room temperature. The activation of mPEG with NPC was confirmed by using proton 400 MHz $^1$H NMR spectrometer (Varian Mercury 400, Varian Inc.) 405.

**Preparation of microdevices:** Glucose-responsive bioinorganic microdevices were prepared as previously described 341,342. Briefly, medical grade silicone tubing (length = 3 cm) was surface treated with oxygen plasma and silanized with 3-aminopropyl trimethoxysilane (0.1 M in ethanol). Silanized devices were crosslinked with a mixture of bovine serum albumin (51 wt.%) glucose oxidase (5.5 wt.%), catalase (1.56 wt.%), MnO$_2$ NPs (11 wt.%) and PNIPAM/MAA NPs (30 wt.%) to create a bioinorganic membrane at one end of the tubing. Glutaraldehyde was used as a crosslinker (0.08:1 mol crosslinker to protein ratio). The other end of the tubing was sealed with EVAC solution (18% w/v in dichloromethane). Surface PEGylation of microdevices was achieved by soaking in 0.1M activated PEG solution in PBS pH 7.4 for 24h. Microdevices were washed several times with DDW water. Buffered insulin solution (human insulin 50 mg.mL$^{-1}$, HEPPS 50 mM, Pluronic F68 0.02M, pH ~ 8) was utilized to fill the devices prior *in vitro* and *in vivo* studies ($\approx$ 50 $\mu$L, 2.5 mg or 67.5 units insulin per device). For this, a thin needle (27 1/2 G) was utilized and air bubbles were prevented by piercing a second needle in the opposite side of the device during the filling step. Devices were stored in pH 7.4 PBS at 4°C prior to use.

**In vitro test of glucose-responsiveness of the device:** Microdevices were placed in glass vials containing pH 7.4 PBS (2 mL) and glucose (100 mg.dL$^{-1}$) as release medium, and placed on rotary mixer at 37°C for the duration of the experiment. Insulin release was determined by monitoring insulin absorbance manually with a UV/VIS spectrophotometer (Lambda 25 UV/VIS spectrometer, Perkin Elmer, USA) every 30 min at $\lambda = 276$ nm. Glucose concentration was
increased from normal (100 mg.dL⁻¹, 0-2 h) to hyperglycemic levels (400 mg.dL⁻¹, 2-4 h) halfway through the duration of the experiment.

**Implantation of insulin delivery device in rats:** All *in vivo* procedures strictly complied with the ethical and legal requirements under Ontario’s Animals for Research Act and the Federal Canadian Council on Animal Care guidelines for the care and use of laboratory animals and were approved by the University Animal Care Committee of the University of Toronto.

**Animals:** Male Sprague Dawley rats (250-300g) were used and induced diabetic by streptozotocin (STZ) i.p. injection (65 mg·kg⁻¹ in sterile saline). Animals were allowed three days of resting to confirm the diabetic state. For all experiments rats were kept in a 12/12 reverse light cycle state to obtain blood glucose and insulin measurements in the fed state and were fed chow and water.

**Biocompatibility and in vivo stability of the microdevice:** For the experiment devices were prepared with different surface treatments (no PEG, 2 kDa PEG and 20 kDa PEG). STZ-rats were randomized into 3 groups (n = 3 per group) and implanted with silanized (no PEG) or PEGylated (2 and 20 kDa) insulin devices subcutaneously in the interscapular tissue of the abdomen. Each animal served as its own control, and was implanted with the three different surface-treated devices, each one in a separate s.c. pocket. After a 15 or 30 day implantation period, animals were sacrificed and the devices with surrounding tissue were carefully explanted. Retrieved devices encapsulated with new-formed surrounding tissue were fixed in 10% buffered formalin and embedded in paraffin for histological analysis. Cross-sectioned slices were stained with hematoxylin and eosin (H&E), MAC2 and Mason’s Trichrome to identify and quantify immune cells, macrophage recruitment and collagen capsule thickness, respectively. To
determine membrane degradation, explanted devices were fixed with buffered formalin and surface morphology was analyzed by environmental scanning electron microscopy (eSEM, Hitachi S3400 microscope, Japan, 15 kV). Wet devices were directly fixed onto a cold stage sample holder with double-sided carbon tape, and frozen at - 24 °C under 90 Pa. Tissue preparation and histology analysis were performed by the CMHD Pathology Core Laboratory at Mount Sinai Hospital, Toronto, Canada.

**In vivo glucose-responsiveness of the device - Glucose challenge test:** Long chain (20 kDa) PEG-treated microdevices were implanted in STZ-rats as described above (3 devices per animal, n = 5) and allowed to rest for 72 hrs to confirm the decrease of blood glucose (BG) to normal levels by the insulin release from the implant. One hour prior to the experiment, food was removed from cages to prevent confounding glycemic overlap. Rats were given an i.p. injection of glucose (1g.kg⁻¹, 50% dextrose), and their BG levels were continuously monitored during 90 min using a One Touch glucometer (LifeScan, Inc., Johnson & Johnson) with blood samples taken from the tail vein. At the end of the experiment food was returned to the cages, and animals were allowed to rest for 24 hrs. The experiment was repeated with the same group of animals for three consecutive days. Non-diabetic healthy animals were used as controls for comparison (n = 3).

**In vivo long-term efficacy of the microdevice:** STZ-rats were implanted with 20 kDa PEG microdevices as described above (3 devices per animal, n = 5). Blood samples were collected daily from the tail vein to determine fed BG and plasma insulin levels. After 21 days, microdevices were surgically retrieved and animals were maintained for further post-implantation BG measurements. Collected blood samples were assayed to determine insulin levels using an antibody radioimmunoassay insulin kit (Linco Research Inc., USA (the assay kit
was used according to manufacturer’s instructions). Diabetic rats without implants were used as controls (n = 3).

**Statistical Analysis:** Student’s t-test or ANOVA followed by Tukey t-test (OriginPro8©) were utilized to determine statistical significance between two or more groups, respectively. A p value < 0.05 was considered to be statistically significant.

### 5.4 Results

**Effect of PEG chain length on the glucose-responsive insulin release of the microdevice**

PEGylation of the device surface was performed through amide bond formation between carboxylate groups of NPC-activated PEG and amine groups in the device surfaces from both protein molecules in the membrane and aminopropyl groups on the silanized device surfaces. PEG chains of different molecular weights (mPEG MW 2 kDa, 20 kDa) were conjugated onto the membrane surface. The effect of PEG chain length on the device performance in vitro and in vivo was investigated to ensure that the chemical treatment would not compromise the ability of the membrane to respond to glucose changes in the environment.

First, the *in vitro* glucose-responsiveness of the devices prepared with 2 kDa or 20 kDa PEG was examined by monitoring the *in vitro* release of insulin as a function of time and glucose levels, with comparison to control devices without surface modification (no PEG). It is seen in Figs. 5.2a and 5.2b, when the glucose concentration in the release medium was changed from clinically relevant ranges of 100 mg.dL\(^{-1}\) (normal) to 400 mg.dL\(^{-1}\) (hyperglycemia), the ratios of insulin release at hyperglycemia versus normal glucose were all above two-fold (R\(_{400}/R_{100}\)) (Table 5.1). As well, the change in insulin release occurred rapidly, with the insulin release rates increasing almost immediately after glucose level was increased at 2 hours, in all three groups.
Release rate in high glucose conditions is the highest initially from 2 to 3 hours, with a slight decrease from 3 to 4 hours, but the net insulin release is still significantly greater than that at the normal blood glucose level, reflecting the device response to glucose levels. This result is comparable to previous observations of the insulin microdevice, with a similar ratio of change in insulin release rate. Moreover, the microdevice modifications with PEG surface grafting did not show a significant difference in insulin release profiles. Insulin formulation remained stable within the devices with no observable fibrillation or aggregation, attributable to the treatment with PEG that increases the surface hydrophilicity of the reservoir.
Fig 5.2 - (a) Insulin release over normal and hyperglycemic glucose concentration for microdevices prepared without PEG treatment (no PEG, ■) and with PEG treatment (2 kDa PEG, ● and 20 kDa PEG, ▲). Glucose concentration changed from 100 mg.dL\(^{-1}\) to 400 mg.dL\(^{-1}\) at 2h. Solid lines indicate trendlines of release rate slopes. (b) Rate of insulin release (mg.h\(^{-1}\)) as a function of glucose concentration, calculated from the slopes of the curves. Error bars represent standard deviation (n=5). Difference between normal to hyperglycemic insulin release rate was statistically significant (p < 0.05).
Table 5.1 – Release rate of insulin from no PEG, 2 kDa PEG and 20 kDa PEG microdevices at 100 mg.dL⁻¹ glucose (R₁₀₀) and 400 mg.dL⁻¹ glucose (R₄₀₀). Release ratio calculated by R₄₀₀/R₁₀₀.

<table>
<thead>
<tr>
<th></th>
<th>No PEG</th>
<th>2 kDa PEG</th>
<th>20 kDa PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>R₁₀₀ (mg.h⁻¹)</td>
<td>0.29</td>
<td>0.22</td>
<td>0.31</td>
</tr>
<tr>
<td>R₄₀₀ (mg.h⁻¹)</td>
<td>0.69</td>
<td>0.8</td>
<td>0.71</td>
</tr>
<tr>
<td>R₄₀₀/R₁₀₀</td>
<td>2.37</td>
<td>3.64</td>
<td>2.29</td>
</tr>
</tbody>
</table>

Effect of PEG chain length on biocompatibility of the microdevice

To study whether PEGylation and PEG chain length would influence biocompatibility of the device, such as cell adhesion and immune response, rats were subcutaneously implanted in the abdomen with microdevices treated with no PEG, with 2 kDa or 20 kDa PEG conjugation. After implantation for 15 or 30 days, the devices were explanted. Histological analysis of surrounding tissue around the microdevices showed apparent differences in the thickness of fibrous capsules, immune response and cell types (Fig. 5.3 and Table 5.2). A reduction of inflammation and fibrosis (tissue encapsulation of the device) was observed in the order 20 kDa PEG > 2 kDa PEG > no PEG.

