IN VITRO SIMULATION OF *IN VIVO* PERFORMANCE OF ORAL DOSED NANOPARTICULATE INSULIN

by

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Abstract

The aim of this study was to evaluate the factors, which affect insulin release and stability in simulated gastrointestinal fluid that ultimately will reduce the bioavailability of insulin in a nanoparticulate oral delivery system. The focus was on nanoparticulate carriers developed by C. Reis, which have the highest level of bioavailability reported in the literature thus far.

Particles observed by TEM were spherical and particle analyzer data showed that the peak of the distribution was 10 nm. Entrapment efficiency of insulin was 85%. Insulin retention/release was evaluated in both enzymatic and enzyme-free simulated digestive fluids. HPLC measurement showed that insulin was stable in acid condition in presence and absence of pepsin. By changing the pH to 6.8 in an intestinal simulation, the amount of insulin decreased such that at the end of the 5 h simulation, 71% of the insulin was measureable in simulated GI fluid in presence of enzymes.

Insulin release profile from nanoparticles was low in gastric condition. After changing the pH to 6.8, an initial release of insulin occurred in the first 1h followed by plateau state in the remaining 2h. After a total of 5h in acidic followed by neutral pH medium, the formulation retained 48% of the insulin in the particles in simulated GI fluid in presence of enzyme. As confirmation of the amount of retained insulin, after 5h, particles were dissolved and the formulation was shown to fully retain up to 45% of the insulin in extended simulated gastrointestinal condition in the presence of enzymes.

Insulin release behavior was investigated in different simulated small intestinal media by incorporating phosphate buffer or bicarbonate buffer and physiological electrolytes. The release rate from particles in the phosphate buffer was faster compared to bicarbonate buffers. KBB-C showed a release profile that was very different from other media, with about 10% released in the first 30 min and 70% of the insulin remaining entrapped within the particles at the end of the experiment. Release in this buffer was reduced due to the decreased sodium to calcium ratio compared to the other KBB media.

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Chapter 1

Introduction

1.1. Insulin

Insulin is a peptide hormone, naturally synthesized and stored in the pancreas. It has become one of the most extensively used therapeutic proteins. Insulin was first isolated by Frederick Banting and Charles Best in Canada in the 1920's (Best and Scott, 1923) and commercial production began soon after. The insulin extraction protocol was developed by Banting who initially isolated it from a dog pancreas, but later it was produced through extraction from the pancreas of mainly pigs, but also cows. Human recombinant insulin from yeast or bacteria presently on the market was developed by two leading pharmaceutical companies, Eli Lilly and Novo Nordisk (Sinding, 2002).

Insulin controls the glucose level in blood through regulating carbohydrate and fat metabolism. Insulin controls the glucose level by signaling the liver, muscle and fat cells to absorb excess glucose. The absorbed glucose is stored as glycogen in the liver and muscles, and as triglycerides in fat cells. When blood glucose levels fall below a certain level, the body begins to use the stored glycogen as an energy source by breaking down the glycogen in the liver and muscles into glucose.

1.2. Diabetes

Diabetes mellitus (DM) or diabetes is a chronic metabolic disease leading to high blood sugar and/or deficiencies of insulin in the body, either because the pancreas does not produce enough

insulin, or because cells do not respond to the insulin that is produced. Long-term complications from high blood sugar can include heart disease, stroke, diabetic retinopathy affecting eyesight, kidney failure requiring dialysis, and poor circulation in limbs that may lead to amputation.

Type 1 diabetes or insulin dependent diabetes (IDDM) affects 10% of diabetic patients (Canadian Diabetes Association 2012). It is an autoimmune disease resulting in damage to insulin producing beta cells and failure of the pancreas to produce enough insulin, leading to increased blood sugar (Raj et al. 2003 and Reis et al. 2009). Patients depend on external insulin for therapy.

Type 2 diabetes or non-insulin dependent diabetes (NIDDM) is the more common form of the disease and covers about 90% of cases (Reis et al. 2009; Raj et al. 2003). It results from failure to properly utilize insulin, or producing insufficient insulin. Type 2 diabetes is managed by increasing exercise and dietary modification in the first stage. If blood glucose levels are not adequately lowered by these measures, it can be controlled using hypoglycemic drugs, insulin therapy or a combination (Reis et. al 2009). Hypoglycemic drugs alone can be effective in managing the condition, but patients may eventually require insulin if other medications and diet failed to control blood glucose levels adequately. Over 40% of patients require insulin as part of diabetes management (Gowthamarajan 2003).

Type 3 diabetes occurs when pregnant women without a previous diagnosis of diabetes develop a high blood glucose level. This type of diabetes may lead to type 2.

Diabetes has become one of the five main causes of death in many countries. It is estimated that the worldwide prevalence of diabetes in adults was 6.6% (285 million people) in 2010, which is projected to increase to 7.8% (438 million people) by 2030. Based on Canadian

Diabetes Association data (2012), more than 9 million Canadians (28% of the population) live with diabetes or prediabetes state and the number of people with type 2 diabetes is increasing dramatically due to factors such as age, obesity and inactive lifestyles.

Diabetes is a major health concern, as it can cause a substantial economic burden on society besides its health impact. In 2009, the Canadian Diabetes Association (CDA) estimated that the cost of diabetes in Canada will rise from $6.3 billion annually in 2000 to $16.9 billion in 2020. A large portion of this cost is related to complications such as limb amputations, blindness, kidney failure, cardiovascular disease and disability (Zimmet 1999). Therefore it is clear that diabetes will be a growing economic burden and extensive efforts are needed to find ways to prevent an increase in the number of patients with diabetes and more effectively treat those already affected.

1.3. Insulin therapy difficulties

Parenteral dosage form of insulin is the only form available on the market, since insulin discovery. In spite of its prevalence, there are several concerns about this mode of administration including injection anxiety, pain, cost, infection, and an overall decrease in patient compliance compared to other, more widely accepted methods of drug administration such as oral, transdermal, or bucal (Cefalu, 2004; Khafagy et al. 2007).

The average insulin dependent diabetic requires approximately 5-6 insulin treatments daily to regulate blood glucose levels, but most patients take just 2-3 injections (Sadrzadeh et al. 2007). As a result, patients are exposed to extended periods of hyperglycemia, which in long term can causes diabetes complications such as blindness, lower limb amputations, heart disease and kidney failure. Moreover, in type 2 diabetic patients, other hypoglycemic medications have failed to control blood glucose levels adequately. Convincing these patients to start insulin

administration is difficult because of the inconvenience related to injections (Raj et al. 2003). The market has responded by developing insulin pens and different insulin formulations, but in reality, patient compliance still remains a significant issue.

This has encouraged researchers to search for alternative and non-invasive delivery routes such as transdermal (Sintov et al. 2007), nasal (Pringels et al. 2006), pulmonary (Todo et al. 2001), rectal (Onuki et al. 2000) and oral administration routes (Damgé et al. 2007; Owens et al. 2003; Sadrzadeh et al. 2007). Unfortunately in spite of extensive research in this field, these methods of administration have had limited success. The only alternative mode of administration that has reached the market was a pulmonary delivery device manufactured by Pfizer (Exubera), which was withdrawn from market after complaints related to its side effects (coughing, shortness of breath, sore throat and dry mouth), and inability to deliver precise insulin dose resulting in insulin overdose (Mathieu et al. 2007).

Among the non-invasive delivery routes of administration, the oral route is preferred due to its simple, non-invasive and convenient administration (Pringels et al. 2006; Sintov et al. 2007). An oral dosage form of insulin would greatly improve the quality of life for millions of diabetics, especially children and the elderly due to ease of administration and not having to self-inject, or use injection equipment (Chalasani et al. 2007; Belminet al. 2003)

There is no commercially available product on the market today, despite the benefits of administrating insulin as oral form. Development of an oral formulation requires overcoming complications, such as low gastrointestinal permeability of large molecules (Sadrzadeh et al. 2007), insulin hydrophilicity, and inactivation or rapid enzymatic degradation in the gastrointestinal tract (Hamman et al. 2005). These unfavorable physicochemical conditions

present enormous challenges in the design of an oral delivery vehicle. Different strategies have been studied for improving bioavailability of oral insulin. Most of the research and strategies developed have been designed to overcome these challenges through various chemical and formulation approaches (Raj et al. 2003).

Chapter 2

Literature Review

2.1 Oral insulin delivery

Insulin is administered parenterally in the treatment of diabetes mellitus. Unfortunately, injections are painful, and can lead to low patient compliance. Amongst the possible non-invasive delivery routes, such as buccal, nasal, rectal and pulmonary, the oral form is preferred due to simplicity and convenience of dosing and administration. (Pringels et al. 2006; Sintov and Wormser 2007; Chalasani et al. 2007).

The liver is the key site of glucose regulation and homeostasis, controlling glucose uptake and release into circulation under different conditions. Oral administration has the same effect of the physiological pathway of insulin, since insulin is absorbed from the GI tract, entering the hepatic portal vein and going directly to the liver enabling glucose homeostasis, (Raj et al. 2003). Oral administration of insulin would greatly improve the treatment of diabetes, and prevent peripheral hyperinsulinemic effects caused by insulin injections, such as hypertension and atherosclerosis (Agarwal and Khan 2001; Reis et. al. 2009).

The main advantages of oral administration are patient comfort and improved lifestyle, as it is noninvasive, convenient, and easily dosed (Reis et al. 2006 and 2009). Despite these advantages, formulation of an oral delivery system is difficult, as there are many challenges that need to be addressed before developing a successful delivery system.

The main obstacle preventing a successful oral product has been insulin's low bioavailability in the gastrointestinal tract (Sadrzadeh et al. 2007; Reis et. al. 2006). Principal factors associated

with low bioavailability are susceptibility to acidic, proteolytic and hydrolytic degradation and poor absorption across the intestinal mucosa (Hamman et al. 2005). The large molecular size relative to metabolites, the electrical charge and hydrophilic characteristic also limits absorption from the GI tract (Cui et al. 2006; Jung et al. 2000).

The combination of these factors limits insulin permeability through the epithelial cells in the GI tract, and presents severe challenges in the design of an oral delivery structure. The main principles for developing an oral formulation is overcoming obstacles such as low gastrointestinal permeability (Sadrzadeh et al. 2007), insulin hydrophilicity, and providing sufficient protection to preserve its biological activity from the acidic environment of the stomach, and rapid enzymatic inactivation or degradation, in the gastrointestinal tract (Hamman et al. 2005).

2.2 Approaches for effective oral insulin delivery

Many methods have been studied for the purpose of improving the bioavailability of orally administered insulin, and preserving insulin physiological activity during the formulation and delivery pathway. Most of the research and strategies developed have been aimed to overcome the challenges through various chemical and formulation strategies (Raj et al. 2003).

Oral strategies for optimizing insulin pharmacological and pharmaceutical properties can be summarized as follows: (Khafagy et al. 2007; Woitiskiet al. 2008)

1. Chemical modifications of insulin (Damgé et al. 2007; Dave et al. 2008)
2. Mucoadhesives: Chitosan (Reis et al. 2008)
3. Enzyme inhibitors (Liu et al. 2003)
4. Absorption enhancers: cyclodextrin (Shao et al. 1994), bile salts (Mesiha et al. 2002), surfactants (Eaimtrakarn et al. 2002), peptides (Morishita et al. 2007)
5. Particulate delivery systems (Khafagy et al. 2007)

Researchers have attempted to modify or attach certain groups to insulin to increase its solubility and stability against gastrointestinal enzymes (Damgé et al. 2007; Dave et al. 2008). For example, Emisphere company developed a method for insulin oral delivery, by attaching non-acyl amino acids to insulin that causes unfolding of insulin's structure and exposing the hydrophobic groups on the surface which promotes insulin penetration from the lipid layer of the GI tract (Sadrzadeh et al. 2007). After permeation, the complex structure is removed and the insulin returns to its basic structure.

Mucoadhesive carriers increase the residence time at the surface of the absorptive epithelial cells, providing a high localized concentration of insulin and improving uptake. The drawback of mucoadhesive carriers is that they may be affected by mucous turnover. Polymers such as polycarbophil and chitosan have bioadhesive properties and could improve the oral absorption of insulin (Bernkop-Schnurch et al. 2004; Gowthamarajan and Kulkarni 2003; Krauland et al. 2004). Chitosan facilitates insulin uptake by modification of intestinal permeability and opening the tight junctions between epithelial cells (Behrens et al. 2002).

Oral administration of insulin and other protein-based drugs is challenging due to the gastric proteolytic enzymes in the GI tract. Aprotinin which is a trypsin inhibitor can enhance absorption of insulin through the GI tract (Ziv et al. 1987). Acarbose as an intestinal α-glucosidase inhibitor (Katavoich and Meldrum 1993) and FK- 448 which is an inhibitor of chymotrypsin (Fujii et al. 1985) have shown positive effects in protecting the biological activity of insulin.

Utilization of enzyme inhibitors with the drug delivery system has negative points due to the inhibition of digestive enzymes, which can cause incomplete digestion or the inhibitory action can cause an increase in the secretion enzymes by a feedback regulation that causes several side effects (Peppas and Kavimandan 2006).

Absorption enhancers facilitate the intestinal uptake of insulin through epithelial membranes. For example, bile salts and fatty acids increase the permeability of the lipid membrane of epithelial cells to insulin, by disrupting the tight junction between adjacent cells (Carino et al. 1999). Also coadministration of insulin with oligoarginine, has shown improvement in insulin intestinal absorption without damage to cellular integrity. Arginine is a positively charged amino acid, which improves permeation by interacting with the negatively charged proteoglycan on the membrane surface (Morishita et al. 2007). Components such as surfactants, citrates, or ethylene diamine tetra acetate (EDTA) can act as absorption promoters (Shao et al. 1994).

The main problem with absorption enhancers is that they cannot specifically improve the target drug absorption, but will enhance absorption of negative factors such as viruses and intestinal microorganisms. In addition, absorption enhancers can disrupt the cell membrane structures, requiring more studies and research before application (Reis et al. 2009).

Among the various strategies for improving oral delivery of insulin, the use of nanoparticulate carriers show promise (Cui et al. 2006; Alonso 2004).

2.3 Particulate Delivery Systems

Encapsulation is a technique in which an active ingredient is enveloped within a particle of a different substance, such as a polymer or phospholipid. This method is used in the pharmaceutical industry and micro or nanoencapsulation refers to the integration of the active material into micron or nano-sized particles respectively.

The main reason for encapsulating an active ingredient is for controlling the release profile and pharmacokinetics (absorption, distribution, metabolism, and excretion) of a drug. In addition, encapsulation is used to enhance drug stability, reduce drug toxicity and health risks, and mask an unpleasant taste or odor (Peniche et al. 2003)

The target for drug delivery and release profile can be controlled through carrier selection, formulation optimization and particle engineering (Issa et al. 2006). For example, chitosan is a polymer that can increase the permeability of particles by opening the tight junctions and promoting mucoadhesivity (Ventura et al. 2008).

Nanoparticle is a term that includes nanospheres and nanocapsules. Nanospheres have a matrix structure where the drug can be released from the surface, or be entrapped within the particle.

Nanocapsules have a membrane type structure, in which the drug can be encapsulated in the particle core and/or be adsorbed on the polymeric membrane as is illustrated in Figure1.

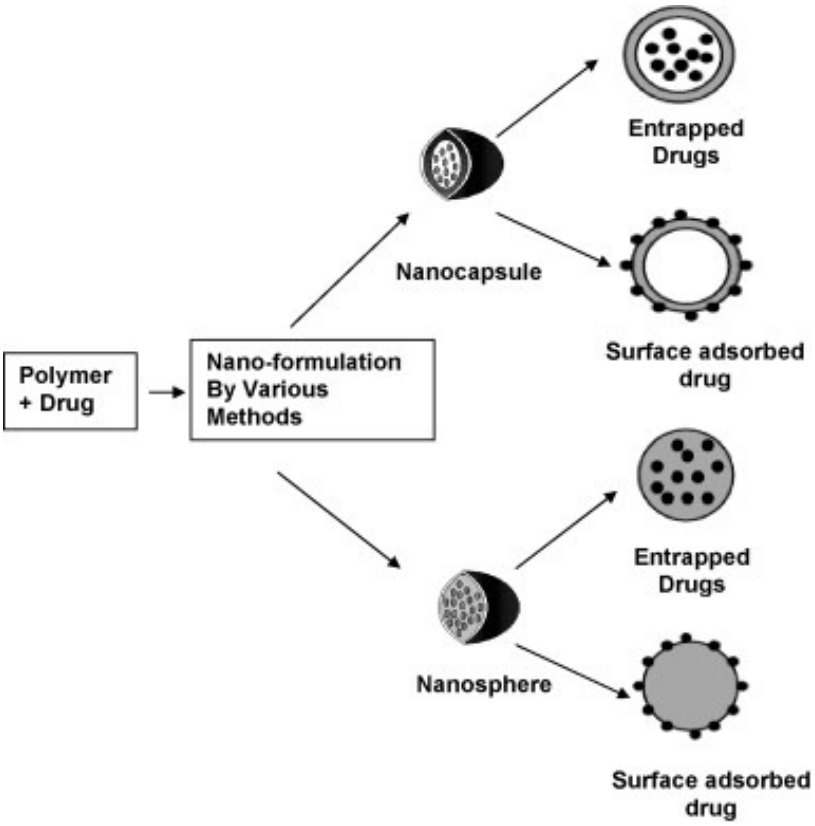

Figure 1. Different types of nanoparticles. Active ingredient is pictured by black dots that can adsorb on the surface or be encapsulated into the nanoparticles (Kumari et al., 2010)

2.4 Polymeric encapsulation strategies for insulin

A number of researchers have developed different types of nanoparticle based oral delivery systems for insulin since Damgé et al. developed the pioneering idea in 1988. Polymeric encapsulation of insulin is a significant advance to oral insulin delivery systems and was found to be effective in prolonging insulin release, preserving the biological activity against enzymes in the GI tract and facilitating insulin permeability and uptake via the epithelial cells in the GI tract (Reis et al. 2006; Schechter et al., 2005).

Different types of polymers are applicable for drug delivery purposes such as natural, semi-synthetic or synthetic. Depending on the polymer combinations, nanoparticles can stabilize insulin, control release profile, increase oral bioavailability and preserve biological activity (Bies et al. 2004; Ré, 1998).

It has been determined that the size of the particle has an important effect on absorption. In oral delivery systems through the GIT, submicron diameter particles have been demonstrated to increase absorption rates by 10-250 times in comparison to larger particles (Chen et al. 1998). Particle morphology and charge can also affect absorption rates. Generally, it has been shown that hydrophobic particles absorb more readily than hydrophilic molecules. Hydrophilic neutral and positively charged particles have a higher affinity for epithelial cells than negatively charged particles, and are consequently more readily taken up (Reis et al. 2006).

The most recent progress in nanotechnology applied to insulin, is toward the use of natural polymers, particularly polysaccharides such as alginate, chitosan and dextran (Tewa Tagne et al. 2006). The advantages of natural materials include biocompatibility, biodegradability, natural abundance, and unique chemical structures (Augst et al. 2006; Orive et al. 2005; Dang et al. 2006). The problem with natural polymers is in the polymer's fixed structures that limit flexibility in application, and ability to modify properties of the molecule (Chan et al. 2009).

Table 1 is a summary of natural polymer based nanoparticles developed for insulin. A wide range of natural polymers have been used, but various forms of alginate, chitosan and dextran appear frequently. The highest bioavailability reported was based on a formulation developed in the Neufeld lab at Queen's University involving insulin within an alginate-dextran core, coated by chitosan-PEG, then by albumin. (Reis et al. 2007 and 2008).

Among different strategies found in this table for formulating oral insulin nanoparticulate delivery systems, emulsification/internal gelation and ionotropic pregelation will be detailed in the following sections.

