Oral Delivery of Glucagon-Like Peptide-1 Using PLGA-COOH Microspheres

by

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A thesis submitted in conformity with the requirements for the degree of Master’s of Science
Graduate Department of Physiology
University of Toronto

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Oral Delivery of Glucagon-Like Peptide-1 Using PLGA-COOH Microspheres. Jamie William Joseph (M.Sc.), University of Toronto, Department of Physiology

Abstract

The insulinotropic hormone, glucagon-like peptide-1 (GLP-1), has been proposed for the treatment of patients with type II diabetes. Since GLP-1 is rapidly inactivated by dipeptidyl-peptidase IV (DP IV), a DP-IV degradation-resistant analog of GLP-1, D-ala²-GLP-1, was tested and found to exhibit enhanced bioactivity when administered subcutaneously to mice. However, the glycemic-lowering effects of this peptide are lost within 4 hour of injection, thus necessitating frequent and invasive administration if it is to be used as a treatment for type II diabetes. To circumvent these problems, we have designed a therapeutic microsphere that is prepared by encapsulating 2% D-ala²-GLP-1 in microspheres made of 50% Olive Oil-48% poly(lactide-co-glycolide)-COOH (PLGA-COOH). The PLGA-COOH microspheres we developed released most of their peptide over a 9 h period in vitro. These D-ala²-GLP-1-microspheres were also effective in delivering therapeutic levels of GLP-1 over an 10 hour period to non-diabetic and diabetic mice, thereby reducing both basal blood glucose levels and the glycemic response to repeated oral glucose tolerance test (OGTT).
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<tr>
<td>5-ASA</td>
<td>5-aminosalicylic acid</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>cyclic-3',5'-adenosine monophosphate</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>COOH</td>
<td>carboxylic acid</td>
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<td>DAG</td>
<td>diacylglycerol</td>
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<td>DCCT</td>
<td>Diabetes Control and complication trial</td>
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<td>DLS</td>
<td>dynamic light scattering</td>
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<td>DP-IV</td>
<td>dipeptidyl-peptidase IV</td>
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<tr>
<td>FFA</td>
<td>free fatty acids</td>
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<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
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<td>GIP</td>
<td>glucose-dependent insulintropic peptide</td>
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<td>GLP-1</td>
<td>glucagon-like peptide-1</td>
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<td>GLP-2</td>
<td>glucagon-like peptide-2</td>
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<tr>
<td>GRP</td>
<td>gastrin-releasing peptide</td>
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<tr>
<td>HPLC</td>
<td>high performance-liquid chromatography</td>
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<tr>
<td>im</td>
<td>intramuscular</td>
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<tr>
<td>IPG</td>
<td>inositolphosphoglycan</td>
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<tr>
<td>IRG</td>
<td>immunoreactive glucagon</td>
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<tr>
<td>iv</td>
<td>intravenous</td>
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<tr>
<td>KRBGD</td>
<td>Krebs-Ringer-2% BSA-Glucose-3% Dextran</td>
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<td>μg</td>
<td>microgram</td>
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<td>μM</td>
<td>micromolar</td>
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<td>NIDDM</td>
<td>noninsulin-dependent diabetes mellitus</td>
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<td>ng</td>
<td>nanogram</td>
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<tr>
<td>OGTTh</td>
<td>oral glucose tolerance test</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PC</td>
<td>prohormone convertase</td>
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<tr>
<td>pg</td>
<td>picogram</td>
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<td>PKA</td>
<td>protein kinase A</td>
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<td>protein kinase C</td>
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<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
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<td>PLGA-COOH</td>
<td>poly(lactic-co-glycolic acid)-carboxylic acid</td>
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<td>RIA</td>
<td>radioimmunoassay</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
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<tr>
<td>sc</td>
<td>subcutaneous</td>
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<td>transmission electron microscopy</td>
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1 Introduction

Compliance with drug therapy is an important component of a patient's health. In the United States alone 10% of hospital admissions are due to a lack of patient compliance (1,2). Poor patience compliance is often a consequence of poor administration of therapeutic compounds, such as peptide and protein hormones, as this has largely been restricted to parenteral routes due to the inherent capacity of the gastrointestinal tract to digest peptide bonds. A number of different approaches have been developed that bypass the digestive system, thereby permitting systemic delivery of peptides and other therapeutic agents (3-7). Some of the most recently developed methods include continuous infusion pumps for peptide hormones such as gonadotropin-releasing hormone (8), insulin (9) and glucagon-like peptide-1 (GLP-1) (10), nasal sprays for peptides including antidiuretic hormone and calcitonin (3,4), and mucosal dosage forms for insulin (11,12). Although these are relatively successful techniques for peptide delivery, these approaches do have various limitations which can lead to poor patient compliance (1,2,13,14). Some of these limitations are due to the invasiveness of the technique used and/or the potential for causing tissue damage at the site of administration [e.g. inhibition of nasal ciliary activity by nasal sprays (15)]. Alternative, less invasive routes of peptide delivery therefore continue to be a focus of investigation.
Oral delivery of drugs has always been the preferred route of administration for most patients. Therefore, it would be advantageous for an oral delivery system for peptides to be developed. Recently, effective methods for encapsulating peptides and other drugs into orally-available, biocompatible polymer-based microspheres have been developed (16-18). One peptide of potential interest for such a method is GLP-1, which has been shown to be useful in the treatment of type II diabetes, predominantly through its actions to stimulate glucose-dependent insulin secretion (19-22). However, there are currently two problems with the therapeutic delivery of GLP-1; its short half-life (23,24) and the requirement for GLP-1 to be present prior to each meal, which currently requires parenteral injections (10,25,26). Therefore, to address these problems, the majority of the work presented in this thesis has focused on testing the biological activity of a newly developed GLP-1 analog and to design an oral delivery system for this peptide. The introduction will thus focus on drug delivery systems, with an emphasis on polymer-based approaches, followed by a brief discussion of GLP-1 and its potential as a treatment for type II diabetes.

1.1 Drug Delivery

Most drugs that are used in medicine today are compounds that are not normally present in the body and that may have adverse effects if not given appropriately. Thus, drug delivery is a very important consideration in the
development of any new therapeutic agent. The most common routes of drug delivery are: parenteral, oral, nasal and lung administration. These methods facilitate drug administration, and most of the delivery systems involved are simple in design. Advanced-drug delivery systems refers to the delivery of drugs using a system that involves aids that help promote bioavailability and/or an increase in the duration of action of the drug. Currently, there are a number of advanced-drug delivery systems on the market or on their way to the market place, including: liposomes (27-29), injectable/implantable polymeric systems (30,31) and transdermal (32,33), and transmucosal drug delivery systems (34-36).

The advantages of improved delivery systems, as outlined by Robert Langer (35), include: 1) maintenance of a continuous level of drug at the appropriate range (this appropriate range can prevent an overdose from occurring); 2) prevention of the harmful side effects of the drugs’ actions on other tissues through targeted drug delivery; 3) reduction of the amount of drug required through targeting of drugs to their site of action; 4) reduction of the number of doses a patient requires through a continuous delivery system, thereby improving patient compliance; and 5) enhancing the ability to deliver drugs with short in vivo half-lives, such as proteins and peptides. In this section I shall give a brief description of each of these advanced-drug delivery systems for therapeutic peptides and then I will focus on the oral delivery of
therapeutic peptides using advanced-drug delivery systems.

Liposomes are a lipid bilayer that can be used to engulf drugs, such as insulin (37,38) and heparin (39), thus protecting the drug until release inside the body. Liposome-drug formulations have been administered by a number of routes, including: injections (29) and orally (27,38). The problem with the oral delivery of liposomes is that they have not yet yielded a high enough bioavailability. There are also problems with variability in the amount of drug absorbed, likely due to the gastrointestinal environment (27,38). Most liposomes are absorbed across the epithelial cells and Peyer's patches, via paracellular and transcellular routes. Improvements on this system have included coating the liposomes with a polymer to protect the drug from gastric acid (40), although, further studies are required to see if this reduces the variability.

To decrease the number of injections a patient requires, a number of injectable and implantable polymeric systems have been developed that release drugs, such as insulin, slowly over time (30). However, these pumps are associated with a number of problems which increase with time, such as postoperative catheter obstruction and pump malfunction (9). On the other hand, newer implantable-degradable drug pellets do not need to be removed after their drug has been dispensed as is the case with implantable pumps.
Such implantable-degradable drug delivery systems are designed to be biocompatible and non-toxic. Some of the compounds used to make the implantable devices include: hydrogels, copolymers of polylactic and polyglycolic acids, polylactic acid, poly(orthoesters), polyanhydrides, poly(ε-caprolactone), and polyurethanes (30,41-43). One problem with these delivery systems, however, is that the pellets still need to be injected or surgically implanted.

Advanced-transdermal drug delivery systems are now being tested to increase the size of molecules that can be absorbed in this fashion and to allow for more non-lipophilic molecules to be absorbed. An increase in the absorption of low-molecular mass molecules has been achieved using electrical approaches such as iontophoresis, which involves low-voltage pulses for long periods of time to deliver the drug (44). To increase the size of the molecules absorbed even further, electroporation (which uses high voltage pulses for a few milliseconds) has been used to delivery heparin to a human model of skin absorption (45,46). Ultrasound has also been used to delivery large molecular mass compounds such as insulin (47).

In the remainder of this section a brief description of some of the transmucosal-delivery systems will be given with a major focus on oral peptide delivery systems.
Buccal delivery of peptides and proteins requires additional factors called permeation enhancers, such as bile salts (11,48-50) and oleic acid (51). With the help of these permeation enhancers insulin (11,50) and GLP-1 (48,52) have been delivered using buccal tablets. It is believed that bile salts act as a surfactant that reduces the hydrophobic barrier to permeation by peptides and proteins (49); they may also act by inhibiting peptidase activity (50). Other permeation enhancers are thought to work in a similar fashion by reducing the hydrophobic barrier to drug absorption. The permeation technique has been used to enhance peptide absorption leading to high bioavailability in the systemic circulation (53,54). An additional advantage of the buccal tablet method of drug delivery is that this route allows for the bypass of hepatic first-pass metabolism and degradation in the stomach and intestines (55,56). The most common problem associated with this delivery system is that the tablet may cause irritation at the site of application, especially after repeated applications (14,55).

Nasal drug delivery has, until recently, been predominantly by aerosolizing lipophilic drugs into the sinus cavity. Current research in the area has focused on increasing drug absorption into the systemic circulation, while at the same time protecting the drug from inactivation by encapsulating it into polymer microspheres (12,57-59). This method of delivery has been used for
a number of peptides such as glucagon, oxytocin and insulin (60). An interesting side note is the recent development of a system that can lead to the absorption of drugs directly into the cerebral spinal fluid via the nasal cavity (61). The problem with these delivery systems, however, is the risk for damaging the mucosal membranes that protect the nasal cavity from infection (55,62). This is of particular concern with the absorption enhancers, which work by opening up gap junctions so that the drugs can be absorbed (55,62).

The problem with current lung-inhalation devices, such as nebulisers, pressurised metered-dose inhalers and dry powder inhalers, is that they are not very efficient in aerosol delivery (only 15-30%) (63). Recent developments in this area have improved drug delivery to about 60% using a non-electrostatic holding chamber and/or an inhaler chamber with the superior hydrofluoroalkane propellant (63,64). These new systems still have problems, such as particle aggregation in the inhaler, drug deposition in the mouth and throat, and/or particle removal from the lungs by the actions of the mucociliary or phagocytic clearance mechanisms. The lungs are also a potential site for the delivery of systemic drugs, such as peptides and proteins, because the lungs have a very thin absorptive mucosal membrane, but this idea is complicated by the complexity of the anatomic structures and the possible influences of the drug on the respiratory tract (65). Some of the
current methods being developed to deliver peptides and proteins into the lungs involve encapsulation into highly porous microspheres, which would protect the drug as it is being absorbed, and which are highly porous to allow for better aerosolization (66).