Capsule thickness was greatest for animals implanted with no PEG microdevices, up to 1000 µm after 15 days implantation (Fig. 5.3a) and 100 – 500 µm after 30 days implantation (Fig. 5.3b). In contrast, PEGylated devices showed thinner fibrous capsule, lower inflammation and little to no immune reaction. Devices treated with 2 kDa PEG showed a thinner fibrous capsule with presence of wavy, mature collagen matrix, 100 – 350 µm (15 days, Fig. 5.3c) and up to 500 µm (30 days, Fig. 5.3d). Capsule thickness was further reduced for devices treated with 20 kDa PEG (<100 µm) at both timepoints, and much more uniform across the entire cross-section (Fig. 5.3e, 5.3f).
After 15 days implantation, heavy inflammation was seen near the internal capsule walls surrounding the device with no PEG, characterized by dark purple staining from active neutrophil recruitment (Fig. 5.4a). Large plasma cells and immature fibroblasts were characteristic of localized and continuous inflammation as well. Inflammation was heavily pronounced after 30 days implantation and numerous eosinophils and vasculature were also present. The heavy recruitment of eosinophils indicates a strong allergy-mediated reaction (immune response) caused by the implant (Fig. 5.4b). For the devices with 2 kDa PEG, the capsule walls under H&E staining showed some darkly stained, inflammatory-mediated neutrophil recruitment closest to internal lumen (Fig. 5.4c), but the presence of eosinophil and vasculature was markedly reduced (Fig. 5.4d), indicating lower allergic response, as compared to no PEG devices (Fig. 5.4b).

With 20 kDa PEG modification, the internal neutrophil recruitment within the capsules was minimal and presence of eosinophils was insignificant (Fig 4e,f) compared with the samples surrounding the devices with no PEG or 2 kDa PEG, suggesting very low or absent inflammation and allergic response. All three groups showed presence of plump plasma cells, an indicator of chronic immune response to foreign material, that however are not highly active in cell recruitment and inflammatory response. These results are summarized in Table 5.2.
**Fig. 5.3** – Transverse cross-sectional comparison of PEG treatments to fibrous capsule thickness around devices under MTS and H&E staining. Thickness indicated by double ended arrows across blue MTS stained capsule. Asterisk indicates location of microdevice within capsule.
Fig. 5.4 – Transverse cross-sectional comparison of PEG treatment to inflammatory response and immune cell recruitment in fibrous capsule under H&E staining. Boxes on left column show capsule inflammation indicated by H&E staining intensity. Boxes inset on right column indicate plasma cells (dark stained) and/or eosinophils (dark stained with red halo) dispersed in fibrous capsule. Asterisk indicates location of microdevice within capsule.
### Surface Treatment

<table>
<thead>
<tr>
<th>Implantation period</th>
<th>Diagnosis</th>
<th>No PEG</th>
<th>2kDa PEG</th>
<th>20kDa PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Capsule thickness range</td>
<td>1000 µm</td>
<td>100 – 300 µm</td>
<td>50 – 150 µm</td>
</tr>
<tr>
<td>15 days</td>
<td>Capsule composition</td>
<td>Mononuclear inflammatory cells (mainly macrophages)</td>
<td>Mature fibrosis with some infiltration of macrophages, lymphocytes and plasma cells.</td>
<td>Thin layer of fibrosis and fibroplasia with fewer macrophages and lymphocytes.</td>
</tr>
<tr>
<td></td>
<td>Inflammation</td>
<td>Extensive</td>
<td>Extensive</td>
<td>Mild</td>
</tr>
<tr>
<td></td>
<td>Immune response</td>
<td>Mild, occasional eosinophils</td>
<td>Low, occasional eosinophils</td>
<td>Low, occasional eosinophils</td>
</tr>
<tr>
<td></td>
<td>Capsule thickness range</td>
<td>100 – 500 µm</td>
<td>30 – 500 µm</td>
<td>30 – 50 µm</td>
</tr>
<tr>
<td>30 days</td>
<td>Capsule composition</td>
<td>Mature fibrosis with large infiltration of macrophages, lymphocytes and plasma cells.</td>
<td>Mature fibrosis with infiltration of macrophages, lymphocytes and plasma cells.</td>
<td>Mature fibrosis with fibrocytes embedded in abundant collagen.</td>
</tr>
<tr>
<td></td>
<td>Inflammation</td>
<td>Severe</td>
<td>Mild</td>
<td>Minimal</td>
</tr>
<tr>
<td></td>
<td>Immune response</td>
<td>Extensive, large infiltration of eosinophils in the fibrous capsule and surrounding tissue.</td>
<td>Low, minimal infiltration of eosinophils in the fibrous capsule.</td>
<td>Absent, extremely rare eosinophils.</td>
</tr>
</tbody>
</table>

**Table 5.2** - *In vivo* comparison of PEG surface treatments to immune parameters for insulin microdevices implanted subcutaneously in diabetic rat models.
**Effect of PEG chain length on the morphology and cell adhesion of bioinorganic membrane**

For long-term applications, minimal host response to the device and low degradation rates of glucose-responsive membrane are important. In order to evaluate the effect of the surface treatments on the *in vivo* degradation of the membrane, devices retrieved from animals after 15 or 30 days implantation were fixed with formalin and their surface morphology was analyzed by environmental scanning electron microscopy (eSEM).

Before implantation (Day 0), devices showed no differences in membrane morphology and integrity as expected between the three groups (Fig. 5.5a, 5.5d, 5.5g). After 15 days implantation, cell attachment was seen on the membrane with no PEG (Fig. 5.5b) while reduced cell attachment and agglomeration was present on the membrane with 2 kDa PEG (Fig. 5.5e). On the 20 kDa PEG-treated devices, negligible cell attachment was observed (Fig. 5.5h), an indication of good resistance to initial non-specific protein attachment and subsequent cascade response. In addition, cellular and protein adhesion on the silicone tubing was easily visible on the devices with no PEG, while little adhesion was observed for both PEGylated samples. After 30 days, complete central degradation of the membrane occurred in the devices with no PEG, with obvious damage of the permeable membrane and heavy cellular and tissue adhesion throughout the surface (Fig. 5.5c). Compared to the device with no PEG, the 2 kDa PEG-treated device showed less membrane degradation and cellular attachment. However, the 2 kDa PEG treatment did not completely prevent intense cell attachment on the membrane surface and the adjacent silicone tubing (Fig. 5.5f). Further reduction of cellular attachment was obtained when 20 kDa PEG was used. More importantly, the images revealed that the integrity of the membrane was not compromised even after 30 days implantation (Fig. 5.5i). Little to no cellular adhesion onto the
silicone surface adjacent to the membrane was observed, indicating a significant improvement of the biocompatibility of the device by addition of long chain PEG to the silanized silicone surface.

The eSEM results are in agreement with the observations by histological analysis, showing that the PEG treatment can greatly decrease cellular adhesion on the device surfaces in the order 20 kDa PEG > 2 kDa PEG > no PEG. Due to the highest biocompatibility and lowest degradation, with negligible change in glucose-responsiveness, the devices modified with 20 kDa PEG were further investigated for their in vivo efficacy.

![ESEM analysis of bioinorganic membrane surface of microdevice from no PEG, 2 kDa and 20 kDa PEG at day 0, 15, and 30. Highlighted area indicates presence of cellular attachment and buildup. Magnification of 50x.](image)

**Fig. 5.5** – ESEM analysis of bioinorganic membrane surface of microdevice from no PEG, 2 kDa and 20 kDa PEG at day 0, 15, and 30. Highlighted area indicates presence of cellular attachment and buildup. Magnification of 50x.
Long chain PEG-modified microdevices show multiple cycle glucose-responsiveness in vivo

An ideal closed-loop insulin device should be able to release more or less insulin in response to real time glucose changes in vivo, similarly to healthy beta cells. In previous work we have demonstrated in vivo efficacy of insulin microdevices implanted i.p. over 5 days and their effect on rapidly decreasing plasma glucose levels in response to a short-term glucose challenge \(^{404,405}\).

Herein, we wanted to investigate whether microdevices implanted subcutaneously would present similar in vivo glucose responsiveness and maintain this activity over several cycles. The short term in vivo performance of the devices was determined by monitoring blood glucose levels in animals with implanted devices over an acute time period immediately after glucose bolus injection, to simulate meal-time glucose challenge and glycemic response. The same experiment was performed simultaneously in healthy animals for comparison. Ideal glycemic response of insulin release should minimize hyperglycemic period and return glucose concentration to euglycemia rapidly. As shown in Fig. 5.6, the implanted insulin microdevices mimic the pancreatic beta cells in healthy rats and bring the glucose level to normal values within 30-60 minutes in three separate glucose challenge tests at different days, indicating consistent and repeatable performance of the devices.
Fig. 5.6 – Multiple cycle glucose challenge testing for healthy (■) and device-implanted STZ-diabetic rats (●). Rats were fed for 12 hours in between glucose challenge cycles and fasted one hour before glucose injection until the end of test. Error bars represent standard deviation (n=3).