Table 1. Summary of insulin natural polymer based particles for oral administration (Sonia and Sharma 2012)

Polymer	Size (nm)	Zeta (mV)	BA (%)	Reference
Alginate crosslinked dextran sulfate poloxamer coated albumin	396	-36.6 to 44.5	13	Woitiski et al. 2010
Alginate–dextran sulfate core, chitosan–polyethyleneglycol-albumin coated nanospheres	<1842	-7 ± 4	42 10*	Reis et al. 2007
Calcium phosphate-PEG-insulin-casein (CAPIC) particles	600		-	Morçöl et al. 2004
Alginate–dextran–chitosan	750		6.8 3.4*	Sarmento et al. 2007
Chitosan–TBA (thio-butyl-amidine)			1.69±0.42	Krauland et al. 2004
Thiolated trimethyl chitosan	100-200	+12 to +18	-	Sandri et al. 2007
Chitosan–polyglutamic acid	218		15.1	Mi et al. 2008
Chitosan–polymethylmethacrylate	150-280			Qian et al. 2006
Lauryl succinyl chitosan	315-1090			Rekha and Sharma, 2009
Dextran sulfate–chitosan	500	-20.6	5.6 3.4*	Sarmento et al. 2007
Vitamin B12–dextran sulfate–chitosan	192		26.5	Chalasani et al. 2007
Chitosan	25-400		14.9	Pan et al. 2002
Aminoalkyl Vitamin B12 dextran sulfate chitosan	150-200		29.4	Chalasani et al. 2007

* Bioavailability (BA) reported for different insulin dose levels

Bioavailability is the portion of an administered dose reaching the systemic circulation and becoming available to the target tissue.

2.4.1 Emulsification/internal gelation as an insulin encapsulation method

Alginate is a natural polymer that forms a network structure in the presence of calcium ions. It has received attention in drug delivery systems due to characteristics that produce a crosslinked network in the presence of divalent cations which can provide sustained release particulate systems for a variety of drugs, proteins and even cells (Raj and Sharma, 2003).

One of the methods for production of these drug delivery systems is emulsification/internal gelation as illustrated in Figure 2. Emulsification/internal gelation is an alternative encapsulation technique, proposed to replace extrusion/external gelation for producing small diameter alginate microspheres in large quantity (Poncelet, 2001). The diameters of alginate particles usually produced by external gelation are typically between 2-5 mm with a low production rate (Poncelet et al. 1995), but considerably smaller diameters can be produced using emulsion dispersion.

An emulsion dispersion is formed by high speed mixing of an alginate solution containing ultrafine calcium carbonate and drug ingredient, within an oil or solvent phase, facilitated by an emulsifier. After emulsification, gelation of the dispersed alginate is initiated by internal release of solubilized calcium from carbonate complex through pH reduction from 7.5 to 6.5. (Poncelet et al. 1995 and 2001). Calcium ions cross-link the alginate, entrapping the drug in the core of the particles (Alexakis et al. 1995; Quong et al. 1996).

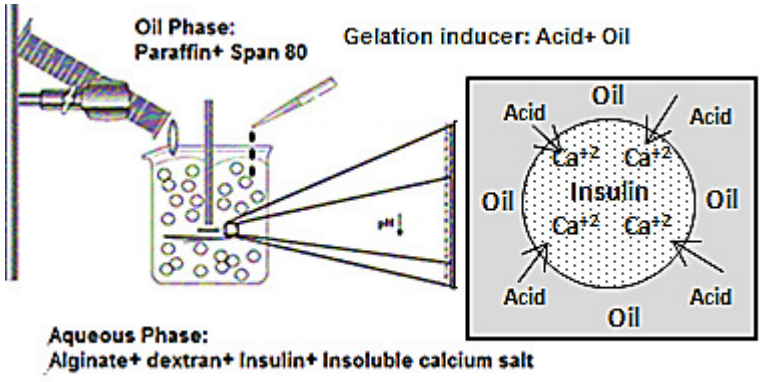

Figure 2. Process of emulsification–internal gelation using alginate. (modified from Reis et al. 2006)

This method is simple and cost-effective, providing the ability to produce small spherical particles on a large scale, with good mechanical stability (Esquisabel et al. 2000) and encapsulation efficiency. Particle size can be controlled by monitoring the conditions under which the water-in-oil emulsion is produced (Poncelet 2001). The method can have potential biological, biomedical and food related applications (Poncelet et al. 1992). The internal gelation method has scale-up potential in production of large quantities of small and controlled size alginate micro (Poncelet et al. 1992) and nanoparticles (Reis et al. 2008). Porosity could be a limiting factor for internally gelled particles, where higher permeability can induce loss or inactivation of drug in comparison to externally gelled particles (Quong et al. 1998).

2.4.2 Ionotropic pre-gelation/ polyelectrolyte complexation

The ionotropic pre-gelation method is based on the ability of polyelectrolyte polymer to crosslink in the presence of counter ions to form polyelectrolyte complexes (PEC) or particulate matrix (Sarmento et al. 2006). Natural polyelectrolytes such as alginate/dextran sulfate (polyanions) contain anionic structures that form a reticulated structure by crosslinking with polyvalent cations such as calcium, inducing gelation (Patil et al. 2010).

Particles are initially produced by dropping drug-alginate solution in to calcium chloride (polyvalent cation) solution with gentle mixing. Polyelectrolyte complexation is then used for improving the stability and permeability of particles by adding another polyelectrolyte with opposite charge. An example of complexation is dropwise addition of chitosan (polycationic) solution to stabilize the pregel nuclei (alginate/dextran crosslinked with Ca^{+2}), forming nanoparticles (Woitiski et al. 2009). Ionotropic gelation is a simple and environmentally friendly pharmaceutical technique since toxic solvents are not used.

2.5. Biopolymers used in insulin encapsulation

2.5.1 Alginate

Alginate is an ideal biodegradable polymer for insulin encapsulation due its availability and chemical and physical properties. It is a natural anionic polysaccharide derived from brown algae. Alginate particles can be formed by various methods including extrusion into calcium chloride solutions, emulsification/internal gelation,

ionotropic pregelation, and spray drying (Coppi et al. 2002; Reis et al. 2006; Sarmento et al. 2006).

Alginate gel is relatively permeable and has low retention capacity for small molecules. The low retention capacity is due to an open pore reticulated structure that depends on the extent of swelling and constriction of the alginate gel (Reis et al. 2007). There have been numerous efforts to reduce the pore size and extend drug release from alginate such as coating with polycationic polymers or blending alginate with other polymers such as dextran to improve its retention capacity.

2.5.2 Dextran sulfate

Dextran sulfate is a biodegradable and biocompatible polyanionic polymer, with a polysaccharide backbone and negatively charged sulfate groups. It has been extensively used as an effective matrix material for controlling release of drugs and as a stabilizing agent (Janes et al. 2001).

Alginate gel is relatively permeable and has low retention capacity. Adding dextran sulphate minimizes loss of the entrapped drug (insulin) in alginate particles. In simulated GI medium, insulin release was prevented at gastric pH when dextran sulphate was added to the alginate, but insulin was rapidly released upon transferring to intestinal medium at pH 6.8 (Reis et al. 2007). It seems that in the presence of dextran, alginate forms a more compact structure that reduces permeability and additionally protects insulin from acidic conditions (Reis et al. 2007). At neutral pH (6.8) both alginate and insulin are negatively charged, leading to electrostatic repulsion and promoting insulin release from the particles (Reis et al. 2007).

It seems that alginate and dextran sulphate in the particle core are not sufficient for preventing insulin release at neutral pH. As a result, other components would need to be used in developing an effective oral delivery system such as adding a coating material like chitosan.

2.5.3 Chitosan

Chitosan is a nontoxic, bioabsorbable and natural polysaccharide based polymer. It has been studied for the controlled release of several drugs as a coating material due to its polymeric cationic characteristics which promotes its interaction with negatively charged molecules or polymers (Draget et al. 1994).

Chitosan is often applied to alginate particles through polyelectrolyte complexation, for improving retention of drug in the porous network structure of alginate. Adding chitosan to alginate particles forms a stable and tight complex membrane coating that improves encapsulation by preventing release of insulin through the less permeable chitosan membrane (Reis et al. 2007). Moreover, chitosan has mucoadhesive properties, which improve adhesion to the mucosal surface facilitating uptake of drug through opening tight junctions between epithelial cells (Vila et al. 2001; Dodane et al. 1999).

2.5.4 Bovine serum albumin (BSA)

BSA particle coating has been proposed as a sacrificial target for gastric and intestinal proteases. (Reis et al. 2008). Albumin coating has the potential to stabilize insulin within the particle core and provide protection to the entrapped insulin from intestinal proteases in the gastrointestinal tract (Reis et al. 2008). Albumin is negatively charged at pH 5.1,

thus will be attracted to positively charged chitosan, producing a more compact polymeric layer that may provide a better protection from acid and proteases (Reis et al. 2008).

The effectiveness of chitosan and BSA coating was studied through polyelectrolyte complex coating on an alginate/dextran/poloxamer core formed by the ionotropic pregelation method (Woitiski et al. 2010). Almost all of the insulin was retained in the particles under simulated gastric conditions, however most of the insulin was released under simulated intestinal condition after 1 h. The insulin-loaded nanoparticles showed an improved biological effect in comparison to free insulin (Woitiski et al. 2010).

2.6. Insulin structure and self-association in different pH media

Human insulin has a molecular weight of 5808 Da and consists of polypeptide chain A (21 amino acids) and chain B (30 amino acids), connected by two disulfide bridges, as shown in Figure 1(a) (Cuatrecasas et al. 1990). Insulin monomers in solution have a tendency to form monomer, dimer or hexameric structures, depended on concentration, pH and the presence of zinc. Zinc is included in most commercially available insulins, so the insulin is found in a hexameric form with two zinc cations in the core of the molecular structure (Sadrzadeh et al. 2007). Accordingly, the flexibility of the insulin conformation and different quaternary structures has been associated with various insulin properties and receptor binding (Derewenda et al. 1991). Human insulin primary structure is shown in Figure (3a).

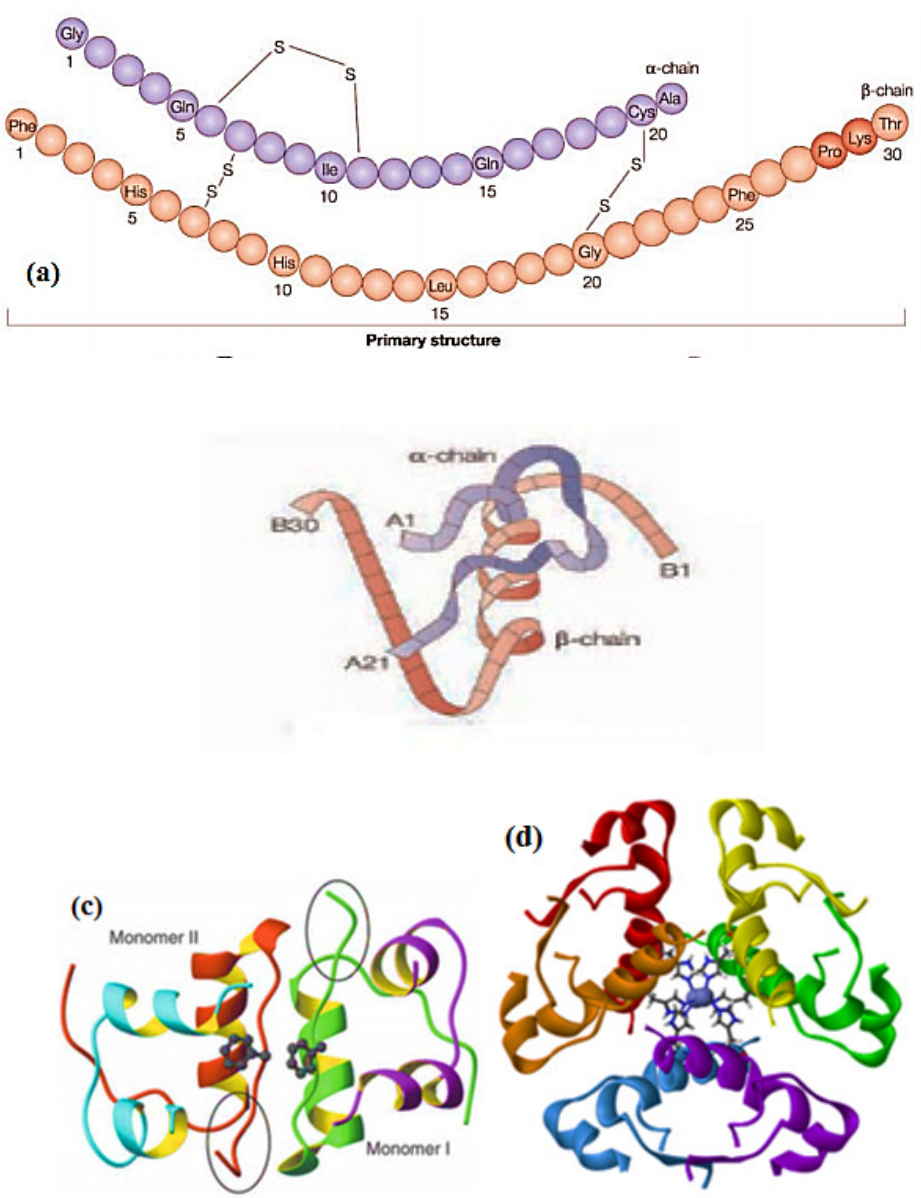

Figure 3. (a) Primary structure of human insulin α- and β- primary chains (b) tertiary structure of Insulin monomer (c) insulin in dimeric form (d) in hexameric form (Owens 2002)

Insulin molecular structure is highly dynamic and flexible due to having the ability to readily associate into dimers and hexamers. Insulin can form a hexamer (6 insulin molecules around 2 zinc ions) or dimer (double insulin molecules). The most stable form of insulin is the hexamer, as insulin is stored as crystalline zinc-hexamers in pancreatic β-cells.

Insulin secondary structure involves chain A that has two antiparallel α-helices A2 to A8 and A13 to A20. The B chain wraps around the A chain and forms a single α-helix from B9 to B19 followed by a turn and a β strand from B21 and B30. The tertiary structure of insulin is formed by disulphide bridges between chains A and B as illustrated in Figure 1b. Three disulphide bridges are formed, two between the A and B chains (A7 and B7, A20 and B19), and one within the A chain (A6 and A11) (Whittingham et al. 2002).

Folding of insulin into a tertiary structure is essential for its biological activity. Insulin molecule folding, including tertiary and quaternary structures form by hydrogen bonding, hydrophobic, van der Waals and electrostatic interactions (Bryant et al. 1993). Studies on insulin analogues have shown that insulin activity is significantly dependent on the integrity of the insulin molecular folds (Gill, 1994). The quaternary structure of insulin (dimer or hexamer) results in changes in side-chain and main-chain structure of insulin, besides preserving the insulin molecule biological activity.

The insulin monomer has a hydrophobic main structure, with polar amino acids residues on the surface, which make it a hydrophilic molecule. Insulin has an isoelectric point (pI) of 5.3, so changing the pH can alter the charge of amino acids (ionizable groups) on the surface and side chains that cause changes in electrostatic interactions of the molecule by changing the surface charge of the insulin molecule. For example, in the pH range 7-11, insulin has a negative charge (-2 to -6) (Elsayed, 2012) and has a positive charge at pH 2 (Jeffrey and Coates, 1966).

In aqueous solution, insulin exists as an equilibrium mixture of monomers, dimers and hexamers. The ability to self-associate depends particularly on concentration, zinc content, pH and solvent (Brange et al. 1987). At low physiological concentrations and neutral pH, insulin is mainly present as a monomer (Frank et al. 1972) and it is the monomer, which is the active form of insulin (Cahill, 1971) as insulin in the neutral pH of plasma is mainly found as monomer (Helmerhorst and Stokes, 1987). By increasing the pH above 8, the proportion of monomer to other forms (hexamer, dimer) increases based on its negative charge and dissociation reaches the maximum by pH 10 (Helmerhorst and Stokes 1987; Frederico, 1953). Also the dissociation value decreases by increasing the insulin concentration and zinc amount (Frederico, 1956). It seems that buffers affect insulin association, as in phosphate buffer, which has a strong complexing effect with zinc, the zinc is removed by precipitation and the association extent of insulin will decrease (Cunningham et al. 1955).

At higher concentrations of insulin at acid or neutral pH, insulin monomer forms dimers and hexamers in the presence of zinc (Jeffrey and Coates, 1966; Carpenter, 1966). As a result, pH changes, protein concentration and presence of zinc can affect the quaternary structure of insulin that can result in changes to the side-chain and main-chain structure. The packing arrangement in dimer and hexamer conformation results in burial of interactive parts from the surface of the insulin molecule (Chothia et al. 1983).

2.7. Review of previous studies on oral insulin nanoparticulate delivery system

Insulin nanoparticles were previously developed in the Neufeld lab by Reis (Reis et al. 2007, Reis et al. 2008), Woitiski (Woitiski et al. 2009, Woitiski et al. 2011) and Sarmento (Sarmento et al. 2007).

Reis et al. (2008) developed a formulation using nanoemulsion dispersion/triggered in situ gelation. The alginate-dextran core was then complexed with a chitosan-polyethylene glycol layer, then an albumin outer shell. It was shown that 50% of the particles had a diameter less than 812 nm. Insulin encapsulation efficiency was 82.5%. It was shown that 25% of encapsulated insulin was released into simulated gastric medium while an additional 45% was released following pH change to 6.8. Eventually, 70% of encapsulated insulin was released after 480 min. The formulation suppressed insulin release in acidic media and promoted a sustained release at neutral conditions. Blood glucose reduction following oral administration in diabetic rats was more than 70% of the basal value in producing an oral hypoglycaemic response. The formulation showed bioavailability around 42% for the dose 25 IU/kg, thus this formulation is able to dramatically improve the intestinal absorption of insulin and will be of interest in the treatment of diabetes with oral insulin.

Woitiski et al. (2009) developed an oral formulation based on the ionotropic gelation/polyelectrolyte complexation method. Insulin-loaded nanoparticles showed a uniform size distribution with mean diameter of 460 nm. An entrapment efficacy of 85% was achieved by this method. Insulin release from nanoparticles in enzyme-free digestive fluids was prevented during 120 min in gastric conditions, and over 80% of insulin was

released after 180 min in simulated intestinal fluid. The pharmacological effect of orally delivered nanoencapsulated insulin (50 IU/kg) in diabetic rats reduced the plasma level to 40% of the basal value and a pharmacological availability of 13% was determined 12 h after oral administration.

Reilly et al. (2011) examined factors limiting the effectiveness of oral insulin such as the role of gastric pH, gut proteases, method and intensity of mixing and premature insulin release in a gastrointestinal tract simulation, by using the insulin nanoparticles developed using the method of Woitiski. In this study, comparisons were made between two different GI simulators, actively mixed and a passively mixed simulator. The actively mixed simulator was a magnetically stirred flask that had more intense mixing resulting in more insulin release. The passively mixed simulator was a flexible container on a rocking stage that provided a gentle and slow mixing pattern that caused an improvement in insulin retention in comparison to the actively mixed simulator. The ability of the nanoparticles to protect insulin was studied in the presence of simulated GI medium, trypsin and pepsin. From the release profile, 70% of insulin was seen to be released in the gastric fluid without enzymes in comparison to 95% of insulin released in the presence of enzyme in the actively mixed simulator. The passively mixed simulator showed an increase in insulin retention.

In the present study, the focus is on the nanoparticulate carriers developed by Reis, which have the highest level of pharmacological availability reported in the literature thus far. The factors responsible for reduced bioavailability were examined, so as to increase levels above the presently reported 42% for future work.