New advanced rectal-delivery systems have been used to deliver a number of therapeutic peptides, such as insulin (67,68), immunization agents (69) and calcitonin (70). The current problems with delivering drugs in this fashion is patient compliance with drug regimens. On the other hand, vaginal delivery systems are also common and are already being exploited in the delivery of contraceptive drugs which last for up to 6 months (35). Other types of vaginal treatments that have recently been designed include the delivery of antibodies, which have already been shown to prevent sexually transmitted diseases (7).

The oral delivery of drugs is by far the most common method of delivery. The major advantage is the ease in which patients can take oral drugs, thereby allowing for better patient compliance (1,2). The two main problems with the development of these systems is increasing the amount of drug absorbed and maintaining a constant steady release profile. Some of the early experiments in the field involved drug excipient complexes such as emulsions, suspensions or coatings that did not dissolve at a gastric pH (1-5)
but did dissolve at the pH of the small intestines (54,71,72). Some of the more challenging drugs to deliver orally include peptides and proteins, because of their sensitivity to the luminal environment. A number of materials have been used to date to develop oral delivery systems, such as polymers (35,73,73-80), liposomes (27) and drug enteric coatings (54,71,72,81,82).

Oral polymer drug-delivery systems have used a variety of polymer types (16,83-92). Polyester microspheres, such as poly(lactic-co-glycolic acid) (PLGA) have been used, but delivery systems using this polymer have a low uptake of less than 1% by the Peyer’s patches (93). As shown in the present study, modifications to this polymer system can increase the drug absorption rate (see discussion). Some lipophilic polymers have been shown to have higher absorption but most of the materials used in these systems to date are not degradable and therefore need to be removed after the drug has been dispensed (94). Although liposomes have also been used orally, they must be polymerized to prevent destruction by bile acids, thereby allowing for about 3% absorption. Covalently attaching ligands with an affinity for Peyer’s patch M-cells to the surface of liposomes can increase their absorption to about 10% (94). Some of the considerations for the future will be to achieve higher bioavailability and reproducibly for practical use in humans.
1.1.2 Upcoming Modes of Therapy for the Treatment of Diseases

Due to the limitations of current drug delivery systems, there has been intense interest in designing new methods of delivering drugs, some of these methods include xenotransplantation, organogenesis and cell engineering and encapsulation. To alleviate the high demands for organs, scientists have been working to develop new ways to either obtain organs from other species as well as to grow organs from human progenitor stem cells. But both of these areas are still in their infancy and it will be a number of years before any such organs will become readily available. The major source of xenotransplant organs is the pig (although monkeys were initially used, there were fears that they could harbor virogens that could infect humans) (95). There are a number of problems with xenotransplantation, the most important being immune rejection of the organ by the recipient (96,97).

The first embryonic stem cells were isolated from humans and grown successfully in culture in 1998 by Thomson (98). Using these cells Thomson was able to show that they could form a wide variety of recognizable tissues when transplanted under the skin of mice. These embryonic stem cells have the potential to be used to repair disease damaged tissues, and are also being studied for their potential in organogenesis. Organogenesis involves growing an whole organ from a few starter cells but there are a number of problems in organogenesis research, the main one being the design of a
culture system that will allow an organ to grow in a three dimensional manner.

Another technology that may become useful in the near future is the encapsulation of cells that secrete therapeutic compounds as a treatment for disease. Cells with plastic membranes or synthetic polymers that shield the cells from immune attack can be inserted into the body which then can release peptide and/or proteins in a controlled manner (99-108). Such systems could be very useful in the treatment of diabetes via encapsulation of either islets or engineered cells that secrete insulin in a glucose-dependent manner (104-108). However, there are still a number of obstacles to overcome. Such as what type and what source of cells should be used, how to maintain the cells in the body for extended periods of time and how to ensure that the cells correctly secrete the right amount of product in response to endogenous stimuli (99,100,103).

1.2 Complex Polymer Preparations as a Drug Delivery System

Over the years polymers have become increasingly important in the delivery of drugs. The study of polymers for the controlled release of pharmaceuticals, pesticides and other bioactive agents was just beginning in 1974 (35). The problem with the early delivery systems was that the polymers used exhibited poor release kinetics, but over the last few decades
polymers have been developed with improved release characteristics. The additional advantage of these degradable polymers is that many are also biocompatible, thus obviating the need for removal after all of the drug has been released. These developments in polymer chemistry have now allowed scientists to design even more sophisticated systems such as those used for tissue engineering (109,110) and drug delivery (111).

Currently, there are a number of types of polymer-based delivery systems (35), including: 1) a polymer membrane system in which the drug is trapped in a reservoir and is release by diffusion; 2) an osmotic polymer system in which the drug is pumped out through a laser-drilled hole (the pump works by drawing water through a semipermeable membrane into a salt-filled cavity where the drug is encapsulated in a non-permeable membrane; with increasing flux of water into the cavity, the drug is forced out through the hole); 3) a polymeric drug-conjugate system which involves chemical bonding of the drug to the polymer and release by enzymatic or other means of cleaving the drug from the polymer (this system is also called the soluble polymer system because the polymer does not need to be in solid form); and 4) a polymer matrix system in which the drug is evenly distributed throughout a solid mass of polymer, and can be released by a number of mechanisms, the most common being diffusion and/or polymer erosion. In this part of the review, I will focus only on the polymer matrix systems particularly in the form
of micro- or nanospheres with encapsulated drugs.

1.2.1 Types and Methods of Preparing Polymers Used in Drug Delivery Systems

Polymers are chains of molecules connected by chemical bonds. The type of molecules, or monomer units, and the bond type are two important factors in determining a polymer's characteristics including degradation rate. The length of the chain and whether or not there is branching of the chain also determines the nature of the polymer.

Some of the more common monomers used in polymers include lactic acid, glycolic acid, sebacic acid, fumaric acid and poly(ε-caprolactone). Polymers can also be classified on the basis of the bond type used to connect the monomers. Some common polymeric bonds include polyanhydrides, polyketals, poly(ortho-esters), polyacetals, polyesters, polyureas, polycarbonates, polyurethanes, and polyamides (112), and newer bonds including polyphosphoesters (113), polyphosphazenes (114) and pseudopolyamino acids (115). The nature of this bond affects the rate of hydrolysis such that a polyanhydride bond is hydrolyzed in approximately 0.1 hours, while a polyester bond has a hydrolysis rate of 3.3 years and a polyamide bond requires 83,000 years for hydrolysis (112,116). It must be noted, however, that the use of the same bond type with a different kind of monomer can also affect the degradation rate (117-120). There are also a number of
other ways to modify polymer degradation, such as using a combination of monomers in a single polymer as well as changing the length of the polymer, the number of branches, using combinations of bond types, and as discussed later, addition of non-polymeric compounds.

1.2.2 Methods of Encapsulating Drugs into Polymers as a Drug Delivery System

Just as there are a number of ways of modifying the nature of a polymer, there are also variations in both the way in which polymer can be made and the method in which a drug can be encapsulated within the polymer. Some of the methods for encapsulating drugs into polymers include: 1) solvent-evaporation (121); 2) hot-melt encapsulation (122); 3) spray-drying (123); 4) double-walled microsphere preparation (124-126); 5) solvent-removal (127) and 6) microencapsulation by phase-inversion (16). It is important to note here that not all of these techniques will work with every polymer type. As the microencapsulation by phase-inversion technique was used in the present study, only this method will be described here.

The microencapsulation by phase-inversion method has characteristics similar to both the double-walled microsphere and solvent-removal preparation techniques. The polymer and drug are first dissolved into a solvent (if the drug is not soluble in the solvent, it can be simply dispersed in the solvent). This solution is then rapidly poured into a non-solvent resulting
in the spontaneous formation of microspheres. The microspheres are isolated by drying off the solvent and non-solvent. The size of the microspheres can be controlled by modifying the non-solvent to solvent ratio, such that the higher the ratio the smaller the microspheres. This general method was used in the present study to prepare GLP-1-containing microspheres for the treatment of diabetes (16). The advantage of this system is the ease with which microspheres can be prepared with basic laboratory equipment, although the disadvantages include the possibility of solvent and non-solvent residue.

1.3 Diabetes and its Treatment

Glucose homeostasis in the body depends on the balance between glucose production and glucose utilization. Glucose production occurs predominantly in the liver, whereas glucose utilization occurs in insulin-dependent tissues, such as the muscle and adipose, and in insulin-independent tissues such as the brain, kidney and red blood cells (128,129). Although there are a number of other factors involved in glucose homeostasis, it is primarily regulated by the pancreatic islet β-cells, which secrete insulin, and α-cells, which secrete glucagon. Maintenance of glucose homeostasis relies on three important events: 1) during changes in glycemia, the body needs to secrete the appropriate amounts of insulin and glucagon in response to a given glycemic level and to maintain these responses until basal glucose is reached; 2)
once secreted both insulin and glucagon must be able to act on their target tissues (nutrient storage in liver or muscle for insulin, and nutrient output in liver and fat for glucagon); and 3) glucose needs to enter cells independent of insulin's action (often referred to "glucose sensitivity" or "glucose effectiveness") (129,130). In the next few sections I will discuss two of the most common forms of abnormal glucose homeostasis, type I and type II diabetes.

1.3.1 **Type I Diabetes**

Type I diabetes is an immune disease that affects 0.3% of the world's population (131). The major cause of the disease is autoaggressive T cells that infiltrate the pancreas and destroy insulin producing β-cells, leading to a rise in basal glucose levels in the body. The target antigen of the autoaggressive T cells has long been debated, but current research suggest that a single protein expressed in the pancreatic β-cell, glutamic acid decarboxylase (GAD), may be involved in the initial developmental steps leading to type I diabetes (132). It appears that the autoaggressive T cells promote the production of autoantibodies against the pancreatic GAD protein. This likely leads to other pancreatic β-cell-specific autoantibodies eventually resulting in the destruction of all of these cells in the pancreas and, thus, type I diabetes. After the diagnosis of type I diabetes (usually at a young age), these individuals are then dependent upon exogenous insulin for the rest of
their lives. Currently, the major routes of delivery include sc and im injections, although oral, inhaled and intranasal insulin's are under investigation.

1.3.2 Type II Diabetes

In general, type II diabetes is characterized by two pathological defects: 1) peripheral insulin resistance (133-139); and 2) an inability of pancreatic β-cells to secrete the appropriate amount of insulin for a given glycemic state (138,140,141). The earliest detectable defect in type II diabetes is usually an impairment in the body’s ability to respond to insulin (“insulin resistance”) (128,142,143). Insulin resistance occurs in liver, peripheral muscle and fat tissues. In the postabsorptive state hepatic glucose output is normal or increased even though insulin levels are elevated. In the later stages of insulin resistance, exogenous or endogenous insulin fails to suppress glucose production in the liver and glucose uptake in the muscle is reduced. The increased glucose production and output in the liver is largely due to gluconeogenesis. The defects in insulin action in the muscle are the primary reason for the decreased glucose uptake; some of these defects may be due to impaired insulin-receptor tyrosine kinase activity, diminished glucose transport, and reduced glycogen synthase and pyruvate dehydrogenase activities. These defects account for all three disturbances seen in the insulin resistant muscle (e.g. glucose disposal, glycogen synthesis and glucose
oxidation) (142). What causes insulin resistance to begin is likely a multitude of defects including genetic and environmental factors (128,144,145). The genetic factors can be classified into primary and secondary subsets. Primary genetic factors, or "diabetogenic factors", are factors that initiate the diabetic process (128,145). Secondary factors are those that are diabetes-related and result in changes in gene expression; they are not usually unique to type II diabetes and may also occur in type I diabetes (145). Environmental factors include: glucose toxicity, diet, activity level, and possibly environmental toxins (128,142).