**Long-chain PEGylated microdevices show long-term in vivo efficacy**

Finally, we wished to determine whether the optimization of the device would lead to prolonged efficacy for the control of blood glucose *in vivo*, as compared to our previous device prototypes. Long-term *in vivo* performance of the microdevice was determined by monitoring glycemia and plasma insulin in subcutaneously implanted diabetic rats over 28 days. As shown in Fig. 5.7a, diabetic rats showed very high glucose levels as expected (> 500 mg.dL\(^{-1}\)) throughout the duration of the experiment. In contrast, animals treated with implanted 20 kDa PEG-treated insulin microdevices modified with 20 kDa PEG showed an immediate decrease in blood glucose levels from hyperglycemia (> 500 mg.dL\(^{-1}\)) to normoglycemia (75 - 140 mg.dL\(^{-1}\)) within 24h. Normoglycemia was maintained over 18 days, without evident episodes of hypo- or hyperglycemia. After 18 days, the glucose level increased outside of normal range (~ 180 – 270 mg.dL\(^{-1}\)), however it was still significantly lower than levels observed in the control rats. Upon device removal from the implanted rats, blood glucose raised to hyperglycemic levels, confirming that the glycemic control was provided by the insulin microdevices. Analysis of
plasma insulin levels shows initial increase post-surgery, but steady levels throughout the duration of the experiment (Fig. 5.7b). A slight increase in plasma insulin levels observed after 15 days (Fig. 5.7b) seems to not cause hypoglycemia (Fig. 5.7a), which might suggest that more insulin is needed to maintain euglycemia due to body weight increase in the rats or partial deactivation of insulin. Upon removal of the insulin microdevice, insulin levels dropped to pre-implantation levels. This result suggests that no endogenous insulin was produced to exert effect on glycemic levels and that the detected circulating insulin was provided solely from the microdevices. All rats in the insulin microdevice treated group remained healthy throughout the duration of the experiment, with slight weight gain over time.
**Fig. 5.7** – *In vivo* performance of subcutaneously implanted insulin microdevices in STZ-diabetic rats. Microdevices were s.c. implanted at Day 0 and retrieved at day 21. Blood glucose (a) and insulin (b) were taken via test strip and blood sample assay, respectively. Error bars represent standard deviation (n=5).
5.5 Discussion

Long-chain PEG Surface modification extended in vivo efficacy and reduced host-foreign body response

The closed-loop insulin delivery microdevice was optimized for extended in vivo efficacy with reduced host-foreign body response to the implant. Previously, we have applied advanced surface modification techniques (e.g. silanization) to the microdevice prototype and covalently attached amino-reactive moieties on both the membrane and insulin reservoir surfaces. These advanced surface treatments improved safety, hydrophilicity and biocompatibility of the device for i.p. implantation in rats. Through this approach we could improve adherence of the membrane to the insulin reservoir and also avoid aggregation of insulin within the interior of the reservoir that may be induced by contact of the hydrophobic domains of insulin with hydrophobic surfaces. However, the previous prototype microdevice was still susceptible to biodegradation of the glucose-responsive permeable membrane in vivo due to tissue encapsulation and immune cell response towards the foreign implant. The characteristic porous morphology of the glucose-responsive membrane and its composition (~ 60 % crosslinked proteins) allow relatively free diffusion of substrates and makes the exposed hydrophilic membrane vulnerable to degradation in vivo due to cellular infiltration, compared with dense, hydrophobic materials, such as silicone. This is typical with crosslinked hydrophilic implant systems, which is attributed to an oxidative degradation mechanism. Also, the adherence of immune cells on the membrane surface may potentially cause nanopore obstruction and the failure of the microdevice to release insulin, compromising efficacy.
To solve this problem, herein we have attempted to further modify the device surfaces with improved polymeric coatings that resist protein absorption, based off of nonfouling poly(ethylene glycol) (PEG)-based surface treatments (PEGylation). Coupling with PEG has been proven to be the most protein-resistant surface modification and remains the standard for biocompatibility comparison\textsuperscript{412-414}. Through PEGylation of the microdevice we decreased the \textit{in vivo} degradation of the glucose-responsive membrane and at the same time improved the biocompatibility of the implant with minimal response to the host. PEG is known for masking the overall surface of implants by creating a hydrophilic, protein adsorption-resistant, nonfouling brush layer. This leads to less immune cell recognition of the foreign implant/biomaterial and improved short and long-term biocompatibility\textsuperscript{412-416}. More importantly, it is also known that PEG chain length strongly influences nonfouling properties through increased steric resistance to attachment proteins (Fig. 5.8)\textsuperscript{417,418}. To this purpose, we have attempted to increase PEG chain length to further disturb immune cell and protein attachment to improve biocompatibility.
Fig. 5.8 – Surface-modification schematic for PDMS (silicone) surfaces. Initial oxygen plasma treatment was followed by silanization and a comparison of three treatment profiles (No PEG, 2 kDa PEG and 20 kDa PEG). Steric resistance to immune cell and protein attachment after in vivo implantation is shown.

The findings showed that the PEGylation of the device leads to marked improvement of short and long-term implant biocompatibility. Moreover, the improved results of devices treated with 20 kDa PEG compared to 2kDa PEG defines the effect of PEG length to the reduction in both acute and chronic immune response. Negligible recruitment of eosinophils and reduced inflammation on 20 kDa treated microdevices confers a biofouling-resistant microdevice surface,
to a lesser extent with 2 kDa. These results agree with previous findings on the effect of PEGylation on implantable materials, which has shown that grafting of a PEG chain layer is an effective deterrent for initial adhesion of blood and immune proteins through dispersion, crosslinking, copolymerization or grafting. Attachment proteins show reduced adhesion loads when exposed to PEG-treated versus non-PEG treated surfaces, stopping initial cascade of immune response to foreign bodies. PEG grafting onto silicone surface has been shown to resist acute bioadhesion and prevent the initial attachment of blood proteins in vivo. The hydrophilic brush-layer shows resistance against attachment protein adhesion, specifically albumin, fibronectin and fibrinogen, presumably from a sterically-mediated mechanism\(^\text{414,416}\). Studies have also shown that extending PEG chain length and density can further enhance this adhesion resistance by increasing the thickness and coverage of the hydrated brush layer, improving ‘stealthing’ of the biomaterial\(^\text{413,414}\). This larger hydration shell of 20 kDa length PEG versus 2 kDa PEG retards both cellular and protein adhesion to the hydrophobic PDMS beneath and stops the initial cascade towards secondary immune cell recruitment. PEG-modified surfaces have shown improved biocompatibility of s.c. implant systems by significantly reducing monocyte and macrophage recruitment within acute time periods\(^\text{414,415}\), which coincides with our histology results. We were able to successfully graft long-chain 20 kDa PEG onto our insulin microdevice and significantly improve long-term biocompatibility with s.c. implantation. It is also important to note the close, inverse relationship between allergy-mediated eosinophil recruitment and PEGylation of the silicone surface, which is promising for potential reimplantation or refilling of our microdevices.
In vivo glucose-responsive performance is improved with 20 kDa PEG-treated devices

Multiple cycle glucose tolerance testing with device-implanted STZ-rats showed rapid glucose-responsive activity. The first cycle was performed after a 4-days implantation period (day 4) with subsequent cycles being performed at day 5 and day 6. As showed in the graphs, for all cycles, immediately after glucose injection, healthy animals showed an increasing sharp peak in BG above 180 mg.dL$^{-1}$ from 0 to 15 minutes and blood glucose rapidly returned to normal levels (80 – 140 mg·dL$^{-1}$) by 30 minutes. Immediate decrease in blood glucose is due to the rapid response of the pancreas, releasing insulin from islet cells in response to blood glucose peaks.

Microdevice implanted animals showed profiles comparable to healthy animals, with a similar return to normal glucose within a range of 30 – 45 min in all three cycles. These results showed the ability of the microdevice to function as a closed-loop system when implanted subcutaneously and its ability to act in real time in response to glucose challenge similarly to a healthy pancreas. The insulin release in response to blood glucose challenge occurs within a tight glycemic window, preventing animals from reaching values above 300 mg.dL$^{-1}$. Repeatability in all three glucose challenge cycles show device consistency, which has not been shown with our system previously. Despite significantly lower blood glucose from 30 to 90 min after the glucose challenge in two of the three days, hypoglycemia (< 65 mg.dL$^{-1}$) was not detected in the discontinuous process, suggesting that insulin was not released unspecifically from the highly concentrated depot, which is desirable for the sake of device safety. Moreover, an overshoot of insulin is not likely, as the device released insulin adequately dampening the hyperglycemic challenge without reaching post-challenge hypoglycemic levels. Such a property of glucose-regulated insulin release is very important, as overtreatment of hyperglycemia with excess
exogenous insulin can lead to symptoms of hypoglycemic unawareness, which is exacerbated with repeated hypoglycemic episodes$^{364,365}$. 

As Type 1 diabetic patients do not have proper glucagon control, often with glucagon oversecretion, hypoglycemia is a major secondary danger that can arise from improperly managed insulin treatment. Chronic hypoglycemic exposure can lead to deleterious effects on CNS, vascular and renal systems$^{361,362,363}$. From the cyclic nature of the nanoparticles, low-glucose states induce low basal release from implanted microdevices as expected, and this prevents excess insulin influx and potential hypoglycemic spike. This provides dual attenuation against extreme glycemic states, without the use of glucagon. Not only are primary hyperglycemic conditions minimized, as expected, but hypoglycemic episodes as well. There is an increase in plasma insulin levels at later time points past 15 days, but they do not correlate with a subsequent reduction in blood glucose. There might exist inactivated insulin in the microdevice from long-term implantation, however, it cannot be distinguished from the active form by the radioimmunoassay. Circulating insulin may be contributed from both active and inactive insulin, which could not sufficiently regulate the blood glucose levels that start to steadily increase in later times. Further tests will be needed to analyze insulin in explanted devices with respect to insulin stability and bioactivity.

Long-term in vivo performance was also improved with the optimized 20 kDa PEG-treated s.c. implant system. Compared to our previous results, the treatment of the device with long-chain 20 kDa PEG extended the efficacy of the implant from 5 to 18 days, over a 3-fold increase, with minimal hyper- and hypoglycemic episodes. Removal of implanted devices induced a rapid return to hyperglycemia, showing the paramount importance of the s.c. insulin implant for glycemic control. Furthermore, the s.c. implantation was achieved through a minor surgical
procedure, which is much less invasive than i.p. implantation. This implantation method also allows for simple removal and potential reimplantation of the device for future extended studies.