Chapter 3

Research objectives

Therapeutic proteins are used for treating numerous diseases due to high levels of specificity and bioactivity. Therapeutic human peptides and proteins are now being widely produced in commercial quantities. With a few exceptions, all are administered via injection. In spite of the widespread prevalence of insulin, there are several concerns associated with parenteral dosage including injection anxiety, pain, cost, infection, and an overall decrease in patient compliance compared to other, more widely accepted methods of drug delivery such as oral or transdermal (Khafagy 2007).

Academic and industrial research groups are working on the development of therapeutic peptides with improved features for non-invasive delivery, as well as on novel drug delivery systems that can improve bioavailability.

Since the pioneering work by Damgé et al. in 1988, a number of researchers have developed nanoparticle-based oral delivery systems for insulin. Polymeric encapsulation is an interesting approach to insulin delivery and has been found to be useful in prolonging insulin retention and controlling release (Boonsongrit et al. 2006; Schechter et al. 2005), providing protection against acid and enzymatic degradation and enhancing permeation of insulin nanoparticles from the gastrointestinal tract (Reis et al. 2008, Woitiski et al. 2011).

The objective of the overall area of research in the Neufeld lab is to design an oral insulin delivery vehicle by integrating biomaterials to improve insulin stability from

acidic and enzymatic degradation in the gastrointestinal tract, and by enhancing intestinal absorption.

Insulin nanoparticles were developed by Sarmento (Sarmento et al. 2007) using ionotropic pregelation/polyelectrolyte complex coating, Reis (Reis et al. 2007; Reis et al. 2008) using nanoemulsion dispersion/triggered in situ gelation, and Woitiski (Woitiski et al. 2009; Woitiski et al. 2011) who developed a hybrid approach. Reilly then examined the role of gastric pH, gut proteases, method and intensity of mixing and premature insulin release in a gastrointestinal tract simulation, by using the insulin nanoparticles developed using the method of Woitiski.

In the present study, the focus was on the nanoparticulate carriers developed by Reis, which have the highest level of pharmacological availability reported in the literature thus far. The specific objectives of the research were to:

1. Design a simple method for evaluating the factors responsible for decreasing the bioavailability of insulin nanoparticles.

2. Examine the different methods of protein assay for measuring insulin concentration stability, when released from nanoparticles.

3. Evaluate the effect of simulated gastrointestinal medium in the absence and presence of proteolytic enzymes, on stability of insulin.

4. Study the effect of simulated gastrointestinal medium in the absence and presence of enzyme on release profile of insulin from nanoparticles.

5. Analyze the effect of different simulated intestinal media and presence of electrolytes on release profile of insulin from nanoparticles.

Chapter 4

Materials and Methods

4.1. Materials

Novolin ge Toronto human recombinant insulin (100 U/mL) manufactured by Novo Nordisk (Mississauga, Ontario, Canada) was purchased from a local pharmacy. Crystalline human-recombinant insulin (expressed in yeast) was supplied by Sigma Aldrich (St. Louis, USA).

Alginic acid sodium salt from brown algae (Mw 81,216 - viscosity 250 cps), dextran sulfate (5 kDa) sodium salt from *Leuconostoc* sp, low molecular weight chitosan (50 kDa, 75-85% deacetylated), bovine serum albumin, calcium carbonate, $CaCl_2$, NaCl, paraffin oil, Span 80 and PEG 4000 were supplied by Sigma Aldrich (Oakville, Canada). Trypsin from bovine pancreas, pepsin from porcine gastric mucosa, trypsin inhibitor from Glycine max (soybean), and pepstatin A, were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada).

Water solution containing 0.1 % (v/v) trifluoroacetic acid and acetonitrile HPLC grade were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada).

Human insulin ELISA kits were purchased from Mercodia (Winston Salem, NC, USA). Micro BCA protein assay kits were purchased from Thermo Fisher Scientific (Ottawa, Ontario, Canada).

4.2 Methods

4.3 Insulin nanoparticle preparation

Insulin-loaded alginate–dextran nanoparticle cores were prepared by nanoemulsion dispersion followed by triggered in situ gelation. Insulin (100 IU/mL, 10 mL) was added to a mixture of sodium alginate (2%, w/v containing ultrafine calcium carbonate; 5%, w/v) and dextran sulfate solution (0.75%, w/v). The mixture was emulsified within paraffin oil facilitated by Span 80 emulsifier (1.5% v/v) at high speed (2000 rpm). After 15 min emulsification, gelation was induced by addition of 20 mL paraffin oil containing glacial acetic acid (acid:Ca molar ratio of 3:1) to solubilize calcium dispersed in the alginate dextran nanoparticles.

After 60 min, mixture of acetate buffer solution (pH 4.5) (USP 31) with dehydrating solvents as acetone, isopropanol, and hexane (70:15:10:5) was added to the oil-nanoparticle suspension and nanospheres were recovered by centrifugation at 12000g for 10 min.

The alginate-dextran nanoparticles containing insulin were then coated with a 0.03% (w/v) chitosan, 0.15% (w/v) PEG and 1.5% (w/v) $CaCl_2$solution at pH 4.5. After 24 h, the supernatant containing unbound polymer was decanted by centrifugation. Finally nanoparticles were coated with 1% (w/v) bovine serum albumin at pH 5.1 as an outer shell. After 24 h, the supernatant containing unbounded polymer was decanted by centrifugation.

Part of nanoparticles was stored in suspension at 4°C for characterization studies and the remainder was frozen at -80°C and lyophilized for further studies.

4.4 Characterization

4.4.1. Size distribution, morphology and zeta potential

Insulin nanoparticles were characterized for diameter, zeta-potential, morphology and insulin encapsulation efficiency.

Aqueous suspensions of fresh nanoparticles at pH 4.5 were collected for particle size evaluation. Diameter was determined with a Zetasizer Nano ZS (Malvern Instruments Ltd.) based on dynamic light scattering to measure Brownian motion, which is related to particle size. A size distribution curve was developed based on dynamic light scattering from which the average diameter of the particle sample was calculated as a function of intensity (%).

Particle morphology was determined by transmission electron microscopic (TEM) (Hitachi H-7000) examination. A drop of aqueous suspension of fresh nanoparticles at pH 4.5 was mounted on a copper grid, dried and viewed under transmission electron microscopic to evaluate shape, morphology and to confirm particle size.

Nanoparticle surface properties can influence the uptake by intestinal epithelium and play an important role in the interaction with intestinal barrier and mucosa. These properties can also influence particle stability and promote or limit aggregation (Jung et al. 2000 and Reis et al. 2008)

Surface charge was evaluated by Laser Doppler Micro-electrophoresis using a Zetasizer Nano ZS (Malvern Instruments Ltd.) on aqueous suspensions of fresh nanoparticles at pH 4.5 and 25°C.

4.4.2. Protein Encapsulation Efficiency

Encapsulation efficiency is a measure of the amount of insulin entrapped in the nanoparticle formulation, relative to the initial amount of insulin used to prepare the formulation.

Encapsulation efficiency (%) was determined in two ways.

1. Encapsulation efficiency was measured based of the amount of insulin recovered in the nanoparticles, relative to the initial amount of insulin added. The supernatants from the various formulation steps including washing, particle recovery and coating were collected and the insulin lost to the supernatants was measured by ELISA.
2. Insulin loading and encapsulation efficiency were also evaluated by dissolving nanoparticles in a solution of 0.1 M PBS and 0.1 M EDTA over 48 h. After 24 h, the particles were separated by centrifugation and resuspended in fresh buffer to ensure that all the insulin was released. At the end of 48 h, insulin released after each 24 h period was measured by ELISA. This measured value was compared to initial amount of insulin added when forming the nanoparticles.

4.5 Insulin quantification

4.5.1 Micro-bicinchoninic acid (BCA) assay

The micro-bicinchoninic acid (BCA) protein assay method is a highly sensitive colorimetric assay based on the reduction of Cu^{2+} to Cu^{1+} by proteins, where the extent of reduction is proportional to amount of protein present in the solution (Smith et al. 1985). It relies on two reactions as are illustrated in Figure 4.

Firstly, formation of a Cu^{2+}-protein complex under alkaline conditions is followed by reduction of the Cu^{2+} to Cu^{1+}. It has been shown that cysteine, cystine, tryptophan, tyrosine, and the peptide bond are able to reduce Cu^{2+} to Cu^{1+}. Next, two molecules of bicinchoninic acid (BCA) chelate with each Cu^{+} ion, forming a purple-colored chelate complex in an alkaline environment that absorbs light at 562 nm. The linear working rage of the assay is 2-40 μg/mL (Smith et al. 1985).

The micro-plate protocol was followed by adding 150 μL of sample to each well followed by the addition of 150 μL working reagent, and shaking for 20-30 sec. After 2 h incubation at 37^{o}C, the plate was cooled at room temperature and the absorbance measured at 562 nm. The measurable range for insulin is 2 – 40 μg/mL. All samples were diluted within this range as was appropriate. Calibration plots for Novolin ge Toronto human recombinant insulin were prepared.

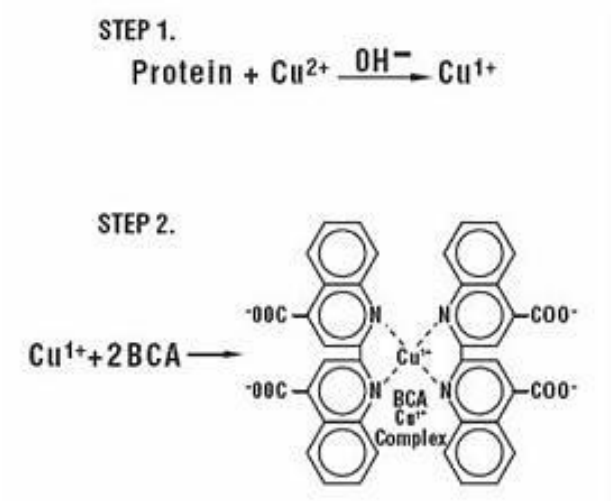

Figure 4. Micro-Bicinchoninic Acid (BCA) protein assay mechanism
(Pierce Technical handbook for the BCA assay)

4.5.2. Human Insulin ELISA

Human insulin ELISA (Enzyme-linked immunosorbent assay) is a solid phase two-site enzyme immunoassay. It is based on binding the insulin between two different monoclonal antibodies as illustrated in Figure 5.

Insulin in the sample binds to insulin specific antibody in the plate well, followed by binding to the peroxidase-conjugated detection antibody. Antibodies are directed against separate antigenic sites on the insulin molecule. Unbound enzyme labeled antibody is then washed away. An enzyme substrate solution is added that is converted to a chromogenic signal by the enzyme linked to the secondary antibody, which can be calibrated to the insulin concentration in solution.

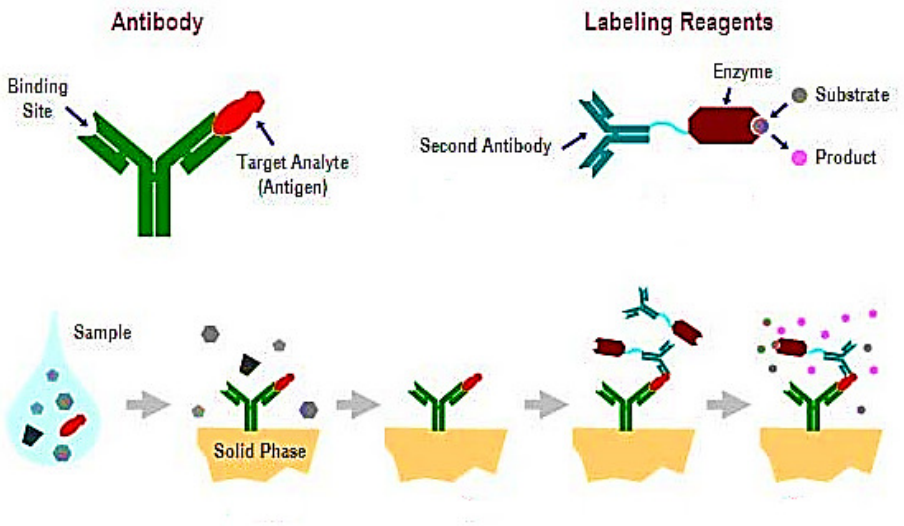

Figure 5. ELISA mechanism: GreenY on surface: capture antibody; floating Blue Y: detection antibody; red part: insulin; Gray circle: enzyme substrate; purple circle - chromogenic signal (http://thefutureofthings.com/articles/37/biopen-senses-biothreats.html, July 8 2013)

Finally, this reaction is stopped by addition of acid to cause a colorimetric endpoint and the chromogenic product is measured at 450 nm. ELISA detection range for insulin is at concentrations of 0.1-9 µg/L.

ELISA detects only intact insulin since it is based on the detection of antibody binding to the whole insulin molecule. Other assays will not be able to detect if the insulin is in its full molecular form or if any breakdown has occurred.

The protocol for this assay begins by adding 25 μL of sample to appropriate wells that contain attached antibody. Detection antibody-enzyme conjugate solution (100 μL) is then added and the plate incubated at room temperature for 1 h on a plate shaker. The plate is then washed 5 times with wash buffer to remove unbound components. After washing, 200 μL of TMB (3, 3′, 5, 5′-Tetramethylbenzidine) substrate is added and incubated at room temperature for 15 min.

Finally, 50 μL of stop solution is added and the color product measured at 450 nm. Samples were diluted to within the working range prior to assay. Calibration plots for Novolin ge Toronto human recombinant insulin were prepared.

4.5.3 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a chromatographic technique used to separate many compounds for identification, quantification or purification. HPLC is based on adsorption, partition, ion exchange or size exclusion, depending on the type of stationary and mobile phase used. There are two different types of HPLC methods, the normal-phase HPLC and the reversed-phase based on the polarity of the mobile phase and the type of the stationary phase. Separation of components is based on the interaction and movement of the sample between the mobile liquid phase and the stationary phase.

Reversed phase HPLC is probably the most used analytical method in pharmaceutics for separation and quantification of drugs. In this method, the stationary phase has a stronger attraction to hydrophobic compounds, and the mobile phase is polar.

The mechanism of the separation is based on strong attraction of the hydrophobic molecules to the hydrophobic stationary phase instead of the polar solvent (mobile phase) and as a result, the hydrophilic molecules in the mobile phase will pass through the column rapidly and will separate first. It is a sensitive and an accurate way to quantify insulin and other protein compounds (Khaksa et al. 1998; Oliva et al. 2000; Moslemi et al. 2003).

The Agilent 1260 Infinity LC (liquid chromatography) with UV-VIS detector equipped with Poroshell 120 SB-C 18 (2.7 μ particle size, 4.6×100 mm length) column was used. The mobile phase consisted of acetonitrile (A) and 0.1% TFA (Trifluoroacetic acid) aqueous solution (B) initially set to 30:70 (A:B, v/v) which was linearly changed to 40:60 (A:B, v/v) over 5 min followed by isocratic elution at 40:60 (A:B, v/v) for 5 min and changed to 30:70 (A:B, v/v) in 1 min followed by isocratic elution at 30:70 (A:B, v/v) for further 1 min. The mobile phase was pumped at a flow rate of 1 mL/min, the injection volume was 20 μL and detection wavelength was 214 nm.

Insulin assay was run at room temperature and the total peak area was used to quantify the insulin. Calibration plots for Novolin ge Toronto human recombinant insulin were determined. This method is a sensitive and an accurate way to quantify insulin. It was developed and validated for the determination of insulin in nanoparticulate dosage forms and nanoparticles composed of alginate and chitosan (Sarmento et al. 2006).

4.5.4 A280 protein absorbance assay

Absorbance assay is the simplest and most convenient method for measuring the

protein content. In this assay, the aromatic side chains of amino acids (tyrosine, tryptophan and phenylalanine) absorb ultraviolet light and exhibit strong UV-light absorption at a wavelength of 280 nm. The protocol for this assay involves measuring the absorbance of protein in solution at 280 nm by cuvette or microplate. Calibration plots for Novolin ge Toronto human recombinant insulin were prepared.

4.6. Evaluation of gastrointestinal simulator components

4.6.1 Design of GI stimulator

The GI stimulator was designed by using a vessel, mixer and paddle as illustrated in Figure 6. The impeller was rotated at 50 rpm (USP 31). Samples were withdrawn and insulin amount measured with an appropriate assay. (USP31-NF26)

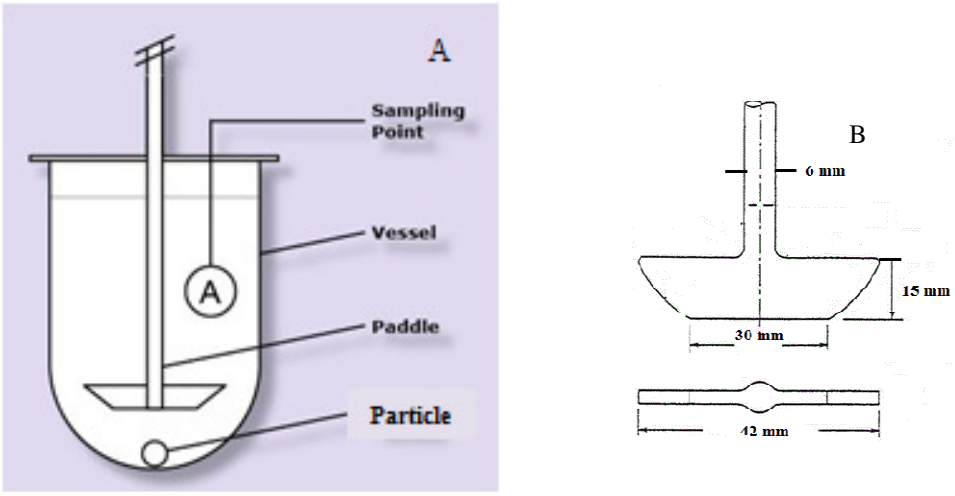

Figure 6: A) GI simulator system, vessel (250 mL volume) and Paddle apparatus B) Paddle dimensions

4.6.1.1. Mixing Characteristics

The mixing characteristics of the GI simulator were evaluated through a tracer study. Vessels were filled with 200 mL of distilled water and 0.5 mL of 1 M HCl injected as tracer. The pH was recorded every 5 sec to determine the mixing pattern.

4.6.2. Simulation of GI medium in absence of enzyme

To stimulate the gastric condition, 75 mL of 0.1 N hydrochloric acid was added to the gastric simulator vessel (pH 1.2). The solution was agitated at 50 rpm for 2 h. Samples for insulin analysis were withdrawn and the collected volume was replaced with fresh gastric fluid. To simulate the progress of moving from stomach to small intestine, the pH of the buffer was changed to 6.8 after 2 h by adding 25 mL of 0.20 M tribasic sodium phosphate. The solution was agitated for an additional 3 h and samples were withdrawn for insulin analysis. The collected volume was replaced with fresh intestinal fluid.

The stability of Novolin ge Toronto human recombinant insulin (100 U/mL) was evaluated at the conditions described in the section above. Insulin was quantified by HPLC and ELISA. Insulin amount was measured by both HPLC and ELISA to measure the amounts of intact insulin present in the solution after exposure to gastric fluid in comparison with insulin in neutral pH water.

4.6.2.1. Release profile of insulin from nanoparticles in simulated GI medium

Insulin release was examined by adding 100 μg of lyophilized nanoparticles to 75 mL of simulated gastric fluid at pH 1.2 without enzymes (USP31-NF26). The particles were agitated in gastric conditions for 2 h at 50 rpm. After 2 hours, the pH was changed to 6.8

by adding 25 mL of tribasic sodium phosphate (0.2 M) for another 3 h. Samples were withdrawn and centrifuged at 5,000 g for 15 min before insulin quantification to remove particle debris. The amount of insulin released was quantified using ELISA.