Early in the development of type II diabetes, the pancreatic β-cell can usually compensate for the insulin resistance by secreting more insulin, allowing glycemia to remain at relatively normal levels. As time passes, the β-cell is unable to compensate for the increasing degree of insulin resistance, which leads to the development of impaired glucose tolerance and eventually type II diabetes. The exact cause of the "β-cell failure" is unknown, but it is likely that an effect of glucose toxicity in genetically predisposed β-cells is a major factor (138). Some of the important characteristics during the development of β-cell failure are the loss of first-phase insulin secretion, altered pulsatility of insulin release, and an enhanced proinsulin to insulin secretory ratio (142).
1.3.3 Current and Upcoming Methods for the Treatment of Type II Diabetes

Most of the long-term complications of diabetes [e.g. macrovascular disease (146-149), cardiovascular disease (131), hyperlipidemia (150,151), retinopathy, nephropathy, neuropathy and foot ulcers (131)] are a result of poor glycemic control, therefore, it is very important to maintain normal or near-normal blood glucose levels. The importance of good glycemic control has been best demonstrated by The Diabetes Control and Complication Trial (DCCT), which was a large randomized, controlled trial performed in type I diabetics. They showed that by aggressively improving glycemic control one can reduce the long-term complications of macrovascular disease (152). However, similar results to these have also been very recently shown in patients with type II diabetes (153-156).

The first-line of therapy for the prevention of hyperglycemia in type II diabetes is exercise, diet and meal planning (157-159). When these non-pharmacological methods fail, then the second-line of therapy that is most commonly prescribed is the sulfonylureas (160). Sulfonylureas, such as chlorpropamide and glyburide, act primarily by increasing pancreatic β-cell insulin secretion, but as they do so in a glucose-independent fashion, they may cause hypoglycemia (161). Biguanides, such as metformin (162,163), and less commonly α-glucosidase inhibitors, such as acarbose (164), are
also used as the second-line of therapy for the treatment of type II diabetes. Metformin lowers blood glucose by improving peripheral insulin sensitivity and decreasing hepatic glucose output (165), but has some associated gastrointestinal side-effects and can’t be used in patients with renal, hepatic or cardiac failure. Acarbose, on the other hand, works by slowing the digestion of ingested carbohydrates thereby delaying the absorption of glucose into the blood stream, but is also associated with gastrointestinal side-effects (164).

A new oral compound on the market is the thiazolidinediones which were designed to enhance the sensitivity of muscle and adipose tissue to insulin (166). However, this new drug has been associated with a number of side-effects, such as liver function abnormalities, reduced hemoglobin and white blood cell counts (166,167). GLP-1 has also been proposed as a treatment of type II diabetes because of its ability to stimulate glucose-dependent insulin secretion (19-22). GLP-1 also possesses minimal side-effects. Therefore, in the next few sections I will first focus on a review of GLP-1 and then a review of GLP-1 as a treatment of type II diabetes.

1.4 Glucagon-Like Peptide-1

In 1983-1984, the mammalian proglucagon gene was cloned and found to encode glucagon as well as several glucagon-like molecules called GLP-1 and glucagon-like peptide-2 (GLP-2) (168-170). There are three known
tissues that express identical proglucagon mRNA transcripts; the pancreatic α-cells, intestinal L-cells and the brain (171). In each of these tissues, however, the translated product is processed differently, to produce unique products including glucagon in the pancreatic islet α-cells, and GLP-1 and GLP-2 in the intestinal L-cell (172). These peptides appear to be the result of tissue-specific processing by pro-hormone convertases (PCs). PC1 is expressed by the intestinal L-cell, and cleaves proglucagon to liberate GLP-1 and GLP-2 (173). PC2, on the other hand, is expressed in pancreatic α-cells and is likely involved in the generation of pancreatic glucagon (173,174).

Nutritional and hormonal factors regulate the biosynthesis and secretion of GLP-1 in the intestinal L-cell. Nutrients have been shown to directly stimulate the synthesis and secretion of GLP-1 (175), but the mechanism is still unknown. Current studies suggest that, due to the rapid rise in plasma GLP-1 levels after nutrient ingestion and the distal location of the intestinal L-cell in the ileum and colon, the stimulation of GLP-1 release by nutrients is at least, in part, likely regulated indirectly by vagal signal from the duodenum (176). GLP-1 secretion has also been shown to be stimulated by cholinergic agonists, gastrin-releasing peptide (GRP), calcitonin-gene-related peptide, and glucose-dependent insulino tropic peptide (GIP), and inhibited by somatostatin (177-180). In humans, the secretion of GLP-1 occurs in a pulsatile manner throughout the day (181), and increases after meal
GLP-1 has a short *in vivo* half-life of 0.9 minutes (24), and the major enzyme contributing to this short half-life is dipeptidyl peptidase IV (DP-IV) (24,184). DP-IV cleaves GLP-1 at the N-terminal penultimate alanine residue to generate GLP-1(9-36)amide. Neutral endopeptidase 24.11 is also involved in the inactivation of GLP-1 but to a lesser degree (185). GLP-1 clearance by the renal extraction has also been shown *in vitro*, but whether this is physiologically relevant remains to be determined (186).

GLP-1 lowers glycemia by stimulating insulin secretion in a glucose-dependent manner (25,187-192). GLP-1 also stimulates β-cell cAMP formation and insulin gene expression (188,190,193-195), and this increase in insulin gene expression is likely via a cAMP-dependent mechanism (196,197). GLP-1 also acts on glucose-resistant β-cells to make them more glucose-responsive (198). Furthermore, GLP-1 also acts on the pancreatic α-cells to inhibit glucagon secretion thereby further lowering blood glucose levels (199,200). GLP-1 augments its direct inhibition of glucagon secretion by stimulating somatostatin release (which inhibits glucagon secretion as well) (201-203). Support for a direct effect of GLP-1 on the pancreatic α- and β-cells is the localization of the GLP-1 receptor to both of these cell types in the islet (204).
GLP-1 may also act on peripheral tissues, such as the liver, adipose and muscle tissues, to control blood glucose levels, although these peripheral effects are still controversial. In the rat liver, GLP-1 was shown to bind to membrane preparations (205), and to stimulate glycogen synthesis (206-208). In contrast other investigators have shown that GLP-1 does not affect hepatic glycogenolysis, or glycogen synthesis (209,210). Therefore, the exact role of GLP-1 in the liver still remains to be determined. In adipose tissue, GLP-1 binding to adipose membranes has been demonstrated in rats and humans (211,212). In addition, GLP-1 has also been shown to enhance insulin-stimulated glucose uptake in 3T3-L1 adipocytes (213) and in isolated rat adipocytes (214). Finally, in muscle, GLP-1 has been shown to enhance insulin-stimulated glycogen synthesis (215-217). This effect may be mediated, at least in L6 myotubes, by a receptor that is different from the GLP-1 receptor found in the pancreas. In contrast to the cAMP-dependent effects of GLP-1 in the β-cell, the signal transduction mechanism mediating the effects of GLP-1 in muscle may involve inositolphosphoglycan molecules (IPGs) and diacylglycerol (DAG) (217).

Expression of the GLP-1 receptor in peripheral tissues still remains to be determined. GLP-1 receptor expression has been shown by RT-PCR in rat and dog muscle, and fat pad (213,218), but other investigators, using a
combination of techniques such as RT-PCR, RNAse-protection assays, and in situ hybridization, could not detect the GLP-1 receptor in adipose tissue, liver, or muscle (211). Therefore the exact role of GLP-1 in peripheral tissues still remains to be determined.

GLP-1 has also been shown to have other effects such as inhibiting gastric emptying (191,219,219-225) and inhibition of food intake via release within the central nervous system (224,226,227). It is likely that the inhibition of gastric emptying slows food absorption which gives the body time to dispose of the nutrients that it has already absorbed, thereby complementing the actions of GLP-1 on insulin and glucose.

1.5 GLP-1 as a Treatment of Diabetes

1.5.1 GLP-1 and Type I Diabetes

GLP-1 has been shown to lower postprandial blood glucose and the insulin requirements of type I diabetics patients. This effects are likely due, in part, to a reduction in circulating glucagon levels (25,228,229) and a delay of gastric emptying (228,230). Therefore, the B-cell-independent effects of GLP-1 are important in the control of blood glucose levels, suggesting that GLP-1 may be useful in the treatment of type I, diabetes as well as in type II diabetes as discussed below.
1.5.2 GLP-1 and Type II Diabetes

GLP-1 has been shown to inhibit gastric emptying, inhibit glucagon secretion and stimulate insulin release in a glucose-dependent manner in type II diabetic patients (220,231) and, therefore, has attracted much interest for the treatment of type II diabetes. A number of short-term studies that have shown that GLP-1, when administered by either iv or sc injection, can normalize both fasting (232,233) and postprandial glycemia (26,233,234) in type II diabetics. This effect is likely a result of enhancing B-cell function, and inhibiting gastric emptying and glucagon secretion (10,235-237). In addition, sc GLP-1 has been shown to improve glycemic control in type II diabetes in combination with sulfonylureas (238) or insulin (239,240) and even in those with secondary sulfonylurea failure or advanced type II diabetes (241), suggesting that even at the later stages of type II diabetes, GLP-1 still is effective. No study to date shows that GLP-1 improves peripheral insulin sensitivity in type II diabetics (242) or normal healthy individuals (243), but more in depth studies are still required.

If GLP-1 is to be used a treatment of type II diabetes it is likely that it will be required continuously due to its very short duration of action (24). This was best shown in a recent in vivo study in which GLP-1 was infused overnight to type II diabetics, normalizing fasting blood glucose levels. When this infusion was stopped in the morning, the effects of GLP-1 on insulin, glucagon and
blood glucose were lost, suggesting that GLP-1 will be required throughout the day and night for the treatment of type II diabetes (244,245). Currently this can only be done either by continuous infusion of GLP-1 or by frequent injection of GLP-1.

The first long-term study examining the effects of GLP-1 given over an extended period of time in type II diabetic patients was done on patients receiving intensive-insulin therapy. Over a 1 week period patients entered into an intensive-insulin therapy regimen, which was followed by either an additional week on insulin alone or on insulin with GLP-1 at meals. The group receiving GLP-1 required less insulin. In addition, the GLP-1-treated group had reduced postprandial hyperglycemia, although they also had an increased preprandial glycemia, likely due to the short half-life of GLP-1 (239). A second long-term study involving a 6 week double-blind crossover trial, showed that sc GLP-1 prior to each meal over a 3 week period significantly improved postprandial glycemic control in those with poorly controlled type II diabetes (240). A similar study also showed that GLP-1 treatment lowered postprandial glucagon levels and improved postprandial glycemic control, with a significant increase in postprandial insulin levels during the first 30 minutes after a test meal (246). Although these results show promise for the long-term treatment of type II diabetes with GLP-1, the pressing concern to date for such use of GLP-1 is to devise an easier route of
administration. Currently, the only non-invasive method to deliver GLP-1 appears to be the buccal tablet, but there are a number of problems associated with the delivery of drugs in this fashion (see section number 1.1.1.5) (48,247,248). Therefore, the overall goal of the current study was to design an oral delivery system for GLP-1 that is effective in the treatment of type II diabetes.

1.6 Rationale and Hypothesis

1.6.1 Rationale of the Current Studies

1.6.1.1 Study I: Design an Oral Delivery System for GLP-1

GLP-1 has been proposed as a therapeutic agent for the treatment of type II diabetes (249,250) because of its ability to stimulate glucose-dependent insulin release (19-22). However, there are two major problems associated with the administration of this peptide: 1) its short in vivo half-life (24), and 2) the current requirement of parenteral administration (22). The active form of GLP-1 is a 31 amino acid peptide that is inactivated by the actions of DP IV (24,251,252). To circumvent this inactivation we have utilized an analog of GLP-1, D-ala²-GLP-1, that was designed to be resistant to DP IV-mediated degradation. Even with this modification we found that this peptide lost its biological activity within 4 hours of subcutaneous (sc) administration. We therefore set out to design an oral delivery system for this new analog of GLP-1.
Recently, effective methods for encapsulating peptides and other drugs into orally-available, biocompatible microspheres have been developed (16-18). In this thesis, I describe a novel approach to the preparation of orally-available peptides, whereby I have enhanced the ability of a commercially-available PLGA-COOH polymer to deliver therapeutic peptides by the inclusion of a naturally occurring ingestable oil during the preparation of the microspheres by phase-inversion method. Although my initial experiments were to design an oral delivery system for the use of GLP-1 in the treatment of type II diabetes, as will become apparent, this delivery system would also likely work for other peptides and possible other therapeutic chemical compounds.

