

**ALGINATE MICROPARTICLES PRODUCED BY SPRAY DRYING FOR ORAL
INSULIN DELIVERY**

by

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Abstract

The aim of this study was to prepare biologically active insulin-loaded alginate microparticles by spray drying. Particles were produced from three alginate feed concentrations of 1, 1.5 and 2% w/v, with respective insulin loadings of 11.8, 7.8 and 5.8 mg/g of alginate and investigated in terms of mass yield, moisture content, particle size, morphology and encapsulation efficiency. The mass yield of the system was determined to be between 15 and 30%, with approximately 3% of the initial dry mass ending up in the exhaust filter. The moisture content of the particles was found to be between 4.9 and 11.1% and the mean size ranged between 1.2 and 1.6 μm . Particulate morphologies were observed to be mostly spherical with some 'divots' present on the surface. Lastly, the encapsulation efficiency determined by absorbance assay was approximately 40%. Particles produced from a 2% alginate feed were further assayed by determining the release of insulin in simulated gastrointestinal conditions and looking at the insulin and alginate distribution within spray dried particles. A steep release profile was observed in the first 120 min of the simulation in a gastric pH of 1.2 and a longer, more sustained release is observed in intestinal conditions, where an additional 20% of the total insulin in the particles is released over 600 min. Fluorescent labels revealed that insulin and alginate are concentrated towards the periphery of the particles. The residual bioactivity of insulin was assessed by an *in vitro* bioactivity assay, which was developed using Fast Activated Cell Based ELISA (FACE™) AKT kits specific for phosphorylated AKT. The bioactivity of insulin in the particles after spray drying was determined to be $87.9 \pm 15.3\%$.

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Chapter 1

Introduction

1.1 Diabetes

According to the World Health Organization, 180 million people worldwide suffer from diabetes mellitus, a number which is expected to rise to 360 million by 2030 (World Health Organization, 2006). There are two forms of diabetes: child onset or type I diabetes and adult onset or type II diabetes. Type I diabetes is an autoimmune disease characterized by the destruction of β -cells in the islets of Langerhans of the pancreas (Castano *et al.*, 1990). β -cells produce and secrete the polypeptide hormone, insulin, which, in conjunction with other hormones, regulates glucose levels in the blood. Without these cells, type I diabetics have no means to produce insulin and are therefore forced into a state of hyperglycemia because their cells cannot absorb glucose from food. Currently, the only effective treatment for type I diabetes is insulin therapy administered to control blood sugar levels (Carino *et al.*, 1999). Type II diabetes, in contrast, is characterized by insulin resistance and glucose intolerance often associated with genetics and poor dietary habits (Leahy, 2005). Generally, type II diabetes can be controlled with diet and exercise; however, in advanced cases, which result in β -cell apoptosis, it is also treated with an insulin therapy regime. Although there are other treatment options available to type I and type II diabetics, such as pancreatic and islet transplants, these procedures currently depend on the availability of human donors and, in the case of islet transplants, do not offer permanent insulin independence (Bretzel *et al.*, 2007; Shapiro *et al.*, 2006). In short, most type I diabetics and approximately 27% of patients with type II diabetes must take parenterally administered insulin treatments daily to manage the

disease, a number that is growing at an astonishing pace around the world (Mayfield *et al.*, 2004; World Health Organization, 2006).

1.2 Insulin

Insulin was first isolated in the 1920's by Frederick Banting and Charles Best to the relief of many diabetic patients who, before that time, would have been subject to rigorous starvation diets and a poor quality of life (Allan, 1972). The crude formulation developed by Banting and Best was initially isolated from a canine pancreas and eventually standardized with support from the American pharmaceutical company, Eli Lilly (Sinding, 2002). Since that time, insulin has become one of the most widely used protein therapies in the world and therefore, very well characterized and studied. Insulin was also the first biopharmaceutical to be successfully mass-produced by recombinant DNA technology.

Insulin is a hormone that is made of two polypeptide chains, named chain A (21 amino acids) and chain B (30 amino acids), connected by two disulfide bridges, as shown in Figure 1 (Cuatrecasas *et al.*, 1990). Insulin can be in monomer, dimer or hexamer form depending on its concentration and the presence of zinc. For most commercially available insulin brands, such as NovoRapid and Novolin GE Toronto, zinc is included in the formulation and the insulin is thus found in a hexameric form with two zinc cations found in the core (Sadrzadeh *et al.*, 2007). A unit of insulin (U) sometimes referred to as an international unit (IU) or physiological unit was originally based on the amount of insulin required to lower the blood sugar of a rabbit to 0.045% within 4 h (Sinding, 2002). It was

later changed to the clinical unit, which is based on a standard preparation where 1 unit is equivalent to roughly 3.5 mg of the standard pure insulin powder.

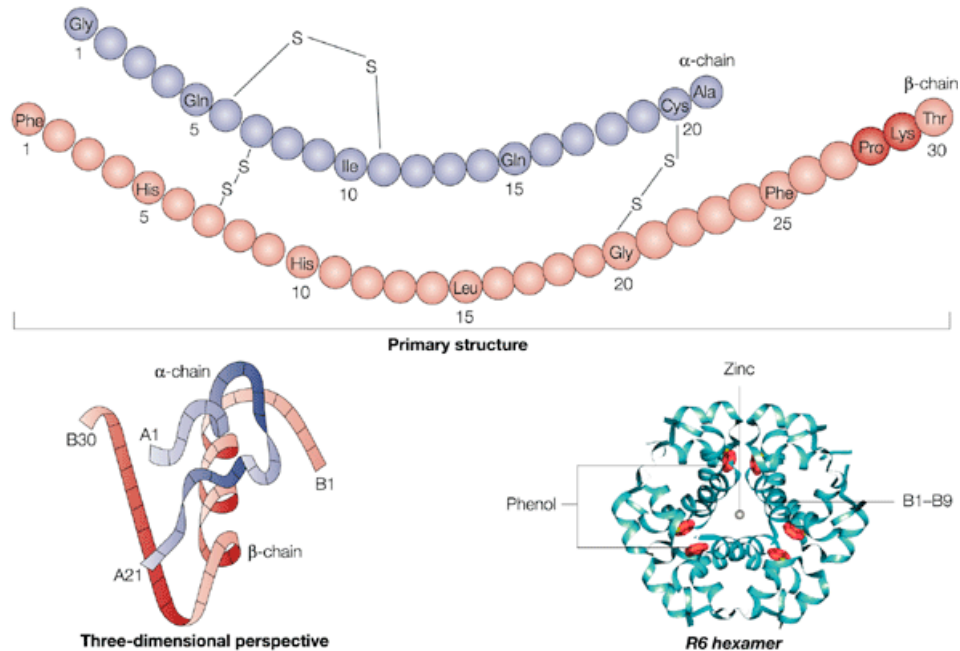


Figure 1 - Insulin α - and β - primary chains, tertiary structure and ribbon representation of phenol Zn insulin in hexameric form (Reproduced from (Owens, 2002))

1.3 Insulin Delivery

In spite of the prevalence of subcutaneous insulin injections, there are several concerns associated with this mode of administration including injection anxiety, pain, cost, infection, and an overall decrease in patient compliance compared to other, more socially accepted methods of delivery such as oral or transdermal methods (Cefalu, 2004; Khafagy *et al.*, 2007). In fact, it has been demonstrated that in order to strictly regulate glucose levels, the average insulin-dependant diabetic requires approximately 5-6 insulin

treatments daily, however most only take 2-3 injections (Sadrzadeh *et al.*, 2007). As a result, patients are subjected to extended periods of hyperglycemia, which is associated with diabetes related complications including blindness, lower limb amputations and kidney failure. Additionally, despite the benefits of tight glycemic control, type II diabetics are less likely to start an insulin regime because of the stigma and inconvenience associated with daily insulin injections (Raj *et al.*, 2003). This has prompted researchers to search for alternative delivery methods including 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). Despite extensive research, these methods of administration have only been met with limited success due primarily to physiological barriers to delivery. Indeed, the only alternative mode of administration to reach the market, a pulmonary delivery device developed by Pfizer, was discontinued after reports of safety concerns associated with insulin overdoses (Mathieu *et al.*, 2007).

Among the possible delivery routes, peroral delivery is particularly interesting because it offers many advantages including ease of administration and high patient compliance (Chalasanani *et al.*, 2007). Good patient compliance and tight glycemic control is correlated with a decrease in complications. This could potentially improve the quality of life for millions of diabetics. As well, people with poor dexterity, such as children and the elderly, would benefit from not having to use cumbersome needles and insulin paraphernalia if an oral insulin system were developed (Belmin *et al.*, 2003).

Despite the advantages of oral insulin, there is no commercially available delivery system on the market today due mainly to its low bioavailability in the gastrointestinal tract (GIT) (Sadrzadeh *et al.*, 2007). There are two principal factors that are attributed to this low bioavailability: enzymatic degradation and poor absorption across the epithelial lining of the GIT (Hamman *et al.*, 2005). Most of the research and strategies developed for oral insulin delivery have been aimed to overcome these challenges through various chemical and formulation strategies (Raj *et al.*, 2003).

Chapter 2

Literature Review

2.1 Oral Insulin Delivery

Among possible systemic absorption routes, peroral delivery is particularly interesting because it offers many advantages including ease of administration and high patient compliance (Chalasani *et al.*, 2007). In addition, oral delivery offers the unique feature of mimicking the physiological path for certain therapeutics such as insulin, that could enter the hepatic portal vein from the intestine and travel directly to the liver (Raj *et al.*, 2003). Accordingly, insulin delivered directly to the liver could decrease complications, such as atherosclerosis, which are associated with high concentrations and build-ups of insulin in the blood. In contrast, insulin injected subcutaneously must circulate through the body before reaching the liver. Despite these advantages, there are few oral protein and vaccine based delivery systems and no insulin delivery systems available due mainly to low bioavailability in the gastrointestinal tract (GIT) (Sadrzadeh *et al.*, 2007). There are two principal factors that are attributed to low bioavailability: enzymatic or acid degradation and poor absorption across the intestinal mucosa (Hamman *et al.*, 2005). Insulin, in particular, is too large and hydrophilic to cross the epithelial lining of the GIT (Cui *et al.*, 2006).

2.2 Approaches to Oral Insulin Delivery

Several strategies have been employed in order to improve the bioavailability of oral insulin aimed both at protecting the peptides against proteolytic degradation in the

GIT as well enhancing their permeability across the intestinal epithelial layer. These approaches can be broken down into four principal categories: chemical modifications to insulin, protease inhibitors, absorption enhancers and particulate delivery systems (Khafagy *et al.*, 2007).

2.2.1 Chemical Modifications

Some researchers have attempted to modify or attach certain molecules to the structure of insulin to increase its solubility and bioavailability in the gastrointestinal tract, and provide protection against proteolytic enzymes (Damgé *et al.*, 2007; Dave *et al.*, 2008). For example, Dave *et al.* (2008) attached a short-chain methoxypolyethylene glycol derivative to improve insulin bioavailability and Xia *et al.* (2000) demonstrated that insulin-transferrin conjugates undergo receptor-mediated endocytosis across intestinal epithelial cells, which lead to a significant hypoglycemic response compared to native insulin (Dave *et al.*, 2008; Xia *et al.*, 2000). Figure 2 shows a diagram representing an insulin-transferrin conjugate linked through disulfide bonds.

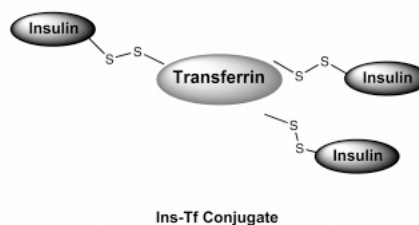


Figure 2 – Insulin and transferrin conjugate developed by Kavimandan *et al.* (2006) to improve oral insulin bioavailability (Reproduced from (N. J. Kavimandan *et al.*, 2006))

Other researchers have examined site-specific oligomeric modifications, which are thought to increase half-lives *in vivo* and provide greater enzymatic resistance compared to native insulin (Clement *et al.*, 2004). Asada *et al.* (1994) found that acyl insulin derivatives were more resistant to intestinal enzymes as the carbon number on the fatty chains is increased (Asada *et al.*, 1994). Cell-penetrating peptides (CPP) have also been investigated to increase cellular translocation. It was found that CPP-insulin conjugates increased transport by 6-8 times across a Caco-2 cell line (Liang *et al.*, 2005).

Emisphere Technologies developed a method, which involves a non-covalent complexation with non-acyl amino acids that causes unfolding of the insulin structure to expose hydrophobic side chains that promotes superior translocation across the lipid bilayer (Sadrzadeh *et al.*, 2007). Once insulin crosses the membrane, the complex dissociates and the protein returns to its native conformation.

2.2.2 Protease Inhibitors

Co-administration of insulin with protease inhibitors is another technique, which researchers have investigated to increase bioavailability in the gastrointestinal tract. Bai *et al.* (1995) conducted a general study on the effects of various protease inhibitors and found that N-ethylmaleimide, 1,10-phenanthroline, ethylenediaminetetraacetic acid, p-chloromercuribenzoate and bacitracin completely inhibited insulin degradation in intestinal enterocytes. Degradation was only weakly inhibited by aprotinin, chymostatin, leupeptin, and diisopropyl phosphofluoridate (Bai *et al.*, 1995). The same group found that N-ethylmaleimide, 1,10-phenanthroline and p-chloromercuribenzoate improved

insulin transport across rat illum, although no mechanism was proposed (Bai *et al.*, 1996).

Since α -chymotrypsin and trypsin are the enzymes primarily responsible for the break down of insulin in the GIT, efforts have also focused on specifically targeting or neutralizing these enzymes in particular (Radwan *et al.*, 2001). Radwan *et al.* (2001) investigated the protease inhibiting effects of known permeation enhancers such as glycocholic acid, taurochenodeoxycholate or dimethyl- α -cyclodextrin, and found that administration with insulin increased the bioavailability of insulin, and capric acid, a fatty acid, was most effective against α -chymotrypsin. Yamamoto *et al.* (1994) found that insulin administered with either soybean trypsin inhibitor or aprotinin increased the hypoglycemic effect in rats (Yamamoto *et al.*, 1994). Some researchers have demonstrated that while the use of enzyme inhibitors increases oral insulin bioavailability, there is still the issue of low permeability, and thus these methods must often be coupled with other approaches (Liang *et al.*, 2005). For example, bile salts and fatty acids have been investigated as a means to retard or impede proteolytic degradation and affect translocation across the intestinal epithelium. This is thought to occur by changing the nature of the cellular membrane or facilitating paracellular uptake by opening tight junctions.

2.2.3 Permeation Enhancers

Mucosal permeation enhancers have also been investigated and co-administered with insulin to improve intestinal permeability (Sakai *et al.*, 1997). Cyclodextrins,

chelating agents, surfactants, bile salts, fatty acids and terpenes have all been shown to increase translocation across the intestinal mucosa. For example, Sakai *et al.* (1997) looked at sodium caprate, a fatty acid, and sodium deoxycholate, a bile salt, and the effect on transepithelial electrical resistance (TEER) across Caco-2 monolayers. They found that both compounds reduced the TEER and reported an increase in transepithelial transport of hydrophilic compounds. Proposed mechanisms include reversibly opening tight junctions and shortening the length of the glycocalyx, which is a physical barrier of glycoproteins that impedes access to the apical membrane of the epithelial cells in the intestine.

Many of these absorption promoters have been applied to insulin delivery systems (Mesiha *et al.*, 2002; Morishita *et al.*, 1993; Scott-Moncrieff *et al.*, 1994; Shao *et al.*, 1994). Li *et al.* (1992) found that a bile salt, sodium glycocholate, increased intestinal permeation by affecting the insulin molecule itself, likely by dissociating oligomers to monomers enabling superior translocation (Li *et al.*, 1992). In another study, Eaimtrakarn *et al.* (2002) found that the surfactant, Labrasol[®], increased the biological availability of insulin following administration to rat intestine; however, the value was still low at around 0.25% (Eaimtrakarn *et al.*, 2002). The naturally derived *Zonula occludens* toxin has also been used to improve intestinal permeability by reversibly opening tight junctions (Fasano *et al.*, 1997).

Shao *et al.* (1994) also looked specifically at co-administration of cyclodextrin derivatives and insulin and found a significant increase in bioavailability compared to insulin alone (Shao *et al.*, 1994). Cyclodextrins are thought to increase membrane

permeability by changing the properties of the cellular membrane, which could lead to irreversible tissue damage, but the study reported no local tissue damage based on light micrographs (Carrier *et al.*, 2007). Regardless, the safety of cyclodextrins and other permeation enhancers must be evaluated if they are to be employed in pharmaceutical delivery systems. It should be noted that chitosan has also been investigated as a means to increase paracellular permeability of hydrophilic compounds; however it will be detailed in the polymer materials Section 2.4.3 (Schipper *et al.*, 1999).