4.6.3. Simulation of GI medium in presence of enzyme

There is a need to improve the effectiveness of *in vitro* studies as an inexpensive and simple method of studying the destiny of the orally administered drugs. Traditional methods used for *in vitro* studies often ineffectively represent the conditions that drugs face in the body condition. The release profile and insulin stability were evaluated in the simulated gastrointestinal fluid in the presence of enzymes as one of components in the GI model. Pepsin is the main protease in the stomach but trypsin, chymotrypsin and other carboxypeptidases are the most important proteases in the small intestine (Schulze 2006).

Pepsin was applied as a representative protease found in gastric fluid. The addition of pepsin was used to evaluate insulin stability and release profile in simulated gastric conditions

In the first 2 h, gastric medium was used with the addition of pepsin that results in an activity of 750,000 Unit/L (USP31-NF26). After 2 h, pepsin was inactivated through the addition of pepstatin A (100,000U/mg) in ethanol to same unit concentration as pepsin solution and mixed for 30 min to stop the reaction. Samples were withdrawn and the collected volume replaced with fresh GI fluid.

Trypsin was chosen as a model enzyme found in intestinal fluid. The effects of trypsin on the stability of insulin and release profile were tested. After 2 h gastric

simulation, the buffer pH was changed to 6.8 by adding 25 mL tribasic sodium phosphate (0.2 M) and trypsin at a concentration of 1.75 USP U/mL (USP31-NF26). After 3 h, trypsin inhibitor was added at the same mass concentration as the trypsin solution (1 mg of trypsin inhibitor will react with 1 mg of trypsin), then mixed for 30 min to stop the reaction. Samples were withdrawn and the collected volume replaced with fresh GI fluid.

The stability of Novolin ge Toronto human recombinant insulin was evaluated at the conditions described in the section above. Insulin was quantified by HPLC and ELISA. The amounts of intact insulin present after exposure to gastric and intestinal fluid containing enzymes were compared with amount of insulin detected in neutral pH water control.

4.6.3.1. Release profile of insulin from nanoparticles in simulated GI medium in presence of enzyme

The effects of protease enzyme on insulin stability within nanoparticles was evaluated by adding 100 μg of nanoparticles to 75 mL of simulated gastric fluid at pH 1.2 with pepsin at a concentration of 750 U/mL (USP31-NF26). The mixture was agitated for 2 h at 50 rpm. Pepsin was inactivated through the addition of pepstatin A (100,000U/mg) in ethanol to the same unit concentration as the pepsin solution and mixed for 30 min. Samples were withdrawn and volume replaced with fresh gastric fluid without enzyme.

Solution pH was adjusted to 6.8 after 2 h, and trypsin added at a concentration of 1.75 USP U/mL (USP31-NF26) for an additional 3 h. The trypsin reaction was stopped by adding the solution of trypsin inhibitor prepared with the same mass concentration as the trypsin solution, for 30 min. Samples were withdrawn and the collected volume was

replaced with fresh GI fluid. All samples were centrifuged at 5,000 g for 15 min before insulin quantification to remove particles. The amount of insulin released in the medium was quantified with ELISA. The release profile was compared to the condition of particles exposed to gastric fluid in the absence of enzymes.

After 5 h, remaining GI fluid was centrifuged at 5,000 g for 15 min to separate particles. Separated nanoparticles were dissolved in a solution of 0.1 M PBS and 0.1 M EDTA over 48 h to evaluate the amount of intact insulin retained in nanoparticles after exposure to simulated GI medium with and without enzyme. After the first 24 h, the particles were separated by centrifugation and resuspended in fresh buffer to ensure that all insulin was released. Insulin released after 24 and 48 h was measured by ELISA. This measured value was compared to initial amount of insulin added when formulating the nanoparticles.

4.7. Effect of different simulated intestinal buffers on insulin release profile

Transmission of drug from gastric to intestinal conditions is considered in typical *in vitro* trials, by changing the pH from acidic (pH 1.2) to neutral (pH 6.8) using phosphate buffer. While phosphate buffers are formulated to mimic the intestinal pH conditions, they don't represent the intestinal environment chemically (McConnell et al., 2008). Physiologically, pH is controlled by bicarbonate secretion through the pancreas and intestinal epithelial cells. Moreover small intestinal fluid is a complex medium containing lipids, bile salts, digestive enzymes, proteins, polysaccharides and electrolytes, none of which are considered in a simple phosphate buffer.

Different simulated intestinal buffers and electrolytes were evaluated for studying insulin release behavior. Three buffer formulations were used: standard phosphate buffer (PB), standard Kreb's bicarbonate buffer (KBB), and phosphate buffer/Kreb's bicarbonate buffer with different ratio of NaCl: $CaCl_2$.

Insulin nanoparticles (100 μg) were added to 10 mL of intestinal buffer. The particles were agitated for 3 h at 50 rpm with a paddle mixer. Samples were centrifuged at 5,000 g for 15 min before insulin quantification to remove particles. The amount of released insulin from nanoparticles to the buffer supernatant was quantified by HPLC. Insulin release profiles in each simulated intestinal buffer was evaluated and compared.

Chapter 5

Results and Discussion

5.1 Insulin nanoparticle development

A promising means of overcoming the obstacles encountered in the oral delivery of insulin is encapsulation within biopolymer based nanoparticles. Numerous researchers have developed various nanoparticulate oral delivery systems. A wide range of natural polymers has been used, but alginate, chitosan and dextran appear frequently in different formulations as shown in Table 1. The highest bioavailability reported for oral insulin was based on a formulation developed in the Neufeld lab at Queen's University involving insulin within an alginate-dextran core, coated by chitosan-PEG, then by albumin (Reis et al. 2007 and 2008).

The main goal in the use of therapeutic peptide/protein oral delivery systems is to overcome low gastrointestinal bioavailability. Oral delivery strategies are designed to prolong release behavior, enhance intestinal retention and permeation, and provide protection against gastric acid and gastrointestinal (GI) enzymes. Nanoparticulate carriers should be designed to improve the bioavailability of insulin through facilitating the uptake of encapsulated drug through the epithelial cells of the GI tract and provide protection against gastric and enzymatic degradation.

The formulation developed by Reis et al. (2006, 2007, 2008) was modified to provide the focus for the research in this study. Insulin-loaded alginate–dextran sulphate nanoparticles were prepared by nanoemulsion dispersion followed by triggered in situ

gelation through release of Ca^{+2} from insoluble carbonate complex by changing the pH in the manner illustrated in Figure 7.

The negatively charged alginate-dextran core containing insulin was then complexed with a positively charged chitosan-polyethylene glycol layer, then an albumin outer shell. The modification to the formulation involved an increase in the mixing energy during nanoemulsion dispersion, so as to form smaller diameter nanoparticles.

Alginate is a polysaccharide that forms a crosslinked structure in the presence of calcium ions that can be used to produce sustained release particulate systems for drugs, proteins and even cells (Raj and Sharma, 2003). Alginate gel is relatively permeable and has low retention capacity, thus dextran sulphate was added to the core to minimize the loss of entrapped insulin.

Dextran is also a polysaccharide, used as a stabilizing agent for controlling the release of drugs in pharmaceutical formulations (Janes et al.2001). Studies have shown that insulin within alginate-dextran alone is unsuccessful in reducing blood glucose after oral administration (Reis et al. 2007). By adding dextran to the alginate, insulin release is prevented at gastric pH, but insulin was rapidly and prematurely released on transferring to intestinal medium at pH 6.8 (Reis et al. 2007).

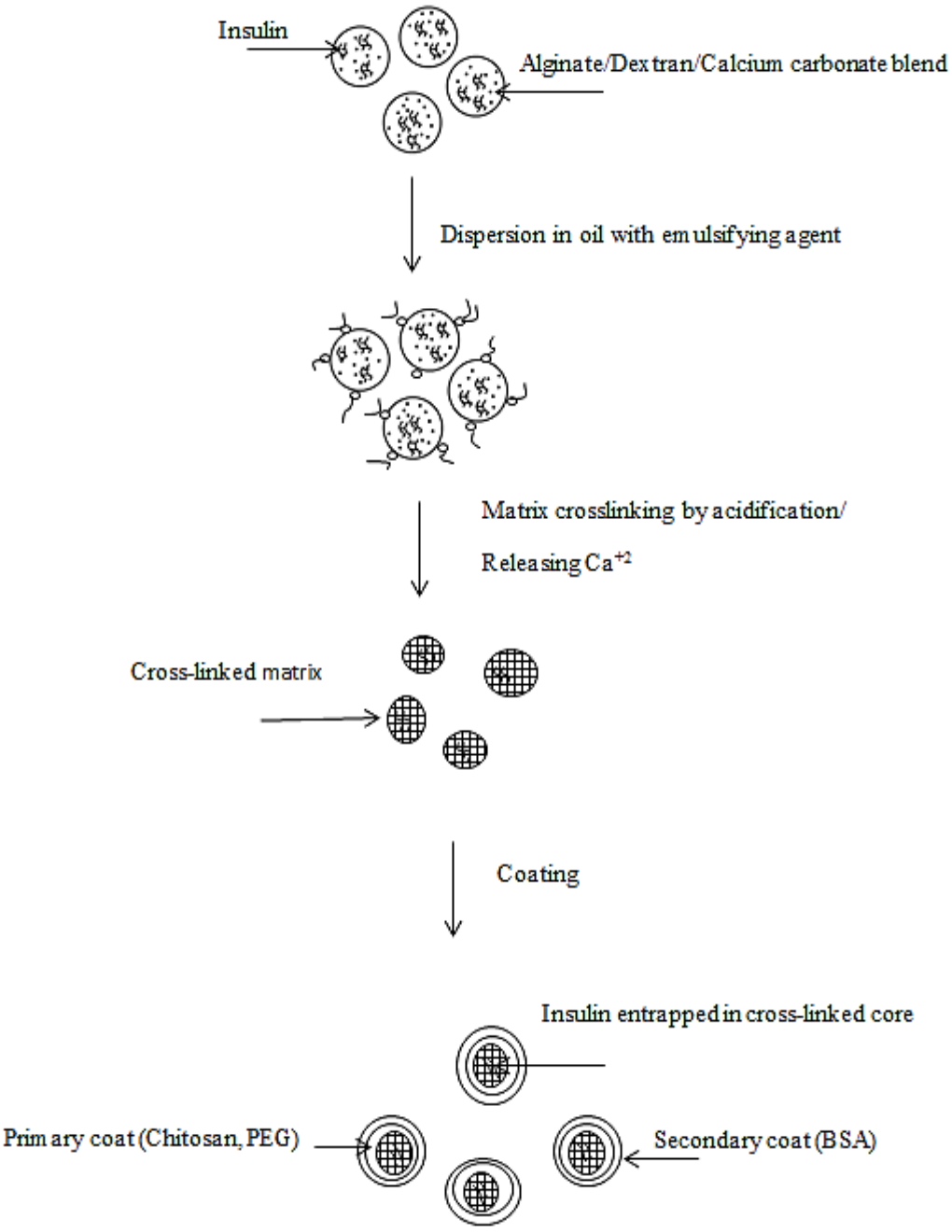

Figure 7. Formulation of alginate-dextran core particles coated with chitosan/PEG, then with albumin. Insulin is contained within the nanoparticle core

Coating strategies were then applied to improve the protection of insulin. The first layer applied was chitosan with PEG. Chitosan as a polycationic polysaccharide stabilizes the polyanionic alginate core through ionic complexation, effectively coating the core particle. The addition of a chitosan coat minimizes the loss of encapsulated insulin, forming a stable and tight complex polymeric membrane on the alginate particulate networks (Reis et al. 2008). Also mucoadhesive and permeability enhancing properties of chitosan facilitate absorption of insulin nanoparticles from the intestinal lumen, through increasing adsorption to mucus lined absorptive cells and opening of tight junctions between the epithelial cells (Schipper et al. 1997).

Polyethylene glycol (PEG) is a stabilizing polymer used to increase the stability of polymeric nanoparticles (Tobio et al. 2000), to improve physicochemical and biopharmaceutical properties (Caliceti et al. 1999), and promote transport of large proteins across nasal and intestinal barriers by increasing the hydrophobicity of the particles (Tobio et al. 1998).

Albumin was added as a second coating layer at pH 5.1. At this pH, negatively charged albumin associates with positively charged chitosan, producing a more compact polymeric layer that may provide a better protection from acid and proteases (Takahashi et al. 1990). Albumin is added to serve as a sacrificial target for gastric and intestinal proteases, potentially stabilizing insulin within the particle core (Reis et al. 2008). After the second layer coating, particles were frozen at -80^{o}C and lyophilized.

Insulin nanoparticles were characterized in the present study for diameter, zeta-potential, insulin encapsulation efficiency and insulin release under simulated gastrointestinal conditions in the presence and absence of gastrointestinal enzymes.

5.2. Insulin nanoparticle characterization

5.2.1. Morphology and size distribution

Particle size is considered an important factor in the efficiency of particulate delivery systems (Norris et al. 1998). Generally, particle uptake by the intestinal epithelium is size dependent and smaller nanoparticles are absorbed to a greater extent than larger particles (Norris et al. 1998). Also smaller particles can reach distant sites more readily, and can be detectable for longer periods of time, thus it enhances retention time in the GI tract (Norris et al.1998). Many factors can affect the size of insulin nanoparticles such as stirring rate, insulin quaternary structure and type of encapsulation method (Reis et al. 2008, Norris et al. 1998, Cournarie et al. 2004, Damgé et al. 1997).

Insulin-loaded nanoparticles were characterized based on size, morphology and zeta potential. Size distribution was evaluated with a zetasizer (Zetasizer Nano ZS) based on dynamic light scattering to measure Brownian motion, which is related to particle size (Malvern Instruments, 2004). Figure 8 displays the result of diameter distribution for a representative suspension of nanoparticles in Tween 80 (1%) based on intensity of the scattered light. A single large intensity peak indicates particles with mean diameter of 10 nm. Particles within this peak, ranged between 5 to 20 nm. The second peak may be related to aggregated particles with aggregate mean diameter of 350 nm.

Reis et al. (2008) who initially developed this formulation showed that 50% of the particles had a diameter less than 812 nm and 90% of the particles had a diameter less than 1 μm (Reis et al. 2008). The difference between the formulations is in the stirring rate for particle formulation. Reis used a stirring rate of 1600, while a stirring rate of 2000 rpm was used in the present study. The higher energy of mixing would explain the dramatic decrease in particle size. Also the diameter of the 3-blade propeller that was used in this study was 52 mm in comparison to 42 mm used by Reis, which results in higher shear stress leading to smaller particles.

Particle morphology was determined by transmission electron microscopic (TEM) examination. Particles in Figure 9 appear spherical and uniform in shape. The dark central core containing insulin appears enveloped within successive layers of chitosan-PEG and albumin, confirming the presence of the coating layers. The distribution of particles measured from the TEM image ranged between 5 to 40 nm with a mean of 20 nm that is similar to the results obtained by light scattering. Also aggregation was observed under the microscope.

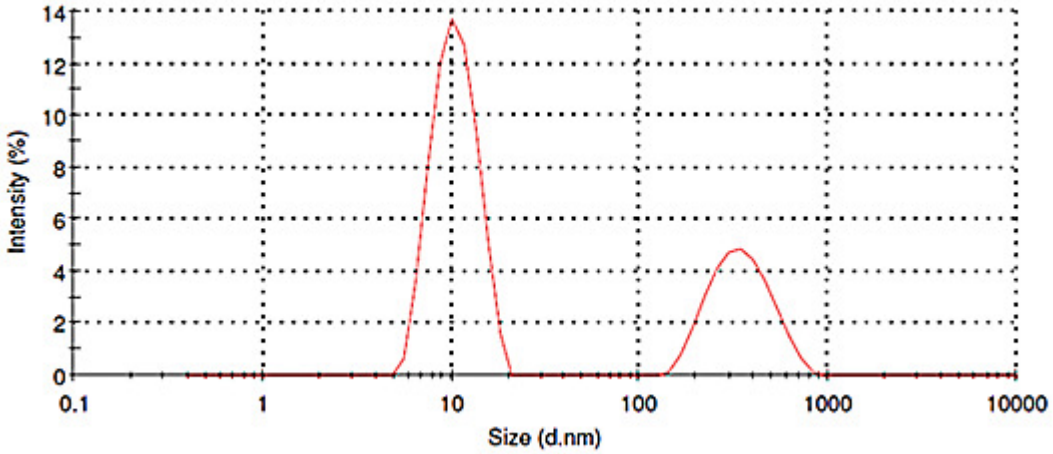

Figure 8. Size distribution of insulin nanoparticles. Large peak indicates particles with mean diameter of 10 nm with particles ranging from 5 to 20 nm. The small peak is related to aggregated particles.

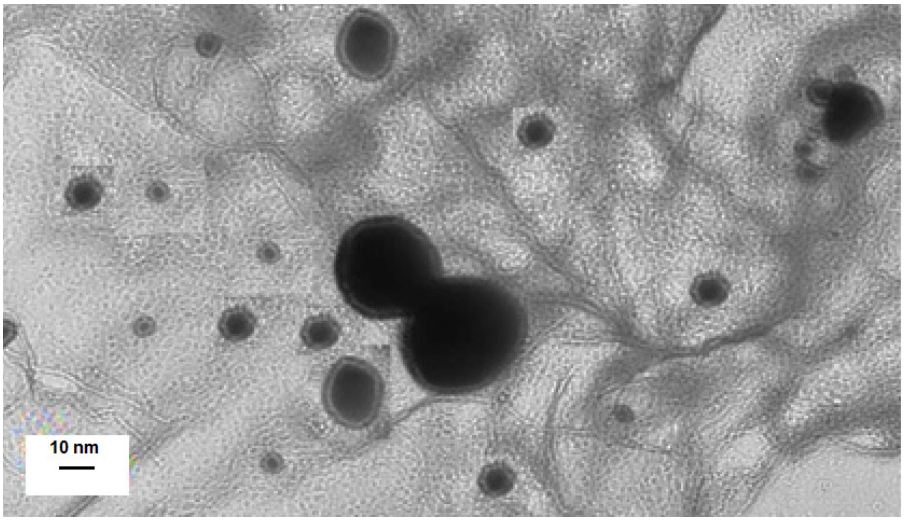

Figure 9.TEM image of insulin nanoparticles

5.2.2. Zeta potential

Nanoparticle surface properties can influence uptake by the intestinal epithelium and promote interaction with the intestinal barrier and mucosa. Zeta potential may also influence particle stability and aggregation (Jung et al. 2000; Reis et al. 2008).

Surface charge was evaluated by laser doppler micro-electrophoresis using a Zetasizer Nano ZS. Surface charge mean value was – 5.8 ± 9 mV due to the presence of negatively charged albumin as the outer coating as is shown in Figure 10. The zeta potential ranged from – 14.8 to+ 3.2 mV.

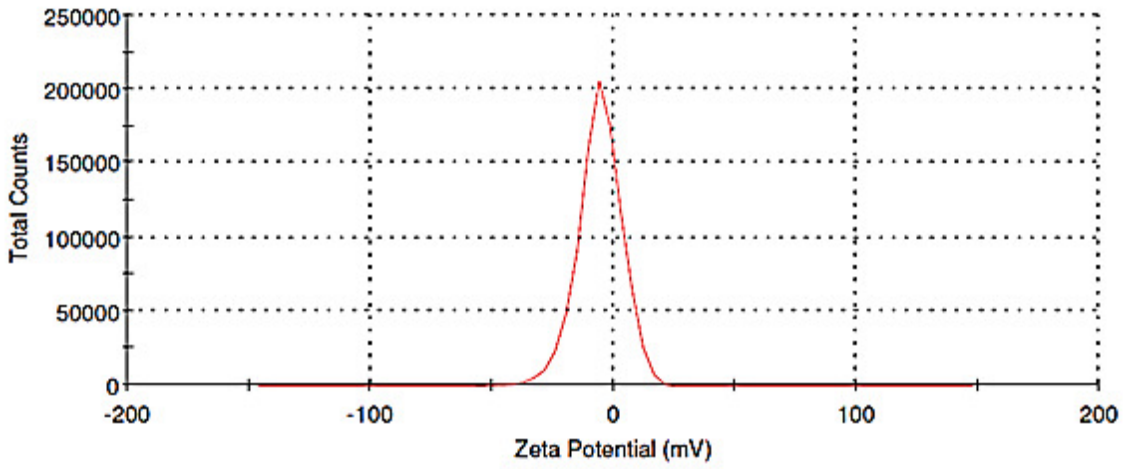

Figure 10. Zeta potential of insulin nanoparticles. Peak of the zeta potential is – 5.8 ± 9 mV for insulin nanoparticles.