1.6.1.2 Study II: GLP-1 Sensitivity in Leptin Receptor mutant db/db Mice

During the course of study I, an interesting observation was made with respect to the effects of GLP-1 on glycemia in db/db mice a mouse model of type II diabetes. The db/db mice have a defect in the leptin receptor, a satiety hormone that also appears to inhibit the release of insulin from the β-cell (253-258). Furthermore, evidence is now accumulating to support the possibility of an interaction of the GLP-1 and leptin signaling systems in the β-cell (253,259). Thus, study II involved an examination of the effects of GLP-1 on insulin secretion from db/db mice using an isolated pancreas model.
1.6.2 Hypotheses

1.6.2.1 Hypothesis for Study I
Encapsulation of a long-acting analog of GLP-1 in modified PLGA-COOH will permit the oral delivery of therapeutic concentrations of GLP-1 for the treatment of type II diabetes.

1.6.2.2 Hypothesis for Study II
Insulin secretion in leptin receptor mutant db/db mice is more sensitive to GLP-1 than in non-diabetic mice that do not have any leptin receptor mutations.
2 Methods

2.1 Materials

Petroleum ether and acetonitrile were obtained from Fisher Scientific Ltd (Toronto, ON), gentamicin sulfate, methylene chloride, KOH, acidic boron trifluoride, α-phosphoric acid and triethylamine (TEA) from Sigma Chemicals (St. Louis, MO), human placental Dipeptidylpeptidase IV (DP-IV) from Calbiochem-Novobiochem (La Jolla, CA), poly(DL-lactide-co-glycolide-COOH; 50/50) from Birmingham Polymers Inc. (PLGA-COOH, $M_w \sim 11.5$ kD; Birmingham, AL), glucagon from Eli Lilly Canada Inc (Toronto, ON), diprotin A from Calbiochem (San Diego, CA), GLP-1(7-36)NH$_2$ from Bachem California Inc (Torrance, CA), and Dextran-Texas Red, 3000 MW, lysine fixable from Molecular Probes, Inc (Eugene, OR). D-ala$^2$-GLP-1 and hexenoyl-his$^1$-GLP-1 were a kind gift from Dr. St. Pierre (UQAM, Montreal, QC, Canada). Spurr’s Resin Kit was purchased from Marivac LTD (Halifax, NS), Cryomatrix from Shandon Inc. (Pittsburgh, PA), 3-O-Methyl-D-[1$^-$3H] glucose from Amersham Life Science (Elk Grove, IL), olive oil from Gallo (100% pure olive oil, from Unico Inc., Concord, ON), and Triheptadecanoin from Nu-Chek Prep Inc. (Elysian, MN). EMLA Cream (2.5% Prilocaine and 2.5% lidocaine) and Xylocaine (5%, lidocaine ointment, USP) were obtained from Astra Pharma Inc (Mississauga, ON), and Scintillation fluid (Ultima Gold MV) was obtained from Packard Instruments Inc. (Meriden, CT).
2.2 In vitro Analysis of D-ala$^2$-GLP-1 Degradation by Dipeptidyl-Peptidase IV (DP-IV)

2.2.1 Incubation of D-ala$^2$-GLP-1 with DP-IV

Incubation of 0.125 mU of DP-IV (specific activity = 5000 mU/mg protein) with either 33 μg GLP-1 (control) or 33 μg D-ala$^2$-GLP-1 for 3, 8 or 24 hours was performed at 37°C in phosphate-buffered saline (PBS). The reaction was quenched by addition of 200 μg of diprotin A (251) ??.

2.2.2 Reversed-Phase High Performance-Liquid Chromatography

The elution position of peptide after incubation with DP-IV was compared to peptide that was not incubated with DP-IV and to an internal standard of $^{125}$I-GLP-1 using reversed-phase high-performance liquid chromatography (HPLC) on a µBondapak C$_{18}$ column (3.9X300 mm) (Waters Associates., Milford, MA). The gradient ran from 45% to 85% of solution B (40% solution A and 60% acetonitrile; solution A: 0.1% o-phosphoric acid and 0.3% triethylamine) over 55 minutes (260).

2.3 In Vitro Preparation and Analysis of the Microspheres

2.3.1 Polymer Preparation

Polymers were prepared using a modification of the microencapsulation by phase-inversion method described by Mathiowitz et al (16). In brief, 250 μg of
peptide and different amounts of albumin (depending on the percent loading desired, from 0-18%) were dissolved in 25 µl of ddH₂O in a borosilicate glass tube. In a second borosilicate glass tube, 0-10 µl of olive oil (density 0.91 g/mL) was added (depending on the percent of the total weight required). In a third borosilicate glass tube, a 500 µl solution of PLGA-COOH polymer was prepared (12.5 mg/ml in methylene chloride). The PLGA-COOH solution was then added to the borosilicate glass tube containing the olive oil, vortexed, and added to the ddH₂O solution made earlier in the protocol. This was vortexed for 5 seconds and then rapidly poured into 50 mL of unstirred petroleum ether in a glass petri dish (methylene chloride: petroleum ether ratio of 1:100), resulting in the spontaneous formation of microspheres. This was allowed to air-dry in a fumehood for approximately 3.5 hours and then the microspheres were harvested by scraping off the petri dish with a razor blade.

2.3.2 Size Analysis

2.3.2.1 Transmission Electron Microscopy

Microspheres consisting of only PLGA-COOH were prepared (e.g. microspheres with no peptide, albumin or olive oil) and then infiltrated with Spurr’s Resin and cured for 2 hours at 65°C. Five to ten µm sections were then prepared by the Microscopy Imaging Laboratory, Faculty of Medicine (University of Toronto, Toronto, ON) and observed at 75 kV.
2.3.2.2 Dynamic Light Scattering
A Nicomp 370/Autodilute Submicron Particle Sizer (Pacific Scientific, Instruments Div., Silver Springs, MD) was used for the analysis of 1 mg of 2% D-ala\textsuperscript{2}-GLP-1-50% Olive Oil-48% PLGA-COOH microspheres. Some of the microspheres were probe-sonicated (8–10 seconds at 16 kc/s with a peak amplitude of 5–7 \( \mu \text{m} \)) prior to analysis. The light source in the Nicomp was a HeNe laser beam at 632.8 nm, and the angle of detection was 90°. All analyses were carried out using a Gaussian analysis method.

2.3.3 Analysis of Olive Oil Content
Teflon-silanized coated glass tubes were used to prevent the fatty acids from sticking to the tubes. Three samples of olive oil alone (2.73, 4.55 and 6.37 \( \mu \)g; controls) or 10 mg of PLGA-COOH microspheres with no olive oil, with 50% olive oil or with 48% olive oil and 2% D-ala\textsuperscript{2}-GLP-1 were analyzed using this method. The triglyceride backbone was broken down using 1 M KOH in methanol under \( \text{N}_2 \) gas at 90°C for 2 hours. \( \text{BF}_3 \) (acidic boron trifluoride in methanol) was then used as a catalyst for methylation of the FFA to give FFA-methyl esters. A Hewlett Packard 5890A Gas Chromatograph was used to separate the different types of FFA. To aid in the determination of the amount of each type of FFA in olive oil, an internal standard of triheptadecanoin was added to each sample. Using the amounts of FFA in each sample and knowing the composition of olive oil, the amount of olive oil
in the microspheres was determined in mg of olive oil / mg of microspheres (261).

2.3.4 *In vitro* Analysis of Peptide Release

A known amount of polymer was weighed into a polypropylene tube and then 10 ml of PBS (pH 7.4) with 0.05 mg/ml of gentamicin sulfate was added. The tube was shaken vigorously for 20 seconds and then centrifuged for 5 minutes at 1300Xg. A sample of the supernatant was collected, then the tube was manually shaken for 20 seconds to resuspend the polymer, after which the suspension was incubated at 37°C. Additional samples were taken every hour for the first 9 hours and thereafter at 24, 32, 48, 56, 72 and 80 hours.

2.4 *In vivo* Testing of the Microspheres

2.4.1 Oral Glucose Tolerance Test

Female CD1 mice (6-8 weeks old) obtained from Charles River Canada (Montreal, QC), or female db/+ or db/db mice (7-11 weeks old) obtained from Jackson Laboratories (Bar Harbor, ME) were used for all experiments. Mice were housed under a light/dark cycle of 12 hours, and were fasted for 16-17 hours prior to the day of experimentation. All experiments were initiated between 0900 and 1000. Mice were given 1.5 mg of glucose per gram of body weight orally through a gastric gavage tube (18 or 22G gavage needle) and blood was collected from a tail vein at 0, 10, 20, 30, 60, 90 and 120
minutes. Blood glucose levels were measured with a One Touch Glucose meter (Lifescan Canada LTD, Burnaby, BC). In some mice, at $t=0$ minutes, polymers were administered orally with the glucose using the 22G gavage needle, or intraperitoneally (ip) using a 25G5/8 needle (note: some of the polymer preparations were probe-sonicated for 4-8 seconds at 16 kc/s with a peak-to-peak amplitude of 5-7 $\mu$m, to allow for smooth delivery), or 5 $\mu$g of D-ala$^2$GLP-1 was delivered by subcutaneous (sc) or ip injection with 25G5/8 needle. At the end of the oral glucose tolerance test (OGTT), EMLA Cream or Xylocaine was applied to the tip of the tail. In some mice, OGTT’s were repeated at $t=4$ and 8 hours, after which several of the mice were anesthetized with Halothane (Medical Industries Inc., Markham, ON), and blood was collected by cardiac puncture for radioimmunoassay (RIA) of plasma GLP-1 levels.

2.4.2 Gastric Emptying

Gastric emptying was measured in CD1 mice using 3-O-Methyl-D-[1-$^3$H] glucose (262). The protocol was the same as above for the OGTT except that 5 $\mu$Ci of 3-O-Methyl-D-[1-$^3$H] glucose was added to the glucose solution given to the mice. Blood samples were taken at 0, 10, 20, 30, 60, 90 and 120 minutes to measure blood glucose concentrations, and an additional 4-5 drops of blood were obtained and placed into a microhematocrit capillary tube, sealed at the end with critseal and then centrifuged to collect 5 $\mu$l of
plasma. Plasma was added to scintillation fluid and the cpm determined in a β-counter.

2.4.3 Site of Absorption and Target Organs of the Microspheres

Dextran-Texas Red (2%) was encapsulated into 50% PLGA-COOH microspheres with 48% olive oil, as described above for the encapsulation of peptides. Dextran-Texas Red, 3000 MW, was chosen because it has a similar MW to that of GLP-1. These microspheres were then given to CD1 mice by oral gavage. Tissues (stomach, duodenum, ileum, large intestine, cecum, kidney, liver and spleen) were collected at 2 or 4 hours after administration and frozen at -70°C in Cryomatrix. After 12-14 hours, fresh unstained tissue sections of 15-20 μm in size were prepared using a cryostat. Tissue sections were viewed immediately using a confocal microscope with a tungsten mercury lamp (filter wavelength at 595 nm).

2.5 Perfused Pancreas

Control CD1 (ages 6-8 weeks) or db/db (ages 9-10 weeks) mice were fasted overnight (15-18 hours) and then anesthetized with 80 mg/kg ip sodium pentobarbital. The surgical procedure for the perfusion of the pancreas was similar to that described in (263,264) with a few modifications. In brief, PE 50 tubing from (Intramedic, Parsippany, NJ) was used for the cannulation of the blood vessels and the perfusate was a modified Krebs-Ringer-2% BSA-
Glucose-3% Dextran (KRBGD) solution. The solution was gassed with 95% O$_2$/5% CO$_2$ to achieve a pH of 7.4 and D-ala$^2$-GLP-1 was delivered in KRBGD solution by switching the tubing from a beaker containing no GLP-1 to a beaker containing 1 nM GLP-1. Glucose concentrations were similarly switched between 1.4 mM and 20 mM. The infusion pump was a Gilson Minipuls 2 (France).