2.2.4 Particulate Delivery Systems

Encapsulation is the term used to describe the incorporation of an active material into a particle of a different substance, such as a polymer or phospholipid (Re, 1998). Two of the main reasons to encapsulate an active material in pharmaceutical delivery are for protection and to control release. The active can be protected from pH extremes or hydrolytic conditions, or the patient may be protected from the active, which may pose toxicity and health risks or have an unpleasant taste or odour (Peniche *et al.*, 2003). The target location for release and the release profile can be controlled through carrier selection, formulation optimization and particle engineering. For example, in mucosal drug delivery, materials are often selected to improve epithelial permeability by reversibly opening tight junctions between cells or by increasing contact time with the mucosa through bioadhesive forces (Issa *et al.*, 2006). Chitosan is an example of a polymer that can both reversibly open tight junctions and promote mucoadhesivity (Ventura *et al.*, 2008).

Micro- or nanoencapsulation refers to the incorporation of the active agent into micro- and nano-sized particles respectively. This technique is often employed in the pharmaceutical industry to affect the pharmacokinetics of a drug, promote stability and reduce toxicity (Tewa-Tagne *et al.*, 2007). “Microparticles” and “nanoparticles” are general terms that include micro- or nanospheres and micro- or nanocapsules. Micro- or nanospheres have a matrix structure in which the protein can be absorbed either at the surface or throughout the interior of the particle. Micro- or nanocapsules, on the other hand, have a shell-type structure in which the drug is encapsulated in the middle of the particle and surrounded by a protein or polymeric coat. Liposomal and polymeric encapsulation will be described briefly in the following sections.

2.2.4.1 Liposomal Delivery

Liposomes have been investigated as oral delivery encapsulation vehicles because they are hydrophobic in nature and exhibit low toxicity (Trotta *et al.*, 2005). Drug loaded liposomes can be made by many methods including solvent evaporation, melt dispersion and film hydration (Degim *et al.*, 2004; Iwanaga *et al.*, 1999; Kisel *et al.*, 2001). Despite this body of work, liposomes typically exhibit poor stability *in vivo*, suffer from low drug loading capacities and poor storage properties and are therefore, less desirable for drug delivery technologies than polymers (Soppimath *et al.*, 2001). Despite liposomal hydrophobicity, Degim *et al.* (2004) failed to show any increase in permeability across Caco-2 cells in dipalmitoylphosphatidylcholine-insulin liposomes compared to free insulin until 30 h had elapsed, which is longer than any particles would remain in the gastrointestinal tract (Degim *et al.*, 2004).

One promising experiment conducted by Trotta *et al.* (2005) demonstrated that cetylpalmitate and glyceryl monostearate liposomes retained over 70% of the total insulin under physiological conditions; however, the particles were placed in a systemic-type simulation as opposed to gastrointestinal simulation (Trotta *et al.*, 2005). Thus, there is still much more work to be done if liposomes are to be used as oral delivery vehicles for insulin.

2.2.4.2 Polymeric Delivery

The large variety of polymers and polymer combinations, as well as the choices of formulation and processing techniques, means that polymeric encapsulation can be used to address a number of delivery challenges and may be readily modified to fit design specifications (Desai *et al.*, 2005; Zalfen *et al.*, 2008). Specifically, the drug-to-polymer ratio, polymer properties and production method can all be used to affect the stability, bioavailability and drug release profiles. Additionally, polymers can be engineered through additional coatings or ligands to be site-specific and thus optimize the dose and subsequent biological response (Bies *et al.*, 2004; Ré, 1998).

In gastrointestinal delivery, the size of the particle has been shown to greatly affect the fraction of particles that actually reach the bloodstream. In some cases, a submicron diameter has been observed to increase absorption rates by 10-250 times compared to the absorption rate of larger particles (Chen *et al.*, 1998). As well, the morphology and charge of the particle can affect absorption rates. In general, hydrophobic particles are more easily absorbed than hydrophilic molecules. Moreover,

hydrophilic neutral and positively charged particles have a higher affinity for epithelial cells than negatively charged particles and thus are more easily absorbed (Pinto Reis *et al.*, 2006).

Particle carriers for oral drug delivery have been researched extensively because of their superior ability to protect peptides against enzymes in the GIT and enhance epithelial permeability as well as for their biocompatibility properties (Pinto Reis *et al.*, 2006). Polymeric particles are also typically better suited to bioencapsulation applications compared to other carriers, such as liposomes, because they have superior controlled release properties, better storage qualities and enhanced stability in physiological fluids (Cook *et al.*, 2005; des Rieux *et al.*, 2006; Soppimath *et al.*, 2001) In addition to a working as a delivery aide through mucosal tissue, polymeric encapsulation has been proposed as a means to control the release profile of some invasive formulations with short half-lives *in vivo* and/or which may be toxic in the native form (Xie *et al.*, 2007). Several physical and chemical modifications have therefore been proposed to overcome this problem including polymeric encapsulation. Polymeric micro and nanoparticles are especially interesting because they can be formulated to target specific areas of the body and prevent the drug from denaturing *in vivo*. Encapsulation will, therefore, increase drug delivery efficiency, which will consequently decrease the amount of drug needed compared to nonencapsulated drugs (Kumar, 2000).

A combination of strategies has also been detailed in the literature (Kavimandan *et al.*, 2008; Radwan *et al.*, 2001). For example Kavimandan *et al.* (2008) looked at

employing pH-dependant hydrogels and insulin-transferrin conjugates to improve oral insulin delivery (Kavimandan *et al.*, 2008).

2.3 Polymeric Encapsulation Methodologies

When considering formulation methods for encapsulated therapeutics, factors such as shear stress, temperature and pH must be considered to avoid denaturation or degradation of the active agent (Baras *et al.*, 2000b; Coppi *et al.*, 2002). Additionally, a successful process must be scalable to produce industrial quantities while minimizing cost (Okuyama *et al.*, 2003; Ré, 2006). Methodologies that are typically found in the literature using preformed polymers include emulsion dispersion, ionotropic pregelation and spray drying, which will be detailed in the following section (Kusonwiriawong *et al.*, 2009; Reis *et al.*, 2007; Sarmiento *et al.*, 2007).

2.3.1 Emulsion Dispersion

The emulsion dispersion methodology to form polymeric particles is typically conducted in two main steps. A polymer and drug in solution is first emulsified with an aqueous phase and the solvent is subsequently evaporated, leaving nanospheres (Reis *et al.*, 2006). This technology enables control over particle size by changing the temperature, viscosity of aqueous and organic solvents and stir rates. Additionally, emulsion dispersion has been shown to reproducibly produce particles in the nano-size range. Problems with emulsion dispersion methods include the ability to scale to an industrial level due to the energy requirements for dispersion and the number of steps

required. As well, an additional drying step, such as lyophilization or spray drying, is typically required to ensure stable shelf life.

2.3.2 Iontropic Pregelation

Iontropic pregelation involves polyelectrolyte complexes (PEC) to form the nanoparticle matrix (Sarmiento *et al.*, 2006). In particular, PECs are produced when polycationic and polyanionic polyelectrolytes are mixed in dilute solution. An example of a polyelectrolyte complex involves alginate, a polyanion, and chitosan, a polycation. The nanoparticles are formed by mixing alginate with calcium carbonate and constantly stirring the mixture to attain a pre-gel state. Next, the drug is added to the solution and finally, chitosan is put into the mixture which forms and stabilizes the desired nanoparticles. Iontropic gelation is advantageous because it a straightforward procedure based on simple techniques, but like emulsion techniques, it is difficult to scale and requires an additional drying step to ensure long-term stability.

2.3.3 Spray Drying

Spray drying is the transformation of an emulsion, suspension or dispersion to a dry state by atomizing the product and dispersing it through a hot gas (Masters, 1976). Spray drying is a well-established technology currently used in a number of industries to produce various food, cosmetic and photoluminescent products (Iskandar *et al.*, 2003). It is also employed in the pharmaceutical industry to produce drug powders and other dry therapeutics (Desai *et al.*, 2005). Both aqueous and organic solvent soluble materials can be dried via spray drying, with the latter conducted in a closed loop operation.

The spray drying process operates in three steps, involving atomization, drying and powder collection (Maa *et al.*, 1998). Initially, a liquid feed is dispersed through an atomizer and dispersed as fine droplets in warm air or inert gas in the drying chamber. Due to the large particulate surface area, the solvent removal step is quick and can take anywhere from a couple to tens of seconds (Maa *et al.*, 2000). Dried particles then pass to a cyclone, where separation occurs under centrifugal force. Particles typically have a narrow size distribution between one and several microns depending on the process conditions and initial formulation (Desai *et al.*, 2005). Figure 3 depicts the main components of a spray dryer and the flow of the air and feed.

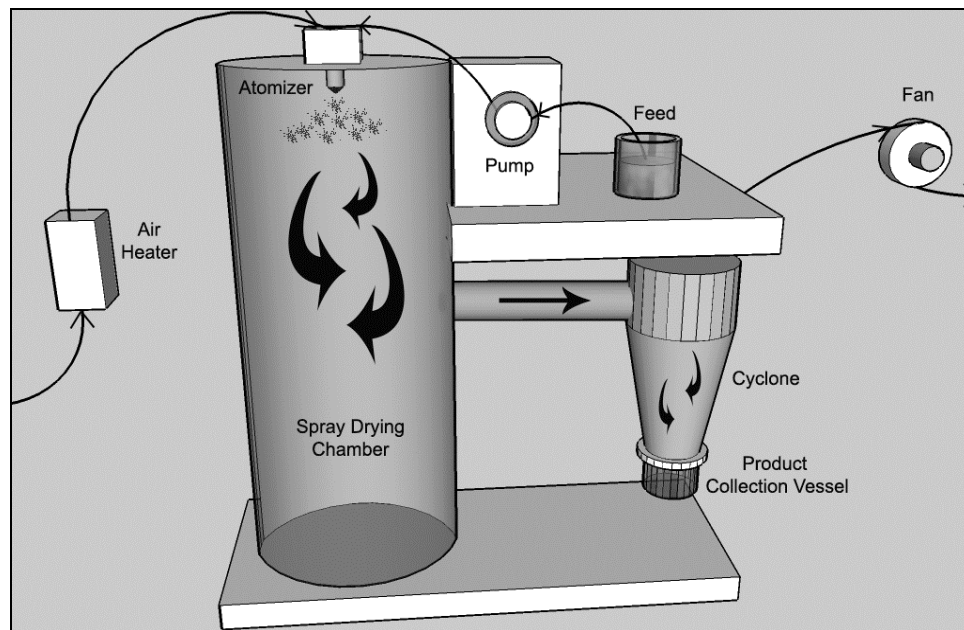


Figure 3 – Principal elements of a spray dryer

2.3.3.1 Spray dryer process parameters

Controllable spray drying process parameters include the atomizing air feed rate (Q_{AA}), liquid feed rate (Q_L), inlet air temperature (T_{in}) and flow of drying air (Q_{DA})

(Ameri *et al.*, 2006; Lee, 2002). These process variables allow control of final particle size, morphology, residual moisture content and bulk density (Huang *et al.*, 2003; Maltesen *et al.*, 2008).

In terms of the optimization of process parameters and product characteristics, there have been several reports describing the effects on the morphology, humidity and size of the final particulate product (Billon *et al.*, 2000; Huang *et al.*, 2003; Kusonwiriawong *et al.*, 2009; Wan *et al.*, 1991). For example, it has been determined that particle size is directly linked to the composition and concentration of the initial formulation, whereas the morphology of the particle is governed by the drying rate as well as initial composition (Kusonwiriawong *et al.*, 2009).

The product yield of spray drying is another important consideration, which can be affected by the process parameters. The mass yield is determined by the amount of solids pumped into the spray drying unit and the mass of particles collected. For laboratory scale operations, the yield is often low, reaching a maximum of 60%, depending on polymer selection and equipment operating parameters (Ameri *et al.*, 2006). Table 1 outlines the relationship between spray drying conditions and product characteristics.

Table 1 - Effect of increase in various spray dryer operating conditions on product characteristics (Based on results from (Buchi Inc., 2009; Prinn *et al.*, 2002))

Parameter	Atomizing Air Feed Rate (Q_{AA})	Liquid Feed Rate (Q_L)	Inlet Temperature (T_{in})	Spray Flow of Drying Air (Q_{DA})
Dependence				
Particle Size	↓	↑	↑	↓
Residual Moisture	↓	↑	↓	↓
Yield	↑	↓	↑	↑

One of the drawbacks of spray drying is the need to frequently operate at inlet temperatures in excess of 100°C, potentially denaturing heat sensitive therapeutics (Ameri *et al.*, 2006). Several studies have shown that optimized conditions or the addition of sugars or amino acids can stabilize proteins (Kusonwiriawong *et al.*, 2009; Patel *et al.*, 2001). Kusonwiriawo *et al.* (2009) showed that BSA spray dried in chitosan matrix

at an air inlet temperature of 120°C retained its integrity and secondary conformational structure.

During the atomization stage, dispersed droplets are moisture saturated and the relative humidity level approaches 100%. The active agent remains at the wet-bulb temperature, which is lower than the temperature of the air inside the drying chamber (Ameri *et al.*, 2006). The active agent is therefore not subjected to the actual inlet temperature. Additionally, as the droplet loses moisture, the temperature in the drying chamber decreases due to the latent effects of evaporative cooling (Shoyele *et al.*, 2006). Regardless, if droplets are subjected to high temperatures, there are several ways to circumvent surface denaturation including the addition of a surfactant (Ameri *et al.*, 2006).

2.3.3.2 Encapsulation via spray drying

Although spray drying is typically considered a drying technology, it has also been used as a method to encapsulate active agents into polymeric microspheres (Reis *et al.*, 2006). Spray drying is particularly advantageous because it can be scaled to an industrial level, is fast, and can be operated as both a batch or continuous basis (Johansen *et al.*, 2000; Masters, 1976).

Alternative techniques such as spray freeze drying, emulsion-solvent evaporation or ionotropic pregelation have been used to produce drug loaded micro- and nanoparticles (Balmayor *et al.*, 2008; Lam *et al.*, 2001; Sadeghi *et al.*, 2008). However, these methods have several disadvantages affecting product quality and downstream processing. Spray freeze drying is an involved process, which requires the selection of suitable

cryoprotectants, and can affect the ultimate bioavailability of a drug (Abdelwahed *et al.*, 2006; Maltesen *et al.*, 2008). Furthermore, organic solvents and multiple processing steps, including an additional drying stage, are routinely required when nanoemulsion dispersion and ionotropic pregelation methodologies are employed. A drying step is often essential to ensure stable shelf life for wet particulate formulations that can lose bioactivity due to aggregation and sedimentation within the therapeutic formulation (Ré, 2006; Tewa-Tagne *et al.*, 2006). In contrast, spray drying offers the advantage of encapsulation and drying in one continuous operation.

Although organic solvents can be used in closed loop spray drying systems, formulations can often be adapted to an aqueous system and thus avoid both the environmental and health risks associated with organic solvents (Ré, 1998). In other cases, when using a poorly water soluble drug or polymer, spray drying leaves low residual solvent levels in the final formulation compared to other encapsulation techniques using the same components (Le Corre *et al.*, 2002). Due to the nature of the spray drying process, the active is only exposed to the solvent for short periods of time (Baras *et al.*, 2000).

Compared to the other methods, spray drying is less dependent on the solubility or hydrophobicity of the active material and matrix polymer and is a good alternative for hydrophilic drugs that cannot be encapsulated using solvent evaporation methods (Baras *et al.*, 2000b; Wagenaar *et al.*, 1994; Wang *et al.*, 2002). These drugs typically leach out during processing, and therefore cannot be efficiently entrapped. Finally, spray drying can be adapted to run under sterile conditions, which is important in pharmaceutical

applications. Spray drying also yields a high active material encapsulation efficiency compared to other microencapsulation methods (Giunchedi *et al.*, 1998; Zgoulli *et al.*, 1999).

2.4 Core Polymers Used in Encapsulation

Formulation materials that are generally used in drug encapsulation via spray drying include biocompatible and biodegradable, high molecular weight polymers. Hydrophobic or amphiphilic polymers, such as methacrylic polymers (Eudragit®), poly(ϵ -caprolactone), poly(lactic acid), poly(glycolide) and their copolymer poly(lactic-co-glycolic acid), are often used in pharmaceutical spray drying because these polymers are non-immunogenic and degrade to products naturally found in the body (Baras *et al.*, 2000b). Natural polymers such as chitosan, cellulose derivatives and sodium alginate are also commonly used because they are water soluble and require minimal processing. Albumin, gelatin and cellulose derivatives have been employed as encapsulation matrices, but have been described to a lesser extent in the literature (Martinac *et al.*, 2005; Nettey *et al.*, 2006; Piao *et al.*, 2008).

2.4.1 Acrylate Polymers

Methacrylate copolymers and their derivatives, commercially known as Eudragit®, are synthetic polymers that have been used in several spray drying applications (Palmieri *et al.*, 2002; Pignatello *et al.*, 2001). Eudragit® polymers are inert and biodegradable, thus are typically used in enteric drug delivery (Esposito *et al.*, 2002). Many chemical analogues of Eudragit® polymers are available and each exhibit specific characteristics

with respect to solubility and permeability (Esposito *et al.*, 2000). Eudragit® L and S are copolymers of methacrylic acid and methyl methacrylate, but are soluble up to a pH of 6 and 7, respectively. Specific solubility characteristics mean that researchers can tailor drug delivery systems to target precise sites in the gastrointestinal tract. The chemical structure of Eudragit® S 100 is presented in Figure 4.