5.6 Conclusions

We have improved the glucose-responsive insulin delivery microdevice and achieved 18 day in vivo efficacy with s.c. implantation. A desirable glycemic profile with minimal hyper- or hypoglycemic episode was obtained. The long chain PEG treatment improved biocompatibility of the device significantly with minimal tissue encapsulation, inflammation and immune responses. Glucose tolerance testing on diabetic rats with implanted insulin microdevices over three cycles on different days showed rapid response to glucose challenge and effective glycemia-regulating capability of the implanted devices, similar to that observed in healthy animals. The results suggest a promising approach to further improve the implantable microdevice to attain a true, long-term ‘artificial pancreas’ system.

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Chapter 6  Thermostable Pluronic F-127 Zinc-Insulin Gel Formulation for Long-term Storage at Physiological Temperature and Use in Implantable Microdevices

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6.1 Abstract

For many diabetes patients, long-term insulin therapy is required to sustain their lives or improve their life quality. In all insulin therapy regimens, insulin formulation is a central component. Because insulin is a protein in nature, it is sensitive to physical and thermal degradation and denaturation, which is exacerbated by increasing protein concentration or temperature. Thus existing commercially available insulin formulations need refrigeration for long-term storage and cannot provide high concentration of insulin for use in implantable microdevices. Therefore, herein we have developed an in situ gelling insulin formulation with amphiphilic polymer Pluronic F-127 to reduce shear forces through gelation at higher physiological temperatures, while remaining liquid at lower temperatures for ease of administration. The combination of this high concentration insulin formulation with zinc allows for an insulin gel with high resistance to thermally induced fibrillation over a 30 day period. Insulin structure and biological activity was maintained after thermal stress when analyzed with RP-HPLC and circular dichroism, demonstrating viability of the in situ zinc-insulin gel as a long-term insulin formulation. Potential in an implantable, sustained release microdevice is demonstrated with a gradient-driven, linear 15 day in vitro release profile, providing potential for pseudo linear release kinetics. These insulin gel microdevices show potential for a ‘hands-off’ basal release system that requires no patient monitoring or electromechanical attachment.

6.2 Introduction

Diabetes mellitus is a global disease that affected around 350 million people worldwide in 2013 and is projected to double by 2030. Insulin-dependent Type 1 diabetes patients are unable to produce their own endogenous insulin, while Type 2 diabetes patients show resistance to insulin
and low insulin sensitivity. Insulin is an endogenous hormone that rapidly induces uptake of blood glucose (BG) into cellular stores. Lack of insulin or low insulin sensitivity from beta cell dysfunction can cause relatively high fasting BG (i.e. higher than 150 mg.dL\(^{-1}\)) from the body’s inability to uptake glucose into cells, resulting in long periods of hyperglycemia. Type 2 patients can change their diet and lifestyle to improve glycemic control, but it is not enough in many cases, requiring administration of anti-diabetic drugs or exogenous insulin\(^{420,421}\). Both Type 1 and severe Type 2 diabetes patients require exogenous insulin to survive, as chronic hyperglycemia can cause a number of cardiovascular, renal and neural complications, and can be fatal if left unchecked\(^{420-430}\). Standard diabetes treatment is typically focused on replacement insulin therapy, primarily via injections, delivering exogenous insulin into the subcutaneous abdominal adipose tissue to decrease glucose levels in the bloodstream via cellular uptake\(^{431-434}\).

Design of insulin formulations for diabetes therapy is of paramount importance to the effective management of diabetes. Along with dosage form and route of administration, the maintenance of insulin bioactivity and stability within the formulation are critical for commercial viability.

With most insulin formulations, native insulin is rarely used, as it is susceptible to denaturation and fibrillation that increases proportionally with higher insulin concentrations. As well, current commercial insulin solutions, which will be discussed later, have relatively low concentrations (up to 500 units per mL) requiring a minimum of once-daily injections for the longest duration of efficacy\(^{431,432,434}\). A high concentration, stable insulin formulation would be a boon for patients, maintaining pharmaceutical potency and extending administration intervals. When considering design of a high concentration insulin formulation, there are critical parameters that must be addressed. Structural instability, short half-lives and physical aggregation are major concerns when preparing a stable formulation. Environmental stresses such as agitation, temperature and
pH are all contributing factors that directly affect insulin stability. This makes storage and transport a potential problem when dealing with these environment-sensitive formulations. Higher temperatures can disturb the folding structure, as free energy can overcome the intramolecular forces (e.g. hydrogen bonding, van der Waals forces) that maintain secondary structure in proteins. Stability of insulin is compromised by low pH and shear forces, as well as detrimental interactions with hydrophobic interfaces such as air-water interfaces. Initial unfolding of insulin proteins from these mechanisms can exacerbate the issue, as partially unfolded insulin can trigger chain fibrillation, and in turn, larger scale aggregation that compromises efficacy of the protein formulation via irreversible precipitation of insoluble fibrils. This aggregation is also a concentration-dependent response, as higher concentrations will lead to increased nucleation and fibrillation stress, which makes stability more difficult with increasingly potent formulations. Agitation and temperature fluctuation during storage can lead to a loss in potency for insulin solutions and a short shelf-life, which increases cost and may compromise therapeutic efficacy.

Advances in protein chemistry and formulation have given us some alternative options besides rapid-acting, native human insulin. The advantage of longer-acting insulins comes from the need for basal insulin levels and a reduction in injection numbers. Commercial intermediate and long-acting insulins such as neutral protamine hagedorn (NPH) and insulin glargine have been shown to have good long-term stability through minimizing the proportion of monomeric insulin. Protamine added to NPH insulin generates formation of a crystalline insulin suspension, while insulin glargine has a chemically modified amino acid residue that shifts the isoelectric point, eliciting reduced solubility and delayed action at physiological pH. These isoforms of insulin provide added protein stability, as well as slow down dissolution of insulin before reaching the
site of action and thus, have a longer duration of bioactivity\textsuperscript{438,441-444}. These stabilized insulin forms have a longer shelf life compared to rapid-acting formulations. However, there are concerns with protamine sensitivity and overall suspension stability compromising accurate dosing with NPH insulin\textsuperscript{443,444}. Insulin glargine solution cannot be premixed with rapid-acting insulins as it is acidic, which can promote irreversible protein deamidation, retarding glycemic efficacy\textsuperscript{445,446}. As well, long-acting insulins have been shown to be less cost-effective as a long-acting strategy compared with standardized fast-acting commercial injections or pumps\textsuperscript{437,438,443-446}. Furthermore, denaturation of insulin formulations may lead to insulin chain fibril formation and HMWPs which are biologically inactive. Overall, all types of commercial insulin solutions require refrigeration to achieve optimal potency and consistency in diabetes therapy. The balance of protein stability and bioactivity is a critical component of an optimal insulin formulation design for clinical use.

Selecting the proper excipient for the delivery of biopharmaceuticals lends itself to novel challenges. Proteins dissolved in solution are limited to linear or burst release kinetics, as well as a short-term window of efficacy, depending on the drug affinity and retention. As a dosage form, gels have distinct advantages versus solutions, such as increased formulation viscosity and extended drug release profile. Biocompatible gels allow for a longer period of retention at the site of action, allowing for a long-term release profile, with slower clearance. Specifically, the polyethylene oxide (PEO)-polypropylene oxide (PPO)-polyethylene oxide triblock polymer gel, polaxomer 407, also known as Pluronic F-127, has been used as a biocompatible pharmaceutical gelation excipient for sustained release of dissolved or suspended drug. A unique property of Pluronic F-127 is that polymer concentrations above 20\% w/v form a micellar lattice structure at a critical gelation temperature, while staying liquid at temperatures below this point, also
known as in situ gelation\textsuperscript{447-449}. This allows for ease of volumetric administration and protein mixing at low fluid temperatures and gelation in physiological environments. This flexibility gives high concentration Pluronic F-127 gels the ability to be injected at cold temperatures and exhibit extended release and stability upon gelation at physiological temperatures. The hydrophobic polypropylene oxide (PPO)-hydrophilic polyethylene oxide (PEO) block copolymer provides a stabilizing presence, acting as a surfactant which prevents exposure of hydrophobic protein domains which can lead to protein unfolding and denaturation\textsuperscript{447,449}. Hydrophilic PEO chain ends associate with hydrophilic protein surfaces, preventing protein aggregation, while the hydrophobic ends interact with other hydrophobic surfaces, like silicone catheters or air-water interfaces. The viscous gel serves as a medium for long-term insulin release or solution application after dissolution and injection. As well, the denaturation stress from shear forces created by agitation and bubbles in solution is negligible with a gel formulation. These properties of Pluronic F-127 make the in situ gelling excipient a good choice for a high concentration insulin formulation. Commercial insulin solutions are susceptible to physical agitation and denaturation, as it can lead to caking, sedimentation and a loss of overall potency. Pluronic F-127 has previously been used as an excipient for sustained insulin release through external buccal and rectal routes, showing good homogeneity with dissolved insulin\textsuperscript{447,450-453}. External application of insulin gels provide a non-invasive method of administration, but still have limited bioavailability and shelf-life of these formulations is undetermined.