Reis et al. (2008) incubated insulin nanoparticles in acidic media with presence of pepsin. Following 2h, the zeta potential changed from -7±4 mV to +21±3 mV, likely due to enzymatic removal of the albumin coating, exposing the underlying positively charged chitosan. It is the exposure of chitosan on the particle surface, which will enable attraction of the nanoparticles to the mucin layer, and thus concentrate particles near the intestinal barrier (Reis et al. 2008).

The intestinal mucin produced by epithelial cells within the GI tract contains sialic acid, L-fucose and sulfate residues that can cause the negative charged layer at the intestinal pH (Attivi et al. 2005). Therefore, positively charged nanoparticles formed by enzymatic removal of the albumin layer, are expected to interact with negatively charged mucin glycoprotein lining the intestinal wall and aid the intestinal uptake of insulin nanoparticles (Reis et al. 2008).

5.2.3. Protein encapsulation efficiency

Encapsulation efficiency is a measure of the amount of insulin recovered in the nanoparticle formulation, relative to the initial amount of insulin. Protein encapsulation (%) was measured based on two methods.

One method was to measure insulin lost to the supernatant solutions during the various formulation steps including washing, particle recovery and coating. Supernatants from the different stages were collected and combined and insulin content was measured by HPLC. Taken as a ratio of the initial amount of insulin used to prepare the formulation, the encapsulation efficiency was 85± 3%, thus most of the insulin was recovered in the particulate formulation.

It has been shown that pH has an effect on encapsulation efficiency (Reis et al. 2007). Insulin has an isoelectric point around 5.3 (Chien et al. 1996) and alginate has pK values of 3.38 and 3.65 for M and G residues (Draget et al. 1994). At pH 4.5, electrostatic attractions of negatively charged alginate polymer to positively charged insulin likely provided the high encapsulation efficiency.

In the second method, insulin loading was measured by dissolving dried particles to release insulin. Each batch yielded 1 ± 0.05 g of dried particles. Particles were dissolved in 0.1 M PBS and 0.1 M EDTA for 48 h. After the first 24 h, undissolved particles were centrifuged and resuspended in fresh buffer for another 24 h to ensure all the insulin was released. Released insulin was assayed by ELISA. Insulin loading was determined to be 29± 2 mg insulin/g of dry particles. The measured value was compared to initial amount of insulin (35 mg) added to the formulation and the encapsulation efficiency was 82 ± 5%.

Similar efficiencies were obtained with the two methods, providing reliable evidence for the amount of insulin contained within the particles, and showing a high level of recovery of the insulin.

5.3. Assay development

Each drug delivery formulation requires analytical techniques to accurately measure the content of active ingredient. Besides the active ingredient, there are excipients that can cause interaction with the assay methods. In this study four assay methods were applied to quantify insulin in the formulation and evaluate the possibilities of interaction. The two forms of insulin used were Novolin ge Toronto human recombinant insulin

obtained as a pharmaceutical preparation, and pure human recombinant insulin in crystalline form. Assay methods included Bicinchoninic acid assay (BCA), human insulin enzyme linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC) and ultraviolet absorption at 280 nm.

5.3.1 Bicinchoninic acid (BCA) assay

The BCA protein assay is based on the reduction of Cu^{2+} to Cu^{1+} by proteins, measured colorimetrically (Smith et al. 1985). The reaction is induced by reduction of Cu^{2+} by peptides containing cysteine, cystine, tyrosine, and tryptophan amino acid residues that form a colored chelate complex with cupric ions in an alkaline environment. In the second step, the reaction of two BCA molecules with one Cu^{1+} ion forms a purple complex that absorbs at 562 nm. The linear working range of the assay is 2-40 μg/mL (Smith et al. 1985).

Crystalline insulin is mainly insoluble at pH around 5.1, but below pH 3.5 or above 6.5, the solubility is ≥ 25 mg/mL (European Pharmacopeia 6.2). Crystals were thus dissolved in acidified water (0.1M HCl) at pH 3.

A calibration plot was produced using different concentrations of human recombinant insulin in liquid injectable formulation (Novolin ge Toronto), and in crystalline form following dissolution in acidified water. Absorbance in Figure 11 was linear for both forms of insulin, but with different slopes up to 50 μg/mL. Differences between the two plots are more noticeable at higher concentration values.

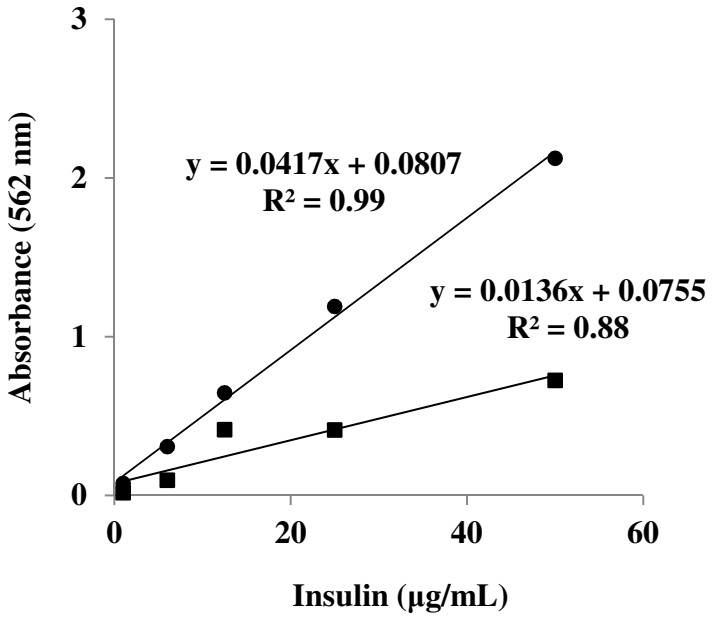

Figure 11. BCA standard plot using human recombinant insulin in injectable formulation (Novolin ge Toronto) (●), and crystalline form following dissolution in acidified water (■).

The slopes of the calibration curves were different between the two formulations, likely due to differences in solution pH and presence of excipients in the Novolin formulation. Cresol is one of the excipients added to Novolin formulation as preservative. It is an aromatic organic compound, which has a structural similarity to some amino acids, likely interfering with the BCA assay and causes the positive response.

Since the nanoparticulate formulation will contain bovine serum albumin (BSA) as a protein, it will also likely interfere with the BCA assay and give a positive response. In addition, there are reports of alginate interfering with the BCA assay, thus alternative assays methods were examined.

5.3.2. A280 protein absorbance assay

Absorbance assay is the simplest and most convenient method for measuring protein content. Ultraviolet light at 280 nm is absorbed by the aromatic side chains of tyrosine, tryptophan and phenylalanine. To use this assay, the protein must contain at least one aromatic side chain based amino acid to generate a measurable signal (Layne et al. 1957, Stoscheck et al. 1990). Proteins and peptides absorb UV-light in proportion to the aromatic amino acid content and total protein concentration. The disadvantage of this method is that any non-protein components that absorb ultraviolet light will interfere with the assay. Because of the structure of alginate, dextran and chitosan, there is a possibility of interference (Omidian et al. 2006, Stoscheck et al. 1990).

A calibration plot at 280 nm was produced using different concentrations of human recombinant insulin in liquid injectable formulation (Novolin ge Toronto), and in crystalline form following dissolution in acidified water. Absorbance in Figure 12 was linear for both forms of insulin, but with different slopes up to 450 μg/mL. Differences between the two plots are more noticeable at higher concentration values.

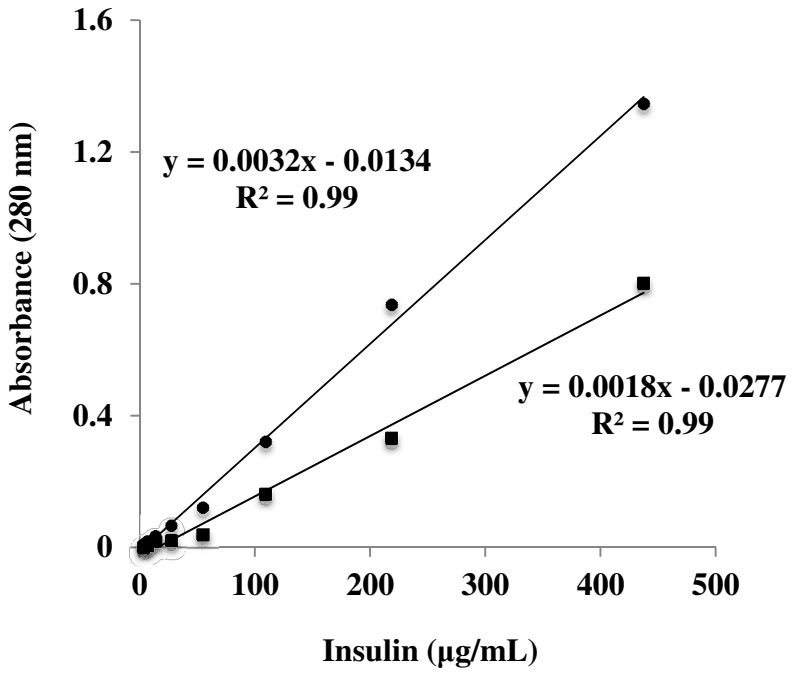

Figure 12. A280 protein assay standard plot using human recombinant insulin in injectable formulation (Novolin ge Toronto) (●), and crystalline form following dissolution in acidified water (■).

Cresol has the potential to interfere with the A 280 assay based on its aromatic structure. The slopes of the calibration curves were different between the two formulations, likely due to differences in presence of excipients in the Novolin formulation. Also having BSA as a protein in nanoparticle formulation, likely can interfere with the A280 assay and give a positive response when assaying insulin in the nanoparticles. The benefit of using ELISA over the A280 assay is to avoid the complications due to presence of albumin and alginate in the nanoparticle formulation structures and avoiding interference.

5.3.3 High performance liquid chromatography (HPLC)

HPLC is a chromatographic technique used to separate many compounds for identification, quantification or purification. HPLC is based on adsorption, partition, ion exchange or size exclusion, depending on the type of stationary and mobile phase used. Separation of components in a solution results from the difference in the relative distribution ratios of the solutes between the two phases.

Human recombinant insulin in crystalline form was dissolved in acidified water (0.1M HCl) at pH 3. This form of insulin was compared to Novolin formulation using HPLC as assay method. A calibration plot was produced using different concentrations of Novolin insulin and crystalline form as shown in Figure 13. The plot was linear between 1-100 μg/mL for both formulations with coefficient of determination (R^2) of 0.999. The HPLC chromatograms are illustrated in Figure 14 showing the retention time for crystalline insulin to be around 3.4 min and for Novolin insulin around 4.5 min.

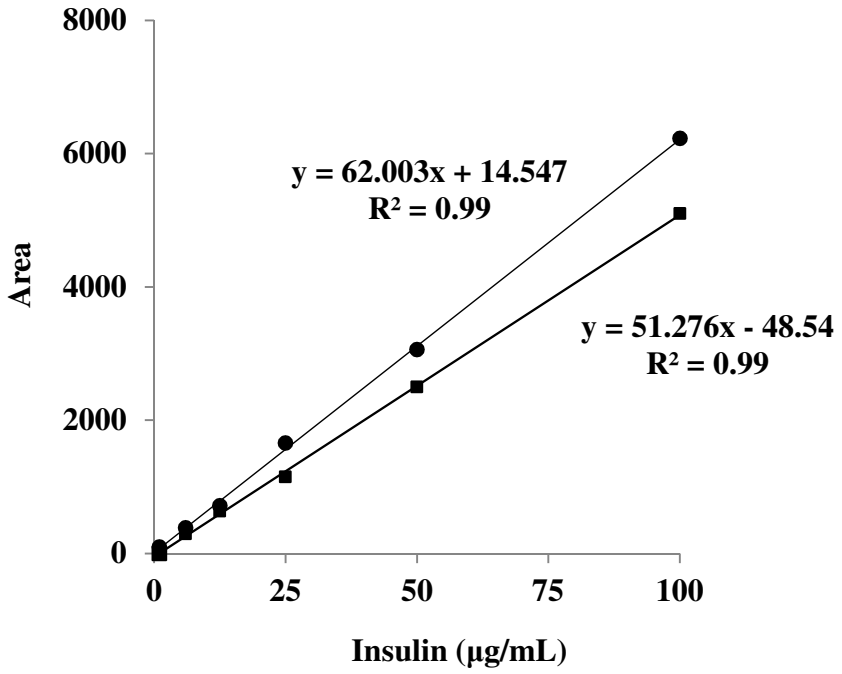

Figure 13. HPLC standard plot using human recombinant insulin in injectable formulation (Novolin ge Toronto) (●) and crystalline form following dissolution in acidified water (■) using UV detector at 214 nm.

HPLC separation of components is based on the interaction and movement of the molecule between the mobile liquid phase and the stationary phase. Insulin self-association in solution can cause different quaternary structures, resulting in molecular weight differences between monomer, dimer and hexamer that would affect the retention time. Also as the charge on the insulin is highly pH dependent, changing the charge affects polarity, hydrophobic/hydrophilic character and interaction with the stationary phase.

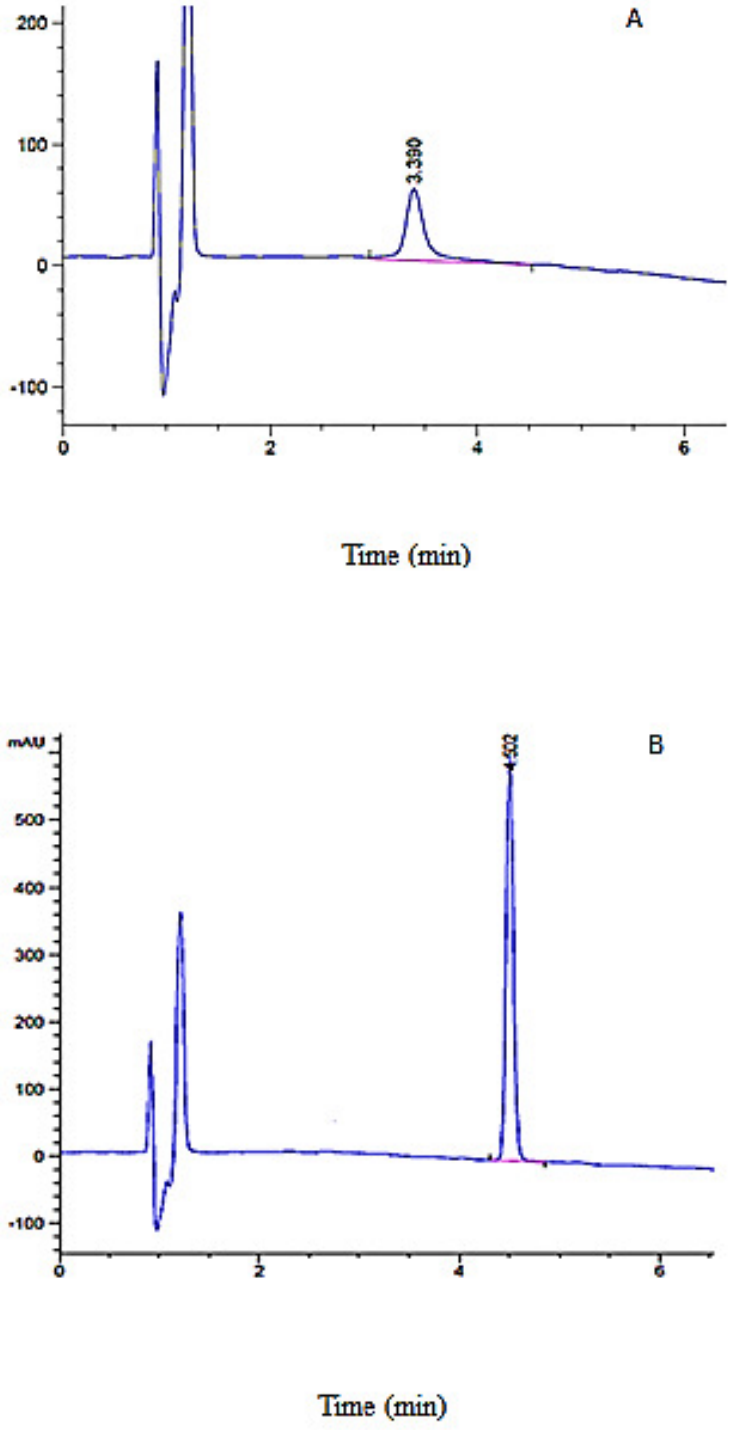

Figure 14. HPLC chromatogram A) Human recombinant insulin crystalline form (50 µg/mL) following dissolution in acidified water B) Human recombinant insulin in injectable formulation (Novolin ge Toronto) at concentration of 50 µg/mL using UV detector at 214 nm.

5.3.4 Human insulin ELISA

A human insulin ELISA (enzyme-linked immunosorbent assay) is based on binding the insulin between two different monoclonal antibodies, directed against separate antigenic sites on the insulin molecule. The primary antibody binds the insulin, while an enzyme linked to the secondary antibody, catalyzes a color reaction, calibrated to the insulin concentration. Insulin at concentrations of 0.1- 9 μg/L can be detected.

ELISA detects only intact insulin since it is based on primary antibody binding to the whole insulin molecule. Other assays will not distinguish whether insulin is in a full molecular form or if any breakdown has occurred. The benefit of using ELISA to BCA assay is to avoid complications due to presence of albumin in the nanoparticle formulation structures, and avoid interference with other formulation components such as alginate, dextran and chitosan.

A calibration plot is presented in Figure 15 for Novolin insulin, crystallin insulin and insulin standard provided by the ELISA supplier. The calibration plot for three types of insulin was linear between the ranges of 0.1- 9 μg/L. Similar plots were obtained with three types of insulin as ELISA detection is based on the interaction between antibody and intact insulin and it seems that the there is no interaction with the excipients.