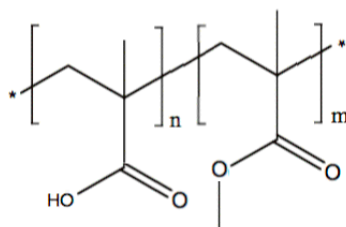


Figure 4 – Chemical structure of Eudragit® S100

2.4.2 Poly(esters)

Polyesters have been widely employed in biomedical spray drying applications (Li *et al.*, 2007; Mok *et al.*, 2008; Murillo *et al.*, 2002; Walter *et al.*, 1999). Poly(ϵ -caprolactone) (PCL), poly(lactic acid) (PLA) and poly(lactic-co-glycolide acid) (PLGA) have been the most widely investigated, and thus will be detailed in this review.

PCL is a low cost, widely available, synthetic polyester that has been employed in a number of studies as a therapeutic delivery carrier (Balmayor *et al.*, 2008; Coccoli *et al.*, 2008; Gibaud *et al.*, 2004). Favorable characteristics include prolonged stability in physiological fluids, negligible toxicity and slow degradation *in vivo* compared to poly(lactic acid) and poly(lactic-co-glycolide acid) copolymers, which means that PCL decomposition does not generate the same levels of local acidity (Sinha *et al.*, 2005). The hydrophobicity of PCL also makes it ideal for mucosal delivery systems (Baras *et al.*,

2000b). Spray drying of PCL has been explored in several studies (Giunchedi *et al.*, 1994; Luzardo-Alvarez *et al.*, 2006; Zalfen *et al.*, 2008). Figure 5 depicts the chemical structure of PCL.

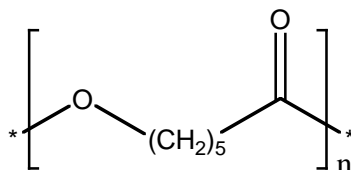


Figure 5 - Poly(ϵ -caprolactone)

PLA and PLGA are often used in encapsulation for biological delivery because these polymers are biocompatible and possess favorable biodegradation properties (Raj *et al.*, 2003). These polymers are often prepared using emulsion/solvent diffusion, solvent displacement or solvent evaporation techniques, which can be used to control the size of the resultant particles, generally ranging from 100 nm - 2 μm (des Rieux *et al.*, 2006; Pinto Reis *et al.*, 2006). These polymers have also been explored for spray drying applications (Bain *et al.*, 1999; Kusunwiriawong *et al.*, 2009; Wang *et al.*, 2002). Figure 6 and 7 depict the structures of PLA and PLGA respectively.

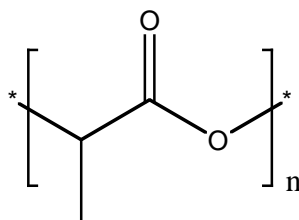


Figure 6 - Poly(lactic acid)

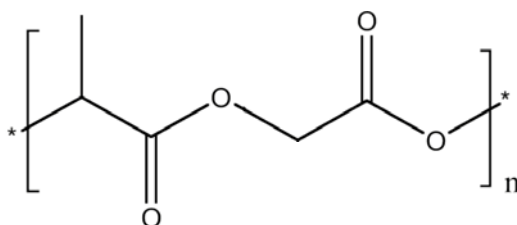


Figure 7 - Poly(lactic-co-glycolide acid)

PLGA is commonly used in encapsulation studies because it is possible to alter the ratio of lactic acid to glycolic acid which alters the physiochemical properties, such as degradation rates *in vivo* (Park *et al.*, 2005). Despite this wide spread use of PLGA, it has been shown to have a higher level of cytotoxicity compared to other biodegradable polymers, such as chitosan and sodium alginate (Sivadas *et al.*, 2008). As well, degradation results in an acidic environment, which could be harmful to the active ingredient as well as to local tissue (Baras *et al.*, 2000a; Raj *et al.*, 2003). PCL, PLA, PGA and PLGA are also problematic because they require the use of harsh organic solvents, such as dichloromethane or ethyl acetate, or surfactants which can damage the therapeutic (O'Hagan, 1998; Vajdy *et al.*, 2001). Additionally, the use of organics in pharmaceutical formulations requires extensive downstream processing and verification measures to ensure the solvents have been removed. Further drying, membrane filtration

or crystallization steps may be necessary and thus many researchers have examined aqueous systems as alternatives, using natural polymers such as chitosan, alginate and cellulose derivatives (Olson, 1995).

2.4.3 Chitosan

Chitosan is a polysaccharide composed of N-acetyl-D-glucosamine and D-glucosamine, derived from the exoskeleton of crustaceans (Hejazi *et al.*, 2003). It is a biodegradable, biocompatible and widely available natural polymer that is soluble in mildly acidic aqueous solutions, and therefore does not require organic solvents in its formulation (Kusonwiriya Wong *et al.*, 2009; Zhang *et al.*, 2008). Many researchers have used chitosan for micro- and nanoparticles in mucosal drug delivery because of mild encapsulation conditions and mucoadhesive properties (Lin *et al.*, 2007; Ma *et al.*, 2005; Pan *et al.*, 2002). Bioadhesion can be attributed to the attraction between negative charges on the glycoproteins of mucin and positive charges on chitosan (Ventura *et al.*, 2008). Spray drying of chitosan has been studied in several papers (Huang *et al.*, 2003; Learoyd *et al.*, 2008; Lin *et al.*, 2007) and its use in biomedical applications has increased several fold between 1994 and 2004, according to a recent review by Issa *et al.* (2006). A drawback to the use of chitosan for delivery systems is the high viscosity however this can be overcome by selecting the appropriate grade and molecular weight (Issa *et al.*, 2006). Figure 8 illustrates the chemical structure of chitosan.

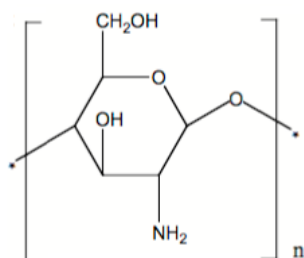


Figure 8 - Chemical structure of chitosan

2.4.4 Alginate

Alginate is a natural copolymer made up of alternating patterns of (1,4)-linked β -D-mannuronic acid (M) and α -L-guluronic acid residues (G), which are depicted in Figure 9 (Wee *et al.*, 1998). Alginate is an ideal encapsulation matrix for micro- and nanoparticles due to wide availability and desirable chemical and physical properties. Alginate forms an inert but biodegradable hydrogel matrix, under mild conditions. The gel porosity enables high drug diffusion rates which can be controlled with polymer coatings (Wee *et al.*, 1998) and like chitosan, alginate possesses mucoadhesive properties owing to its anionic carboxyl groups (Raj *et al.*, 2003). Alginate is a biocompatible and water-soluble polymer, which is categorized in the generally regarded as safe (GRAS) classification by the FDA (George *et al.*, 2006). Alginate particles have been successfully manufactured by many methods including nanoemulsion dispersion, ionotropic pregelation, and spray drying (Coppi *et al.*, 2002; Reis *et al.*, 2006; Sarmiento *et al.*, 2006).

Alginate can be ionically crosslinked with multivalent cations, such as calcium, to form a gel network that dissolves in neutral or high pH environments and is stable in acidic pH (Augst *et al.*, 2006; George *et al.*, 2006). These pH sensitive hydrogels are

particularly attractive for oral delivery because they can contract in the stomach (acid) and protect their payloads. As they pass through the gastrointestinal tract, they subsequently swell and release as the pH increases. The stability of an alginate gel is typically dependant on the M:G ratio, where higher concentrations of G result in stronger gels (Gacesa, 1988). However, crosslinking is conducted after the spray drying process because viscous gels cannot be properly atomized. Spray drying of alginate has been investigated in the literature (Coppi *et al.*, 2002; Crcarevska *et al.*, 2008).

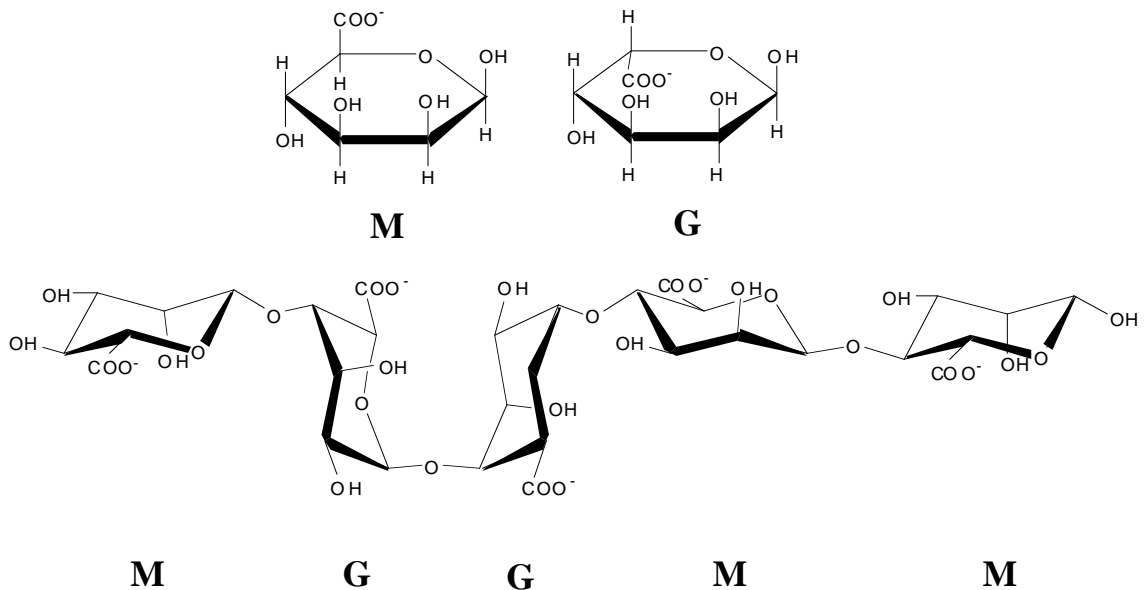


Figure 9 - Alginate structure of β -D-mannuronic acid (M) and α -L-guluronic acid (G) monomers in their Haworth conformation on top and sample polymer block on the bottom. (Reproduced from (Chan, 2009))

Chapter 3

Project Objectives

Developing an oral insulin delivery system remains at the forefront of diabetes research today. An oral dosage could radically change the way diabetes is treated and improve the quality of life for many patients. It is for this reason that scientists have investigated molecular modifications, protease inhibitors, permeability enhancers and encapsulation as a means to improve the bioavailability of insulin in the gastrointestinal tract. Our lab has focused primarily on the use of polymeric particulate carriers to overcome intestinal epithelial barriers and protect insulin from proteolytic enzymes. Catarina Pinto Reis, Bruno Sarmiento and Camile Woitiski have each worked to develop multistep systems based on emulsion dispersion and ionic pregelation methodologies with alginate as the core polymer matrix. Their work has yielded promising results and successfully increased the bioavailability of insulin in the GIT (Reis *et al.*, 2008; Sarmiento *et al.*, 2007; Woitiski *et al.*, 2009).

Several groups have investigated the use of microparticles produced by spray drying for various drug delivery applications (Gavini *et al.*, 2008; Nettey *et al.*, 2006; Oster *et al.*, 2005; Youan, 2004). Spray drying is of interest as an encapsulation technology because it is a single step process that, unlike emulsion dispersion and ionic pregelation, yields dry particles. Based on the fundamental spray drying work of researchers like Broadhead (Broadhead *et al.*, 1995) and Ré (Ré, 1998) it was determined that spray drying would be an important technology to investigate for producing

polymeric particles formulated loosely on the system developed by Reis, Sarmiento and Woitiski using alginate as a core polymer to encapsulate insulin.

The following summarizes the principal objectives of this study:

1. Produce alginate micro- and nanoparticles containing active insulin using a spray dryer, based on parameters previously optimized in the literature for spray drying of proteins.
2. Characterize the particles to determine: the average size, morphology, insulin loading and distribution within the polymer, as well as insulin release in a simulated gastrointestinal environment.
3. Develop an *in vitro* assay to quantify the effects of spray drying on the residual bioactivity of insulin.

In addition to characterizing the particles, targets were set for each assay. Specific goals included obtaining particles with low residual moisture levels, narrow size distributions and an average size below 10 μm . High encapsulation efficiencies and limited release in gastrointestinal simulations, in addition to retaining full bioactivity after spray drying were desirable.

Chapter 4

Materials and Methods

4.1 Materials

Novolin[®] GE Toronto recombinant human insulin from Novo Nordisk (100 U/mL) were purchased from a local pharmacy. Insulin ELISA kits were purchased from Mercodia (Winston Salem, North Carolina, USA) and Fast Activated Cell Based ELISA (FACE[™]) AKT kits produced by Active Motif (Carlsbad, California, USA) were purchased from MJS BioLynx (Brockville, Ontario, Canada). Low viscosity sodium alginate (MW: 147,000; Batch number 112K0931; viscosity of 2% solution at 25°C: 250 cps), fluorescein isothiocyanate, rhodamine B isothiocyanate, bovine insulin, FITC-labeled bovine insulin and Dulbecco's modified eagle medium nutrient mixture were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). HyClone[®] phosphate buffered saline and fetal bovine serum were purchased from Thermo Scientific (Asheville, North Carolina, USA). GIBCO[®] 0.25% trypsin-EDTA was purchased from Invitrogen (Burlington, Ontario, Canada). All other reagents were of analytical grade and used as received.

4.2 Methods

4.2.1 Spray Drying Technique

An alginate solution was prepared by dissolving the appropriate amount of alginate in distilled water using a magnetic stir bar. Insulin (100 U/mL) was then added and mixed for an additional 15 min. The dispersion was fed into a Büchi Mini Spray

Dryer B-290, which is shown in Figure 10, at a feed rate of 5 mL/min, an atomization air flow rate of 357 L/h, an inlet temperature of 150°C and an aspirator rate of 40 m³/h. Particles were collected from the product vessel using a soft brush in the fume hood and transferred to glass containers for storage.



Figure 10 - Büchi Mini Spray Dryer B-290 (Reproduced from (Buchi Inc., 2009))

Table 2 outlines the alginate feed concentration and insulin loadings employed for various spray drying runs.

Table 2 – Formulation alginate feed concentrations and insulin loading

Alginate Concentration (w/v %)	Insulin Loading (mg insulin/gram alginate)
1	11.8
1.5	7.8
2	5.8

The resulting dry particles were collected and stored at 2-6°C. Exhaust particles were also collected for characterization by fitting the filter with a polytetrafluoroethylene (PTFE) membrane purchased from Büchi (Newcastle, Delaware, USA). Additionally, polymeric particles were prepared without insulin to use as experimental controls.

4.2.1.1 Determination of the Spray Dryer Mass Yield

The mass yield was determined by weighing the particles located in the collecting vessel using an analytical balance (PC2000, Mettler) after each spray dry run.

4.2.2 Particle Characterization

In pharmaceutical formulations, the particle properties that are of primary interest are the mean size, encapsulation efficiency, moisture content, zeta potential, release profile and bioactivity of the active material. Particle size for example, is important for pulmonary delivery applications, which require a size of 1-3 μm (Stahl *et al.*, 2002). Particle morphology is also relevant because it affects drug diffusion and particle degradation (Vehring, 2008).

4.2.2.1 Residual Moisture Concentration

The moisture content of the particles was determined by weighing a sample of particles before and after drying in an oven at 80°C for 24 h.

4.2.2.2 Size Distribution

The particle mean and size distribution were determined by laser diffraction using a Malvern Mastersizer 2000 with the dry particle accessory, Scirocco 2000. A standard operating procedure was developed using Malvern's Dispersion Technology Software (Version 5.10) and 200 mg of particles were placed in the sample tray for each run. The system was operated at an air pressure of 3 bars and 30% of the maximum for the vibration speed of the micro-plate from which the sample is dispersed. The size results are based on a number distribution.

4.2.2.3 Particle Morphology

Particles were placed in a desiccator overnight prior to all scanning electron microscopy assays to remove any residual moisture. A sample of particles was then mounted on aluminum pins and coated with a thin layer of gold. The morphology was analyzed by visualizing particles using a scanning electron microscope (JEOL, JSM-840). Several representative micrographs were obtained to examine particulate shape and surface profile.

4.2.2.4 Protein Encapsulation Efficiency

4.2.2.4.1 Absorbance Assay

The encapsulation efficiency (EE) was determined by suspending 150 mg of particles in 10 mL of phosphate buffer (pH 7.4, USP XXXI). The suspension was incubated at room temperature under magnetic stirring (250 rpm) to dissolve the particles. After 24 h, a sample of the solution was removed and the protein content was

quantified by ultraviolet absorbance (280 nm) using a quartz plate and multi-plate spectrophotometer (SpectraMax 250, Molecular Devices). A sample calibration curve of bovine insulin is illustrated in Figure 11. A control trial was also run simultaneously using blank particles.