Implantable insulin pumps have been in use for over two decades as a method of basal insulin release. Patients using commercial, catheter-based insulin pumps for continuous insulin delivery have shown a marked reduction in glycated hemoglobin (HbA1c) levels, a measure of long-term, average blood glucose and fewer hypoglycemic events overall compared with multiple insulin
injection regimens $^{454-458}$. In conjunction with mealtime dosing, pumps can provide baseline levels of insulin to mimic physiological conditions and provide proper glycemic control during non-fed periods, such as sleep. Although very promising, commercial pump systems have inherent technical issues. Catheter or pump blockages, catheter site infection and electronic failure have been some concerns with the semi-external pump systems $^{454,457,458}$. Also, since pump systems are reliant on current insulin formulations, efficacy of the system is directly linked to formulation stability, regardless of pump design, patient compliance and dosing profile. As an alternate form of long-term insulin delivery, subcutaneous implantable microdevices have been explored. Utilizing biocompatible materials to form a delivery vehicle for drug payload allows for potential long-term release method for chronic diseases $^{459-461}$. Specifically, silicone rubber, PDMS, has been used as an implantable material for biocompatible drug reservoirs, tissue scaffolding and biosensing $^{461-465}$, with good mechanical properties and non-reactive surface groups. Our group has previously used modified PEGylated PDMS tubing microdevices as a reservoir for glucose-responsive insulin delivery, with 4-week efficacy and low immunogenicity $^{466,467}$.

Herein, we have designed a zinc-stabilized insulin solution with Pluronic F-127 to create an in situ gelling formulation that has both chemical and physical stability at high insulin concentrations. Our current studies focus on the formulation and efficacy of a novel zinc-insulin gel as a neutral, slow-releasing, sustained insulin formulation with a long shelf-life. We have developed an insulin formulation that does not require refrigeration, maintains bioactivity over time, and has a wider tolerance of storage conditions with exceptional shelf-life. For a sustained release insulin delivery system, we have used technology from our bioinorganic membrane microdevice reservoir as a vehicle $^{470,471}$ for the aforementioned high-concentration, thermostable
zinc-insulin to control gel dissolution. As well, we have utilized a novel gradient conformation of low-to-high insulin concentration within the microdevice reservoir to achieve a linear release profile. This allows for a long-term basal insulin release profile for low-level, ‘background’ glycemic control.

6.3 Methods

Materials

All chemicals were analytical grade and used without further purification. Recombinant human insulin (r-DNA origin, 27 U.mg⁻¹) was purchased from Wisent (Canada). Streptozocin (98%, anomer basis), bovine serum albumin (99%), catalase (solution, 13 mg.mL⁻¹), glutaraldehyde (Grade I, 25%) and trifluoroacetic acid (TFA) (99%) were purchased from Sigma Aldrich (USA). NIPAM:MAA hydrogel nanoparticles were prepared as previously described. Glucose oxidase enzyme (225u.mg⁻¹) was purchased from Calzyme Laboratories Inc (USA). Silicone tubing (PDMS, Nalgene 50 silicone tubing, ID = 1.6 mm, OD = 3.2 mm, wall = 0.8 mm) was purchased by Nalgene (USA). Methanol and distilled deionized water (HPLC grade, 99.7%) were purchased from Caledon Labs (Canada). Humulin® R insulin solution was purchased from Eli Lilly Canada. Ethylene-vinyl acetate copolymer resin (EVAC, ELVAX-40W) was provided by E. I. Du Pont. Pluronic F-68 and F-127 were provided by BASF Corporation (Germany). Distilled and deionized (DDI) water were obtained from a Milli-Q water purifier (Milli-Pore Inc.).

Insulin Gel Preparation

Insulin gels were prepared by dissolving human recombinant insulin in solution prior to gel addition. Briefly, 0.1M NaOH with Pluronic F 68 at ½ CMC was used to dissolve insulin
concentrations at 100 mg.mL$^{-1}$. Turbidity was observed to determine insulin saturation and overall solubility, where 100 mg.mL$^{-1}$ was the maximum soluble concentration without visible precipitates. For NaOH dissolved insulin, 1 M HCl was added dropwise to adjust pH to 8 and the solution was shaken to resolubilize aggregates. Pluronic F-127 (25% w/v) was added to the insulin solution under stirring and refrigerated at 4°C for 24h, utilizing a ‘cold method’ preparation technique to form insulin gel (Fig. 6.1). For zinc modified gels, the insulin gel formulation was stirred at 250 rpm in a glass vial under ice, while ZnCl solution (1% w/v in 0.02 M HCl) was added dropwise and allowed to stir for 10 minutes until fully dispersed. Zinc:insulin molar ratios of 1:3 were used to give a 2-zinc per insulin hexamer ratio (2:6) as reported in literature 434,436.

Fig. 6.1 – Comparison of insulin solution (top) and insulin gel (bottom) after incubation at 37°C.
**Insulin Gel Thermal Stability Assay**

Insulin gels were prepared as described (100 mg.mL⁻¹) with and without zinc as control. Under ice bath, 200 uL of liquid gel solutions were pipetted into glass microtubes and sealed with Parafilm. Gels were kept at 37°C to induce gelation and incubated for 10, 20 and 30 day intervals. After each incubation period, gels were cooled slowly to room temperature, and then refrigerated at 4°C prior to dilution and testing.

**Circular Dichroism Insulin Secondary Structure Analysis**

An Aviv Biomedical Model 62A DS xenon-lamp circular dichroism (CD) spectrometer was used for insulin analysis. Upon cooling, the insulin gels were diluted to 0.5 mg.ml⁻¹ in PBS (pH 7.4) for testing. Secondary insulin structure was analyzed through far UV analysis in the 200-250 nm wavelength range to determine peptide bond conformation with 1 cm glass cuvette and 0.045 um slit width. Fresh insulin and long-term insulin incubated at 10, 20, and 30 day time points of both control and zinc diluted insulin gels were analyzed for comparison. Data was normalized for protein concentration and 5 repeats per test were performed.

**RP-HPLC Insulin Stability Analysis**

Separation column was a NovaPak C-18 column from Waters, 3.9 mm x 150 mm (4 um pore size). Distilled deionized water (0.1% TFA) (A) and acetonitrile (0.1% TFA) (B) were filtered and degassed before using as the mobile phase solvents. A linear gradient elution profile was used, with A/B ratios from 65/35 to 15/85 over 20 minutes. Detection was performed with a 2899 Photodiode Detector Array (Waters) at 215 nm wavelength and the column was kept at 37°C throughout the experiment. Flow rate was 1 ml.min⁻¹ and injection volume was 20 uL. Zinc and control zinc-free insulin samples incubated at 0, 10, 20 and 30 day timepoints were filtered
and diluted to 0.5 mg.ml\(^{-1}\) in pH 7.4 PBS for injection and spectra were compared to determine protein stability. Between sample groups, a concentrated 1% w/v TFA solution was used to wash out columns.

**In vivo Bioactivity Assay for Incubated Insulin Gels**

Sprague-Dawley rats were injected with streptozocin (STZ) at a dose of 65 mg.kg\(^{-1}\) to induce hyperglycemia over a two day period. After diabetes was confirmed (blood glucose \(>20\) mmol.L\(^{-1}\)), hyperglycemic rats were injected with with 250 ul of 8 u.ml\(^{-1}\) diluted insulin sample in saline to give 2 units of insulin per rat. Blood glucose was measured via glucose strip using an OneTouch® meter (LifeScan Canada) measurements from tail prick blood. Measurements were taken at 5, 10, 15, 30, 45, 60, 75 and 90 minute intervals post-insulin injection. Fresh zinc and zinc-free insulin gels were compared with 37°C incubated gels after 10, 20 and 30 day timepoints. Rats injected with fresh Humulin® R, a commercial human recombinant insulin formulation, were also tested as a positive control. Relative insulin activity ratios were determined by comparing net glucose change in diabetic rats between injections of fresh insulin samples versus incubated insulin samples after 90 min period. All *in vivo* procedures strictly complied with the ethical and legal requirements under Ontario’s Animals for Research Act and the Federal Canadian Council on Animal Care guidelines for the care and use of laboratory animals and were approved by the University Animal Care Committee of the University of Toronto.

**FITC-gel reservoir release within bioinorganic tubing microdevices**

Bioinorganic tubing microdevices were prepared as previously described\(^{470,471}\). Insulin gel release was compared using homogenous and gradient modified gels in reservoir with
fluorescence analysis. FITC-dextran (Mw = 10 000) was used as a model drug for analysis of gel migration inside reservoir. To prepare gels, FITC-dextran (1 mg.mL⁻¹) was mixed with Pluronic F-127 (25% w/v) in saline and cooled overnight to dissolve. Gels without FITC were also prepared as a blank control gel. Homogenous gel microdevices were completely filled with cold FITC-dextran gel and warmed to induce gelation. Gradient gel microdevices were filled in the back section with cold FITC-dextran gel halfway up the microdevice, while the remaining front section was filled with blank gel, and then warmed to induce gelation. Fluorescence was measured with Xenogen fluorescence microscope after 5, 10 and 15 day incubation in PBS (pH 7.4) with 100 mg.mL⁻¹ glucose, to determine gel movement and depletion.