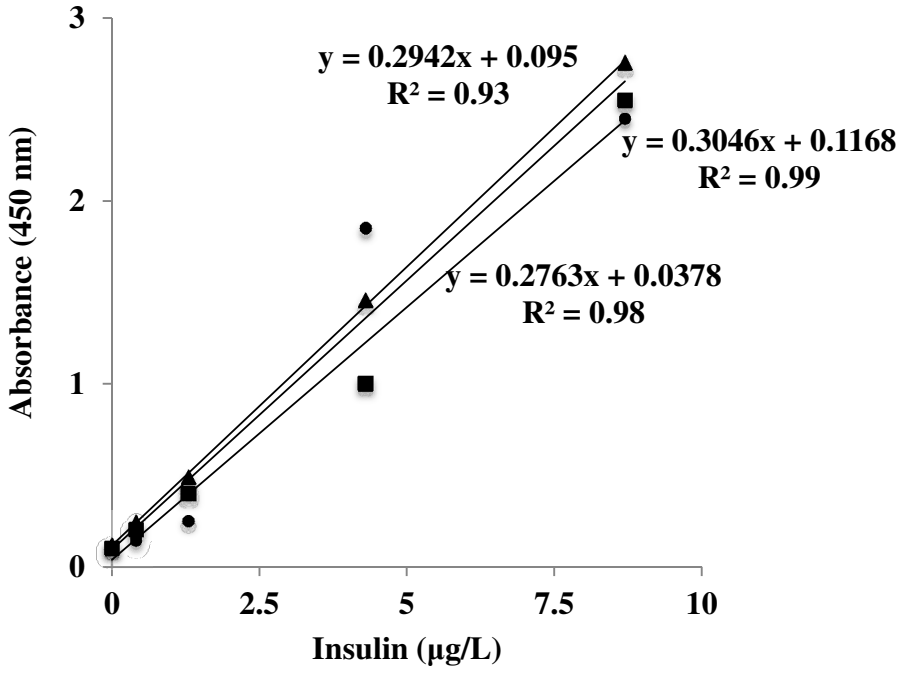

Figure 15. Human insulin ELISA calibration plots using Novolin ge Toronto insulin (R^2= 0.93) (●), crystalline form following dissolution in acidified water (R^2= 0.98) (■) and insulin standard solution provided with Mercodia commercial assay kit (R^2= 0.99) (▲)

Among the assay methods described, ELISA and HPLC offered the most advantages, due to possible interferences with excipients and particle components, observed with BCA and UV assays. ELISA and HPLC methods do not demonstrate interactions with the ingredients in the formulation. ELISA detection is based on the interaction between antibody and intact insulin but the other assays will not distinguish between intact insulin and degradation products. In HPLC, insulin is separated from other components, thus reducing interferences.

5.4 Evaluation of gastrointestinal simulation factors

5.4.1 Mixing pattern of GI simulator

Mixing has an impact on how delivery devices will behave in simulated GIT fluids as stronger mixing likely promotes insulin release. Mixing conditions that more closely mimic the GIT condition may enable better retention of insulin in the delivery device.

Gastrointestinal simulator mixing pattern was evaluated using a mixer paddle agitated at 50 rpm (USP 31) and pH tracer. Vessels were filled with 200 mL of distilled water and 0.5 mL of 1 M HCl injected as tracer. The H^+ concentration was used to evaluate the mixing pattern. The pH was measured every 5 sec until stable but plotted as H^+ concentration with time, to avoid the exponential function of pH.

The pH response illustrated in Figure 16 showed the simulator reached steady state after 150-170 sec. The mixing profile changes gradually and the response was irregular. A previous release study used a simulator operating at 100 rpm in a magnetic bar-stirred flask (Reilly, K, Master's thesis). The time to steady state was 15-20 sec, thus much faster than that observed in the present simulation.

Mixing in the stomach and upper small intestine is gentle and slow. The mixing provided by the vessel and paddle mixer is considerably more representative of *in vivo* conditions than a 100 rpm magnetically-stirred flask. Also, in the magnetic bar mixed system, there is the possibility of crushing nanoparticles trapped between the bar and the bottom of the flask, which is avoided with the paddle mixer.

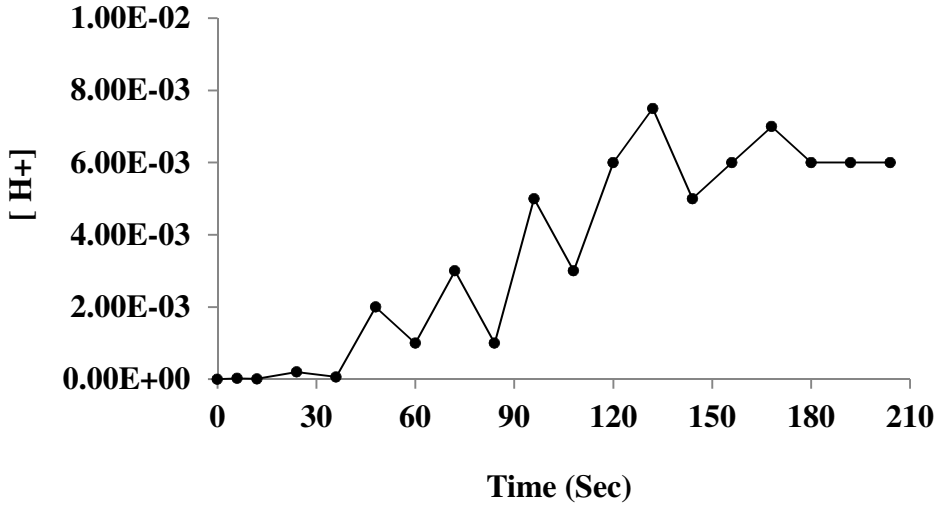

Figure 16: pH response when 0.5 mL of 1.0 M HCl was injected into 200 mL of water, using paddle mixer agitated at 50 rpm (USP 31).

5.4.2. Evaluating insulin stability in gastric and intestinal condition

Insulin stability was examined in simulated gastric (pH 1.2, 2h) followed by intestinal (pH 6.8, 3h) media, both in the presence and absence of pepsin (gastric) and trypsin (intestinal) enzymes. The amount of measurable insulin was analyzed by HPLC and ELISA in two separate experiments.

Methods used for *in vitro* studies commonly inadequately represent the conditions that drugs are exposed to *in vivo*. There is a need to improve the efficiency of *in vitro* studies as an inexpensive and simple method for exploring the destiny of orally administered drugs.

The objective of the GIT simulations was to determine if nanoparticulate insulin is stable in acidic gastric condition and in neutral intestinal condition, and in the presence of proteases. It is also of interest to determine the extent to which insulin released from nanoparticles into the GIT is stable.

Pepsin is the main protease in the stomach, while trypsin, chymotrypsin and other carboxypeptidases are the most important proteases in the small intestine (Schulze 2006). Proteases in the stomach and intestine are responsible for breaking down ingested proteins.

Prior to looking at insulin stability in nanoparticulate form, the stability of unprotected insulin was examined as a baseline. Novolin ge Toronto insulin stability under gastric conditions was determined by adding insulin to 75 mL of HCl buffer at pH 1.2 (USP 31). The solution was agitated using a paddle mixer at 50 rpm for 2 h in the presence and absence of pepsin. To simulate the progress of moving from stomach to small intestine, after 2 h, the buffer pH was changed to 6.8 by adding 25 mL of tribasic sodium phosphate with concentration of 0.2 M and the study was continued for another 3 h. Trypsin was used as the model GI tract enzyme.

Results shown in Figure 17 illustrate the HPLC measureable amounts of Novolin insulin under gastric followed by intestinal simulation. Data points represent the mean of triplicate experiments, and standard deviations are presented showing good repeatability between experimental replicates.

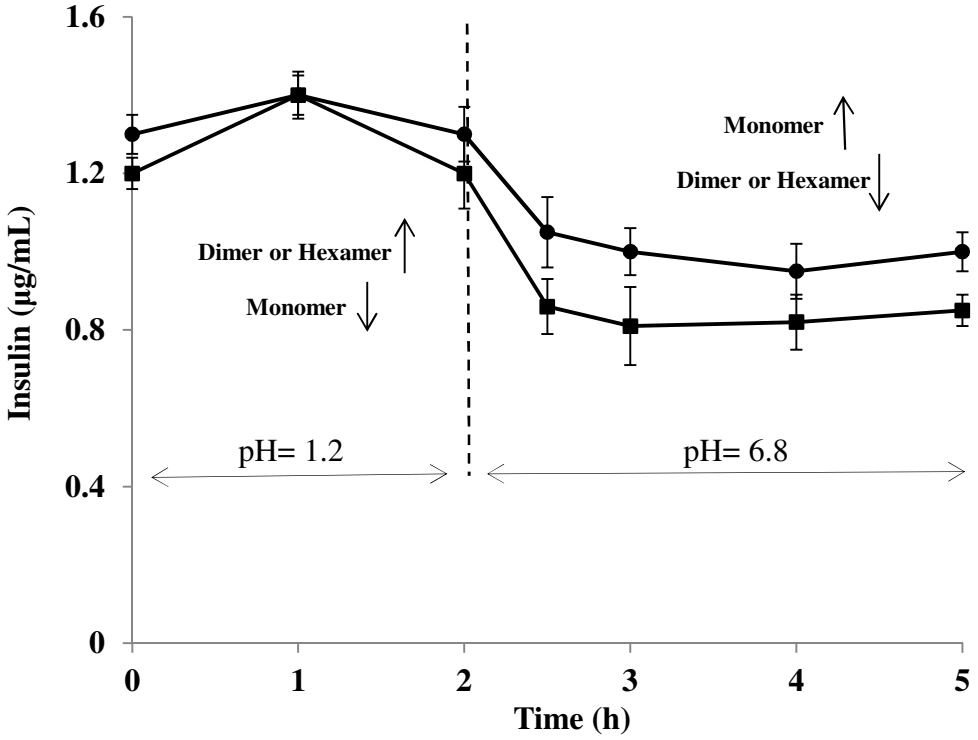

Figure 17. Novolin insulin measureable by HPLC, 2h under simulated gastric condition followed by 3h under simulated intestinal condition in the absence (●) and presence of pepsin for gastric condition and trypsin for intestinal condition (■). Results are mean and standard deviation of triplicate experiments. Acid condition causes conformational changes in insulin quaternary structure in the way that the amount of dimer and hexamer increased in comparison to monomer. Following the pH changes to 6.8, the amount of monomer to dimer or hexamer increased.

Two sets of data are presented, one in the absence of proteases, and the other in the presence of pepsin (gastric) and trypsin (intestinal simulation). It may be seen that the free insulin appears to be stable under gastric conditions, even in the presence of pepsin. After transition to intestinal conditions, insulin concentration dropped steadily over a period of about 1h, with a larger drop in concentration observed in the presence of trypsin. At the end of the simulation, about 78% of the initial amount of insulin at time

zero was still measureable in the absence of proteases, and 71% of the initial amount of insulin at time zero was measureable in the presence of proteases.

While HPLC is not able to distinguish between fully intact and thus active insulin, there is some evidence that the insulin remaining at the end of the simulation was intact. One point of evidence is that only one peak appeared on the HPLC chromatogram consistent with the insulin peak, thus there did not appear to be peaks associated with fragmented insulin. The other point of evidence is that some researchers have observed a small but measureable glycemic response with free oral insulin. (Reis et al. 2007 and Woitiski et al. 2010), possibly pointing to the potential for some insulin to remain intact and stable, even under harsh GIT conditions. Given the amount of residual insulin measured following this simulation, it does appear that the insulin is to some extent stable under these conditions, even when not protected by the nanoparticulate structures.

Bryant et al. (1993) showed that acid stabilization of insulin is attributed to protonation of histidine at position 5 and 10 on the B chain as determined by H-NMR. The role of histidine on stabilizing insulin in acid was examined by changing histidine with other amino acids which showed decreases in insulin stability. By acidification, the net charge of insulin is increased and charge repulsion would destabilize the H^{B5} and H^{B10} folded state due to charge denseness which caused unfolding at that part of the molecule, resulting in insulin stabilization due to changes in the tertiary and quaternary structure. UV spectra of insulin in different pH conditions showed that absorbance at neutral pH is different from that in acidic pH due to changes in the position of aromatic amino acids, side chains and disulfide bonds which cause changes in tertiary or quaternary structure

(Bryant et al. 1993). *In vivo*, insulin is stored in an acidic environment of granules in the β-cells of the pancreas (Halban, 1991) maintained by an ATP-dependent proton pump, thus it seems that the acidic environment of granules is important for insulin stabilization. Also in pharmaceutical formulations, preservative agents such as m-cresol have an important stabilizing effect on insulin (Brange, 1992).

One observation in Figure 17 is that free insulin appears to be stable under gastric conditions, even in the presence of pepsin. After changing the pH to 6.8, insulin decreased in the first hour. It has been reported that insulin exists in solution as an equilibrium mixture of monomers, dimers and hexamers depending on concentration and solution pH (Jeffrey and Coates, 1966; Carpenter, 1966). Dimers are formed by hydrogen bonding, and hexamers are formed in the presence of zinc ions. Insulin at neutral pH and low concentration exists as a monomer (Frank 1972), but in acid (gastric) conditions, insulin monomers can self-associate forming dimers and in the presence of zinc as is the case for Novolin, can form hexamers (Jeffrey and Coates, 1966; Carpenter, 1966).

Changing the quaternary structure of insulin may explain the apparent decrease in insulin concentration under intestinal condition. Altering the pH causes insulin to change mostly to monomer (Chothia et al. 1983). In a study of insulin stability in plasma, it was shown that most of the insulin was present as monomer, with half-life of 4 to 6 min (Duckworth et al. 1998), causing rapid clearance of insulin from blood circulation. Therefore, it is possible that insulin is unstable and susceptible to degradation under neutral pH conditions due to dissociation of the quaternary structure to monomer form.

The amount of measurable insulin in the GIT simulation was also analyzed by ELISA in a separate experiment. Results shown in Figure 18 illustrate the ELISA measureable amounts of Novolin insulin under gastric followed by intestinal simulation. The conditions of the experiment are the same as the previous test except the method of assay, which was done by ELISA so the difference between these two results should be related to the method of assay. Data points represent the mean of two experiments, and standard deviations showed good repeatability between experimental replicates.

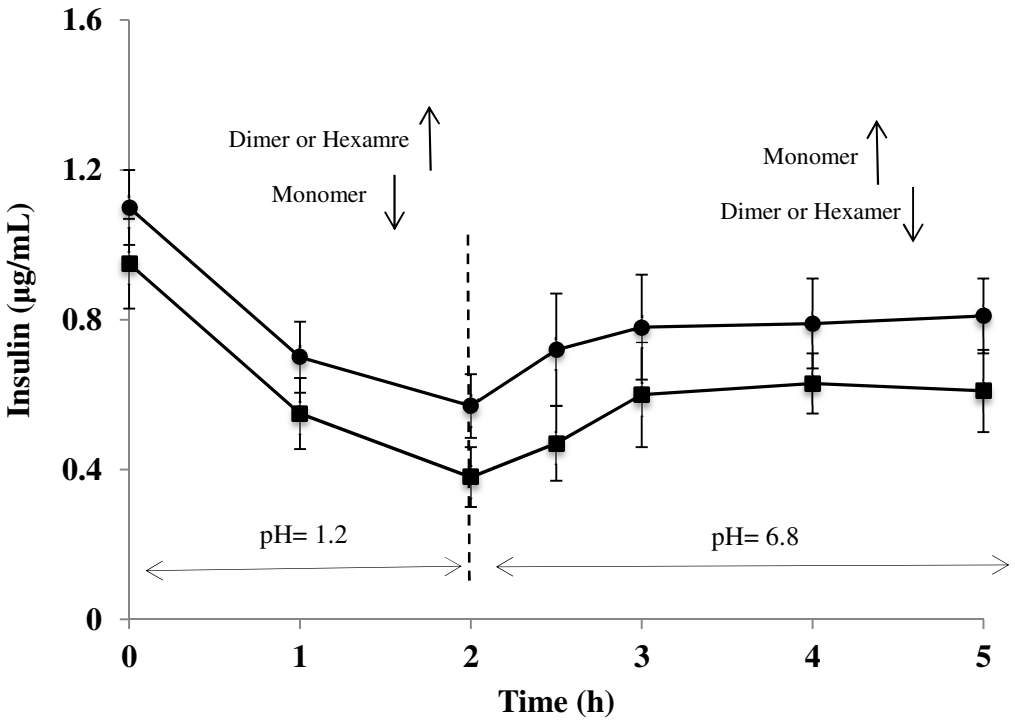

Figure 18. Insulin stability was monitored for 2 h at simulated gastric condition followed by an additional 3 h at simulated intestinal condition. Insulin concentration was measured by ELISA. Stability was tracked in the absence (●) and presence of enzymes (■). Results are mean± SD of two experiments. Acid condition results in conformational changes in insulin quaternary structure in the way that the amount of dimer and hexamer increased in comparison to monomer. Following the pH change to 6.8, the amount of monomer to dimer or hexamer increased.

It can be seen in Figure 18 that the amount of free insulin detectable by ELISA decreased gradually under gastric conditions in presence and absence of pepsin during 2 h. After transition to intestinal conditions, insulin concentration increased during the first hour followed by a stable plateau state over the remaining 2h.

It seems that some portion of the insulin is being degraded due to the acidic environment of the simulated gastric fluid (pH 1.2) and pepsin effect. After 2 h, about 56% of the initial amount of insulin in the absence of pepsin was still measureable, and 42% of the initial amount of insulin in the presence of pepsin was measureable. The difference then between 56 and 42% would be attributable to enzyme degradation.

Under intestinal conditions, insulin concentration appeared to increase during the first hour, followed by a plateau state over the remaining 2h. At the end of the simulation, about 73% of the initial amount of insulin at time zero in gastric medium in the absence of pepsin was still measureable, and 64% of the initial amount of insulin in the presence of pepsin was measureable. It is seen in Figure 18 that the measurable amount of detectable insulin increased from 56% at the end of the 2 h gastric condition to 73% at the end of the 3 h intestinal condition in absence of enzyme and from 42 to 64% at same times and conditions in presence of enzyme. It seems that the apparent increase in the amount of detectable insulin would be related to changing the quaternary structure of insulin and insulin-antibody interaction as no more insulin is added to the medium in intestinal condition.

ELISA is based on the binding of insulin between two different antibodies, so there is the possibility that changes in insulin conformation affects the insulin and antibody interaction, possibly explaining an apparent increase in insulin concentration. In addition, antigen-antibody complex affinity can be affected by pH (Reverberi et al. 2007).

Insulin quaternary structure change is dependent on its concentration, solution pH and presence of zinc ions (Jeffrey and Coates, 1966; Carpenter, 1966). Structural analysis of insulin reveals that in solution at neutral pH and low concentrations, insulin exists as a monomer (Frank et al. 1972) and by increasing pH, the proportion of monomer to other forms (hexamer, dimer) increases (Helmerhorst and Stokes 1987). This may be seen as an increase in ELISA detectable insulin following gastric to intestinal transition, apparent in Figure 18. On the other hand, at higher concentrations of insulin, in acid and in the absence of zinc, the insulin monomer self-associates to form dimers and in the presence of zinc, hexamers (Jeffrey and Coates, 1966; Carpenter, 1966).

Thus, the flexibility of insulin conformation and different quaternary structures has been associated with insulin's various properties and receptor binding (Derewenda et al., 1991). According to results in Figure 18, it can be suggested that pH changes can affect charge of the insulin, which alters the tertiary and quaternary structure of insulin, influencing the extent of antibody binding, likely due to variations in side-chain and main-chain structure because of unfolding/folding in specific areas. The packing arrangement in dimer and hexamer conformation, results in burial of interactive parts of the insulin molecule (Chothia et al. 1983) that are responsible for antigen antibody interaction. As in neutral pH, insulin quaternary structure mostly changes to monomer.

This is the active form of insulin which better interacts with the antibody and provides better detection properties, in comparison to acid condition where insulin is present as dimer and hexamer, likely lowering antibody binding. In principle, one antibody binds with 1 or 2 insulin monomers. Thus when insulin oligomers dissociate to monomer, the stoichiometry of binding is increased, giving the appearance of increasing overall concentration of insulin.

Insulin stability assessment with HPLC and ELISA methods showed important differences. In the HPLC method, insulin appeared stable in acid and by changing the pH to 6.8, insulin decreased to 71% of initial amount of insulin in the presence of enzyme at the end of 5 h. In the ELISA method, the apparent amount of insulin decreased in the acid condition, and by changing the pH to 6.8 the apparent amount of insulin increased. At the end of 5 h in simulated GI medium in presence of enzyme, 64% of initial amount of insulin was detectable.

The amount of measurable insulin at the end of both simulations is different for the two assay methods, likely due to two possibilities. One is that the amount of detectable insulin is seen to be decreased by ELISA, as its detection is based on the interaction between antibody and intact insulin, while HPLC analysis is unable to distinguish between intact insulin and degradation products. The other possibility is the way each detection method interacts with insulin molecules that results in different responses.