The surgical set-up of the perfused pancreas was modified slightly to increase the speed at which the surgery could be performed. In general, the object of the dissection was to isolate the pancreas from its surrounding tissue so that the pancreas and not the intestine was perfused during the experiment and so that fractions could be collected before the perfusate entered the liver. First, a heating pad was used to maintain the body temperature at 37°C and then the small intestine and large intestine were isolated, by ligation of the small intestine 3-5 cm above and below the bile duct and by double tying the mesentery (including the mesenteric artery and vein). The intestine and blood vessels were cut so that the surgical suture material was left inside the body cavity along with 3-5 cm of duodenum, and the remaining intestine was removed from the body cavity. The esophagus was then ligated just above the stomach and cut above the suture. The stomach and attached organs were then detached from the surrounding cavity so that only the hepatic vein, bile duct and celiac axis remained
attached to the remaining internal organs. The portal vein was then isolated and two sutures were tied loosely around the portal vein. A suture was then placed around the aorta just below the celiac axis and tied to prevent any further blood flow past that point. A timer was then set to record the time to initiate the perfusion. The thoracic cavity was opened and the aorta isolated with two sutures, the upper one being tied. A cut in the aorta was placed close to the upper suture and then the aorta was cannulated down to the celiac axis. The lower suture was then tied and the perfusion started. The perfusion was then initiated at a flow rate of 0.5 minutes, and 1 minute fractions were collected. The maximum time allowed for these steps was 8 minutes, although most surgeries were done in 5 minutes. The portal vein was then cannulated so that the perfusate could be collected from the pancreas, and the bile duct was tied off (this was done within 20 minutes after the start of the timer).

2.6 Radioimmunoassays (RIAs)

RIAs for immunoreactive glucagon (IRG) and immunoreactive GLP-1(7-36)amide were carried out (265) using antiserum 04A (Dr. R.H. Unger, Dallas, TX), which cross-reacts with the free C-terminal end of glucagon, and antiserum GLP-1(7-36)amide (Affinity Research, Nottingham, UK), which detects C-terminally amidated forms of GLP-1, respectively. Plasma was prepared for GLP-1 RIA by reversed-phase extraction on a C18 SepPak
(Waters Associates, Milford, MA) and extracts were dried in vacuo prior to assay (265,266). Insulin assays on perfused pancreas were performed as previously described (267).

2.7 Statistics

Statistical significance was assessed by ANOVA using n-1 custom hypotheses tests, or by Tukey's studentized range test, as appropriate, using a Statistical Analysis System program (SAS Institute, Cary, NC). All data are expressed as the mean±SEM.
3 Results of Study I

3.1 Analysis of D-ala\textsuperscript{2}-GLP-1

3.1.1 In vitro Analysis of the DP-IV Resistance of D-ala\textsuperscript{2}-GLP-1
To establish whether the GLP-1 analog, D-ala\textsuperscript{2}-GLP-1, exhibited resistance to the actions of DP-IV, reversed-phase high-performance liquid chromatography (HPLC) was used to compare the degradation of D-ala\textsuperscript{2}-GLP-1 to that of native GLP-1 by DP-IV in vitro. HPLC analysis of D-ala\textsuperscript{2}-GLP-1 showed no DP-IV cleavage over a 24 hour period, whereas native GLP-1 appeared to be cleaved within 3 hours when incubated with DP-IV in vitro (P<0.05, n=3) (Figure 1).

3.1.2 In vivo Analysis of the Biological Activity of this New Analog of GLP-1
To determine whether D-ala\textsuperscript{2}-GLP-1 possessed biological activity in vivo, mice were given a sc injection of saline or 10 \( \mu \)g of native GLP-1 or D-ala\textsuperscript{2}-GLP-1, and their response to an oral glucose tolerance test (OGTT) was determined. Native GLP-1 significantly reduced the glycemic area under the curve (AUC) over a period of 2 hours in comparison to saline (from 337\pm80 to 209\pm29 mM\textbullet{}120 minutes; P<0.05, n=6), and D-ala\textsuperscript{2}-GLP-1 further reduced the AUC significantly in comparison to both saline and native GLP-1 (-5\pm52 mM\textbullet{}120 minutes; P<0.001, n=6) (Figure 2).
Figure 1. High Performance Liquid Chromatography (HPLC) analysis of native GLP-1 and D-ala²-GLP-1 after incubation with DP-IV in vitro. Upper: UV_{214nm} profiles of undigested and DP-IV-treated native GLP-1 and D-ala²-GLP-1 (after a 3 hour digest). I-125 indicates the elution position of the internal standard, $^{125}$I-GLP-1. Lower: Combined HPLC data for the change in elution position with time of native GLP-1 (black bars) and D-ala²-GLP-1 (grey bars) (*P<0.05 vs. native GLP-1 incubated with DP-IV in vitro).
Native (undigested)  D-ala\(^2\)-GLP-1 (undigested)

Native (3h digest)  D-ala\(^2\)-GLP-1 (3h digest)

Change in Elution Position (min)

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<th>Time</th>
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<th>24 Hours</th>
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Figure 2. The effects of GLP-1 and D-ala$^2$-GLP-1 on the glycemic response to oral glucose in mice. **Upper:** Changes in glycemia in response to 1.5 mg glucose/g body weight with PBS (diamonds), 10 μg native GLP-1 (squares), or 10 μg D-ala$^2$-GLP-1 (triangles). **Lower:** Area under the curve (AUC) for the glycemic responses. * P<0.05, ** P<0.01, *** P<0.001 for D-ala$^2$-GLP-1 vs. PBS; # P<0.05 for GLP-1 vs. PBS; and +++ P<0.001 for D-ala$^2$-GLP-1 vs. GLP-1.
3.2 **Designing a Microsphere Preparation for the Delivery of D-ala^2-GLP-1**

3.2.1 *In vitro* Analysis of Peptide Release from Various Preparations of Microspheres

Peptides were encapsulated into microspheres consisting of PLGA-COOH with or without albumin or olive oil and 25 μl of ddH₂O to dissolve the peptide. The degradation rate of PLGA alone is very slow, and it is therefore not considered to be useful in the delivery of therapeutic levels of compounds (268). Consistent with these findings, initial studies using PLGA-COOH microspheres alone showed that the release of encapsulated glucagon was near background levels over 9 hours of incubation *in vitro* (n=2; Figure 3), and did not increase even after 408 hours of incubation (data not shown).

In an attempt to increase the rate of release of peptide from PLGA-COOH microspheres, I tested the effects of incorporating albumin. Using a range of concentrations to obtain up to 60% percent of the total weight of the microsphere with albumin, the best release obtained was only 9.8% of the total glucagon (250 μg of peptide/ 12.5 mg of microspheres) encapsulated within the microspheres over a 9 hour period (Figure 3, only 18% albumin shown; n=2). In a separate series of studies, olive oil was also tested for its effects on the release of peptide from PLGA-COOH microspheres. The addition of olive oil consisting of 30 to 50% percent of the total weight of the
Figure 3. Percent of total peptide released from PLGA-COOH microspheres over a 9 hour incubation period in vitro. Microspheres were incubated in PBS with gentamycin for 80 hours, with hourly sampling for RIA of peptide content in the supernatant. **Background #1 and #2**: 50% PLGA-COOH- 50% olive oil microspheres (n=1); **2% Glucagon**: PLGA-COOH microspheres with 2% glucagon (n=2); **18%alb/2%Gluc**: PLGA-COOH microspheres with 18% albumin and 2% glucagon (n=2); **30%olv/2%Gluc**: PLGA-COOH microspheres with 30% olive oil and 2% glucagon (n=4); **50%olv/2%Gluc**: PLGA-COOH microspheres with 50% olive oil and 2% glucagon (n=5); **50%olv/2%D-ala²**: PLGA-COOH microspheres with 50% olive oil and 2% D-ala²-GLP-1 (n=4); and **50%olv/2%Hex**: PLGA-COOH microspheres with 50% olive oil and 2% hexenoyl-GLP-1 (n=2).
microspheres increased the release of encapsulated glucagon to 45.4 ± 12% (n=4) and 78.5 ± 13% (n=5) of total peptide over a 9 hour period, respectively (Figure 3).

The 50% olive oil-48% PLGA-COOH microsphere preparation was selected for further studies using the D-ala²-GLP-1 and hexenoyl-His¹-GLP-1 analogs of GLP-1. In preliminary studies, we determined that D-ala²-GLP-1 is detected in our GLP-1 radioimmunoassay (RIA) in a similar fashion to that of wild-type GLP-1 (data not shown). When encapsulated within PLGA-COOH microspheres containing 50% olive oil, the release of D-ala²-GLP-1 and hexenoyl-His¹-GLP-1 reached 104 ± 21% (n=4) and 89.2% (n=2) of the total peptide within 9 hours, respectively (Figure 3). The release profiles for 48% PLGA-COOH microspheres containing 50% olive oil and either 2% glucagon (n=5) or 2% D-ala²-GLP-1 (n=4) demonstrated an initial burst phase at about t=1-4 hours, followed by a decline (likely due to loss of peptide via sticking to the test tube or peptide degradation), a second release phase with a peak at t=7-9 hours, and then a further decline over the ensuing 48 hours (Figure 4). A similar profile was observed for hexenoyl-His¹-GLP-1, with the only apparent difference being the absence of the ‘decline phases’. Based upon the release kinetics, microspheres consisting of 50% olive oil- 48% PLGA-COOH- 2% peptide were utilized for all in vivo studies.
Figure 4. Peptide release profiles for 48% PLGA-COOH-50% olive oil microspheres loaded with either 2% glucagon (n=5), 2% D-ala²-GLP-1 (n=4) or 2% hexenoyl-GLP-1 (n=2). Microspheres were incubated in PBS with gentamycin for 80 hours, with hourly sampling for RIA of peptide content in the supernatant. Although these experiments were carried out over an 80 hour period, only the first 48 hours is shown as there was no further release of the peptide after this period.
3.2.2 Size of the Microspheres

Two methods were used to determine the size of the microspheres, both of which gave similar results. The first method was transmission electron microscopy (TEM). These experiments were carried out with microspheres without any peptide or olive oil, giving a diameter of 1.2±0.2 μm (Figure 5; n=3). A Dynamic Light Scattering (DLS) method was also used for determining the size (269,270) of microspheres containing 50% olive oil, 48% PLGA-COOH and 2% D-ala²-GLP-1, which were either sonicated (1.0±1 μm) or unsonicated (2.3±2) (n=4). The size of the microspheres were for the sonicated and μm for the unsonicated.

3.2.3 Olive Oil Content of the Microspheres

Microspheres made of 100% PLGA-COOH had no measurable amount of olive oil; those made of 50% PLGA-COOH and 50% olive oil had 0.22±0.07 mg of olive oil/ mg of microsphere and those made of 48% PLGA-COOH with 50% olive oil and 2% D-ala²-GLP-1 had 0.30±0.02 mg of olive oil/ mg of microsphere (Figure 6). As 0.50 mg of olive oil was added per mg of microsphere during the preparation, this data suggests that 60% of the olive oil added to the preparation was incorporated into the microspheres.
Figure 5. Transmission electron microscopy (TEM) analysis of PLGA-COOH microspheres. These experiments were carried out using microspheres without any peptide or olive oil. The average size of the microspheres was 1.2±0.2 μm in size (n=3).
Figure 6. Analysis of the olive oil content of microspheres. **Polymer:** 100% PLGA-COOH microspheres with no olive oil or peptide (n=3); **Olive Oil alone:** 50% PLGA-COOH-50% olive oil microspheres (n=3); and **GLP-1 & OO:** 48% PLGA-COOH-50% olive oil-2% D-ala$^2$-GLP-1 microspheres (n=3). The line represents the theoretical olive oil content of the microspheres.
Amount of Olive Oil Added to Preparation

mg of olive oil/ mg of microsphere

Polymer  Olive oil alone  GLP-1 & OO
3.3 Effects of D-ala²-GLP-1-Containing Microspheres in Non-Diabetic Mice

3.3.1 Glycemia

When 2.5 mg D-ala²-GLP-1-microspheres (equivalent to 50 µg of peptide at 2% loading) was injected ip into non-diabetic CD1 mice at t=0 hours, followed by repeated OGTT at 0, 4 and 8 hours, the glycemic AUC was significantly reduced as compared to vehicle-injected controls at 0, 4 and 8 hours (from 346±53 to 93±59 mM*120 minutes, P<0.001 at 0 hours; from 424±24 to 219±50 mM*120 minutes, P<0.001 at 4 hours; from 461±29 to 282±49 mM*120 minutes, P<0.001 at 8 hours; n=12 and 6 respectively) (Figure 7).