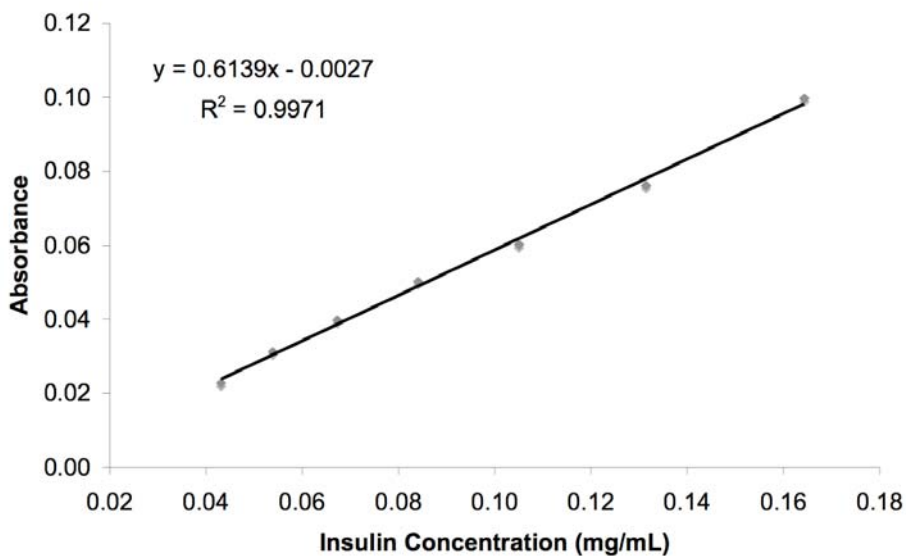


Figure 11 – Absorbance Assay Standard Curve

4.2.2.4.2 Enzyme-Linked ImmunoSorbent Assay

The encapsulation efficiency was also quantified with an insulin Enzyme-Linked ImmunoSorbent Assay (ELISA) kit purchased from Merckodia. The supernatant obtained after centrifugation of the dissolved particles was diluted to the appropriate range (0 - 200 mU/L or 0 - 7×10^{-6} mg/mL) using a Sample Diabetes Buffer also purchased from Merckodia. The kit was run as described in the instruction manual. The colourimetric reaction was quantified by a multi-plate spectrophotometer at a wavelength of 450 nm. A sample calibration curve with the recombinant human insulin standards provided in the

ELISA kit is illustrated in Figure 12. A control trial was also run simultaneously using blank particles.

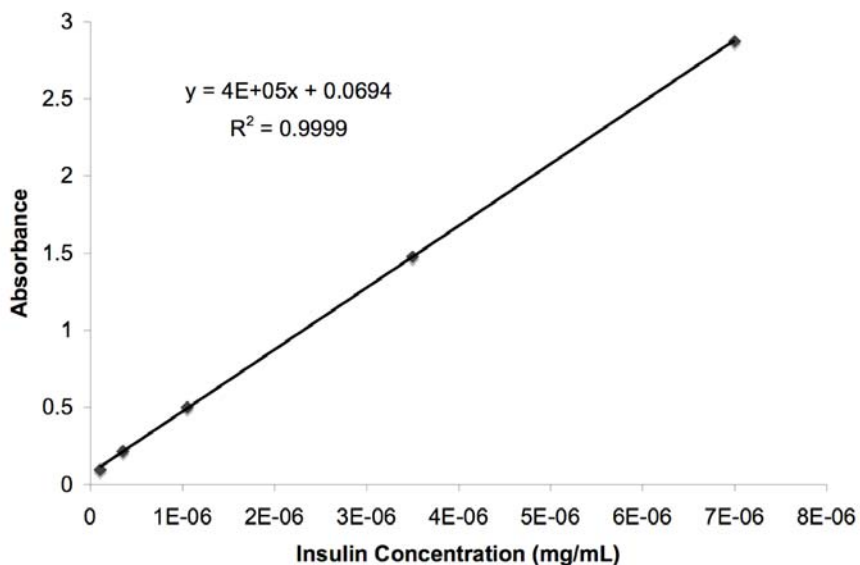


Figure 12 - ELISA Assay Standard Curve

4.2.2.5 Protein Release in Gastrointestinal Simulation

Insulin release in the gastrointestinal tract was simulated *in vitro* by placing 375 mg of particles into 25 mL of hydrochloric acid solution (pH 1.2, USP XXXI). The suspension was then incubated at 37°C and stirred by an orbital shaker (100 rpm). After 2 h, the gastric simulation fluid was centrifuged (12500g, 10 min) and the supernatant was discarded and replaced with 25 mL of phosphate buffer (pH 6.8, USP XXXI). At appropriate time intervals throughout the simulation, 1mL aliquots were removed and centrifuged (12500g, 10 min). The insulin content in the supernatant of these aliquots was quantified using a multi-plate spectrophotometer (280 nm). Fresh hydrochloric acid or

phosphate buffer was replaced as needed so as to maintain a constant volume. A control trial was also run simultaneously using blank particles.

4.2.2.6 Protein and Polymer Distribution

Two fluorochromes, fluorescein isothiocyanate (FITC) and rhodamine B isothiocyanate (RBITC), were selected to label insulin and alginate respectively, with the aim of determining the distribution within the particles. Confocal laser scanning microscopy (TCS SP2 Multi Photon, Leica) was used to visualize dry particle samples and the results were analyzed using *Leica Confocal Software* (Version 2.61).

4.2.2.6.1 Insulin Fluorescence

The distribution of insulin within the particles was determined by substituting unlabelled insulin with FITC-labeled bovine insulin from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada) or with Novolin® GE Toronto insulin labeled with fluorescein isothiocyanate (FITC) using a procedure supplied by Sigma-Aldrich Canada Ltd. and described by Clausen *et al.* (2001). Briefly, 2 mg of FITC was dissolved in 1 mL dimethylsulfoxide and added dropwise to a solution of insulin under magnetic stirring (100 rpm). The solution was then allowed to react in the dark at 4°C for 8 h. NH₄Cl was added to total 50mM and the reaction was left for an additional 2 h at 4°C. The labeled insulin was added to an alginate solution and subsequently spray dried as described in Section 4.2.1.

4.2.2.6.2 Alginate Fluorescence

Alginate was similarly labeled with rhodamine B isothiocyanate (RBITC) using a procedure previously described by Mladenovska *et al.* (2007). 1 mg of RBITC was dissolved in 1 mL dimethylsulfoxide and added to an alginate solution. The reaction was incubated at 40°C for 1 h and stopped by adding NH₄Cl to a final concentration of 50mM. Unreacted RBITC was removed by dialysis (MW 8,000 – 10,000) until free label diffusion was no longer detected. Both insulin and FITC-insulin were added to RBITC-alginate solutions and spray dried as previously outlined.

4.2.2.7 Protein Bioactivity

4.2.2.7.1 Cell Culture

Cell culture techniques were based on the work of Houg *et al.* (2006). Rat L6 myoblasts, kindly donated by Dr. Peter Greer (Department of Pathology and Molecular Medicine, Queen's University), were thawed by placing cryovials in a 37°C water bath. The cells (1 mL) were subsequently transferred to a T-75 flask and cultured in 14 mL of medium containing 78% Dulbecco's modified eagle medium (DMEM), 20% fetal bovine serum, 1% pen-strep, and 1% L-glutamine. The flask was then placed in an incubator for 24 h, and maintained at 37°C and 5% CO₂ levels. Next, the medium was aspirated and the cells were washed with 10 mL of phosphate buffered saline (PBS) to remove any debris. The medium was replaced with 15 mL of fresh medium every 2 days thereafter. Additionally, the cells were split 1:10 once they reached 80% confluence by first washing with 10 mL PBS. Next, 5 mL of trypsin-EDTA (0.25% trypsin, 0.1% EDTA) was added

to release the adherent cells and the flask was incubated at 37°C for 5 min. Following incubation, 5 mL of media was added to deactivate trypsin and the suspension was transferred to a 15 mL tube and centrifuged (1200g, 5 min). The supernatant was aspirated to retain the cell pellet and resuspended in 10 mL of media to achieve a 1:10 split. Finally, 1 mL of the cell/media suspension was placed in a new T-75 flask with 14 mL of fresh media. Strict aseptic conditions were maintained throughout the study to ensure optimal cell growth and prevent bacterial contamination.

4.2.2.7.2 Seeding 96 Well Plates

Cells were seeded onto sterile 96 well plates by following the cell passage procedure outlined in Section 4.2.2.7.1 from a flask that had reached 80% confluence. Instead of culturing the cells in a T-75 flask, a 10 μ L sample of cell suspension was stained with 10 μ L trypan blue and a hemocytometer was used to obtain an estimate of the cell count. The cell/media volume to obtain a predetermined cell per well count was then calculated based on a total volume per well of 200 μ L. This calculation was then used to plate the cells with the appropriate cell suspension to media ratio, which was optimized and detailed in Section 5.2.7.1.1. The cells were allowed to reach 80% confluence in the wells before proceeding to the insulin stimulation step.

4.2.2.7.3 Insulin Stimulation

Once the cells reached 80% confluence, roughly 24 h later, the medium was aspirated and the wells were washed twice with PBS. 200 μ L of starvation medium

consisting of 98% DMEM, 1% pen-strep, and 1% L-glutamine was placed in the wells for 6 h prior to all stimulation trials.

After the starvation period, either (i) Novolin® GE Toronto insulin, (ii) a sample of dissolved insulin-alginate particles or (iii) a sample of dissolved blank alginate particles were diluted within the assay range (0-600 mU/L) with starvation medium. 200 μ L of free insulin or appropriate particulate solution was then applied to each well for a preset stimulation time, which ranged between 5 and 45 min. The dissolved particles were treated as in the encapsulation efficiency assay: 150 mg of particles were suspended in 10 mL of phosphate buffer (pH 7.4, USP XXXI) for 24 h. The protein content was also quantified by absorbance assay to ensure that the solution was within the assay detection range. The absorbance reading was also used to calculate the percent bioactivity based along with the measured cell based ELISA results (See sample calculation in Appendix B).

4.2.2.7.4 Cell Fixation

After the stimulation, the medium was aspirated and 100 μ L of 4% formaldehyde in PBS was pipetted into each well to fix the cells. The wells were sealed with two layers of parafilm, placed in two Ziploc bags, left at room temperature for 20 min and then stored at 4°C until the next step of assay.

4.2.2.7.5 FACE™ AKT Assay

The insulin bioactivity was determined by quantifying the ratio of phosphorylated AKT to total AKT for each well by running an ELISA kit containing specific antibodies

developed for this purpose. The Fast Activated Cell Based ELISA (FACE™) AKT kit used in this study was purchased from Active Motif (Carlsbad, California, USA) and run according to the protocol described for adherent cells in the instruction manual. To start, the cells were permeabilized using a series of wash steps with a 1X PBS and Triton X-100 solution. A primary antibody specific for phosphorylated AKT or total AKT was added to each well and incubated overnight at 4°C. After further washing, a secondary antibody was subsequently applied for 1 h and a developing solution was added to trigger a colourimetric reaction. After 20 min, a stop solution was added and the plate was read using a multi-plate spectrophotometer at 450 nm. Negative controls were run to ensure adequate washing by omitting the secondary antibody.

In order to normalize the results with respect to cell numbers, a crystal violet stain was then applied to the cells and incubated for 30 min at room temperature. The plate was subsequently reread with the spectrophotometer at 595 nm.

4.2.2.7.6 Freezing Cell Cultures

An additional step of this assay was storing an adequate stock of the rat myoblast cell line. This was achieved by freezing a batch of cells at low passages, typically at passage #2 or #3, in order to maintain a consistent cell line. Once the cells were allowed to reach confluence in a T-75 flask, the medium was aspirated and washed with 10 mL of sterile PBS. 5 mL of trypsin-EDTA was then added to release the adherent cells and the flask was incubated at 37°C for 5 min. Following incubation, 5 mL of media was added to deactivate trypsin and the suspension was transferred to a 15 mL tube and centrifuged

(1200g, 5 min). A 10 μ L sample of this suspension was also collected to be used for cell counting with a hemocytometer. After centrifugation and based on the cell count, the pellet was resuspended in sufficient freezing media to obtain a density of 1×10^6 cells/mL. The freezing medium was composed of 7% dimethylsulfoxide and 93% culture medium. Finally, 1 mL of the cell suspension in freezing medium was put into a cryovial, which was subsequently placed in a freezing container filled with isopropanol. After cooling, the cells were placed in a -70°C freezer for long term storage.

4.3 Statistical Analysis and Data Collection

One-way analysis of variance (ANOVA) with *post hoc* Tukey's test were used to detect significant differences in multiple comparisons ($p < 0.05$). The data points presented in this work are expressed as mean \pm standard deviation (S.D.) and were calculated based on Equation 1 and Equation 2, respectively.

$$Pooled\ Mean = \sum_{i=n} \frac{\mu_i}{n} \quad (1)$$

$$Pooled\ Standard\ Deviation = \frac{\sqrt{\left(\sum_{i=n} (n_i - 1) s_i^2 \right)}}{\left(\sum_{i=n} n_i - n \right)} \quad (2)$$

where n is the number of repeated experiments, μ is the sample mean, and s is the sample standard deviation.

Chapter 5

Results and Discussion

5.1 Particle Production via Spray Drying

Alginate particles encapsulating insulin were produced by spray drying with a Büchi Mini Spray Dryer B-290. This formulation technique was selected because it is a continuous and fast process compared to other methods, such as emulsion and ionotropic gelation. The spray drying input variables for this study, which are summarized in Table 3, were optimized by Erdinc *et al.* (2007) for various proteins, and based on technical literature provided by Büchi (Büchi Inc., 2009; Erdinc, 2007).

Table 3 – Spray dryer input values

Parameter	Value	Unit
Liquid Feed Rate	5	mL/min
Atomizing Air Feed Rate	357	L/h
Inlet Temperature of Drying Air	150	°C
Drying Air Flow Rate	40	m ³ /h

The spray dryer operates with a nozzle-type atomizer running under co-current flow, where both the liquid feed and drying air are pumped in the same direction. Figure 13 illustrates the two-fluid nozzle atomizer through which compressed air and liquid feed are pumped co-currently. A concentric type geometry results in mixing of the gas and liquid outside of the nozzle, subsequently contacting warm air in the drying chamber.

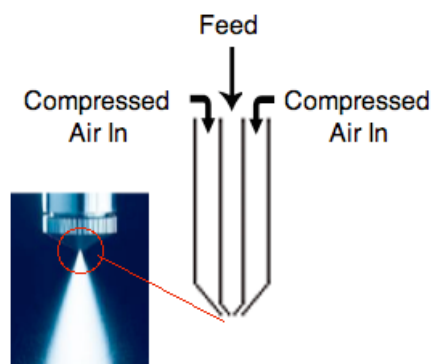


Figure 13 - Schematic of co-current spray flow and two-fluid nozzle

The liquid feed rate (Q_L) can affect droplet drying rate and residence time in the spray drying chamber and thus, final particle characteristics. Erdinc *et al.* (2007) studied the effects of Q_L on residual enzyme activity, particle size and product recovery (Erdinc, 2007). Based on these prior results, the pump was operated at 5 mL/min because this feed rate resulted in the highest residual activity of a model protein, smallest particle size and highest product recovery.

Atomizing air feed rate (Q_{AA}) dictates the flow of air needed to disperse the feed solution into fine particles. Prinn *et al.* (2002) determined that high atomizing air flow rates correspond to a decrease in particle size, likely attributed to the fact that a higher atomization rate results in smaller droplets and consequently, smaller particles (Prinn *et al.*, 2002). Based on this information and the technical data provided by Büchi, an atomizing air feed rate of 357 L/h was selected (Buchi Inc., 2009).

The inlet drying air temperature (T_{in}) setting is that of the drying air just before it enters the spray dry chamber. As outlined in Chapter 2, the droplets do not actually reach

T_{in} , but can be more closely approximated to the outlet temperature, which was recorded between 70-80°C throughout these experiments. An inlet temperature of 150°C was selected based on experiments conducted by Erdinc *et al.* (2007) where it was determined that 150°C resulted in the highest residual activity, while maintaining a high mass yield and low residual moisture content (Erdinc, 2007).

The final controllable spray dry variable is the aspirator setting, which controls flow of drying air (Q_{DA}). In order to obtain adequate particle separation in the cyclone, the highest aspirator rate of 40 m³/h was used. A higher aspirator setting decreases the moisture content, subsequently increasing the yield as a larger amount of air works to evaporate the same amount of solvent in the feed (Geffen, 2006).

Other controllable parameters include the nature, concentration, viscosity and temperature of feed. The concentration and composition of the initial formulation can have an appreciable effect on the final particulate structure. If the active material is dissolved in the polymer, it will result in a matrix structure, termed a microsphere. If the therapeutic is suspended in the polymer, it will be coated, resulting in a microcapsule. A 1-2% alginate feed concentration was selected as higher concentrations were deemed to be unsuitable for spray drying, as the viscous solution tended to clog feed lines. Three alginate concentrations, 1, 1.5 and 2% w/v were selected for preliminary testing. The temperature of the feed was not changed and allowed to equilibrate to room temperature before each trial.