**Sustained release testing with bioinorganic tubing microdevice**

Long-term release kinetics were tested for zinc-insulin gels injected into bioinorganic membrane tubing microdevices prepared as previously described \(^{470,471}\). Insulin release kinetics were measured to compare homogenous gels versus gradient gel formulations in microdevices. Control gel microdevices were filled with 30 uL of 100 mg.mL⁻¹ zinc-insulin gel and heated at 37°C to induce gelation. For gradient preparations, the back end of devices were filled with 15 uL of 100 mg.mL⁻¹ zinc-insulin gel and heated at 37°C to induce gelation. Remaining front end of microdevices were filled with 15 uL of 50 mg.mL⁻¹ zinc-insulin gel and heated at 37°C. Devices were placed into vials with 2 mL pre-warmed PBS (pH 7.4) and incubated on rotary stirrer at 37°C. Buffer medium was measured at 276 nm on UV-Vis spectrometer and replaced with fresh buffer daily after each measurement. Cumulative insulin release was measured daily over a 15 day period.
6.4 Results and Discussion

We have used 0.1 M NaOH as a solvent for insulin, as it is capable of dissolving high concentrations of insulin above its pKa of 5.3. Typically, acidic insulin preparations, although commonly used in commercial formulations, are readily susceptible to hydrolytic deamidation compared with neutral preparations, specifically asparagine breakdown into a carboxyl group, a consequence of low pH. This chemical denaturation mechanism is exacerbated by both increasing temperature and insulin concentration and usually requires a stabilizer to retard this process. Alkaline conditions are known to reconstitute insulin, however, long-term stability has not been assessed with respect to physiological activity. Combining a pH 8-adjusted, 0.1 M NaOH-dissolved insulin gel formulation with zinc complexation provides an appropriate medium for resistance to concentration-dependent protein aggregation seen with high concentrations of insulin, as well as avoiding unfavorable deamidation conditions in physiological environments. Insulin denaturation leading to fibrillation is a major complication for reservoir insulin systems, as insoluble precipitates can block the devices or catheters. Moreover, the loss of potency over time during storage can lead to shorter formulation shelf-lives and higher costs. These slightly basic insulin solutions were mixed with 25% Pluronic F-127 under cold method to induce gelation for the subsequent tests (Fig. 1). Complexation of zinc was performed under cold stirring of dissolved insulin gels, forming an amorphous zinc-insulin gel preparation.

Zinc-Insulin Interaction and Insulin Hexamer Properties

A unique property of insulin is its ability to self-assemble at higher concentrations from monomers into dimers and, in the presence of zinc ions at neutral pH, hexamers. Zinc has been
used as a complexation agent to reversibly bind with insulin to minimize surface exposure of protein residues to denaturation stresses. High-affinity electrostatic interactions allow for a low-energy, stable state for the insulin-zinc complex, through association with the B10 His residue of the insulin molecule, in both crystalline and aqueous preparations.\textsuperscript{472,473} The insulin-zinc complex is comprised of six insulin molecules in a planar hexamer conformation with a dual zinc ion core that provides improved stability compared with zinc-free monomer, dimer and hexamer forms.\textsuperscript{473,474} Keeping non-polar, hydrophobic surfaces isolated from the hydrophilic environment (Fig. 6.2). Typically, intermediate and long-acting insulin formulations use low concentrations of zinc ions in solution to provide a stable, delayed onset of insulin activity for extended release profiles, owing to the slower absorption of insulin hexamers.\textsuperscript{432,434,444,445} In comparing zinc and zinc-free insulin gels, we would determine effect of hexamer complexation on the protein structure and residual activity after subjected to long-term physiological temperatures.

\textbf{Fig. 6.2} – Diagram of insulin hexamer formation from monomeric insulin with and without zinc ion complexation.
Addition of Zn$^{2+}$ ions improved the secondary structure retention of insulin gel formulations

Determining secondary structure of insulin can give a useful interpretation of protein activity, which suggests ligand-receptor affinity has a direct correlation with insulin protein 3-D structure. Freshly diluted insulin gels (Fig. 6.3a) showed expected spectra before incubation, with two characteristic peaks, at 209 and 222 nm, which represent the primarily alpha helical structure of native insulin, representative of both the A and B-subunits. Modified zinc-insulin gel had a slightly flatter spectrum than native insulin gel between 209 and 222 nm peaks, showing the zinc complexation did not affect the alpha helical peaks as expected (Fig. 6.3a). Metal ion perturbation of the 209 nm peak is expected with zinc-insulin formulations but the 222 nm peak is unaffected. After 10 day incubation at 37°C, native insulin gel without zinc gradually lost the 209 nm peak, while still retaining the 222 nm peak. This is typically seen with partially unfolded proteins, which denotes a loss in alpha-helical structure. After 20 day incubation, non-zinc insulin samples lost both defined 209 and 222 nm peaks and after 30 day incubation, no defined alpha helical spectrum remained (Fig. 6.4b). With both 20 and 30 day samples, diluted zinc-free gels required filtration before analysis with some cloudy aggregates, indicative of insoluble fibril formation. Alternately, zinc-insulin gel samples maintained both 209 and 222 nm peaks after 10, 20 and 30 day incubation, confirming maintenance of secondary structure, with overlaid spectra (Fig. 6.4a).
Fig. 6.3 – Far-UV CD spectrum of zinc-insulin gel and non-zinc insulin gel at 0 days (a) and 30 days (b). Spectra normalized according to concentration.

Fig. 6.4 – Far-UV CD spectrum of zinc-modified insulin gel (a) and non-zinc insulin gel at) over 30 day period. Spectra normalized according to concentration.

**Modified zinc-insulin gels have improved thermal stability at 37°C**

The presence of zinc ions in an insulin formulation promotes zinc-insulin hexamer complexation over dimer and free monomer forms. It is clear that incubation at 37°C causes fibrillation through unfolding of alpha helical protein secondary structure, as shown in Fig. 6.4b. Gelation of both formulations at physiological temperatures indicates the denaturation stresses are independent from physical causes (shear forces, air-water interfaces). Structure is maintained through
preferential electrostatic complexation of insulin molecules with zinc ions to form hexamers. The strength of electrostatic interactions between insulin hexamers and the zinc ion core prevent the unfolding and exposure of non-polar protein residues which can occur from an increase in overall free energy provided by higher temperatures. Under 30 day incubation at physiological temperatures, zinc-free insulin gels showed a complete loss in secondary structure. The initial loss of alpha helical structure occurs in zinc-free formulation after only 10 days (Fig. 6.4b), which is due to a reduced population of insulin hexamers in deference to the less stable dimer and monomer forms.

Although high concentration insulin is ideal for a long-term reservoir formulation and more potent therapy, increasing concentration is positively correlated with a higher tendency for fibrillation\textsuperscript{435}, as seen after only 10 days with the zinc-free gel formulations. Peak shape at 209 and 222nm was lost and 20 and 30 day incubated zinc-free samples became cloudy even after dilution and dispersion (Fig. 6.4b). This indicates a thermally-mediated loss of insulin stability, inducing partial unfolding of protein, specifically the alpha helical characteristics of the 209 and 222nm peaks seen in fresh insulin (Fig. 6.3a). As well, this single peak shift seen in 20 and 30 day samples shows beta-sheet character, which promotes the initiation of insulin nucleation and fibrillation\textsuperscript{436,438}.

Over 30 days, zinc-insulin gels showed good stability over time after incubation at 37°C, with far-UV CD spectra being almost identical after all time periods (Fig. 6.4a). Maintenance of peaks at 209 and 222 nm show alpha helical structure in incubated zinc-insulin gel was undisturbed from fresh zinc-insulin gel after 30 days. Zinc ion addition allows for complexation of insulin formulations in a highly stable, less active hexamer form\textsuperscript{473,474} with resistance against thermal and concentration-dependent denaturation stresses. For our bioactivity assay, dilution of the gel
to lower concentrations is essential for physiological glucose response, as a primarily hexameric formulation would have a very slow onset of activity.

During incubation, all insulin samples were maintained in the gel state, suggesting that unfolding and denaturation processes occurred independent from shear forces and air-water interactions typically seen with agitated protein solutions. At 37°C and above, temperature-mediated unfolding occurs when free energy is higher than the intermolecular forces that maintain protein structure (van der Waals, hydrogen bonding). Thermal stress-induced partial unfolding of insulin molecules can initiate nucleation, and, in turn, large scale fibrillation\textsuperscript{434,435,437,438}. As the concentrations tested were over 5 times commercial insulin formulations, protein fibrillation can occur rapidly after initial nucleation lag, ending up in complete irreversible aggregation of the formulation after 10 days of incubation. Conversely, hexamers formed from insulin-zinc complexation have a much stronger electrostatic conformation that is resistant to thermally-induced unfolding, and thus do not undergo fibrillation. They can form stable hexameric aggregates with lower solubility than free monomers\textsuperscript{434,435,478}. This complexation process is reversible by dilution and dispersion of insulin hexamers in saline and the active monomeric insulin conformation can be recovered.

\textit{Zinc-insulin gels have consistent HPLC spectra after 30 day incubation at 37°C}

Insulin in HPLC analysis is typically an analysis of monomeric species, as both dimer and hexamer forms are relatively insoluble in both aqueous and organic mobile phase and do not show appropriate peaks within a reasonable elution time. To this effect, dilution and filtration is performed to isolate and promote the monomeric conformation, allowing for chromatic analysis of the active monomer species. When comparing HPLC spectra in zinc-free insulin samples, we
saw immediate loss in peak sharpness and quality after 10 days incubation at 37°C. The defined peak shape from fresh, native insulin at 9 minutes was gradually lost and a very broad spectra was seen, potentially due to unfolded insulin moieties giving rise to erratic separation behavior (Fig. 6.5a). It is likely this wide, delayed secondary peak corresponds with desamido insulin, a byproduct of deamidation reaction known to be caused form thermal stress. The native insulin peak was lost after 30 day incubation and only artifacts remained. With zinc-insulin diluted gel samples, peak shape remained from all four timepoints, with negligible artifacts (Fig. 6.5b). Retention times were delayed compared with native, zinc-free insulin samples, but peak quality was good. The lag in retention time is expected, from effects of trace zinc ions, which equilibrate in solution with insulin to slightly reduce overall solubility, slowing elution time. This indicated consistent protein structure and similar adsorption characteristics from fresh and incubated zinc-insulin samples.
Fig. 6.5 – RP-HPLC spectra of diluted insulin gels after 0, 10, 20 and 30 days of incubation at 37°C. Comparison of zinc versus non-zinc insulin gel samples.