In particular, the assay methods respond differently to quaternary changes of insulin resulting from pH changes. To understand the possible effect of pH on the quaternary structure of insulin, which causes different detectable amounts of insulin in each method, the stability of insulin was examined in neutral water.

5.4.3. Evaluation of insulin stability in gastric condition in comparison to neutral water

Novolin ge Toronto insulin was monitored for 5h in neutral pH water to examine insulin stability in water in comparison to GI simulation medium and GI medium with presence of enzyme, as presented in the previous section. The aim of this experiment was to understand the stability behavior of insulin in water by HPLC and ELISA methods to determine if changes in apparent insulin concentration in the previous section are related to pH changes and methods of assay, or simply due to changes to insulin in neutral water over time. It was also desired to understand the extent of the effect of simulated GI medium and enzyme on insulin stability in comparison to neutral water.

Insulin stability in neutral water as detected by HPLC during 5h simulation is illustrated in Figures 19 and 20. The results are compared to that of insulin stability in simulated GI medium in presence and absence of enzymes as was presented in Figure 17.

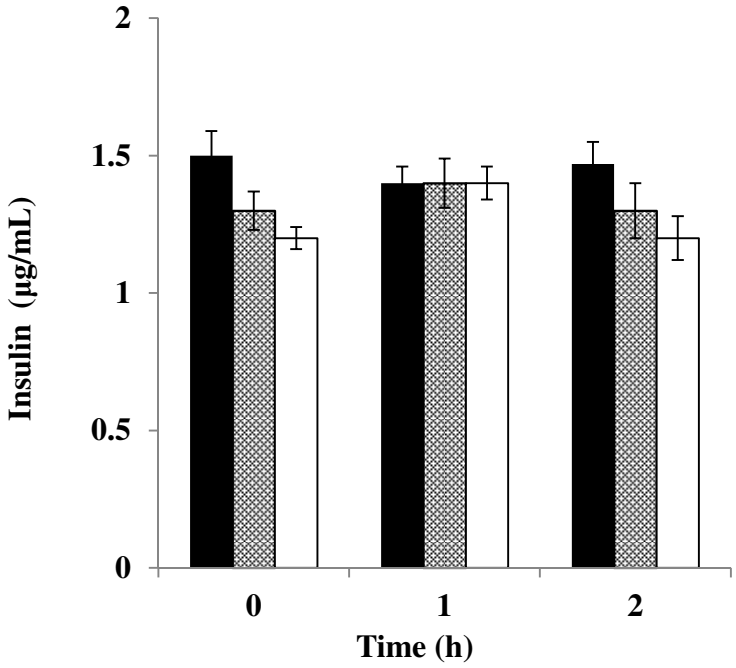

Figure 19. Novolin ge Toronto insulin stability was monitored for 2 h under simulated gastric condition as measured by HPLC. Stability was tracked in neutral pH water (closed bar), acidic medium (hatched bar) and acidic medium with presence of pepsin (open bar). Results are mean± SD of three experiments.

Insulin amount in neutral water seen in Figure 19 was largely unchanged over 2 h and 98% of the initial insulin added to water control was measureable after 2h. It appears that Novolin is stable in water, as was essentially the case in simulated gastric conditions, with and without enzyme.

Insulin in neutral water was assayed for an additional 3h representing intestinal transit. Results in neutral water were compared with insulin in intestinal medium in the presence and absence of trypsin as illustrated in Figure 20.

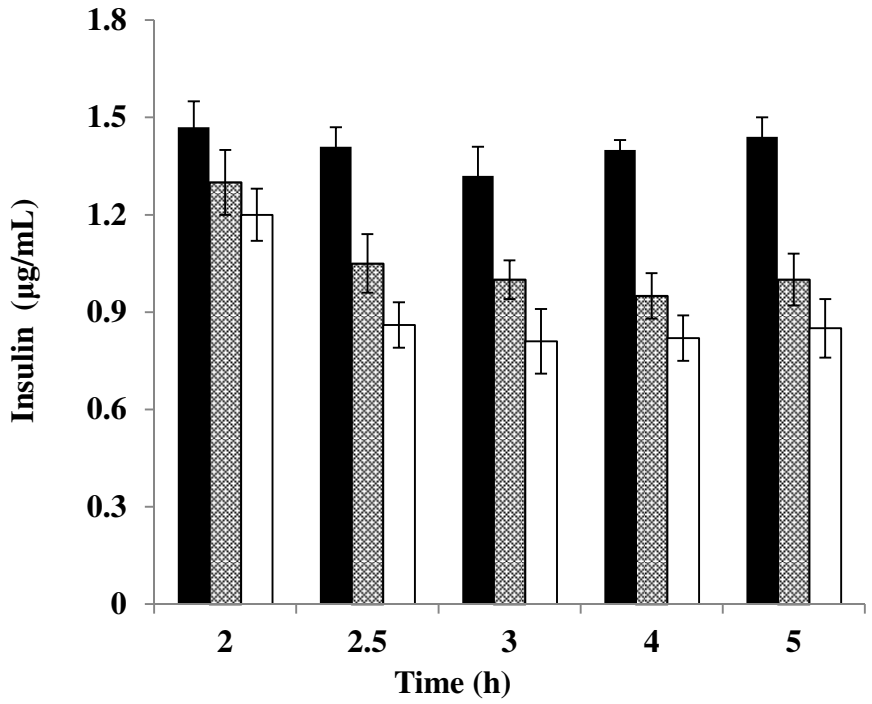

Figure 20. Novolin ge Toronto insulin stability was monitored for 3 h at simulated intestinal condition. Insulin concentration was measured by HPLC. Concentration was tracked in neutral water (closed bar), simulated intestinal medium (hatched bar) and simulated intestinal medium with presence of trypsin (open bar). Results are mean± SD of three experiments.

It may be seen that insulin concentration remained unchanged in neutral water, although there was a gentle dip in insulin, which gradually recovered to the end of the experiment. The measureable concentration of HPLC detectable insulin at the end of 5 h, was 96% of the initial amount.

The amount of measurable insulin in neutral water condition during 5 h was also monitored using ELISA in a separate experiment. The results are compared to Novolin stability in simulated GI medium with and without enzyme that was previously described in Figure 18. The results illustrated in Figures 21 and 22 represent the mean of two separate experiments, and standard deviations are presented showing good repeatability between experimental replicates.

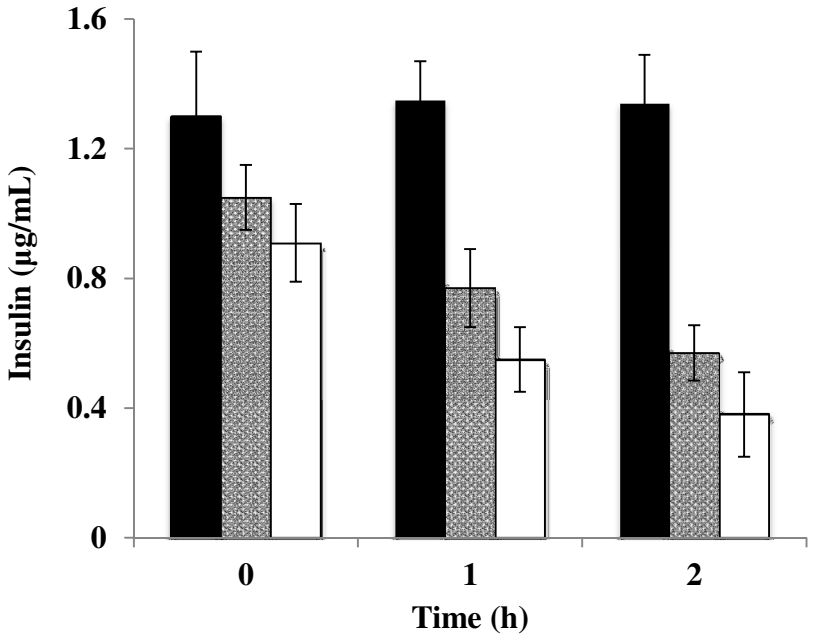

Figure 21. Novolin ge Toronto insulin stability was monitored for 2 h under simulated gastric condition. Insulin concentration was measured by ELISA. Stability was tracked in neutral pH water (closed bar) acidic medium (hatched bar) and acidic medium with presence of pepsin (open bar). Results are mean± SD of three experiments.

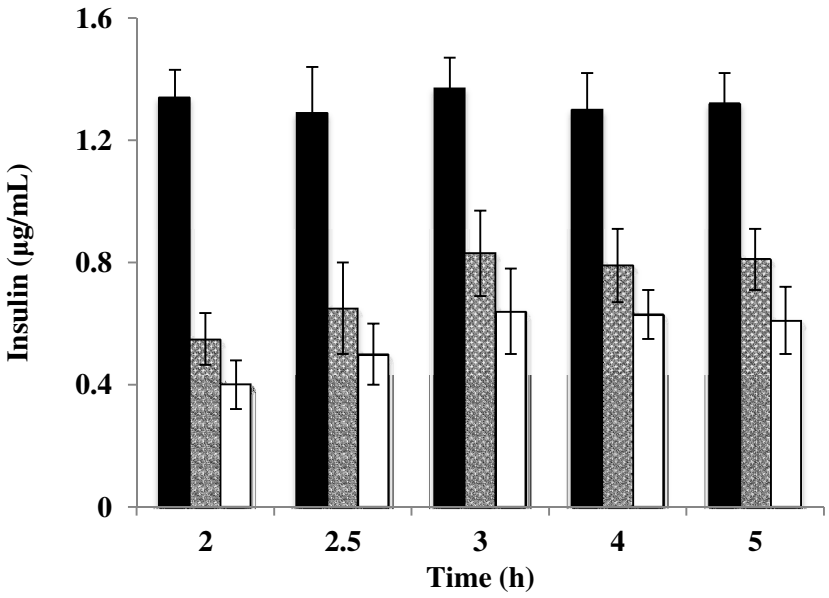

Figure 22. Insulin stability was monitored by ELISA for 3 h under simulated intestinal condition. Stability was tracked in neutral pH water (closed bar) intestinal simulation medium (hatched bar) and simulation medium with presence of trypsin (open bar). Results are mean± SD of three experiments.

Insulin was stable during total of 5 h in neutral water with almost 97% of insulin detectable by ELISA in comparison to the initial amount added. These results are consistent with those observed by HPLC.

Base on the consistent results in neutral water as measured by HPLC and ELISA, it can be suggested that the contradictory results between HPLC and ELISA analysis in simulated GI medium are likely due to insulin association or dissociation behavior in different pH conditions that affect the result, due to differences in the detection mechanism of the two methods. The pH plays an important role in tertiary and quaternary changes to insulin which cause different responses to insulin by the different methods of analysis.

5.5. Insulin release profile in stimulated gastrointestinal medium

The focus of this part of the investigation was to evaluate the stability of nanoparticulate insulin in simulated GI medium, in the presence and absence of proteases.

An insulin release experiment was performed by incubating 100 µg of lyophilized nanoparticles in 75 mL of HCl buffer at pH 1.2 (USP 31), simulating gastric conditions. Particles were agitated using a paddle impeller at 50 rpm for 2 h without enzyme or by adding 750 U/mL of pepsin to simulate the enzymatic gastric medium (USP 31). After 2 h, reaction was stopped by adding pepsin inhibitor (Pepstatin A).

To simulate the progress of nanoparticles moving from stomach to small intestine, the buffer pH was changed to 6.8 after 2 h to simulate intestinal condition without enzymes or by additionally adding 1750 USP U/L of trypsin to simulate the enzymatic intestinal medium (USP 31). Reaction was stopped by adding trypsin inhibitor. Samples were withdrawn at specific points and the released insulin assayed using ELISA. Cumulative insulin release was then calculated as percentage of initial insulin content.

Figure 23 illustrates the insulin release profile in the absence and presence of protease enzymes in stimulated gastrointestinal fluids. Insulin release was low in gastric condition due to the acid protective nature of the nanoparticle complex and only 8% of the encapsulated insulin was released in the gastric fluid in comparison to 14% in gastric medium in presence of enzyme during the first 30 min.

At low gastric pH, insulin is retained likely due to alginate compaction during precipitation of alginic acid forming a compact acid-gel structure (George et al. 2006, Reis et al. 2007, Cheng et al 2004). Most of the insulin is retained due to the particles collapsing in acid, reducing particle permeability, promoting insulin retention. Subsequent decrease in the amount of insulin during the 2 h is likely caused by the hydrolysis effect of pepsin and acid on insulin. The formulation would be able to protect the remaining 92% of insulin retained in the particles in acidic condition without enzyme and 86% with presence of enzyme.

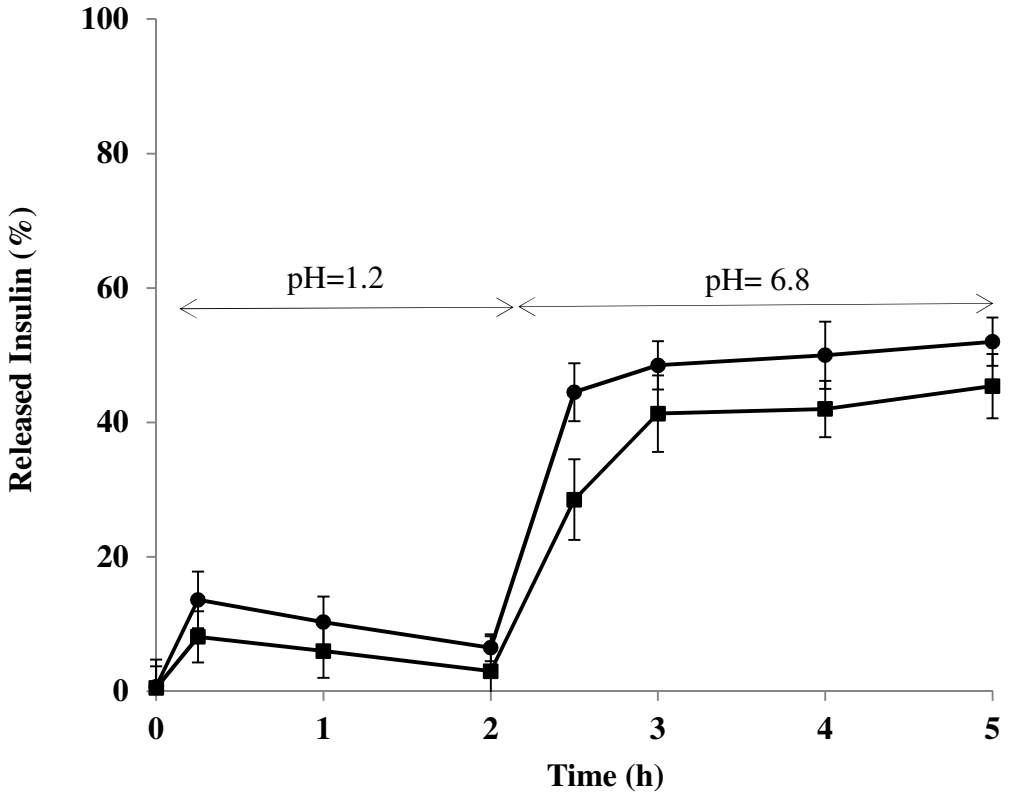

Figure 23. Insulin release profile was monitored for 2 h at simulated gastric condition followed by an additional 3 h in simulated intestinal condition. Insulin concentration was measured by ELISA in the presence (●) and absence of enzymes (■).

After changing the pH to 6.8, an initial rapid release of insulin occurred in the first hour, followed by a plateau state during the remaining two hours. At neutral pH, alginate matrix swells and becomes more permeable promoting insulin release (Reis et al. 2007). The alginic acid formed under gastric conditions will become increasingly soluble and thus unstable at neutral pH, enabling release of encapsulated insulin (George et al. 2006). Also Ca^{2+} released from the gel promotes destabilization of the particles. At neutral pH, alginate and insulin are both negatively charged and electrostatic repulsion promotes insulin release (Reis et al. 2007). After a total of 5 h in acidic followed by neutral pH medium in presence of enzyme, the formulation retained 48% of insulin within the particles.

Evidence for the possibility that particles are collapsing and reswelling as they transition from acid to neutral pH conditions was obtained by measuring particle diameter under such conditions. The results illustrated in Figure 24 indicate that the mean particle size was 48.5 nm in acetate buffer, 34.7 nm in simulated gastric fluid and 80.3 nm in simulated intestinal fluid, demonstrating that the particle size is dependent on pH, consistent with the explanation provided in the previous section. Collapse of particles in acid medium, then promotes insulin retention and protection. Subsequent transfer to neutral pH medium results in particle swelling, promoting release of insulin.

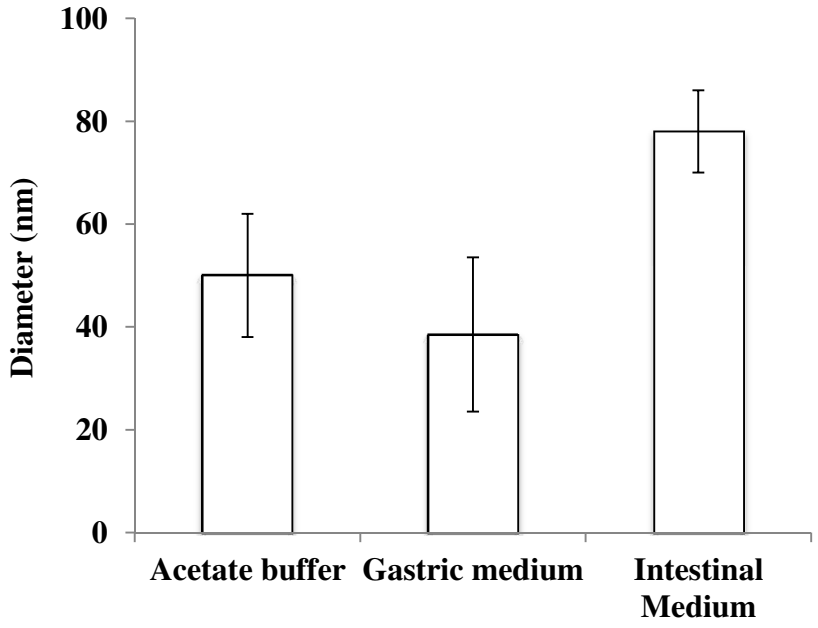

Figure 24. Mean size of insulin nanoparticles in acetate buffer pH 4.5, simulated gastric medium pH 1.2 and intestinal medium at pH 6.8. Data are mean ± SD of two separate experiments.

After the end of 2 h gastric simulation followed by 3 h of intestinal simulation, 52% of the insulin is released in presence of enzymes as shown in Figure 23. The balance (48%) is retained in the particles.

The retention amount was verified by dissolving the particles after the 5h simulation, in 0.1 M PBS and 0.1 M EDTA for 48 h. After 24 h, the particles were separated through centrifugation and resuspended in fresh buffer. At the end of 48 h, undissolved particles were separated by centrifugation and resuspended in water for 2 h, vortexed and the supernatant was again separated by centrifugation for ELISA analysis. Insulin released

for each time point was measured to determine the amount of intact insulin retained inside the particles after the simulation. The measured amount was compared to the amount of insulin initially added to the formulation.

The results illustrated in Figure 25 show insulin retained in nanoparticles after 5 h suspension in simulated GI medium with and without enzyme. It may be seen that 53% of the initial amount of insulin was detectable after 5 h suspension in simulated GI fluid in comparison to 45% of insulin after 5 h suspension in simulated GI fluid in presence of enzyme.