5.1.1 Mass Yield

There are a number of factors that can affect the yield in a spray dryer including feed viscosity and the shape of the cyclone and particle collection vessel (Maa *et al.*, 1998). In this study, the mass yield was investigated with respect to initial alginate formulation and was determined by taking the ratio of the mass of particles in the product, relative to the mass of the initial solution formulation per batch. The particles from the collection vessel were weighed directly after spray drying and the results are shown in Figure 14.

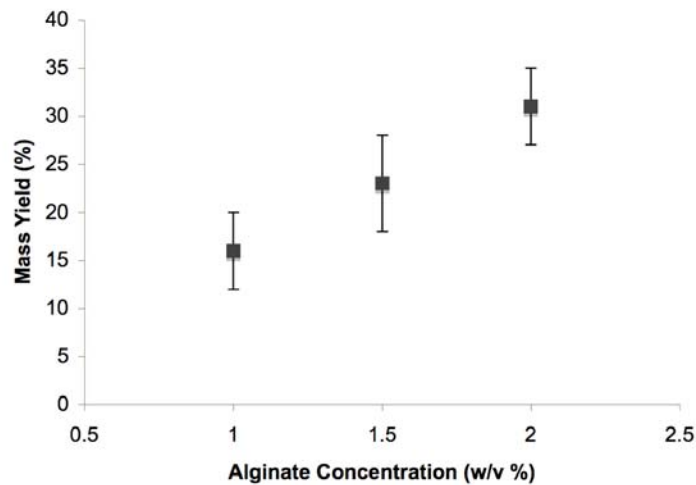


Figure 14 - Effect of alginate concentration in feed on the product mass yield (mean \pm S.D., n=3).

A significant increase in yield was observed between 1% and 2% alginate concentrations in the fluid feed. The increase in yield as a function of polymer concentration could be due to a corresponding increase in feed viscosity, which has been shown to affect particle size (Wang *et al.*, 2002). Higher viscosities tend to be more

difficult to atomize and thus, result in larger droplets and resultant particle sizes. Larger particles are easier to separate in cyclones and thus, a higher yield would be observed (Prinn *et al.*, 2002).

The yields determined are within range of that reported by other groups using the Büchi Mini Spray Dryer to encapsulate active materials. Rassau *et al.* (2008) achieved higher yields by spraying Eudragit[®] and ketoprofen resulting in a 60% yield, whereas Bain *et al.* (1999) reported yields as low as 2% for poly(L-lactide) particles encapsulating rifampicin (Bain *et al.*, 1999; Rassu *et al.*, 2008).

The fines or the fraction of particles trapped in the PTFE filter were collected and weighed after a 2% alginate feed run. The mass yield of the fines was determined to be 3.3% of the total mass yield. Thus, approximately 35% (3.3% from the fines and 32% from the collection vessel) of the mass was accounted for in the spray dryer. The remainder was deposited on the walls of the drying chamber, cyclone, and various fittings within the unit, or exited with the exhaust air.

In terms of pharmaceutical proteins, a low yield translates into high production cost and it would therefore be advisable to investigate methods to maximize the yield (Wang *et al.*, 2003). As shown in Figure 14, increasing the solids concentration is one way of increasing the yield, but the use of excipients or alternative product separation systems could also be investigated. For example, Maa *et al.* (1998) altered the geometry of the product collection vessel and/or cyclone to maximize particulate collection (Maa *et al.*, 1998).

5.2 Particle Characterization

5.2.1 Moisture Content

The water content of the particles was determined by weighing before and after drying at 80°C for 24 h, and determining the percent difference in mass. Results are shown in Table 4.

Table 4 – Residual moisture content of spray dried alginate particles (mean ± S.D., n=3)

Alginate Feed Concentration	1%	1.5%	2%
Moisture Content (%)	4.9 ± 2.1	5.8 ± 3.5	11.1 ± 5.7

Even though a trend toward increasing residual moisture content with increasing polymer concentration is evident from the results, a one-way ANOVA did not show a statistically significant difference and thus a change in alginate concentration from 1 – 2 w/v % does not appear to have an affect on resulting particle moisture content. Regardless, the moisture content is within range with that determined in other spray dryer studies, where moisture content has been reported anywhere between 2 - 20% for various drug and polymer formulations (Learoyd *et al.*, 2008; Maa *et al.*, 1998; Sivadas *et al.*, 2008). In general, particles with moisture contents above 15% cannot be recovered from the spray dryer because adequate drying is not achieved and particles tend to stick to the spray drying chamber and cyclone (Billon *et al.*, 2000). The mean moisture content

determined was under 15%, and thus the formulation and process parameters selected resulted in dry particles below the upper limit.

High moisture content is associated with loss of protein integrity in pharmaceutical formulations over prolonged storage times (Nguyen *et al.*, 2004). Although the moisture content after spray drying is an important consideration, the relative humidity of the storage environment also plays a role in determining long term protein stability. Maa *et al.* (1998) found that particles stored in refrigerated and humidity controlled conditions (temperature 4-6°C, relative humidity 38%) maintained biochemical stability for one year. It would be advisable to determine whether the combination of the moisture content range determined, between 4.9 and 11.1%, and long term storage has an effect on residual activity of insulin in a future study.

5.2.2 Size Distribution

Particle size was determined by laser diffraction spectrometry. Particles produced from 1, 1.5 and 2% initial alginate concentrations were assayed and the size distribution is presented in Figure 15.

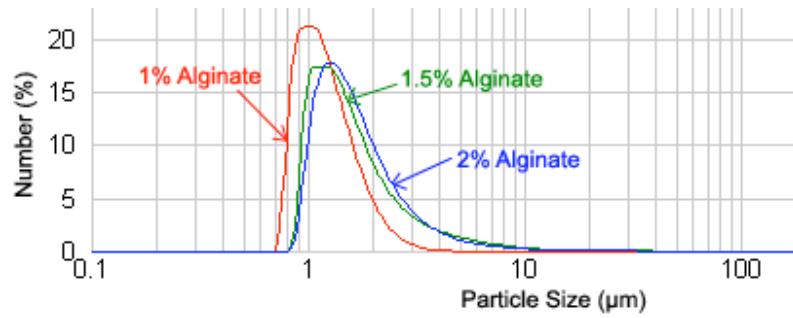


Figure 15 - Size distributions of particles collected from spray dried 1, 1.5 and 2% alginate feed concentrations

Particles produced show monomodal distributions, with slightly higher particle sizes resulting from higher viscosity feed solutions. The span is a measure of the breadth of the distribution, defined in Equation 3 (Gavini *et al.*, 2008; Le Corre *et al.*, 2002);

$$Span = \frac{D_{90} - D_{10}}{D_{50}} \quad (3)$$

where D_x represents the mean particle diameter at which X% by volume of the particles are smaller and (100-X)% are larger. For example, if D_{10} is 1.5 μm then 10% of the sample is smaller than 1.5 μm and 90% of the sample is larger. Table 5 gives the span and calculated size distributions parameter for particles formed from 1, 1.5 and 2% alginate feed formulations.

Table 5 - Size distribution of spray dried formulations measured by laser diffraction

where 10, 50 and 90% of the particles are smaller than D_{10} , D_{50} and D_{90} respectively.

[Alginate] w/v %	D_{10} (μm)	D_{50} (μm)	D_{90} (μm)	Span
1	0.7	1.2	1.8	0.8
1.5	1.0	1.5	3.1	1.5
2	1.1	1.6	3.0	1.3

Table 5 shows that the width of the size distribution, represented by the span value, is lower for 1% alginate feed than the span of the particles produced from 1.5 and 2% alginate feed concentrations. Size distributions of spray dried particles are influenced by the evaporation rate (Kusonwiriawong *et al.*, 2009), thus droplets that are not uniformly dried as a result of changes in heat energy in the dryer typically yield particles with broader size distributions. It could be postulated that an increase in polymer feed concentration, which is associated with a decrease in atomization efficiency, produces a less uniform spray that dries to yield particles with larger size variations (Wang *et al.*, 2003).

Similarly, an increase in polymer feed concentration is typically associated with a larger product particle size due to the increase in viscosity, which decreases the atomization efficiency (Masters, 1976; Mosen *et al.*, 2004; Wang *et al.*, 2003). A one-way ANOVA confirmed that there is a statistically significant increase in sample means between 1 and 2% alginate feed concentration. Mosen *et al.* (2005) suggested that lower

polymer concentrations result in droplets with less dry material so when the solvent evaporates, smaller particles are produced (Mosen *et al.*, 2004).

A problem often identified is the inability of spray dryers to produce particles small enough to be used in drug delivery applications. Specific to peroral delivery, particles less than 10 μm have been shown to cross the intestinal epithelial layer (Eldridge *et al.*, 1990). It can be difficult to optimize a spray drying system that can yield particles in this size range due to the large number of process parameters and types of equipment available, plus the wide range of polymer choices and formulations (Maa *et al.*, 2000). Although other factors, such as hydrophobicity and charge also play a role in determining particulate translocation, it can be concluded from the results presented in Table 5 that the alginate system developed can produce particles that meet the maximum size requirements for oral drug delivery vectors, via transepithelial particle transit.

Fines passing through the cyclone separator were collected from the PTFE membrane filter following a run with 2% alginate feed. Figure 16 compares the size distribution of the fines to the product collected from the cyclone.

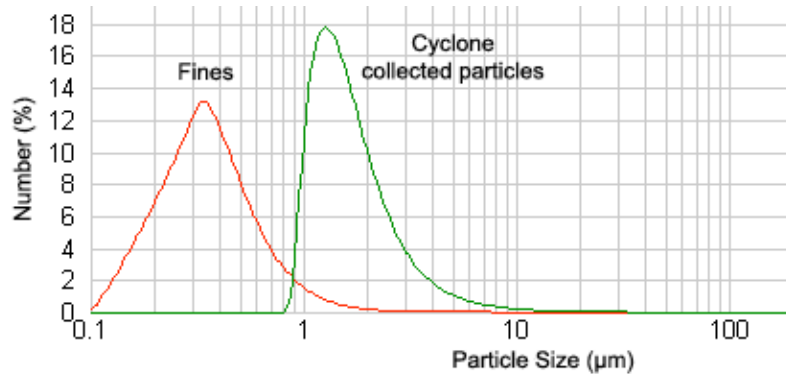


Figure 16 - Particle size distributions of particles collected from the product vessel and from the PTFE filter

Distribution parameters for the fines are presented in Table 6.

Table 6 - Size distribution of particles recovered from PTFE filter

Derived Diameter	D₁₀ (µm)	D₅₀ (µm)	D₉₀ (µm)
Size	0.18	0.34	0.68

The mean size of the fines appears to be considerably smaller than the particles in the product collection vessel, and the distribution profile is monomodal. A t-test confirmed that there is a statistically significant difference in the mean particle size between the fines and cyclone-collected product. This result demonstrates that submicron, nano-sized particles are indeed being produced, but with the present equipment configuration, are not being separated in the cyclone and are instead entrained with the exhaust gas.

5.2.3 Particle Morphology

The morphology of the particles was determined under a scanning electron microscope (SEM). Figures 17 through 19 show three representative micrographs from separate runs consisting of 1, 1.5 and 2% initial alginate feed concentrations.

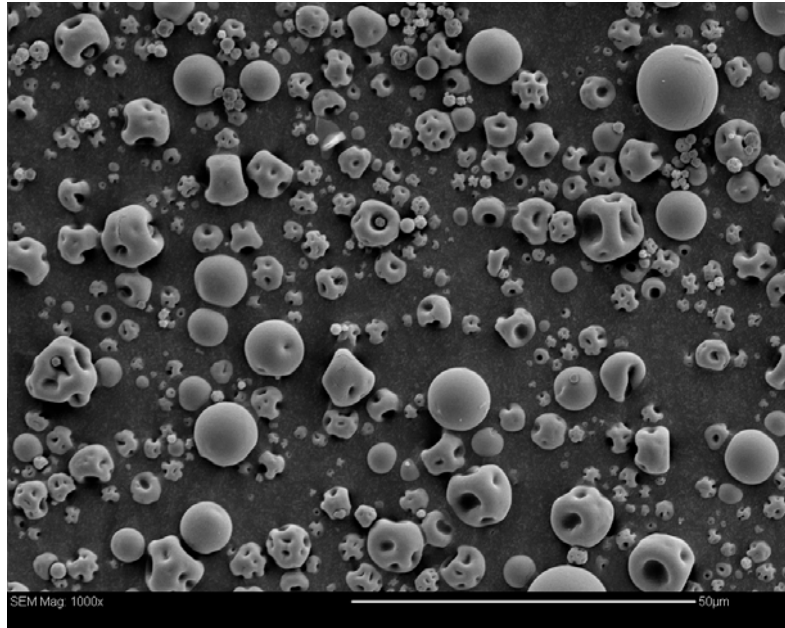


Figure 17 - SEM micrograph of particles prepared from 1% alginate solution at 1000x magnification

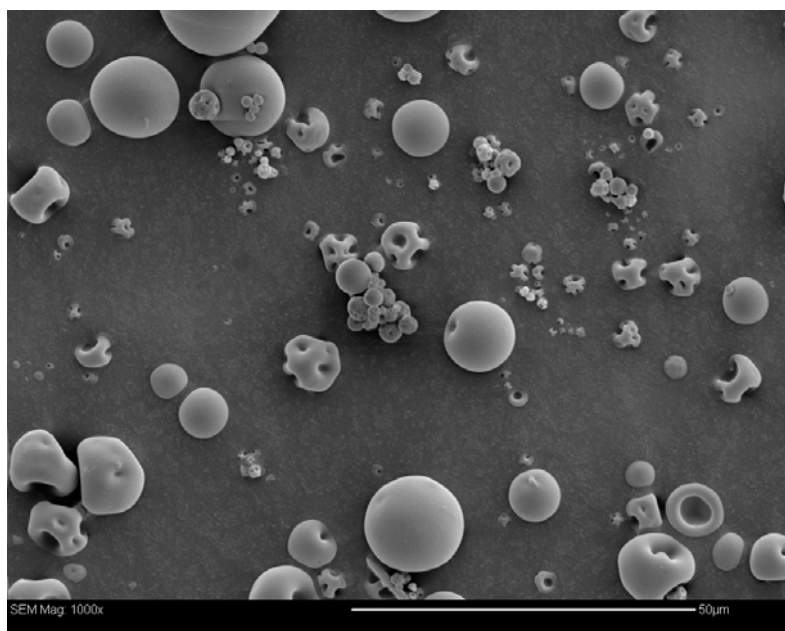


Figure 18 - SEM micrograph of particles prepared from 1.5% alginate solution at 1000x magnification

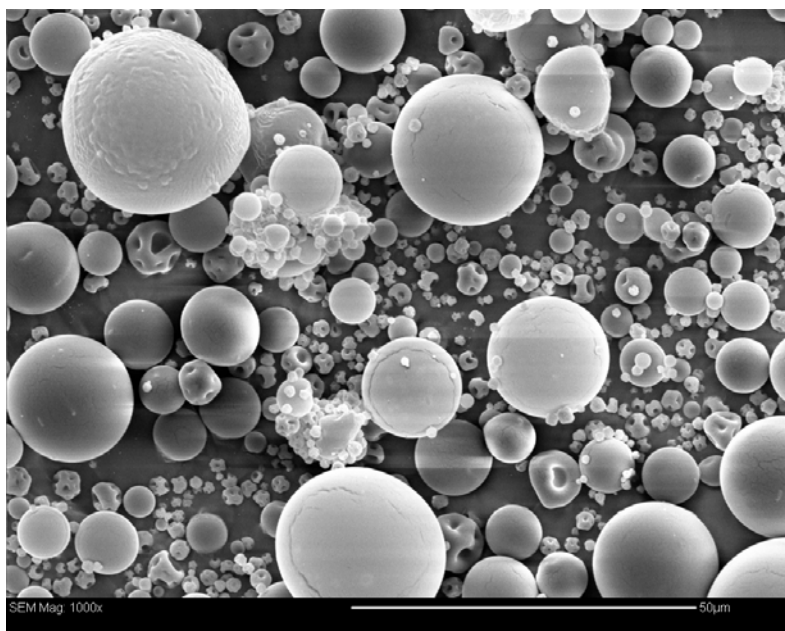


Figure 19 - SEM micrograph of particles prepared from 2% alginate solution at 1000x magnification

Prinn *et al.* (2002) outlined a classification system for spray dried particle morphology as: (I) smooth spheres, (II) collapsed or dimpled particles, (III) particles with ‘raisin-like’ appearance and (IV) highly crumpled folded structures (Prinn *et al.*, 2002). Based on this scale, most of the particles illustrated in Figure 17 and 18 appear to be roughly spherical with ‘divots’, and thus can be considered Class II. There are also some spheres in the sample that would fall into Class I. In contrast, a larger portion of particles in Figure 19 appear spherical and would fall into Class I, though there are smaller dimpled particles present in the background that would be considered Class II. Both spherical and collapsed particles appear to have fewer pores compared to other spray dried formulations. For example, particles from this study are compared to poly(lactic-co-glycolic acid) particles encapsulating etanidazole produced by Wang *et al.* (2002) in Figure 20.