When 5 µg of D-ala²-GLP-1 was given alone by ip injection at t=0 hours, there was a significant difference as compared to controls only at t=0 hours, but not at any of the other time points (from 346±53 to 161±67 mM*120 minutes, P<0.001 at 0 hours; n=6).

When 12.5 mg D-ala²-GLP-1-microspheres (2% loading; equivalent to 250 µg of peptide) was given orally to mice at t=0 hours, followed by repeated OGTT at 0, 4 and 8 hours, the glycemic AUC was significantly reduced as compared to controls at the 4 and 8 hour time points (from 424±24 to 247±50 mM *120 minutes, P<0.001 at 4 hours; and from 461±29 to 371±53 mM *120 minutes,
To determine the effectiveness of the microspheres in protecting the orally-administered D-ala$^2$-GLP-1, 250 µg of D-ala$^2$-GLP-1 alone was given orally to mice. The glycemic AUC was not significantly affected as compared to controls at any of the time points studied (295±54 mM *120 minutes, at 0 hours; 340±28 mM *120 minutes, at 4 hours; 514±38 mM *120 minutes, at 8 hours; n=9) (Figure 7). To ascertain the approximate amount of bioactive peptide present at each time point, mice were given an ip injection of 5 µg of D-ala$^2$-GLP-1 just before each OGTT (one injection at each of t=0, 4 and 8 hours). The glycemic AUC was significantly reduced as compared to controls at all of the time points studied (from 346±53 to 198±56 mM*120 minutes, P<0.001 at 0 hours; from 424±24 to 128±37 mM*120 minutes, P<0.001 at 4 hours; from 461±29 to 170±32 mM*120 minutes, P<0.001 at 8 hours; n=4) (Figure 7).

Plasma levels of immunoreactive GLP-1 were determined in mice after the 8 hour OGTT (10 hours after oral administration of 48% PLGA-COOH- 50% olive oil- 2% D-ala$^2$-GLP-1 microspheres). In mice given nothing or 5 µg of D-ala$^2$-GLP-1 sc at t=0 hours, plasma GLP-1 levels were 179±45 pg/ml (n=7) and 181±19 pg/ml (n=3), respectively (Figure 8). GLP-1 levels were elevated in mice given ip 50% PLGA-COOH- 50% olive oil microspheres or oral 48%
Figure 7. Area under the curve of the delta blood glucose levels following repeated OGTT in normal CD1 mice at 0, 4 and 8 hours. Peptide alone or microspheres made of either 50% PLGA-COOH-50% olive oil (empty microspheres) or 48% PLGA-COOH-50% olive oil-2% peptide were given at t=0 hours: **First Bar:** empty microspheres given ip (control; n=12); **Second Bar:** 5 μg D-alal-GLP-1 given sc (n=6); **Third Bar:** ip injection of : 48% PLGA-COOH- 50% olive oil- 2% D-alal-GLP-1 microspheres (equivalent to 50 μg of peptide) (n=6); **Fourth Bar:** orally administered 48% PLGA-COOH- 50% olive oil- 2% D-alal-GLP-1 microspheres (equivalent to 250 μg of peptide) (n=9); **Fifth Bar:** 250 μg of D-alal-GLP-1 given orally (n=9); or **Sixth Bar:** 5 μg of D-alal-GLP-1 given at t=0, 4 and 8 hours prior to OGTT (n=4). * P<0.05, *** P<0.001 vs. control mice.
Figure 8. Plasma levels of immunoreactive GLP-1 in mice after the 8 hour OGTT. First bar: empty 50% PLGA-COOH-50% olive oil microspheres given ip (n=7); Second bar: 5 μg D-ala²-GLP-1 given sc (n=4); Third bar: 50 μg D-ala²-GLP-1 administered ip in microspheres (n=6); Fourth bar: 250 μg of D-ala²-GLP-1 given orally in microspheres (n=5); and Fifth bar: 250 μg D-ala²-GLP-1 given orally alone (n=6).
PLGA-COOH- 50% olive oil- 2% D-ala<sup>2</sup>-GLP-1 microspheres (465±124 pg/ml, n=6; and 346±63 pg/ml, n=9; respectively). Plasma GLP-1 levels in mice given 250 μg of D-ala<sup>2</sup>-GLP-1 orally were 381±85 pg/ml (n=9).

3.3.2 Gastric Emptying
To test whether oral administration of 48% PLGA-COOH- 50% olive oil- 2% D-ala<sup>2</sup>-GLP-1 microspheres inhibited gastric emptying, 5 μCi 3-O-Methyl-D-[1-3H] glucose was added to the glucose given at either t=0, 4 or 8 hours to control CD1 mice (n=4-6), mice given an ip injection of 5 μg of D-ala<sup>2</sup>-GLP-1 (n=4), oral 48% PLGA-COOH- 50% olive oil- 2% D-ala<sup>2</sup>-GLP-1 microspheres (n=4-5) or 250 μg of D-ala<sup>2</sup>-GLP-1 alone orally (n=4-6). The results showed no significant effect of the microspheres, or any other treatment, on gastric emptying at any time point (Figure 9).

3.4 Effects of D-ala<sup>2</sup>-GLP-1-Containing Microspheres in a Mouse Model of Type II Diabetes
When 48% PLGA-COOH- 50% olive oil- 2% D-ala<sup>2</sup>-GLP-1 microspheres were given orally to diabetic db/db mice (at 0 hours), followed by repeated OGTT at 0, 4 and 8 hours, the basal blood glucose values were reduced at 4 hours (from 13±1 to 10±1 mM) and significantly reduced at 8 hours (from 12±1 to 8±1 mM, P<0.05, n=6) in comparison to controls, db/db mice given no microspheres (Figure 10). The OGTT response at t=0 hours for mice
Figure 9. Gastric emptying measured by appearance in the plasma of 3-O-methyl-[3H]-glucose (3-O-MG) given during repeated OGTT in normal CD1 mice at 0, 4 or 8 hours. Peptide alone or microspheres made of either 50% PLGA-COOH-50% olive oil (empty microspheres) or 48% PLGA-COOH-50% olive oil-2% peptide were given at t=0 hours. **Diamonds:** empty microspheres given ip (control; n=4-6); **Cross:** 5 µg D-alal²-GLP-1 given sc (n=4); **Triangle:** orally administered 48% PLGA-COOH-50% olive oil-2% D-alal²-GLP-1 microspheres (equivalent to 250 µg of peptide) (n=4-6); **Square:** 250 µg of D-alal²-GLP-1 given orally (n=4-6). **Top:** Results of the 0 hour OGTT; **Middle:** Results of the 4 hour OGTT; and **Bottom:** Results of the 8 hour OGTT.
Figure 10. Basal blood glucose values at the beginning of each OGTT (at t=0, 4 and 8 hours) in diabetic db/db mice. Squares: control mice given no microspheres; and Open circles: mice given 12.5 mg of 48% PLGA-COOH-50% olive oil-2% D-ala²-GLP-1 microspheres (* P<0.05, n=9).
treated with the D-alα₂-GLP-1 microspheres were not distinguishable from controls. At t=4 and 8 hours however, the OGTT curves were markedly different, with the curve for the diabetic mice receiving D-alα₂-GLP-1 microspheres being shifted downward (Figure 11). Determination of the absolute glycemic AUC (AUC calculated including basal blood glucose values) indicated that the D-alα₂-GLP-1 microspheres significantly reduced the glycemic response as compared to controls, at the 4 and 8 hour time periods (from 2589±105 to 1921±138 mM*120 min, at 4 hours, P<0.001; and from 2460±152 to 1835±88 mM*120 min, at 8 hours, P<0.001; n=6) (Figure 12a). In addition, the delta glycemic AUC (AUC independent of the initial basal blood glucose value) was also significantly reduced in comparison to controls at the 4 hour time point (from 1000±140 to 714±49 mM*120 min, P<0.05; n=6) (Figure 12b).

3.5 Route of Absorption and Target Organs of the Microspheres

Dextran-Texas Red (2%) was encapsulated into 48% PLGA-COOH microspheres with 50% olive oil and given orally to CD1 mice. No fluorescence was seen in tissues from mice treated with microspheres containing no Dextran-Texas Red. Diffuse fluorescence was found 2 and 4 hours after administration in the duodenum, ileum, liver, kidney and spleen of mice given 250 μg of Dextran-Texas Red alone orally, and in mice given microspheres containing Dextran-Texas Red. However, there was markedly
Figure 11. Glycemic response following repeated OGGT at 0, 4 and 8 hours in db/db mice. Mice were treated at t=0 hours with nothing (controls, diamonds; n=9) or with 12.5 mg of orally administered 48% PLGA-COOH-50% olive oil-2% D-ala^2-GLP-1 microspheres (squares; n=9).
Figure 12. AUC for the results shown in figure 11. A: Values expressed as the absolute AUC (area under the curve of the values including the basal blood glucose values). B: Values expressed as the delta AUC (area under the curve of the values independent of the basal blood glucose values) (dAUC). **Black bars:** *db/db* mice receiving no microspheres (controls); and **grey bars** *db/db* mice given 48% PLGA-COOH-50% olive oil-2% D-ala^2^-GLP-1 microspheres. * P<0.05, *** P<0.001 vs. controls.
increased fluorescence in the microsphere-treated mice as compared to mice receiving the 250 μg of Dextran-Texas Red alone (Figure 13a and b).

4 Results of Study II

As the glycemic response to 48% PLGA-COOH- 50% olive oil- 2% D-ala\textsuperscript{2}-GLP-1 microspheres in \textit{db/db} mice decreased the delta AUC (363±49 mM*120 minutes; Figure 12) to a significantly greater extent than that observed for CD1 mice (177±50 mM*120 minutes; Figure 7) (P<0.05), it was hypothesized that \textit{db/db} mice may be more sensitive to the effects of GLP-1 than CD1 mice. To determine the response of \textit{db/db} mice to D-ala\textsuperscript{2}-GLP-1, \textit{db/db} mice or their heterozygote littermates (7-9 weeks of age) were given 0, 5, 10 or 25 μg of \textit{ip} D-ala\textsuperscript{2}-GLP-1 and an OGTT was performed. The \textit{db/db} mice had elevated basal, peak and final blood glucose levels as compared to their heterozygote littermates (basal glucose of 3.0±0.1 and 7.1±1.5, P<0.05; Peak glucose of 10.3±1.3 and 24.6±2.3, P<0.001; final glucose level 3.6±0.8 and 17.2±1.8, P<0.05; CD1 and \textit{db/db} mice respectively) (Figure 14).

However, the glycemic response to glucose was reduced for both \textit{db/+} and \textit{db/db} mice by increasing doses of \textit{ip} D-ala\textsuperscript{2}-GLP-1 (Figure 14). When the AUC was calculated for the mice given vehicle alone, the \textit{db/db} mice had an AUC of 2522±223 mM*120 minutes as compared to 768±68 mM*120 minutes on their lean littermates (Figure 15). D-ala\textsuperscript{2}-GLP-1 dose-dependently
Figure 13. Tissue fluorescence after administration of Dextran-Texas Red alone or 48% PLGA-COOH-50% olive oil-2% Dextran-Texas Red microspheres. Row A: duodenum; Row B: ileum; Row C: spleen; and Row D: liver. Column 1: shows representative light microscopy pictures for each tissue (100X); Column 2: shows the results from mice 2 hours after being fed 250 µg of Dextran-Texas Red alone; Column 3: shows the results from mice 2 hours after being fed 48% PLGA-COOH-50% olive oil-2% Dextran-Texas Red microspheres; and Column 4: shows the results from mice 4 hours after being fed 48% PLGA-COOH-50% olive oil-2% Dextran-Texas Red microspheres.
Figure 14. Glycemic response of db/db mice and their heterozygous littermates (db/+) (7-9 weeks of age) to an OGTT after an ip injection of 0, 5, 10 or 25 µg of D-ala²-GLP-1. A: db/+ mice (n=4); and B: db/db mice (n=4??). **Diamonds**: 0 µg; **squares**: 5 µg; **triangles**: 10 µg; and **circles**: 25 µg. * P<0.05.
Figure 15. AUC for the results shown in figure 14. A: *db/+ mice; and B: *db/db mice.* $P<0.05$ vs. 0 μg controls.
decreased the AUC of both the \textit{db/db} and their heterozygote littermates (Figure 15). The 5 µg dose decreased the \textit{db/db} mice AUC by 40±6% as compared to only a 24±2% for their heterozygote littermates (P<0.05). Further suggesting that the \textit{db/db} mice may be more sensitive to GLP-1. It is noted however, that another possibility is that GLP-1 could simply be exerting a glucose-dependent effect in the \textit{db/db} mice due to their abnormally high basal glucose levels. To control for the difference in glycemia, which are prominent, we therefore used a perfused pancreas model which allowed us to measure the insulin response to D-ala$^2$-GLP-1 infusion at the defined level of glycemia.