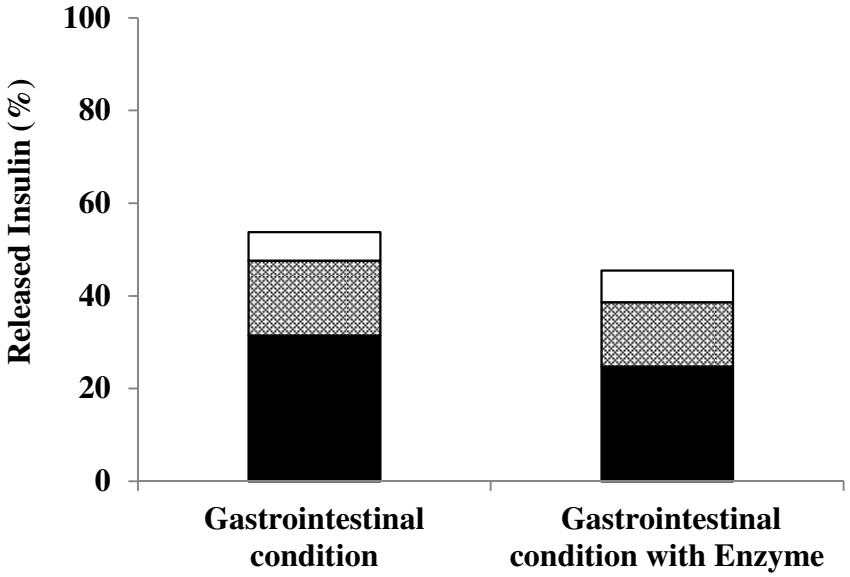

Figure 25. Retained insulin concentration was measured by ELISA after 24 (closed bar) and 48 h (hatched bar) of suspending particles in solution of 0.1 M PBS and 0.1 M EDTA. After 48 h, undissolved particles were separated and dispersed in water for 2 h (open bar). The supernatant of each step was separated by centrifugation and analyzed by ELISA. Released insulin was calculated based on the percent of insulin entrapped in particles compared to the amount added in the initial formulation.

Based on the initial amount of insulin entrapped in the nanoparticles, it is confirmed that the formulation can fully retain up to 45% of the insulin in extended simulated gastrointestinal condition in the presence of proteolytic enzymes. The alginate-dextran nanoparticles coated by chitosan and albumin can play a role in preserving the biological activity of a protein drug under gastrointestinal conditions in comparison to other formulations by protecting it during formulation and from proteolytic and acidic degradation during gastrointestinal transit. Reis et al. (2008) studied the glycemic response of insulin nanoparticles following subcutaneous injection to diabetic rats. Results showing that the nanoparticulate insulin was as effective as the same dose of free insulin, demonstrating full retention of insulin activity in nanoparticles. The 45% retention of insulin in nanoparticles is similar to the 42% bioavailability that was reported by Reis et al. (2008) based on animal studies.

5.6 Evaluating the effect of simulated intestinal buffers and electrolytes on release profile

Conventional drug release tests, examining transit from stomach to intestine are normally considered by changing the pH from acidic (1.2) to neutral (6.8) by using phosphate buffers. Although phosphate buffers are formulated to accurately capture intestinal pH conditions, they are not chemically representative of the *in vivo* environment (McConnell et al. 2008). In the intestine, pH control is achieved by bicarbonate secreted from the pancreas and intestinal epithelial cells. In addition, small intestinal fluid is a complex medium containing lipids, bile salts, digestive enzymes, proteins, polysaccharides and electrolytes, none of which are modeled in a simple phosphate buffer.

Insulin release behavior was investigated in different stimulated small intestinal media by incorporating bicarbonate buffer and physiological electrolytes, as summarized in Table 2.

Table 2. Components (mM) of simulated intestinal buffers (Adjusted to pH=7.4)

Constituents (mM)	**Phosphate Buffers**		**Krebs Bicarbonate Buffers**		
	PB-A	**PB-B**	**KBB-A NaCl: $CaCl_2$ (47:1)**	**KBB-B NaCl: $CaCl_2$ (10:1)**	**KBB-C NaCl: $CaCl_2$ (1:1)**
KH_2PO_4	1.8	1.8	1.2	1.2	1.2
Na_2HPO_4	10	10	–	–	–
NaHCO3	–	–	24	24	24
NaCl	–	118	118	110	60
KCl	–	4.7	4.7	4.7	4.7
$CaCl_2$	–	2.5	2.5	11	60
$MgSO_4•7H_2O$	–	1.2	1.2	1.2	1.2

PB-A (Standard Phosphate Buffer)
PB-B (Standard Phosphate Buffer with Electrolytes)
KBB-A (Standard Kreb's Bicarbonate Buffer)
KBB-B, KBB-C (Standard Kreb's Bicarbonate Buffer with Different Amounts of Electrolytes)

Phosphate Buffer A (PB-A) was selected as one of the commonly used intestinal simulation mediums. Phosphate Buffer B (PB-B) was a more complex version of A with four salts added in equivalent concentrations to those in Krebs Bicarbonate Buffer A (KBB-A). Due to presence of bicarbonate as the buffering agent and inclusion of common physiological electrolytes found *in vivo* (Fadda et al. 2009), KBB-A is used as a model to simulate conditions in the small intestine. Krebs Bicarbonate Buffers B and C

(KBB-B and KBB-C) were modified forms of KBB-A with different sodium chloride to calcium chloride molar ratios (the total molar amount of sodium chloride and calcium chloride was kept constant). *In vivo*, the ratio of sodium to calcium varies based on the composition of recent meals (Lindahl et al. 1997). For example after a high-sodium meal, sodium levels increase in the small intestine that can cause an increase in the amount of insulin released prematurely in the intestinal lumen, thus reducing the drug pharmacological availability or the opposite effect might occur after a high calcium meal, improving the level of pharmacological availability.

5.6.1. Effect of buffer and electrolytes on insulin release from nanoparticles

Insulin nanoparticles were added to 10 mL of stimulated buffer, and mixed at 50 rpm in a magnetically-stirred Erlenmeyer flask for 180 min. Released insulin was analyzed by HPLC. Insulin release profiles are shown in Figure 26.

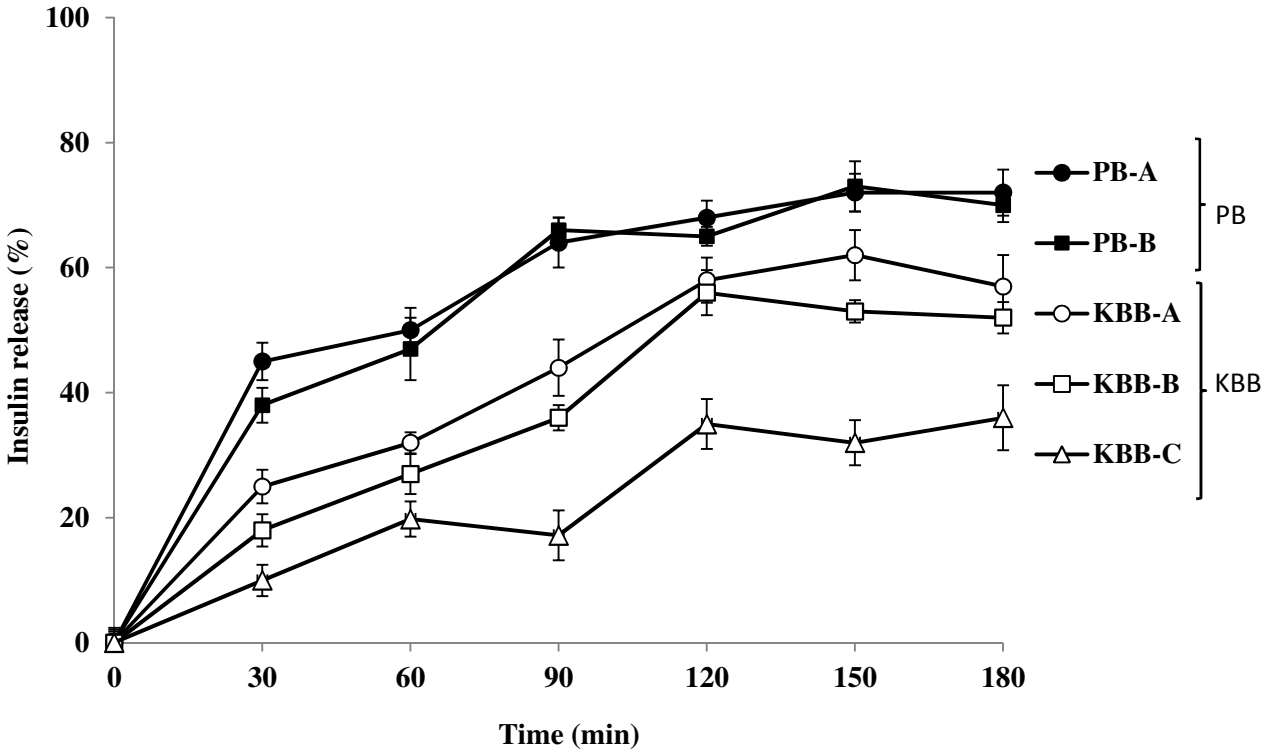

Figure 26.Comparison of insulin nanoparticle release profiles measured by HPLC resulting from the use of PB-A (●), PB-B (■), KBB-A (O), KBB-B (□) and KBB-C (Δ) as the small intestinal simulation media. Results are the mean± SD of three experiments.

The release profiles from particles in the two phosphate buffers (PB-A and PB-B) were similar, as observed through overlapping error bars. In both cases, the initial rate of release was faster compared to the bicarbonate buffers where about 40% of insulin was released in the first 30 min and 70% after 180 min. Release was slower in KBB-A and KBB-B, with 20% released in the first 30 min and 50% released after 180 min. KBB-C yielded a release profile that was very different from the other media, with approximately 10% released in the first 30 min and 70% of the insulin remaining entrapped within the particles at the end of the experiment. For all buffers, some amount of the insulin

remained entrapped within the particles, but the largest amount of insulin was retained in KBB-C medium.

Important differences were observed between the release profiles in PB-B and KBB-A, which represent media with the same electrolyte composition but were buffered with different electrolytes. In the first 30 min, release was 25% in KBB-A, much less than the 40% in PB-B. Full release was not observed in KBB-A and about 50% of the insulin remained entrapped. It seems that phosphate was more effective at disrupting the calcium-alginate matrix of the particles than was bicarbonate. The negative effect of phosphate on alginate gels is known, as phosphate buffers are a commonly used method for dissolving these polymers. Phosphate as a trivalent anion, complexes calcium cations, which stabilize the polymer network, causing the structural breakdown (Pillay et al. 1998). Whether a similar effect occurs with carbonate is unknown, but the results suggest that it is not as strong an effect as that of phosphate.

It is also seen that release was slowed as the ratio of NaCl:$CaCl_2$ decreased in the bicarbonate buffered media. Sodium can disrupt cross-linking between alginate chains by displacing calcium. KBB-A was used in this study as a model to most closely simulate conditions in the small intestine which has a NaCl:$CaCl_2$ ratio of 47:1 mM. The destabilizing effect of sodium overcame the stabilizing effect of calcium, promoting insulin release and particle swelling. By changing the NaCl:$CaCl_2$ ratio in KBB-B to 10:1 mM, particle swelling was depressed and insulin release rate was reduced as result. At the end of the experiment, around 50% of the insulin remained entrapped within the particles. In KBB-C (1:1), insulin release rate was decreased significantly as the amount of entrapped insulin in particles was 70% at the end of the experiment. It seems that by

increasing the amount of calcium in the medium, it is possible to increase the stability of the particles and thus improve retention of insulin.

Comparison of release profiles in Figure 27 reveal that the rate of insulin release from nanoparticles is dependent upon both the buffer type and the electrolyte composition of the simulation medium. For buffer type, the phosphate buffers promoted a faster rate of release than the bicarbonate buffers. For electrolyte composition, release was slowed in the bicarbonate buffers as the sodium to calcium ratio decreased. The presence or absence of physiological electrolytes had no clear effect on the release behavior in the phosphate buffers.

Chapter 6

Conclusions and recommendations for future study

The overall goal of the present study was to focus on the nanoparticulate insulin carriers developed by Reis et al. (2008), which showed the highest level of oral pharmacological availability reported in the literature thus far. The specific objective of the research was to evaluate the factors responsible for limiting the bioavailability of insulin in nanoparticulate oral dosage form and understand the factors that affect insulin stability in gastrointestinal (GI) medium.

Each drug delivery formulation requires analytical techniques to accurately measure the content of active ingredient. In this study, four different methods of protein assay were examined for measuring insulin concentration, when released from nanoparticles. The aim was to understand the possible interactions between the excipients or particle components in the formulation with the assay methods, to minimize the chance of an interfering response in insulin measurement. Two forms of insulin used were Novolin ge Toronto human recombinant insulin obtained as a pharmaceutical preparation, and pure human recombinant insulin in crystalline form. Between the four different assay methods, ELISA and HPLC presented the most advantages, due to possible interferences with excipients and particle components observed with BCA and UV assays. ELISA and HPLC methods do not show interactions with the ingredients in the formulation. ELISA detection is based on the interaction between antibody and intact insulin but the other assays will not distinguish between intact insulin and degradation products. In HPLC, insulin is separated from other components, thus reducing interferences.

Different factors could affect the insulin release profile from particles, reducing bioavailability and thus the effectiveness of oral insulin. Methods used for *in vitro* studies usually inadequately represent the conditions that drugs are exposed to *in vivo*. On the other hand, the *in vivo* test is expensive, complicated and cannot be applied to patients, so there is a need to improve the efficiency of *in vitro* studies as an inexpensive and simple method for exploring the destiny of orally administered drugs. In this study, different factors that could affect the release profile, and thus the bioavailability, were examined such as mixing pattern, simulated GI medium in the presence and absence of enzyme, and different simulated intestinal buffers and electrolytes.

The main goal of the GI simulations was to determine the extent that nanoparticulate insulin could be stable in acidic gastric condition and in neutral intestinal condition, in presence and absence of proteases and to evaluate the stability of insulin released from nanoparticles into the GIT.

Insulin stability assessment with HPLC and ELISA methods showed contradictory behavior of insulin. In the HPLC method, insulin appeared stable in acid and by changing the pH to 6.8, insulin decreased to 71% of initial amount of insulin in the presence of enzyme at the end of 5 h. In the ELISA method, the apparent amount of insulin decreased in the acid condition, and by changing the pH to 6.8, the apparent amount of insulin increased. At the end of 5 h in simulated GI medium in presence of enzyme, 64% of the initial amount of insulin was detectable. It seems that the released insulin from the nanoparticles could be stable and detectable in GIT medium as at the end of 5 h in presence of enzyme, 64% of the insulin was detectable by ELISA.

To understand the contradictory behavior of insulin as assayed by HPLC and ELSA, the results were compared with insulin stability in neutral water. The results showed that 97% of insulin was detectable over 5h by both ELISA and HPLC. It can be suggested that the contradictory behavior of insulin in the gastric and intestinal simulations are likely due to insulin association or dissociation behavior that cause changes in tertiary and quaternary structures of insulin at different pH conditions. The changing pH plays an important role on insulin structure, which caused the inconsistent assay results. Insulin exists in solution as an equilibrium mixture of monomers, dimers and hexamers depending on concentration and solution pH (Jeffrey and Coates, 1966; Carpenter, 1966). Dimers are formed by hydrogen bonding, and hexamers are formed in the presence of zinc ions. Insulin at neutral pH and low concentration exists as a monomer (Frank 1972), but in acid (gastric) conditions, insulin monomers can self-associate forming dimers and in the presence of zinc as is the case for Novolin, can form hexamers (Jeffrey and Coates, 1966; Carpenter, 1966). As a result, pH changes, protein concentration and presence of zinc can affect the quaternary structure of insulin that can result in changes to the side-chain and main-chain structure. The packing arrangement in dimer and hexamer conformation results in burial of interactive parts from the surface of the insulin molecule (Chothia et al. 1983) that will ultimately affect the test results. ELISA detection is based on the binding of insulin between two different antibodies, so there is the possibility that changes in insulin conformation affects the insulin and antibody interaction. In addition, antigen-antibody complex affinity can be affected by pH (Reverberi et al. 2007).

This is likely due to how the methods interact with insulin based on the quaternary changes of molecules for detection. Thus the difference between the remaining insulin in

the media after 5 h simulation should be related to the difference in the mechanism of detection. Also there is another possibility that the difference between the detectable amounts of insulin at the end of 5 h simulation, is due to ability of ELISA to detect intact insulin based on antibody binding preferentially to the monomeric form, while HPLC is unable to distinguish between the different forms of insulin including degradation products.

Insulin release profile showed that release was low in gastric condition due to the acid protective nature of the nanoparticle complex, resulting in collapse of the alginate-core particles in acid reducing particle permeability. After 2h simulation, 86% of the insulin was retained in the particles in acidic condition in presence of enzyme. By changing the pH to 6.8, an initial release of insulin occurred in the first 1 h followed by plateau state due to swelling of the alginate matrix at neutral pH, increasing permeability promoting insulin release.

Finally after 5h in acidic followed by neutral pH medium and presence of enzyme, the formulation retained 48% of insulin in the particles. The retention amount was verified by dissolving the particles after the 5 h simulation and measuring the amount of intact insulin. It was shown that 45% of the initial formulated ELISA detectable insulin remained entrapped in particles following the simulation, thus accounting for nearly 100% of the initially formulated insulin.

Insulin release behavior was also investigated in different stimulated small intestinal media using bicarbonate buffer, phosphate buffer and physiological electrolytes. The release profile from particles was slower in bicarbonate buffers in comparison to

phosphate buffer. In the two phosphate buffers (PB-A and PB-B) the initial rate of release was faster in comparison to the other media where about 40% of the insulin was released in the first 30 min and 70% after 180 min. Release was slower in the Kreb's bicarbonate buffers KBB-A and KBB-B, with 20% released in the first 30 min and 50% released after 180 min. KBB-C showed a release profile that was very different from other media, with about 10% released in the first 30 min and 70% of the insulin remaining entrapped within the particles at the end of the experiment. The largest amount of insulin was retained in KBB-C medium.

It can be concluded that the rate of insulin release from nanoparticles is dependent on both buffer type and electrolyte composition of the simulation medium. The phosphate buffers promoted a faster rate of release than the bicarbonate buffers. For electrolyte composition, release was slowed in the bicarbonate buffers as the sodium to calcium ratio decreased. It can be concluded that the type of meal based on the Na or Ca content would be able to affect the amount of insulin released in the intestinal lumen, thus potentially reducing/increasing the drug pharmacological availability.

In this study, alginate-dextran nanoparticles coated by chitosan-PEG and albumin showed the potential to protect 45% of the insulin in the particles in stimulated GI medium with presence of proteases. The main part of insulin release from the delivery device occurred after changing the pH to that of intestinal medium.

The rate of insulin release from nanoparticles is dependent on buffer type and electrolyte composition of the simulation medium as by changing the phosphate buffer to bicarbonate buffer, the rate of insulin release decreased.

Future research should be directed to improvements to the oral insulin drug delivery system by addressing the premature insulin release after changing the pH to 6.8. Different protective coatings or techniques that could retain encapsulated insulin or reduce insulin breakdown in the GI tract, should enable improved bioavailability. Also studying the conjugation of vitamin B_{12} to insulin structure is a promising method to preserve stability of insulin in the GI tract and improve its bioavailability, as there is a an active transport mechanism in the GIT for absorption of large vitamin B_{12} molecules. It is possible that conjugating insulin with B_{12} will protect the insulin from digestion and improve uptake and absorption. Additionally, enhancements to the *in vitro* model should be developed, to evaluate the release profile using more complex simulated media that better represent GIT conditions. Intestinal fluids contain lipids, bile salts, digestive enzymes, proteins, polysaccharides and electrolytes that normally are not modeled in the regular *in vitro* method. Also, further examination of the quaternary structures of insulin as function of pH that will play important role in insulin stability may be provided by Gel Permeation Chromatography (GPC) based on the changes of the molecular weight of insulin.

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