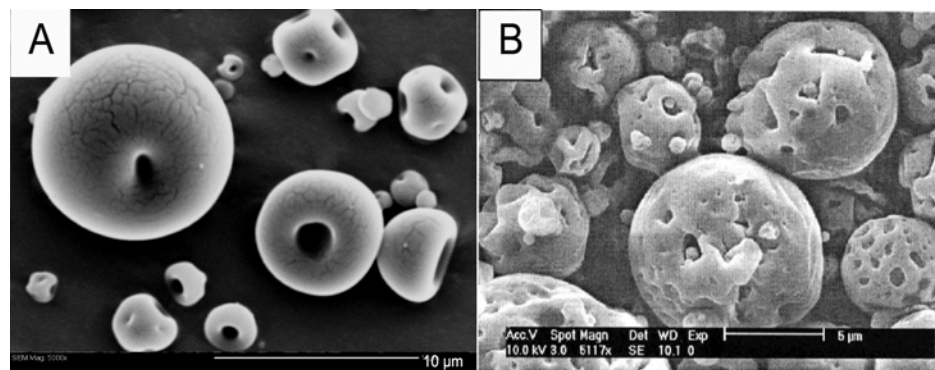


Figure 20 - (A) SEM micrograph of alginate particles at 5000x magnification, produced in the present study (B) SEM micrograph of PLGA particles at 5117x magnification, reproduced from (Wang *et al.*, 2002))

Ameri and Maa (2006) proposed a mechanism, which could occur during the drying process, to explain the dimples present on some spray dried particles (Ameri *et al.*, 2006). They describe the formation of a polymer film at the external surface of the droplet during the early stages of drying caused by rapid evaporation of solvent at the surface. The subsequent increase in the concentration of the polymer at the surface could impede the diffusion of water to the periphery of the droplet and cause a build up of water vapour pressure inside the particle. At a certain point, the film would burst resulting in particles that have dimples or holes.

The rate of drying also affects final particulate morphology and faster drying tends to yield more deformed particles. The difference in morphologies observed in Figures 17 – 19 could be due to the possibility that solutions with lower viscosities tend to produce smaller droplets, which experience faster rates of evaporation in the drying chamber (Wang *et al.*, 2002).

Fines collected from the PTFE filter using 2% alginate as feed were also examined under SEM, as shown in Figure 21.

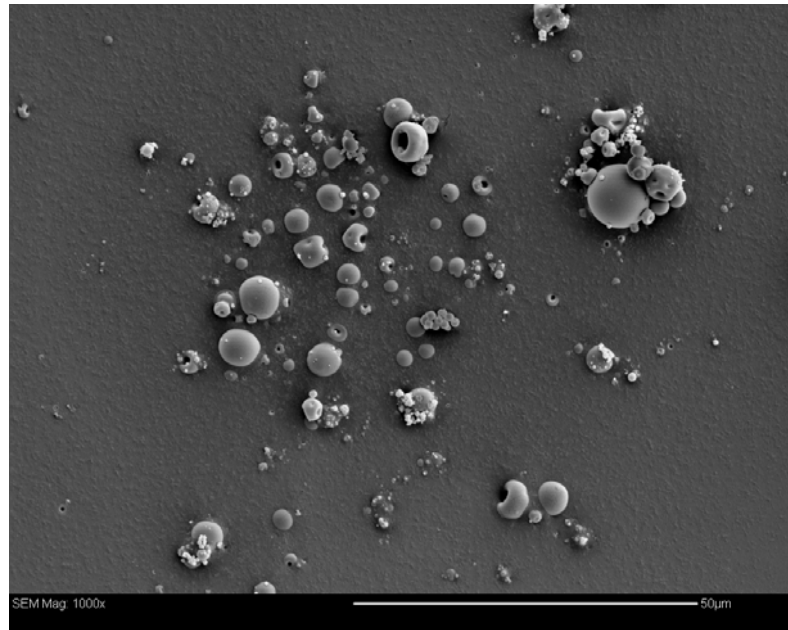


Figure 21 - SEM micrograph of fines at 1000x magnification

A visual examination of Figure 21 reveals that the fines can be considered both Class I and Class II particles, which have both spherical and dimpled morphologies. The morphology of the fines seems to be similar to the particles collected from the cyclone, though the particles appear considerably smaller.

5.2.4 Protein Encapsulation Efficiency

5.2.4.1 Absorbance Assay

The absorbance assay is based on ultraviolet absorbance by the amino acids tyrosine and tryptophan at 280 nm (Layne, 1957). The assay is a convenient measurement of protein concentration because it does not require the addition of reagents or dyes, and does not involve incubation time.

Encapsulation efficiency (EE) was determined from the supernatant after dissolving particles in phosphate buffer. An insulin standard calibration plot (Figure 11), was used to determine insulin per gram of particle and compared to the theoretical loading per gram, based on the initial insulin to alginate concentrations in the feed solution. A sample calculation can be found in Appendix B. The results obtained are summarized in Table 7.

Table 7 – Insulin encapsulation efficiency of spray dried particles determined by absorbance assay (mean \pm S.D., n=3)

Alginate Feed Concentration (w/v %)	Encapsulation Efficiency (%)
1	39.3 \pm 4.7
1.5	40.6 \pm 6.3
2	38.2 \pm 9.5

Efficiency values of approximately 40% are low, but within the range observed by other groups who produced drug-loaded polymeric particles by spray drying. For example, Martinac *et al.* (2005) recorded EE as low as 10% for loratadine-loaded chitosan-ethylcellulose particles (Martinac *et al.*, 2005). On the other hand, Kusonwiriawong *et al.* (2008) reported essentially full recovery of BSA in chitosan particles produced by spray drying (Kusonwiriawong *et al.*, 2009). The variation in EE observed in various drug-loaded particles produced by spray drying could be due to both the nature and/or the concentration of the polymer and active (Wang *et al.*, 2002). An increase in solubility of the active in the feed is associated with an increase in

encapsulation efficiency since undissolved drug will likely be removed through the exhaust system (Wang *et al.*, 2003). Thus, as polymer feed concentration increases, there is less solvent available for the drug to dissolve. As well, the interaction between the polymer and the active could affect the extent of encapsulation, where oppositely charged materials could increase association and subsequently improve the EE (Reis *et al.*, 2007). The molecular weight of the active may also affect EE, since drugs with a smaller molecular weight, such as loratadine (MW: 0.4 kDa), could be more easily removed with water during the evaporation stage compared to a larger molecule, such as BSA (MW: 70 kDa).

Due to the relatively low insulin loading (presented in Table 2), it is unlikely that the encapsulation efficiencies observed are a result of the solubility of insulin in the feed concentration, since insulin is likely completely dissolved in solution. It is more probable that low encapsulation efficiencies are due to the interaction between alginate and insulin, which are both negatively charged in the feed solution. It would be proposed to investigate the use of a positively charged polymer, such as chitosan, in a future study to determine the effect of charge interaction on EE. The molecular weight of insulin (MW: 5.8 kDa) could also contribute to the low EE, since it is relatively small compared to the molecular weight of alginate (MW: 147 kDa) and could be pulled out with water during drying (Wee *et al.*, 1998).

A one-way ANOVA test was used to determine that there was no statistically significant change in EE with polymer concentration, which would be expected since the protein loading is small compared to the amount of solvent and polymer used in the

formulation. Thus, no variation in drug solubility would occur between different feeds and no change in encapsulation efficiency would be observed.

5.2.4.2 Enzyme-Linked ImmunoSorbent Assay

The enzyme-linked immunosorbent assay (ELISA) was employed to determine EE as it is an assay specific to insulin. ELISA is based on a ‘sandwich’ technique in which two specific antibodies are employed for insulin antigen quantification as shown in Figure 22. The primary antibody, specific to insulin is fixed to a substrate, while the detection antibody is added after the insulin antigen is bound by the primary antibody. An enzyme linked to the secondary antibody reacts with a substrate, generating a colorific signal, measured spectrophotometrically.

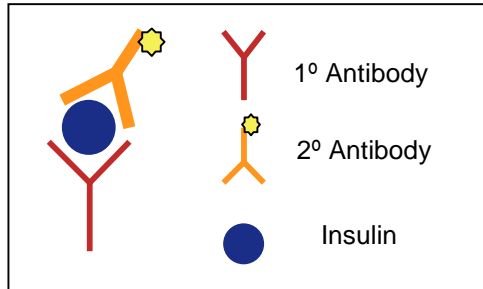


Figure 22 - Diagram of ELISA sandwich technique with primary antibody, enzyme linked secondary antibody and insulin molecule

Table 8 summarizes the EE results obtained from particles produced from 1, 1.5 and 2% alginate feed concentrations.

Table 8 - Insulin encapsulation efficiency of spray dried particles determined by ELISA (mean \pm S.D., n=3)

Alginate Feed Concentration (w/v %)	Encapsulation Efficiency (%)
1	64.9 \pm 7.1
1.5	65.7 \pm 7.6
2	48.1 \pm 12.1

A one-way ANOVA test determined that there was no statistically significant increase in encapsulation efficiency based on feed concentration for the results presented in Table 8, which is consistent with the results obtained using the absorbance assay. In spite of this, the EE results determined by ELISA are higher than as those presented in Table 7. The difference in EE observed could be due to the high sensitivity of the ELISA assay, since it is designed to measure physiological insulin levels in human plasma. Since the insulin levels expected in the present assay are at considerably higher levels extensive and accurate sample dilution is required to bring the measurable concentrations to within the detectable range, between 0 and 200 mU/L (or 7×10^{-6} mg/mL). Since there is a larger variation in the results associated with the ELISA kit and because ELISA cannot be run online, the absorbance assay was employed to determine *in vitro* GI release and residual bioactivity.

Based on the results presented thus far, there was little difference observed in the characteristics of particles from 1, 1.5 or 2% alginate feeds. Therefore, subsequent assays were conducted using particles produced from 2% alginate feeds.

5.2.5 Protein Release in Gastrointestinal Simulation

Insulin release in the gastrointestinal tract was simulated by suspending a sample of particles in simulated gastric fluid (pH = 1.2) for 120 min, which is the approximate residence time in the stomach. The particles were then resuspended in simulated intestinal fluid (pH = 6.8) for another 600 min. The results in Figure 23 are presented as cumulative insulin release.

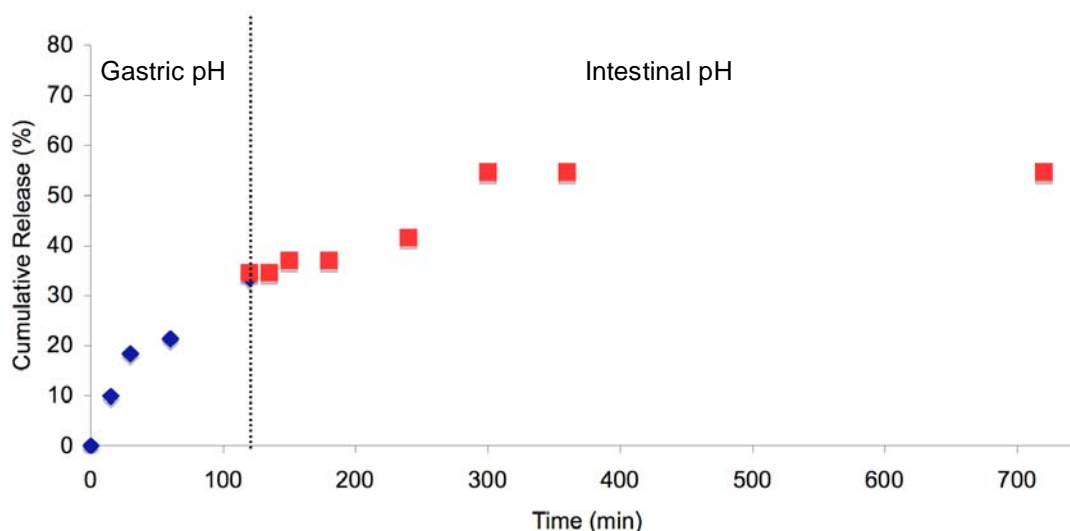


Figure 23 - Cumulative release of insulin from spray dried particles in simulated gastrointestinal tract. (◆) Represents cumulative release from particles suspended in pH 1.2. (■) Represents cumulative insulin release from particles suspended in pH 6.8.

A steep release profile was observed in the first 60 min of the gastric simulation, which starts to plateau at the end of 120 min. The total release in the gastric fluid was approximately 35% of the total insulin present in the particles. This initial release could

be attributed to the release of insulin contained on or near the surface of the particles (Kim *et al.*, 2003).

In the intestinal simulation starting at 120 min, it can be seen that there is little additional release in the first 30 min. A higher pH would cause the particles to swell, promoting release of insulin, however particles are still in a compact state after being suspended in the gastric fluid. With swelling, the particles absorb water, providing little opportunity for further insulin release. Once a swollen equilibrium is established at approximately 300 min, insulin may be released through a diffusional or particle erosion mechanism. After swelling equilibrium, there was a steady increase in cumulative release, reaching a plateau at approximately 55%. The remaining insulin was ultimately released beyond the 12 h period.

Insulin released prior to particle translocation across the epithelial layer of the intestine can be subject to proteolytic enzymes and therefore be inactivated, although insulin released from particles that adhere or are in close proximity to intestinal epithelial cells could be transported without being subject to degrading enzymes. An oral insulin delivery vector should release as little protein as possible in the stomach and the lumen of the intestine (George *et al.*, 2006). Based on the high initial rate of release observed, it would be recommended that methods to slow or stop insulin from diffusing out of the polymer be investigated. Lemoine *et al.* (1998) demonstrated that a coating, such as poly(L-lysine), can retard the release of bovine serum albumin from alginate microparticles, which could be investigated in future studies involving insulin (Lemoine *et al.*, 1998).

5.2.6 Protein and Polymer Distribution

Another important characteristic of microparticles is the distribution of the active throughout the polymer matrix. The location and distribution can affect release characteristics, and potentially provide protection (or not) from enzymatic and hydrolytic conditions. In order to determine protein distribution, confocal laser scanning microscopy (CLSM) was used on fluorescent labeled insulin, contained within the microparticles. At the same time, the polymer matrix was labeled, so as to potentially track the gastrointestinal distribution and uptake of insulin, independent of the particle matrix for *in vivo* tracking (Borges *et al.*, 2006; Damgé *et al.*, 2007).

FITC and RBITC were selected to allow simultaneous detection through CSLM, where FITC emits a characteristic green colour between 505 nm and 540 nm using an excitation wavelength of 280nm, and RBITC emits a characteristic red colour between 570 nm and 700 nm at an excitation wavelength of 560 nm.

5.2.6.1 Insulin Fluorescence

Novolin GE Insulin was labeled with FITC by a employing a procedure described by Clausen *et al.* (2001). FITC is known as an amine reactive fluorescent probe, and thus is very commonly used to label proteins (Gok *et al.*, 2004; Ye *et al.*, 2006). CSLM light and dark field micrographs of FITC-insulin and unlabelled alginate particles are presented in Figures 24 and 25.

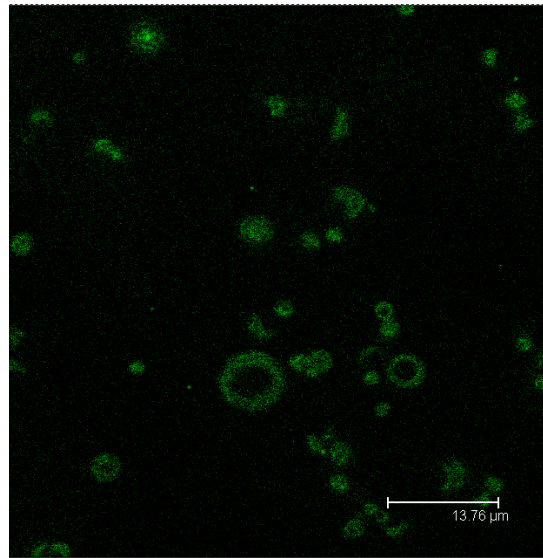


Figure 24 - Dark field confocal micrograph of FITC-human insulin alginate particles

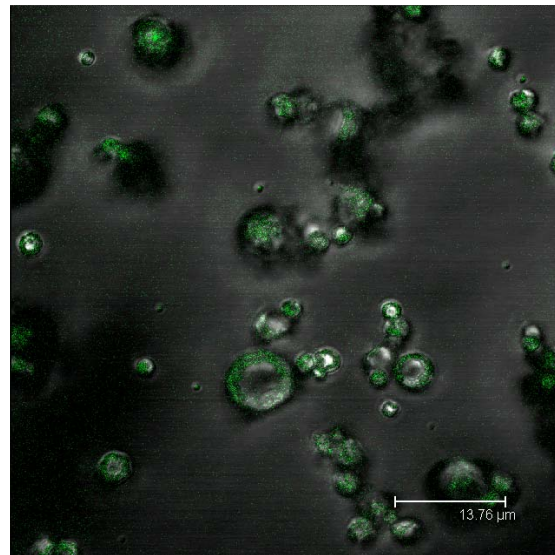


Figure 25 - Dark and light field confocal overlay of FITC-human insulin alginate particles

Figures 25 and 26 indicate that FITC was successfully labeled to Novolin GE Toronto insulin. Figure 25 is included as a control to show that all particles visualized in the micrograph contain labeled insulin. It is apparent from Figure 24 that insulin is concentrated toward the periphery in some of the larger particles present in the sample. The insulin distribution within the particles was examined further by employing commercially available FITC-bovine insulin in the feed formulation.