Retention times with hydrophobic column packing surfaces (C-18) are repeatable with fresh insulin, as Pluronic F-127 surfactant-solubilized insulin molecules have native, undisturbed folding structure. As partial unfolding occurs at increased temperature, exposed hydrophobic surfaces would give rise to increased hydrophobic-hydrophobic interactions with column packing material, which has two major effects on elution profiles. Firstly, unfolding is not a binary mechanism and degree of unfolding governs the amount of interaction with hydrophobic
column material, leading to a lag in retention time, giving a broad, inconsistent peak shape. Also, as mentioned before, thermally-induced unfolding may lead to hydrophobic interactions with other unfolded insulin monomers, and this protein-protein adsorption would compromise elution time from a sterically-mediated mechanism, which compounds the loss of secondary structure.

These results are promising for a high-concentration, thermostable gel insulin formulation. Combating the concentration-dependent effect of insulin fibril formation with stabilizing hexamers allowed us to achieve an in situ gel that is over five times more concentrated than commercial ‘high potency’ U-500 insulin solution.

**Zinc-insulin gels maintain bioactivity after long-term incubation at 37°C**

After long-term incubation, dilution and dissolution of zinc-insulin hexamer gel into sterile saline can be performed before injectable administration. This is an important assay to correlate the retention of native insulin secondary structure with bioactivity for clinical application. The protein stability of incubated gels were determined by circular dichroism, measuring changes in ellipticity in the far UV range, a factor in protein secondary structure analysis. To correlate this with in vivo bioactivity, incubated gels were diluted to physiological relevant concentrations and injected into islet cell-deficient STZ-rats to measure insulin activity on plasma glucose. As shown in Fig. 6.6b, fresh, zinc-free insulin gels showed rapid response after injection, as expected. Upon injection of thermally incubated zinc-free insulin from day 10 to 30, a steady decrease in relative bioactivity was seen. Day 10 samples only decreased blood glucose level to 200 mg.mL\(^{-1}\) after 75 minutes, more than twice as long as fresh gels after 30 minutes. Both day 20 and 30 incubated zinc-free insulin formulations did not reach a normal glycemic range after 90 minutes (> 300 mg.dL\(^{-1}\)) (Fig. 6.6b). Relative insulin activity ratios for zinc-free gels
decreased to 0.73, 0.41, 0.38 after 10, 20 and 30 day incubation, respectively, after measuring STZ-rat blood glucose at 90 minutes post-injection (Fig. 6.6d). This coincides with the loss of native alpha-helical structure (209 and 222 nm peaks) which could lead to a loss of insulin receptor-ligand interaction and thus, glycemic activity. We can see that protein unfolding of zinc-free insulin gels leads to inactive insulin solution after gel dilution, which is worsened with longer incubation times.

Alternately, zinc-insulin showed maintenance of relative bioactivity ratios over all three incubation periods compared with fresh zinc-insulin gel. The bioactivity ratios were similar for zinc-insulin gels (>0.95), with a slight decrease towards 30 day incubation; However there was no statistical significance (p > 0.05) between all samples (Fig. 6.6a, 6.6d). The slope of decrease in blood glucose for fresh, zinc-free insulin gel was sharper within the initial 30 minute time period, compared with fresh zinc-insulin gel. This was expected, as presence of dilute zinc ions in insulin solution will lead to a greater proportion of insulin hexamers, leading to a delayed glycemic effect, compared with zinc-free monomer solutions. Dissociation of hexamers into dimers and active monomers occurs after dilution in the interstitial fluid, allowing for absorption and onset of activity. Also, diluted insulin solution promotes dissociation of zinc-insulin hexamers into active monomers, a concentration-dependent process. This shift towards monomeric insulin is necessary to elicit glycemic effect, as hexamers, and to a lesser extent dimers, cannot readily pass through epithelial barriers. As zinc is still present in the diluted preparations, it can be expected that some zinc insulin hexamers are present as well. Humulin R, a zinc ion containing commercial insulin solution, showed similar effect as diluted zinc-insulin gels at identical injection concentrations (Fig. 6.6c). These pharmacokinetics are consistent with zinc-containing intermediate-acting insulins, reaching onset of activity within 30 to 60 minutes.
There is a slight delay from dissociation of residual zinc-insulin hexamers before insulin can cross physiological membranes and elicit glycemic effects. Comparing incubated zinc-insulin gel bioactivity with fresh samples showed a good maintenance of activity, showing negligible activity loss from long-term 37°C incubation. This suggests the retained insulin secondary structure with zinc hexamer complex seen from CD analysis translates into *in vivo* glycemic bioactivity. Use of zinc insulin gels in a sustained release microdevice system was explored as an application, with no noticeable loss of efficacy or insulin stability over long-term physiological conditions.

**Fig. 6.6** – Bioactivity of insulin samples in STZ-diabetic rats. (a) zinc-insulin gel formulation over 30 days, (b) insulin gel formulation over 30 days, (c) comparison of fresh insulin samples with Humulin R, and (d) relative insulin activity of zinc-insulin and insulin gel. Error bars represent standard deviation (n=3).
Injectable gels are exposed to immune cell interactions and inconsistency in gel morphology upon injection can give a variable dissolution rate and insulin release. Hydrophilic Pluronic gels have a relatively rapid dissolution rate, making them inadequate for direct subcutaneous injection for extended release beyond a few hours \(^{447,448}\). Moreover, the dissociation step of insoluble zinc-insulin hexamers would retard the diffusion across physiological barriers, adding difficulty in controlling basal release kinetics. For this purpose, we used previously designed bioinorganic membrane tubing microdevices to minimize potential immune response and control the desired insulin release profile. Release of insulin is controlled by hydrophilic membrane permeability, which allows for soluble monomer diffusion, but resists insoluble hexamer release. This prevents burst release of the high concentration zinc-insulin gel and promotes a sustained release profile. As insulin content within the gel reservoir decreases, this is compensated by a shift in zinc hexamer to monomer dissociation. Additionally, long-term insulin release can be controlled with a directional gradient design, utilizing lower concentration insulin in the membrane half of the microdevice reservoir, which in turn affects overall gel dissolution (Fig. 6.7b). The concentration gradient driving diffusion across the bioinorganic membrane is kept consistent with gradient mixing of higher concentration zinc-insulin preparation in the back end of microdevices, which would slowly weaken in a homogenous system. Microdevices with homogenous and gradient gels were compared, and long-term total insulin release was measured to determine optimal release kinetics.
Fig 6.7 – Comparison of insulin release from bioinorganic microdevices with homogenous and gradient insulin gel (a). Error bars represent standard deviation. Schematic of homogenous versus gradient formulation design within microdevice reservoir (b).

Homogenous insulin gels show an initial zero-order release profile from microdevices, likely due to viscosity of gel formulation. Up to 5 days and 35% total insulin released, daily release rate begins to decrease over time, with 20% released in the next 5 days. Slight plateauing occurs up to day 15, showing a weakening of insulin concentration gradient (Fig. 6.7a). A homogenous gel formulation allows for depletion of not only the reservoir insulin, but Pluronic F-127 polymer as well, albeit at a reduced rate, owing to the size of the polymer chains ($M_w = 12\,500$). This leads to increasing hexamer and polymer gel dissolution rate, as the reservoir viscosity decreases. Total released insulin coincides with concentration gradient weakening, as 50% is released by 8 days, leading to a reduced diffusion rate at later time points.
Gradient insulin gels show slight lag in relation to total insulin release at early time points, which is expected with a reduced concentration insulin gel used at the anterior, membrane compartment (50 mg.mL\(^{-1}\)). Compared with homogenous gels, gradient-driven gels have a more linear profile, with 25% total insulin released after 5 days, and 20% release over the next 5 days. This ‘pseudo’ linear profile is better suited to a sustained model, which requires a steady release of insulin over a 24 h period. Release from day 10 to 15 show slightly under 20% total insulin released, similar to days 5 to 10. Retention of a strong concentration gradient is crucial for any membrane-based reservoir system, which we achieve with a delayed, diffusion-mediated high concentration gel (100 mg.mL\(^{-1}\)) in the posterior compartment of insulin microdevices (Fig. 6.7a). This compensates for the depletion of the anterior, low concentration gel, providing an improved maintenance of the insulin concentration gradient. One should note the total insulin between homogenous and gradient devices is not identical (5 mg vs 3.75 mg, respectively), but for theoretical purposes, near linear release kinetics are achieved and net release can be tailored to clinically relevant levels by total number of devices used.

The linear profile of the insulin gradient gel microdevice can provide basal release, giving ‘pseudo’ zero-order kinetics for low-level insulin therapy. Further improvements would include increasing viscosity in gel formulations to slow gel mixing and potentially reach true zero-order release, in conjunction with directional, multiphasic concentration partitions. Studies have shown that an insulin therapy regimen using a combination of fast-acting combined with slow-acting insulin gives better glycemic control than fast-acting insulin alone. As a result, intermediate and long-acting insulins are often combined with fast acting insulin for tight glycemic control and reduced HbA1 levels over time. Our thermostable, sustained insulin gel implant can be a useful
pillar for Type 1 diabetic therapy and optimal glycemic control with minimal patient intervention.

6.5 Conclusions

We have combined a zinc-complexed insulin formulation with Pluronic F-127 gel to create a thermally stable and long-term insulin gel that remains bioactive after 30 days. We have integrated this in situ thermostable, zinc-insulin gel with a long-term release PDMS microdevice, which can provide basal release of insulin and may be combined with a less severe acute insulin regimen. Thermal stability is high due to long-term incubation at physiological temperatures and retention of protein stability and bioactivity in this zinc-insulin gel. A bioinorganic microdevice filled with zinc-insulin gel showed a 15 day linear, sustained release profile, which shows potential for a low-level implantable insulin system.