Basal insulin secretion (1.4 mM glucose) was lower in CD1 than in \textit{db/db} mice, (Figure 16). Increasing glucose to 20 mM stimulated a biphasic insulin response in the pancreas from CD1 mice, but had only a marginal effect on insulin secretion from the \textit{db/db} pancreas (Figure 16). D-ala$^2$-GLP-1 stimulated a further insulin response in both CD1 and \textit{db/db} pancreas when given in combination with high glucose (20 mM). When the D-ala$^2$-GLP-1 perfusion was stopped, insulin secretion fell in both CD1 and \textit{db/db} mice (n=4 and n=6, respectively) (Figure 16).

When the data were analyzed as AUC for high glucose and D-ala$^2$-GLP-1 as compared to high glucose alone (Figure 17), D-ala$^2$-GLP-1 was found to
Figure 16. Perfused pancreas from CD1 and db/db mice with and without 1 nM D-ala²-GLP-1. The pancreas was perfused with LOW glucose (1.4 mM) for 5 minutes; HIGH glucose (20 mM) for 10 minutes; HIGH glucose±1 nM D-ala²-GLP-1 for 20 minutes; HIGH glucose (20 mM) for 10 minutes; and LOW glucose for 10 minutes over a 55 minute period. Top: Response of CD1 mice (n=6). Bottom: Response of db/db mice (n=4-6). Circles: - D-ala²-GLP-1; and Squares: + 1nM of D-ala²-GLP-1. Values were corrected for pancreatic insulin content.
Figure 17. Fold increase in insulin secretion induced by high glucose + 1nM D-alα²-GLP-1 as compared to high glucose alone, in CD1 and db/db mice (values determined from figure 16). **Black bars:** CD1 mice; and **grey bars:** db/db mice. * P<0.05 vs. CD1 mice.
increase insulin secretion by 1.5±0.5 fold in CD1 mice and by 3.0±0.6 fold in
$db/db$ mice ($n=6$ and $n=4$ respectively). Each phase of the insulin secretion
was then separated into two parts, with the first phase of the insulin secretion
being the first 10 minutes and the second phase being the last 10 minutes.
When this was done the second phase of the insulin secretion was increased
by 1.5±0.5 fold in CD1 mice, and by 3.8±0.7 fold in $db/db$ mice ($P<0.02$)
(Figure 17). This data suggests that the pancreatic $\beta$-cell in the diabetic $db/db$
mice is more sensitive to GLP-1, as compared to non-diabetic CD1 mice, at
the same level of glycemia.
5 Discussion

5.1 Study I: Development of an Oral Delivery System for GLP-1

Delivery of therapeutic peptides to date, has predominantly involved invasive means (e.g. sc, im, iv injections) that may not be optimal with respect to patient compliance (13) and may cause tissue damage (3,8,13,14). This is particularly true for peptides with short half-lives that require repeated administration. A number of less invasive strategies for peptide delivery have also been reported in the literature, including buccal, nasal, transdermal and vaginal administration (3-6,48). The limitations of peptide delivery have led us and others (271-274) to re-examine the oral route of delivery, using a variety of methods to protect the peptides from degradation by gastric acid and gastrointestinal proteases. The results of the present study indicate that encapsulation of GLP-1 in microspheres composed of 48% PLGA-COOH, 50% olive oil and 2% peptide permits delivery of bioactive GLP-1 through the oral route in non-diabetic mice and diabetic mice.

The major contributor to the relatively short half-life of GLP-1 (0.9 minutes) (24) is the enzyme DP-IV, which cleaves the first two amino acids (His\(^1\)-ala\(^2\)) from the N-terminus of GLP-1, thus inactivating the peptide (184,251,252). To overcome this short half-life, Dr. Serge St. Pierre from the University of Quebec in Montreal, synthesized an analog of GLP-1 that was designed to
be DP-IV-resistant. We have shown that this novel GLP-1 analog, D-ala$^2$-GLP-1, is resistant to DP-IV cleavage over a 24 hour period in vitro (figure 1) and is more potent than native GLP-1 in lowering glycemia in vivo (Figure 2). It has been shown by our group that D-ala$^2$-GLP-1 does not exhibit enhanced GLP-1 receptor binding or activation (275). Since D-ala$^2$-GLP-1 did not exact any effects on gastric emptying, at least at the doses used in the present study (Figure 9), the enhanced effects of D-ala$^2$-GLP-1 in vivo are therefore likely due, at least in part, to its ability to: 1) resist DP-IV degradation; and 2) stimulate insulin secretion (figure 17). Therefore, this peptide may potentially be useful in the treatment of type II diabetes. However, even with the resistance to DP-IV cleavage, the biological activity of D-ala$^2$-GLP-1 was lost within 4 hours after administration to mice (Figure 7), thus still necessitating repeated injections of the peptide if it is to be used as a treatment of type II diabetes.

To avoid the need for repeated injections of GLP-1, we have designed a novel oral delivery system for this peptide using a based upon a method similar to that developed by Mathiowitz et. al. (16). The polymers that Mathiowitz et. al. used for drug encapsulation consisted of poly(fumaric acid):PLGA or poly(fumaric acid)/poly(sebacic acid):PLGA, all of which are biodegradable and biocompatible. Using this method to encapsulate insulin, Mathiowitz et. al. (16) has been able to deliver bioactive insulin orally to rats.
These results suggested that polymers similar to these could be used to deliver GLP-1 orally. More specifically, we hypothesized that oral delivery of GLP-1 in modified PLGA-COOH microspheres may be a useful technique for delivering therapeutic levels of GLP-1 to type II diabetics.

Although a number of investigators have utilized PLGA and related polymers for oral peptide delivery (13,16,276), several novel aspects of the present study warrant discussion. First, no previous study has reported on the use of PLGA-COOH as the base polymer for microsphere preparation. The addition of a -COOH group to the polymer was done in the view of recent data suggesting that such an end group decreases the passage of microspheres through the gastrointestinal tract, thus increasing their chance of being absorbed (277-279). PLGA has been shown to be taken up into the systemic circulation (16,276), and its degradation products are non-toxic (17), although this has not been established for PLGA-COOH. Second: as we and others (280,281) have demonstrated (Figure 3), the use of PLGA or PLGA-COOH alone does not permit release of peptide within a time frame that is reasonable for peptide therapeutics. The preferred time frame for the delivery of GLP-1 to type II diabetics would be over a 9-12 hour period because patients could rely on a single dose to maintain therapeutic levels of GLP-1 throughout the day.
Thus, in an attempt to increase the rate of release of peptide from these PLGA-COOH microspheres, the PLGA-COOH polymer was combined with other biocompatible compounds, including albumin and olive oil. While both are non-toxic, olive oil is also soluble in methylene chloride, but not in petroleum ether, which are important considerations for the preparation of microspheres. Albumin had only a marginal effect on increasing the rate of release of peptide \textit{in vitro} (Figure 3). The addition of increasing concentrations of olive oil, on the other hand, up to 50% of the total weight of the microspheres, clearly increased the ability of peptide to be released from the microspheres; approximately 70-100% of the peptide was consistently recovered within 9 hours of incubation \textit{in vitro}, regardless of the peptide being tested. Interestingly, despite the excellent recovery of peptide during these incubations, microspheres were still clearly visible, even after 17 days of incubation (data not shown). These findings suggest that the major mechanism of release of peptide from these microspheres is via diffusion, rather than through degradation and/or bulk erosion, as reported for other polymer preparations (282,283). Finally, although the recovery of immunoreactive peptide appeared to decrease with time \textit{in vitro}, we speculate that this occurred due to non-specific adsorption of the peptide to the tube walls and/or to the surface of the microspheres during incubation.

As no other study has previously reported on the use of olive oil in their
polymer preparation or any other commercially available ingestable oils for that matter, these novel findings clearly require further testing. There are two studies to date that have used oleic acid, a major constituent of olive oil (70%), to affect polymer function. In one study (284), a solution of 6% oleic acid did not enhance the absorption of polystyrene microparticles into the lymph of rats and, in fact, decreased their absorption as compared to saline in a closed intestinal-loop model. What is interesting is that lecithin (another lipid delivery vehicle) did marginally enhance the absorption of polystyrene microparticles in this model, suggesting that this lipid could be used as a substitute for olive oil in our microspheres. In another study, oleic acid, and other low molecular weight compounds, were applied to the surface of poly(ε-caprolactone) and PLGA (280). The rate of PLGA hydrolysis was increased with oleic acid application suggesting that the intrinsic reactivity of the glycolate linkage in PLGA was modified by this process.

Third: what is unique about our experiments in comparison to previous studies using oleic acid is that we have used olive oil as an integral component of our microspheres as opposed to a means of delivery or as a topical application. Support for olive oil actually being incorporated into the microspheres comes from our experiments using gas chromatography (Figure 6). Also, in view of the study (280) showing that oleic acid increases the rate of degradation of PLGA, it is likely that the addition of olive oil also
increased the rate of degradation of our PLGA-COOH microspheres, possibly from the inside out, thus allowing for an increase in the release of peptide (as seen in our in vitro release studies; Figures 3 and 4). Also the olive oil may diffuse out of the microsphere itself, which could leave behind a number of holes throughout the remaining parts of the microsphere, allowing for peptide to be released by diffusion.

One disadvantage of adding olive oil is that it may decrease the rate of absorption across the gastrointestinal tract (284), which may explain why there was only a small effect of our microspheres as compared to the amount of peptide actually administered in the microspheres. Therefore, substituting olive oil with a commercially available oil that has lecithin in it may improve the absorption of our microspheres. It is also feasible to substitute any other oil that can be ingested orally in an effort to enhance the absorption of our microspheres, but these possibilities still remain to be tested. Enhancement of the absorption of the microspheres can also be done by adding other compounds that actively work to increase absorption by binding to sites along the gastrointestinal tract (285-287,287-289), such as conjugating lectin molecules to the PLGA-COOH polymer (286,290,291). Lectin has been previously shown to improve the absorption of some types of microspheres (286,290,291), and may therefore be useful as part of our microsphere preparation.
In non-diabetic mice the duration of action of a single dose of \textit{sc/ip} D-ala$^2$-GLP-1 was extremely short (<4 hours) and orally administrated D-ala$^2$-GLP-1 (250 µg) was biologically inactive. When the D-ala$^2$-GLP-1-microspheres were given orally, however, this permitted the delivery of bioactive peptide that resulted in a significant reduction of the area under the curve in response to OGTT at t=4 and 8 hours, but not at t=0 hours. In addition, plasma levels of immunoreactive GLP-1 levels were elevated after 10 hours in the mice given \textit{ip} and oral microspheres, providing some evidence for the release of GLP-1 from the microspheres over the course of the experiment. Unexpectedly however, oral peptide alone also elevated plasma GLP-1 levels after 10 hours, but this did not result in any changes in the OGTT responses at any time point suggesting that this peptide was biologically inactive.

The amount of biologically active peptide at t=4 hours in mice treated with oral microspheres containing a total of 250 µg of D-ala$^2$-GLP-1 was approximately 5 µg of peptide (determined by comparing the response of oral microspheres at t=4 hours to a 5 µg \textit{ip} injection given at t=4 hours; Figure 7). Therefore, in these mice the microspheres permitted the oral delivery of therapeutic levels of peptide over a 10 hour period equivalent to repeated injections of 5 µg of D-ala$^2$-GLP-1 (representing approximately 1-2% of the total peptide given in the microspheres at each of t=4 and 8 hours). It must
be noted, however, that the possibility of a slow continuous release mechanism precludes estimation of the total bioavailability at the present time.