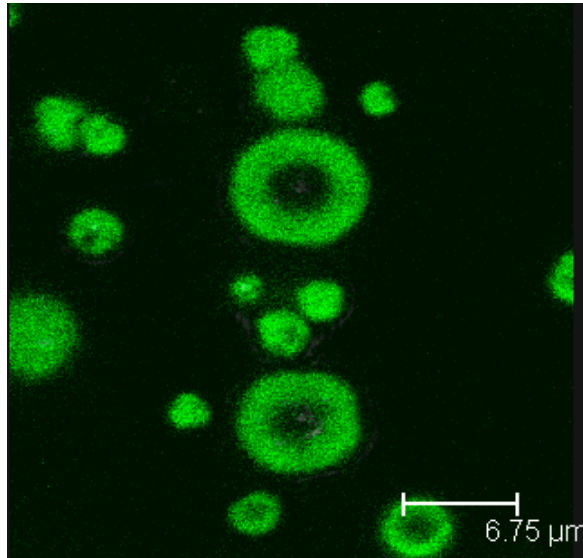


Figure 26 - Dark field confocal micrograph of FITC- bovine insulin alginate particles

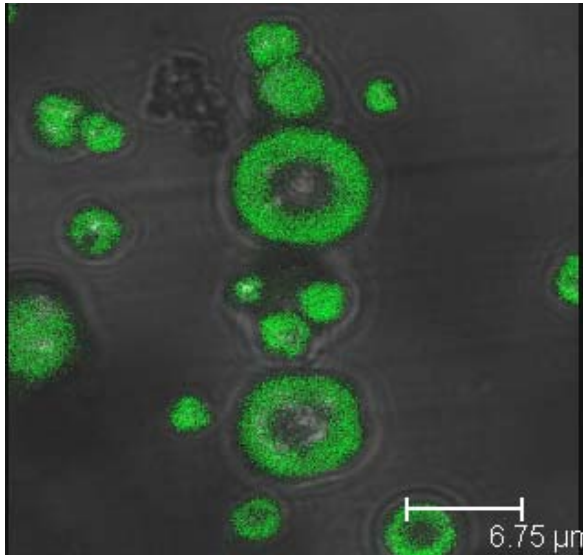


Figure 27 – Dark and light field confocal micrograph overlay of FITC- bovine insulin alginate particle

As in Figure 25, Figure 27 shows that insulin is evenly distributed throughout smaller particles. Larger particles on the other hand, show insulin localized to the outer edges. This apparent insulin-free core region may be explained by the divots or indentations observed on some larger particles as seen in SEM micrographs or that the particles in fact have a hollow core. Despite the uniformity of the label observed in the smaller particles, if particles are indeed hollow, fluorescence may appear evenly distributed because smaller particles rest at different focal depths compared to larger particles on the slide. Since insulin appears to concentrate from the core of the particle toward the surface, this may explain the burst release observed in Figure 24 during the first 60 min of the gastrointestinal simulation, involving insulin located at or near the particle surface. The dark and light field micrographs superimposed in Figure 27 show that particles appearing in the sample image are labeled.

5.2.6.2 Alginate Fluorescence

Alginate was labeled with RBITC and CLSM micrographs are presented in Figures 28 and 29.

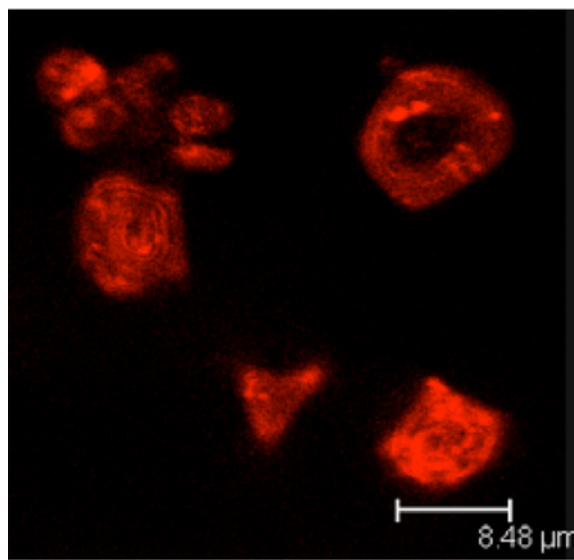


Figure 28 – Dark field confocal micrograph of RBITC-alginate

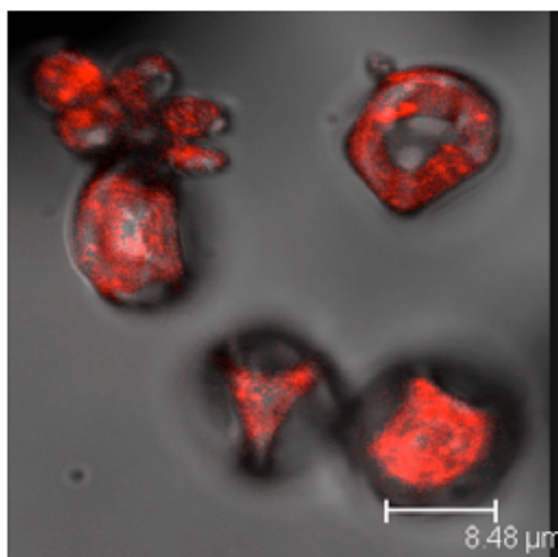


Figure 29 - Overlay confocal micrograph of RBITC-alginate

Figures 28 and 29 indicate that RBITC successfully labeled alginate. The micrographs obtained from RBITC-alginate particles show that dimpled particles appear to have the polymer fluorescent signal concentrated toward the outer surface of the

particles. As previously explained, this could be a result of the drying process where water is removed by evaporation from the particles at a fast rate. As the water is being pulled from the particle, it may effectively drag both alginate polymer and insulin toward the periphery of the drying particle, forming a concentration gradient from the core.

5.2.6.3 Composite Insulin and Alginate Fluorescence

Both FITC-labeled insulin and RBITC-labeled alginate were spray dried together to provide a picture of the protein and polymer distribution throughout the particles. Figures 30 and 31 show the dark field micrographs of the same particles for the green FITC and red RBITC channels, respectively.

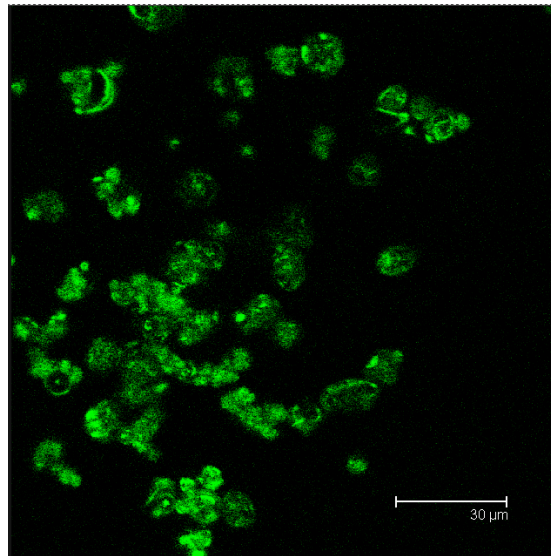


Figure 30 - Dark field confocal micrograph of FITC-insulin channel for RBITC-alginate and FITC-insulin labeled particles

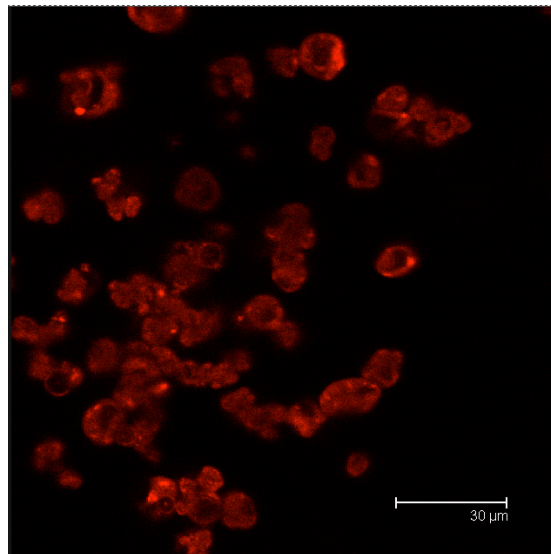


Figure 31 - Dark field confocal micrograph of RBITC-Alginate channel for RBITC-alginate and FITC-insulin labeled particles

Figures 31 and 32 show that insulin and alginate can be fluorescently labelled in the same formulation and visualized using confocal microscopy. The results of this experiment are especially important for future *in vivo* studies when it may be necessary to assess whether insulin is encapsulated or released from the alginate matrix.

5.2.7 Insulin Biological Activity

Bioactivity of entrapped and released insulin is an important consideration in the development of a particulate formulation for oral delivery. The integrity of the insulin molecule, including tertiary folding and specific 3D conformation, is necessary to correctly bind to insulin receptors located on the surface of cells. Successful coupling of insulin and insulin receptor causes a cell-complex signaling cascade resulting in the transport of glucose into the cell for use as a carbon and energy source. The cell-signaling cascade is illustrated in Appendix A. Any changes to the structure, renders the insulin inactive, as it has detrimental effects on the receptor coupling process. Ensuring insulin bioactivity is important, since spray drying subjects the protein to high temperatures, potentially causing irreversible structural damage.

Insulin bioactivity is normally tested using laboratory animals such as diabetic mice and rats. For oral dosed insulin, the subsequent glycemic response is then compared to the equivalent subcutaneous insulin dose (Damgé *et al.*, 2007). However, this method has several drawbacks including the cost to buy, care and house animals and the time required to set-up and maintain animal facilities. Additionally, *in vivo* tests present ethical and animal standards issues, especially with animals that must be induced with diabetes,

and subsequently sacrificed. *In vitro* bioactivity assays were therefore proposed as an alternative and is the focus of the research conducted and detailed in this section.

There has been little research detailed in the literature regarding *in vitro* insulin bioactivity assays and only two papers may be found on the subject (Patel *et al.*, 2001; Reis *et al.*, 2007). The assay detailed by Reis *et al.* (2007) was developed in our lab, and is based on binding to the insulin receptor generating the well-characterized signaling cascade (shown in Appendix A). One of the downstream reactions triggered by insulin binding to the insulin receptor is the phosphorylation of protein kinase (PKB), also known as AKT. Reis *et al.* (2007) were able to quantify the extent of phosphorylation by Western blot correlated to bioactive insulin concentration (Reis *et al.*, 2007). Since that time, a Fast Activated Cell Based ELISA (FACE™) AKT kit has become commercially available. The ELISA kit is preferable to Western blot, because it is faster and simpler, requiring non-specialized equipment or personnel. The ELISA kit is also a whole cell assay whereas Western blot requires an additional cell lysis step. Thus, the FACE™ AKT ELISA was investigated as a means to detect insulin bioactivity.

5.2.7.1 Biological Activity Assay Optimization

The FACE™ AKT ELISA incorporates two specific antibodies, one for total AKT, which includes phosphorylated AKT, and one antibody specific for phosphorylated AKT (p-AKT). The secondary or detection antibody is a non-specific horseradish peroxidase-conjugated antibody used for detection. Insulin responsive rat L6 myoblast cells are thus grown, fixed to a support, then stimulated with insulin. One of the primary

antibodies (specific to AKT or p-AKT) is then added and the cells incubated overnight. Cells are then washed and the HRP-conjugated detection antibody is added resulting in a colorimetric reaction measured spectrophotometrically. Finally, the cells are stained with crystal violet and the absorbance is measured to normalize the results to cell number per well.

5.2.7.1.1 Cell Number

The first step in optimizing this assay was to determine the number of cells required per well to achieve 80% confluence over a 24 h period in a standard 96-well plate. Between 30,000 and 70,000 cells/cm², were seeded onto 96-well plates and incubated overnight. Plates were then examined using a light microscope, and it was determined that 70,000 cells/cm² or roughly 22,500 cells/well gave the closest to 80% confluence.

5.2.7.1.2 Insulin Concentration

The insulin concentration was then examined to determine the limits of the assay. Based on work conducted by Houg *et al.* (2006) using Western blot, a preliminary insulin concentration range was selected between 0 – 600 nM (Houg, 2006). Figure 32 shows the results obtained for standards prepared with free Novolin GE Toronto insulin using a 10 min cell stimulation time. The normalized absorbance is the ratio of p-AKT absorbance to total AKT absorbance divided by the absorbance measured by crystal violet cell staining in order to normalize for cell number.

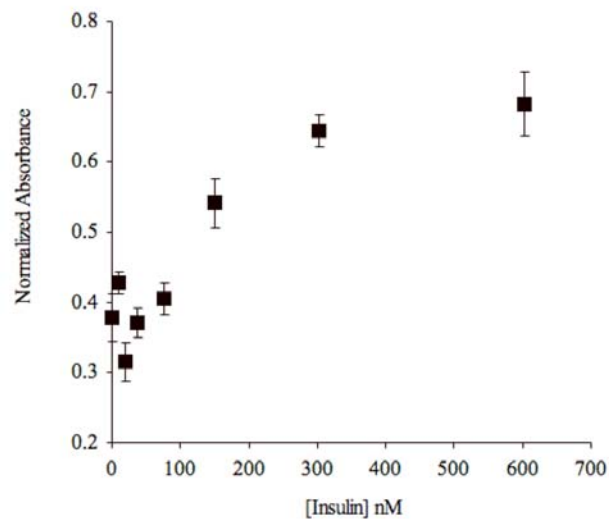


Figure 32 - FACE™ AKT free insulin stimulation concentration range

Figure 32 shows a correlation between insulin concentration and normalized absorbance, which appears to scatter around a baseline below 100 nM, steadily increase and then plateau after roughly 300 nM. The normalized absorbance readings for wells that were stimulated with blank standards indicate that there is a low level background of normalized absorbance of roughly 0.375, thus the detection range for this assay is 100 - 300 nM. Examining lower concentration levels and verifying the detection limits as shown in Figure 34, further demonstrated this range of detectability.

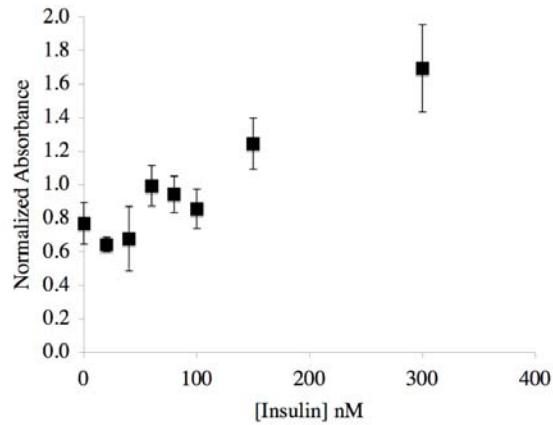


Figure 33 - FACE™ AKT ELISA at low range free insulin concentration

Figure 33 confirms the lower limit of the assay to be about 100 nM. The scattering at lower concentrations suggests that the assay is not picking up detectable signal between 0 – 100 nM insulin.

5.2.7.1.3 Stimulation Time

The standard trials depicted in Figures 33 and 34 indicate that there is a correlation between bioactive insulin concentration and normalized absorbance and thus, the stimulation time was varied to determine the time to peak cellular p-AKT levels. Two insulin concentrations, 300 nM and 600 nM, and a blank were examined at times ranging between 5 and 25 min. The results are presented in Figure 35.

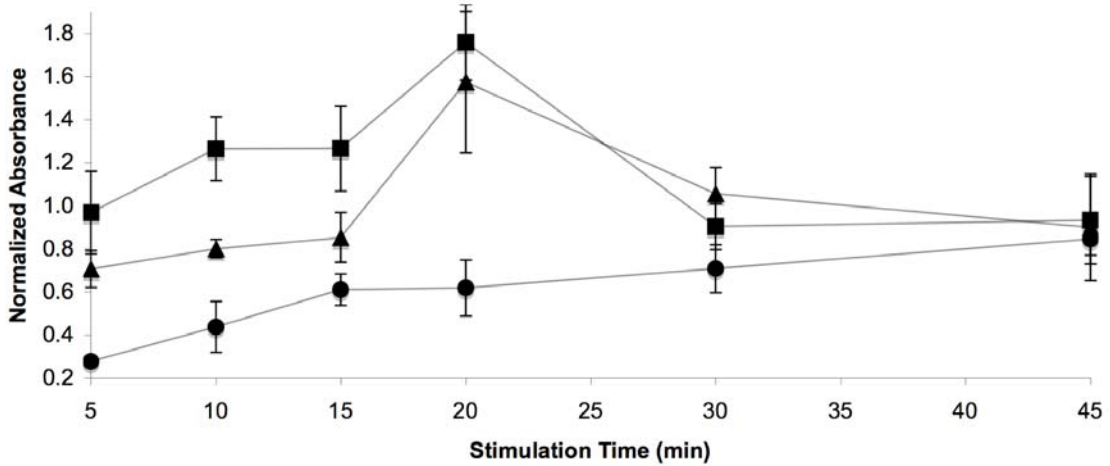


Figure 34 - Free insulin stimulation time at (●) 0 nM (■) 300 nM (▲) 600 nM

It appears in Figure 34 that a maximum in p-AKT is reached at 20 min for both insulin concentrations, which is not seen in the blank sample. From this data, it was determined that a 20 min stimulation time would furnish the highest p-AKT absorbance values and thus this stimulation time was used for all subsequent assays.