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Chapter 7  Conclusions and Future Perspectives

7.1 Overall Conclusions from this Thesis

The work described in this thesis includes a number of steps towards a true, closed-loop insulin delivery system. Traditional insulin therapy behooves patients to maintaining strict records of blood glucose levels to optimize their individual insulin regimen. Although the glucose-responsive implant prototype is still a long way from commercialization, first-in-man trials are not necessarily the final goal. Use of responsive insulin implant devices may be considered for diabetic animal application, as they cannot self administer insulin and difficulty with multiple injection regimens may be difficult to overcome. Alternately, with the elderly who may not monitor their own blood glucose regularly or may be averse to sophisticated pump systems, a hands-off, non-electronic implant microdevice may be ideal. As outlined in this thesis, the current progress towards glucose-responsive systems gives hope for improved quality of life for diabetes patients in the near future.

The goal of this work was to develop a glucose-responsive insulin therapy system for in vivo application. To this end, PDMS-based microdevices were designed with incorporation of the glucose-responsive membrane with insulin reservoir. Through embedded pNIPAM/MAA nanoparticles, pH- and glucose-responsive release was attained with ethylcellulose or albumin-based nanocomposite membranes. With PDMS microdevice prototypes integrated with PNIPAM/MAA-embedded ethylcellulose membranes previously developed by our group, rapid, step change in Vitamin B\textsubscript{12} release was attained in response to pH changes in the environment. The in vitro results with model drug and novel prototype microdevices showed potential for translation towards an insulin-based system.
Existing glucose oxidase-based systems for insulin therapy have been explored\textsuperscript{141,144-148}, although \textit{in vivo} efficacy has been limited. We were able to achieve glucose-responsive efficacy with the first implantable, insulin microdevice when tested in a diabetic rat model. This was successfully demonstrated with a robust analysis of both short and long-term assays of blood glucose, plasma insulin and c-peptide, which confirmed our hypothesis. To improve \textit{in vivo} efficacy, we modified the first prototype implantable microdevice to a slimmer, tubing microdevice for subcutaneous application. Biocompatibility was improved with a long-chain PEGylation surface treatment on the PDMS and bioinorganic membrane surface, conferring reduced immune cell recruitment and capsule formation through histological analysis. This allowed us to achieve an unprecedented 18-day \textit{in vivo} efficacy in a diabetic rat model, showing both multi-cycle glucose-responsiveness and long-term maintainence of euglycemia.

As mentioned before, this implantable, ‘smart’ insulin system has potential for use in patients such as children, the elderly, or patients in the intensive care unit, where they lack the ability to reliably self detect glucose level, inject insulin, or operate complicated insulin pump equipment. Also, the design of the tubing prototype lends itself to refill capability, which may be explored in the future. The slend cylindrical shape of the device also allow for easy retrieval of the device in case of sudden change in patient’s condition.

With advances in glucose-responsive microdevice design, we also explored the insulin formulation itself. In the clinic, a variety of commercial insulin solutions are used as the first line of insulin therapy. Limited to maximum concentrations of U-500 and below, this makes them adequate for multiple dosing regimens\textsuperscript{33,34}. However, when utilized with pump systems and other long-term insulin delivery systems requiring continuous administration, there are some concerns about maintaining therapeutic efficacy. The inevitable loss of potency from
denaturation of insulin formulations is present, limiting the effective shelf life of commercial products. This is exacerbated upon vial opening, agitation and temperature fluctuations \textsuperscript{258-270}. Advancements in insulin analogs have not excluded them from this loss of therapeutic efficacy over time \textsuperscript{253-255}, which is exacerbated with the increased costs associated with their use \textsuperscript{251,253}. We have explored a novel, high potency, \textit{in situ} gelling insulin formulation to combat these potential issues, with a specific focus on maintaining physical and chemical stability of the insulin molecule within its unique formulation design. Not only does this high concentration, thermostable insulin gel have clinical application in medical institutions, but it may also have use in countries where proper insulin storage and handling is not possible. Tropical, developing countries where heat and lack of accessible refrigeration are major issues may be a target for these insulin gels. A proper insulin dose can be a simple dilution and injection away.

\textbf{7.2 Original Contributions in this Thesis}

\textit{Development of the first monolithic, polymeric microdevice for pH-responsive drug delivery}

Chapter 4 outlines the first prototype microdevice system integrated with pH-responsive ethylcellulose membrane. The swelling ratio of PNIPAM/MAA nanoparticles showed a 1.5 increase in particle diameter from pH 4 to pH 7.4. \textit{In vitro}, embedded PNIPAM/MAA nanoparticles in ethylcellulose membrane at 30\% w/w gave an over ten-fold increase in permeability to VB\textsubscript{12} from pH 7.4 to pH 4. Using patterned PDMS microfabrication, we were able to seamlessly integrate the pH-responsive ethylcellulose membrane to a PDMS microdevice to create a monolithic reservoir system. Biocompatible reservoir microdevices
gave ‘on-off’ pH-responsive release kinetics in vitro, showing the potential for a monolithic implant system for pH-controlled drug release.

**Development of Glucose-Responsive Insulin Delivery Microdevices Showing Efficacy in Diabetic Rats**

In chapter 5, microdevice systems were reengineered to utilize an albumin-based hydrophilic membrane for glucose sensing and insulin release. With this change, we were able to covalently bond the membrane with the body made of PDMS to form an integrated microdevice for implantation. A GOX-based glucose-responsive albumin membrane was crosslinked to a PDMS grid and integrated with PDMS reservoir to give a glucose-responsive implant prototype. Release of insulin from the microdevice reservoir at hyperglycemia showed a two-fold increase from normal conditions, demonstrating their suitability for use under clinically relevant physiological condition. The early in vivo tests were conducted via i.p. implantation in a diabetic rat model. A rapid increase in plasma insulin following glucose bolus challenge was observed in treated rats, demonstrating in vivo glucose-responsiveness of the devices. The in vivo efficacy was evident with maintenance of normal glucose levels in a diabetic rat up to one week. This is a promising demonstration of an implantable, glucose-responsive microdevice system showing in vivo efficacy in a diabetic rat.

**Improvement in Long-term Efficacy and Biocompatibility of a Subcutaneous Implant for Real-Time Glucose-Responsive Insulin Delivery**

In chapter 6, a number of improvements were made to the initial glucose-responsive prototype. A new, silicone tubing microdevice shape was used to confer ease of s.c. implantation for simpler insertion and removal of the device. PEGylation with various chain lengths of PEG was
performed to modify the surface of the device to improve long-term efficacy, biocompatibility, and device longevity. Histological analysis of retrieved implant and surrounding tissue after a 30 day implantation indicated much better resistance to inflammation and immune cell recruitment was obtained by using long-chain 20 kDa PEG for the surface modification as compared with 2 kDa and untreated microdevices. The 20 kDa PEG-treated microdevices provided rapid response of insulin release to glucose challenge in device implanted rats, equivalent to healthy rats. Such performance was repeatable over three-cycles, showing the consistency of the device. Long-term efficacy of the device was extended to 18 days, showing clear improvement of the glucose-responsive implant prototype.

A Thermostable In situ Zinc-Insulin Gel for Long-term Sustained Release with an Implant Microdevice

The previous manuscripts outlined the core design and improvements made to the microdevice integrity with respect to a successful long-term implant for controlled insulin release. Chapter 7 focuses on the insulin formulation itself, and the specific protein stability and denaturation stresses that affect insulin activity and potency. We developed a novel Pluronic F-127 in situ gel with zinc-hexamerized insulin that shows high resistance to thermal and physical denaturation that can be present in insulin solutions. Using HPLC and CD analysis, native insulin secondary structure was maintained in zinc-hexamerized insulin gels over a 30 day period at 37°C. As well, reinjection of diluted zinc insulin gels in diabetic rats showed full bioactivity. Potential for use in a sustained implant system was demonstrated with a gradient formulation design in the bioinorganic silicone rubbing microdevice, showing pseudo linear in vitro release over a 14 day period.
7.3 Future Work and Directions

Closed-loop insulin therapy is the holy grail of diabetes treatment and has been explored extensively, commonly utilizing integrated glucose sensor/implantable pump systems in clinical trials to achieve ‘smart’ insulin release profiles \(^{68-70}\). Our integrated GOX-based bioinorganic membrane and PDMS insulin reservoir form a monolithic design, without extraneous wiring or electrical systems. As this is a prototype device in a small-scale testing environment, commercialization is still not attainable within this scope, although there are many routes that have yet to be explored.

**Refill/catheter potential for insulin microdevices?**

We have presented s.c. implant microdevices with long-term, glucose-responsive insulin release over an 18 day period. Device efficacy is inherently limited by insulin depletion, which must be restored to remain viable. Potential refill strategies could be considered, including transdermal catheter integration to refill microdevice reservoirs. Multi-refill cycles can be performed and solution measured to determine extension of device efficacy, while avoiding invasive explantation procedures. Immune system 'memory', causing increased inflammatory response, can be avoided, while providing fresh insulin, with optimal potency and stability.

**Improve glucose-responsive membrane integrity?**

Hydrophilic membranes inherently have higher bioadhesion to interstitial proteins and immune cells. Although optimal for integration of enzyme and hydrogel nanoparticle, long-term degradation of membrane can be occur, even with PEG treatment. Modifying membrane systems with inorganic or hydrophobic additives to create a ‘hybrid’ membrane to allow large molecule
diffusion may be the next step to improving membrane integrity. Layer-by-layer film formation or biphasic membranes may be considered to achieve this. Various non-degradable materials (PMMA, mesoporous silica) have been used in a controlled insulin delivery scope, but may be redesigned with a glucose-responsive focus.

**Combination of sustained release device with glucose-responsive device to improve efficacy?**

Ideally a glucose-responsive implant system will have a long duration of efficacy to minimize surgical intervention. The previous sections detail microdevice improvements, but potential for short and long-acting insulins within the reservoirs could be explored, to achieve a robust insulin therapy regimen. A two-device insulin reservoir system, one glucose-responsive, rapid-acting and one sustained, long-acting microdevice, may be worthwhile to combine to solve individual problems of bolus and basal insulin release.
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