The apparently low bioavailability of bioactive GLP-1 seen in the mice receiving the D-ala²-GLP-1 microspheres may also be due, in part, to gastric acid digestion of the microspheres and the peptide within them. If this is the case then a mechanism to protect the microspheres from gastric acid may be advantageous. Such a mechanism could involve an enteric coating (71,72), such as shellac or cellulose acetate phthalate (CAP) (54). CAP has been used as a protective enteric coating since 1940 (81), because of its resistance to gastric acid. Raising the pH to 6-7.5, however, such as occurs in the intestinal lumen, results in the slow disintegration of the CAP coating. Furthermore, CAP is also likely degraded by the actions of luminal esterase (292). Therefore, if necessary using a protective compound such as CAP may be useful in protecting our microsphere preparation from the actions of gastric acid.

There are four proposed sites of absorption of microspheres, which include the villus tips, intestinal macrophages, enterocytes and Peyer's patches (293). The mechanism of absorption at each of these sites dictates the size of particles that can be absorbed. The proposed mechanism of absorption
across villus tips is persorption, which allows for particles up to 150 \( \mu m \) in size to be absorbed. In contrast, intestinal macrophages can absorb <1 \( \mu m \) particles by phagocytosis, enterocytes can absorb <200 nm particles by endocytosis, and Peyer’s patches can absorb <10 \( \mu m \) particles by trans- and/or paracellular means. Although all of these sites likely play role, the major site of absorption of most microparticles, including PLGA microspheres of 1-5 \( \mu m \) in size, has been suggested to be Peyer’s patches (276,294,295).

In addition, other studies have shown that microspheres that are <5 \( \mu m \) in size are suitable for absorption across the gastrointestinal tract (276,293,296,297). The microspheres that we produced were about 1 \( \mu m \) in size (after sonication; Table 1), suggesting to us that they were a suitable size for absorption across the gastrointestinal tract. If the major site of microsphere absorption is the Peyer’s patches then for an effect to be seen one must wait until the microspheres reach the ileum where the majority of the Peyer’s patches are located (298-303), suggesting one possible reason why there was no effect of our microspheres at the 0 hour time point. After absorption, PLGA microspheres have been found in the lymph nodes, spleen, kidneys and liver (276). Furthermore, consistent with these findings, our microspheres were found in the duodenum, ileum, spleen, kidney and liver at 2 and 4 hours after administration, as determined by encapsulating Dextran-Texas Red into our microspheres (figure 13).
In vivo studies with D-ala\textsuperscript{2}-GLP-1-microspheres clearly indicated that the peptide was released into the circulation of the mice and retained its biological activity when delivered through the ip and oral routes of administration. An ip injection of D-ala\textsuperscript{2}-GLP-1-microspheres permitted the delivery of bioactive levels of peptide that resulted in a significant reduction of the area under the curve in response to OGTT at t=0, 4 and 8 hours (Figure 7). The time course of action for these effects differed markedly from that of non-encapsulated peptide, such that a much more prolonged duration of action was observed for the D-ala\textsuperscript{2}-GLP-1-microspheres (for at least 10 hours). The effectiveness of this peptide over the entire 10 hour period tested is ideal for the delivery of a therapeutic peptide that is required throughout the day, although clearly both the total duration of action and the dynamics of the peptide release from the microspheres in vivo still remain to be completely established.

Of some note was the finding that, when injected ip, the D-ala\textsuperscript{2}-GLP-1-microspheres induced an immediate fall in glycemia during the 0 hour OGTT (Figure 7). This was somewhat unexpected, and suggests that some of the peptide may have not been encapsulated within the microspheres, but rather, likely remained as free peptide coating the outside of the microspheres. As compared to the effects of sclip 5 and 10 μg D-ala\textsuperscript{2}-GLP-1 alone at t=0 hours, the decrease in the glycemic response with ip D-ala\textsuperscript{2}-GLP-1
microspheres was similar to that observed with the lower load. This suggests that, of the 50 μg of peptide that was administered in the ip injected microspheres, less than 10% (5 μg) was not encapsulated within the microspheres. Although this free peptide could be eliminated by washing the microspheres prior to administration, the peptide is not likely to be biologically active when the microspheres are administered orally, due to the low pH of the gastric acid and the proteolytic actions of digestive enzymes of the small intestine. This supposition was borne out by the data obtained for oral administration of the microspheres, whereby the biological actions of the D-ala²-GLP-1 were not observed prior to the 4 hour OGTT. Supportive evidence that most of the peptide was indeed encapsulated within the microspheres is the fact that the Dextran-Texas Red did not change the color of the water added to a 2% Dextran-Texas Red microsphere preparation (data not shown), as would be expected if a good proportion of the molecules were not encapsulated within the polymer.

In addition to non-diabetic mice, D-ala²-GLP-1-microspheres were also given to db/db mice, a model of type II diabetes. db/db mice have a leptin receptor mutation that results in hyperphagia, hyperglycemia, hyperinsulinemia and obesity (304-306). The association of hyperglycemia and hyperinsulinemia in these mice is a similar characteristic found in type II diabetes (305). There was no difference seen between control db/db mice and db/db mice treated
orally with D-ala\(^2\)-GLP-1-microspheres at \(t=0\) hours, as expected. However, the response to orally administered D-ala\(^2\)-GLP-1-microspheres at \(t=4\) and \(8\) hours was a downward shift in both basal and stimulated glycemia as compared to control mice. Therefore, in addition to being able to deliver bioactive D-ala\(^2\)-GLP-1 orally to non-diabetic mice, we were also able to deliver therapeutic levels of peptide over a 10 hour period in a model of type II diabetes. These results suggest that this technique for orally delivery of peptides may be useful in the treatment of type II diabetes and possibly other disorders requiring the continuous presence of therapeutic peptides.

5.2 Study II: GLP-1 Sensitivity in Leptin Receptor Mutant \(db/db\) Mice

The perfused pancreas studies have shown that D-ala\(^2\)-GLP-1 stimulates insulin secretion from the islets of both non-diabetic CD1 mice and diabetic \(db/db\) mice. In addition, the \(\beta\)-cell of \(db/db\) mice appeared to be more sensitive to D-ala\(^2\)-GLP-1 independent of glycemia, as compared to those of the CD1 mice (Figure 16). The increase in GLP-1 sensitivity in \(db/db\) mice may be explained by an interaction of the leptin and GLP-1 receptor signaling pathways in the pancreatic \(\beta\)-cell. This possible interaction has been suggested by a number of investigators (253,255,258,259,307), but the exact mechanism has yet to be determined. In the next few paragraphs, a brief summary of some of the studies aimed at determining this interaction will be discussed, followed by a focus on how this data explains my results.
Leptin has been shown to inhibit insulin secretion and gene expression in pancreatic β-cells. These effects are supported by the fact that the leptin receptor is expressed in islet β-cells derived from mouse, hamster and rat pancreas (254,308). Leptin is able to reduce proinsulin mRNA levels after they are increased by GLP-1 during both euglycemia and hyperglycemia (255,258), is able to inhibit GLP-1-stimulated insulin release from the isolated perfused rat pancreas (253-255) and also reverses hyperinsulinemia in leptin-deficient ob/ob mice (258). These results suggest that the lack of leptin or a lack of leptin signaling in response to leptin, called “leptin resistance”, may be involved in the pathogenesis of diabetes in mice.

How leptin exerts its inhibitory effects on both insulin secretion and insulin gene expression are not understood (258). Under GLP-1-stimulated and hyperglycemic conditions (16.7 mM), intracellular Ca^{2+} levels rise in the β-cell; this rise can be rapidly reduced by leptin (253-255), suggesting that the ability of leptin to modulate insulin secretion may be mediated by decreasing cytosolic Ca^{2+} levels. Leptin can also activate phosphodiesterase 3B, leading to a marked inhibition of GLP-1-stimulated insulin secretion (259). This effect of leptin was abolished when insulin secretion is induced with cAMP analogues that cannot be hydrolyzed by PDE3B and when selective inhibitors of PDE3B and PI-3-K are used (259). However, others have shown that
GLP-1-induced cAMP generation remains unchanged after leptin exposure (253), suggesting that further studies are still required to determine the exact role of PDE3B. A third possible mechanism whereby leptin inhibits insulin secretion is by activating the ATP-sensitive K⁺ channel, which would prevent membrane depolarization and the subsequent stimulation of insulin secretion (255). Therefore, GLP-1- and hyperglycemia-stimulated insulin gene expression in pancreatic β-cells may be inhibited/reduced by a leptin-induced decrease in cytosolic Ca²⁺ or cAMP levels, and/or by activating the ATP-sensitive K⁺ channel. Therefore, the loss of leptin signaling, as seen in the db/db mouse, may increase the sensitivity to GLP-1 in the β-cell. The results presented in this thesis provide evidence for this hypothesis with the demonstration that GLP-1 sensitivity is greater in the leptin receptor mutant mice.

Interestingly, the converse side of this story may be true; as leptin appears to be more potent in GLP-1 receptor knockout mice (GLP-1R -/- mice) (307). Pre-treatment with leptin improves glucose tolerance and the glycemic response to an OGTT in GLP-1R -/- mice and this effect is not seen in GLP-1 receptor +/- mice (307). Although not looked at directly, leptin also appears to inhibit glucose-stimulated insulin release to a further degree in leptin-treated GLP-1R -/- mice as compared to GLP-1R +/- mice (307). Therefore, the greater effects of leptin on glucose and insulin in leptin-treated GLP-1R -/-
rnice provides support for the possibility that disruption of GLP-1 signaling modifies the sensitivity to leptin in vivo (307). In combination, therefore both of these studies present evidence that support the hypothesis that GLP-1 and leptin signaling pathways converge in the pancreatic β-cell to control insulin release.

5.3 Future Directions

Future experiments may involve testing the microspheres in larger animals, such as fa/fa diabetic rats. These rats are a model of type II diabetes, due to a leptin receptor mutation similar to that in db/db mice (306,309). In chronically cannulated fa/fa rats, measurements of plasma GLP-1, insulin and glucose levels over an extended period of time could be performed in response to oral microspheres containing D-ala²-GLP-1, thus permitting exact determination of bioavailability.

Additional types of ingestable oils may be useful to further increase the absorption of the microspheres. Another possibility would be to conjugate molecules such as lectin to our microspheres, that may also increase the absorption of the microsphere preparation. With improved absorption of microspheres this method will be extremely effective in orally delivering therapeutic levels of peptides.
Finally, the mechanism underlying the enhanced GLP-1 sensitivity in db/db mice remains to be determined. This may be accomplished by perfusion of the pancreas from db/db mice with downstream stimulators of the leptin receptor signaling pathway, to determine whether this modulates the insulin response to GLP-1, and at which level.

5.4 Summary and Conclusions

In summary, the results of this study have shown that D-ala\(^2\)-GLP-1 is resistant to DP-IV degradation in vitro and, because of this resistance, D-ala\(^2\)-GLP-1 is more potent than native GLP-1 at lowering the glycemic response to oral glucose in non-diabetic mice. We have designed a delivery system for therapeutic peptides using PLGA-COOH and olive oil to produce microspheres that are about 1 \(\mu\)m in size and that release most of their peptide within a 9 hour period in vitro. These microspheres are able to lower the glycemic response to repeated OGTT over an 10 hour period when administered orally to normal and diabetic db/db mice. The decrease in the glycemic response seen in these mice is likely due to the ability of D-ala\(^2\)-GLP-1 to stimulate insulin release, as shown using a perfused pancreas model.

In conclusion, oral delivery of therapeutic peptides can be accomplished through a novel approach to encapsulate peptides within biocompatible
PLGA-COOH microspheres containing olive oil. Although only a few peptides were tested in the present study, the approach is clearly feasible for delivery of a wide variety of bioactive peptides for the treatment of type II diabetes and, possibly, other disorders requiring the continuous presence of therapeutic peptides.
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