5.2.7.2 Microparticle Insulin Activity

The next step was to determine the bioactivity of insulin in spray dried alginate particles. Particles were dissolved in phosphate buffer for 24 h and assayed for insulin concentration by absorbance assay. The sample was then diluted to within the established range of the FACE™ AKT ELISA, between 100 – 300 nM and percent of bioactive insulin determined by calculating the ratio of bioactive insulin determined by FACE™ AKT ELISA to the concentration from the absorbance assay. The bioactivity was determined to be $87.9 \pm 15.3\%$ (mean \pm S.D., n=3; see Appendix C for sample

calculation). Figure 35 shows a sample standard curve of normalized absorbance, obtained from the bioactivity assays. Basal levels of normalized absorbance for the blanks are included in the plot.

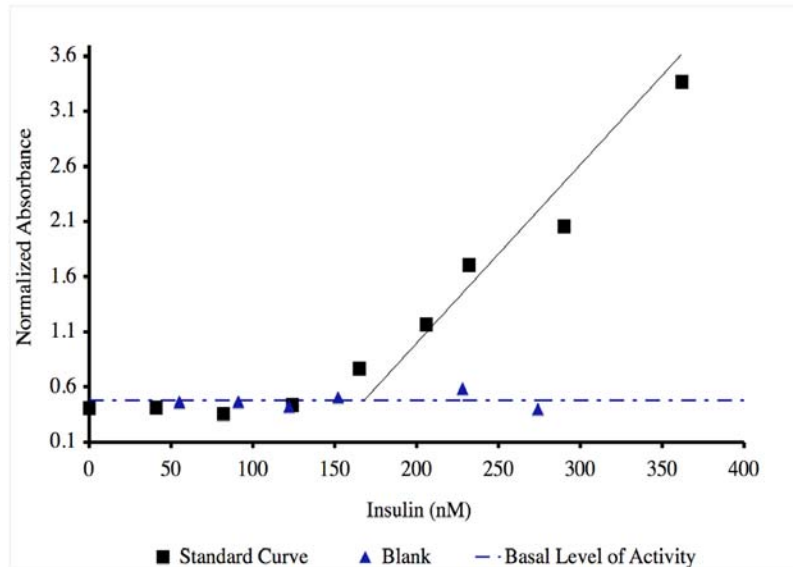


Figure 35 - Sample standard curve of normalized absorbance for the FACE™ AKT ELISA bioactivity assay of insulin. The basal level of normalized absorbance is plotted for the blank runs with each insulin assay.

These results would indicate that spray drying does not have a significant effect on the residual bioactivity of insulin in the alginate particles as a high level of bioactivity can be demonstrated.

Given that this is the first time a FACE™ AKT ELISA has been used to assay the bioactivity of insulin, it is not possible to compare to other results. Reis *et al.* (2007) did determine activity of insulin-loaded alginate-dextran particles produced by emulsion dispersion using the Western Blot immunodetection technique (Reis *et al.*, 2007). The

bioactivity of insulin was determined to be 55% of the theoretical value assuming full activity retention, which was subsequently confirmed with *in vivo* tests using diabetic rats.

Chapter 6

Summary and Conclusions

The aim of this research was to produce insulin encapsulated alginate micro- and nanoparticles by spray drying, which could potentially be applied in peroral delivery. Moisture content, mean size and distribution, morphology, insulin loading and release characteristics, insulin and alginate distribution and bioactivity were determined. As well, the fines were assayed for particle size, distribution and morphology.

The mass yield of the insulin-alginate product collected was determined to be between 15 and 30% of the initial solids concentration in the feed, depending on the initial feed formulation. Yields were low because alginate polymer deposited on vessel and cyclone walls, and submicron sized particles were lost in the fines and exhaust. The extent of drying, characterized by the residual moisture in the particles, may affect the mass yield since wet particles are more likely to stick to the walls of the spray dryer. The balance between the extent of drying, which is affected by the inlet air temperature and/or feed rate, and mass yield must be carefully considered when developing drug delivery systems formulated by spray drying to ensure that the active does not experience high temperatures for extended periods. The results from this study indicate that the combination of the formulation and spray dryer settings do not affect residual activity. Since the product yield is important for pharmaceutical formulations at an industrial level, it would be recommended to investigate the capture efficiency of the spray dryer through electrostatics or by changing the geometry of the cyclone or collection vessel instead of altering spray drying parameters (Maa *et al.*, 1998).

Particle moisture content was found to be between 4.9 and 11.1%, independent of polymer feed concentration. The moisture content of spray dried particles is important because it can affect protein stability, though the storage conditions of dried particles may be just as important in determining long term activity (Ameri *et al.*, 2006). In any case, the advantage of spray drying over wet encapsulation techniques such as emulsion dispersion and ionotropic pregelation is that it combines drying and encapsulation in a single step. Particles produced by wet methods would likely require a drying step such as spray freeze drying or lyophilization in order to achieve the levels of residual moisture determined in this research.

Mean particle sizes were 1.2, 1.5 and 1.6 μm for 1, 1.5 and 2% alginate feed concentrations respectively and were determined to be under the upper limit of particle translocation in the gastrointestinal tract. Even so, it would be recommended to test the permeability of particles using an inverted Caco-2 intestinal model since, even in the micron range, size has been shown to have a large effect of the fraction of particles that cross the epithelial layer of the intestine (Liang *et al.*, 2001).

The morphology of the particles was spherical with divots that may have been caused by the formation of a polymer film during drying that eventually bursts leaving deformed particles.

Encapsulation efficiency was determined using an absorbance assay and an immunoassay involving ELISA. It was concluded that the ELISA assay had larger sources of error due to the variability introduced by extensive dilution and samples, and

thus encapsulation efficiencies determined as 40% determined by absorbance assay regardless of alginate feed concentration. An EE of 40% is low for pharmaceutical systems involving high value proteins such as insulin. It would therefore be recommended to study the effects of charge interactions between the protein and encapsulating polymer and the molecular weight of the active and polymer on EE in spray drying processes.

Both insulin and alginate were successfully labeled with FITC and RBITC, respectively and a concentration gradient of both polymer and insulin observed originating from the centre of the particle toward the outer surface. The concentration gradient could be due to the movement of polymer and insulin drawn toward the particle surface during rapid and almost instantaneous removal of water during evaporation. These results are consistent with the release observed in the simulated gastrointestinal tract where insulin release was steep at the beginning of the gastric simulation possibly due to the insulin located at or in proximity to the surface.

An *in vitro* bioactivity assay was developed and optimized by investigating the detection limits and stimulation time. Based on this assay, it was determined that insulin is highly bioactive following spray drying, despite the temperature in the drying chamber.

The use of spray drying as a technology to encapsulate drugs is often overlooked in the field of drug delivery due to the belief that spray dryers cannot produce particles sufficiently small for drug delivery and because the active is denatured by the high temperatures within the drying chamber. Based on the system developed and

characterized in this research, it would appear that spray drying can in fact produce particles small enough to be used in oral delivery, which contain active protein. Insulin encapsulated alginate particles were successfully produced by spray drying. Overall, it was concluded that the particle characteristics determined in this study warrant further investigation into application as a peroral delivery vector, to be tested in animal models.

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Appendix A

Cell Signaling Cascade

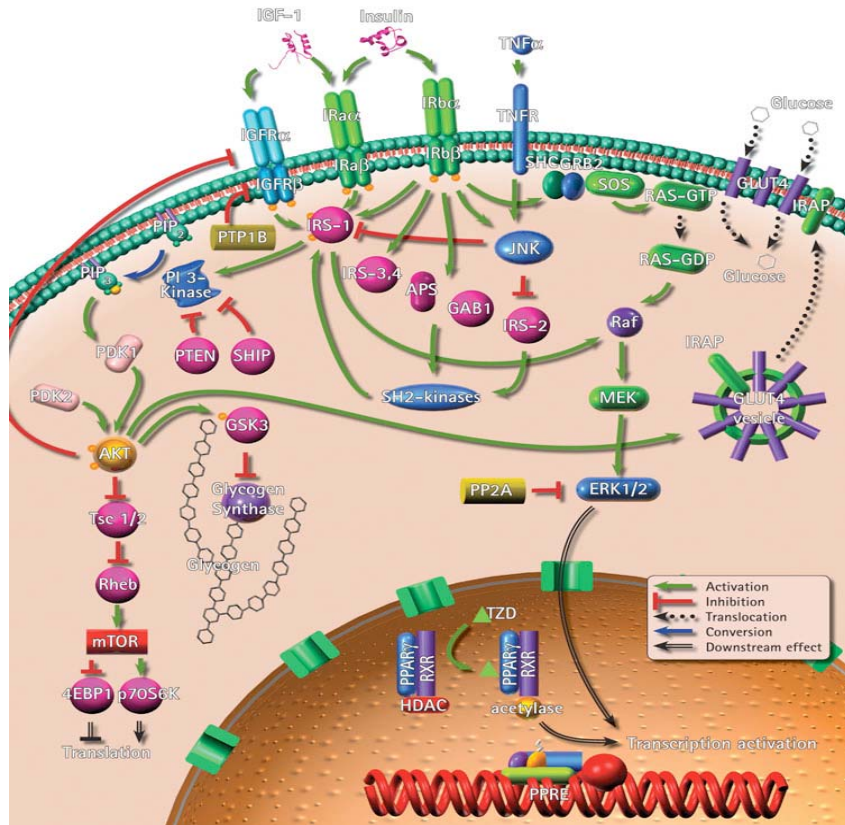


Figure 36 - Insulin cell signaling cascade (Reproduced from (CalBioChem, 2009))

Appendix B

Sample Calculation - Encapsulation Efficiency

Theoretical Protein Loading

Assuming all the insulin is evenly distributed throughout the particles, the theoretical insulin loading per gram of particle is:

$$\textit{Theoretical Insulin Loading} = \frac{\textit{mass}_{\textit{insulin in feed}} \textit{(g)}}{\textit{mass}_{\textit{alginate in feed}} \textit{(g)}}$$

To make a 1% alginate feed, 3 g of alginate was dissolved in 300 mL of distilled water and 35mg of insulin was added. After spray drying, the theoretical protein loading per gram of particle could then be determined as:

$$\textit{Theoretical Protein Loading} = \frac{35 \textit{ mg}}{3 \textit{ g}} = 11.67 \textit{ mg/g}$$

Actual Protein Loading

$$\textit{Actual Protein Loading} = \frac{\textit{m}_{\textit{insulin in product}} \textit{(g)}}{\textit{m}_{\textit{product}} \textit{(g)} - \textit{m}_{\textit{water}} \textit{(g)}}$$

To determine $m_{\text{insulin in product}}$, a protein concentration assay was performed. The absorbance was measured with a spectrophotometer at 280 nm for insulin-encapsulated alginate particles dissolved in phosphate buffer. From the standard curve presented in Figure 11, the slope and intercept can be used to calculate insulin concentration.

$$\textit{Insulin Concentration} \left(\frac{\textit{mg}}{\textit{mL}} \right) = \frac{\textit{Absorbance} - \textit{Intercept}}{\textit{Slope}}$$

$$\textit{Insulin Concentration} \left(\frac{\textit{mg}}{\textit{mL}} \right) = \frac{0.05 - 0.0027}{0.6139} = 0.077$$

Since 0.15 g of particles were dissolved in 10mL of phosphate buffer, we can determine the insulin loading per gram of particle:

$$\text{Insulin Loading } \left(\frac{\text{mg}}{\text{g}} \right) = 0.077 \frac{\text{mg}}{\text{mL}} \times \frac{10 \text{ mL}}{0.15 \text{ g}} = 5.13 \frac{\text{mg}}{\text{g}}$$

And the encapsulation efficiency can be calculated as:

$$\text{Encapsulation Efficiency} = \frac{\text{Actual Insulin Loading}}{\text{Theoretical Insulin Loading}} \times 100$$

$$\text{Encapsulation Efficiency} = \frac{5.13 \frac{\text{mg}}{\text{g}}}{11.67 \frac{\text{mg}}{\text{g}}} \times 100 = 43.95\%$$

Appendix C

Sample Calculation - Insulin Biological Activity

The insulin concentration in 0.15 g of particles dissolved in 10 mL of phosphate buffer was first determined by 280 nm, as outlined in Appendix B. For this batch, the particles were derived from an initial alginate concentration of 2% and the absorbance was 0.022. Based on the standard curve, the insulin concentration can be calculated as:

$$\text{Insulin Concentration} \left(\frac{\text{mg}}{\text{mL}} \right) = \frac{\text{Absorbance} - \text{Intercept}}{\text{Slope}}$$

$$\text{Insulin Concentration} \left(\frac{\text{mg}}{\text{mL}} \right) = \frac{0.022 - 0.0027}{0.6139} = 0.031 \frac{\text{mg}}{\text{mL}}$$

This concentration can then be converted to nM, which is the base unit of the cell-based assay.

$$0.031 \frac{\text{mg}}{\text{mL}} \times \frac{1000 \text{ mL}}{1 \text{ L}} \times \frac{\text{g}}{1000 \text{ mg}} = 0.031 \frac{\text{g}}{\text{L}}$$

Using the MW of insulin (5808 g/mol):

$$0.031 \frac{\text{g}}{\text{L}} \times \frac{\text{mol}}{5805 \text{ g}} \times \frac{1000 \text{ mmol}}{1 \text{ mol}} \times \frac{1000 \text{ } \mu\text{mol}}{1 \text{ mmol}} \times \frac{1000 \text{ nM}}{1 \text{ } \mu\text{mol}} = 5337.47 \text{ nM}$$

This sample is then diluted with starvation media to a concentration of 274 nM, for example. This solution is applied to the cells for 20 min and the FACE™ AKT ELISA kit is run according to the prescribed protocol.

A normalized absorbance is obtained, in this example 2.329, from dividing the phospho-AKT and total-AKT absorbance and normalizing for cell number using the crystal violet readings. The standard curve for this run is shown in Figure 37.

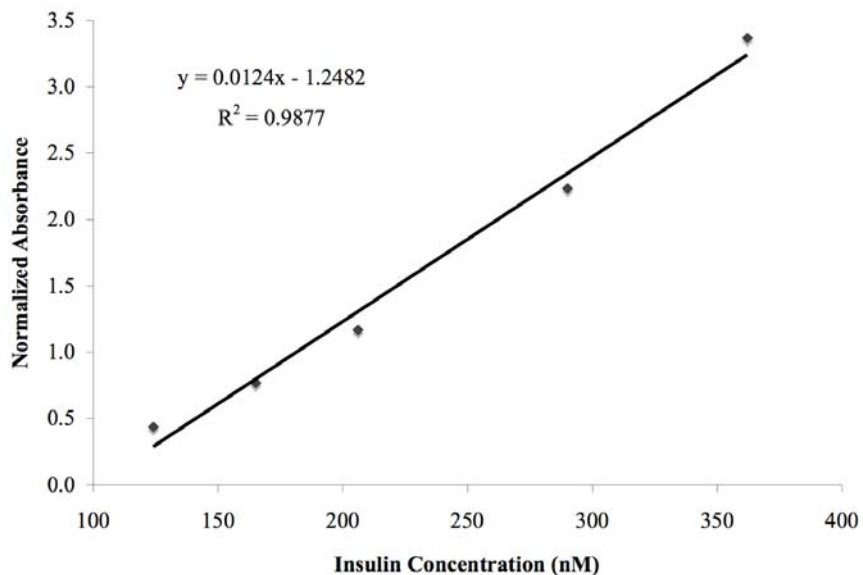


Figure 37 - Sample standard curve for FACE™ AKT ELISA bioactivity assay

$$\text{Insulin Concentration (nM)} = \frac{\text{Absorbance} - \text{Intercept}}{\text{Slope}}$$

$$\text{Insulin Concentration (nM)} = \frac{2.329 + 1.2482}{0.0124} = 288.48 \text{ nM}$$

The bioactivity can then be calculated as the ratio of concentrations:

$$\text{Insulin Bioactivity} = \frac{\text{Insulin Concentration from FACE AKT ELISA}}{\text{Insulin Concentration from absorbance assay}} \times 100$$

$$\text{Insulin Bioactivity} = \frac{288 \text{ nM}}{274 \text{ nM}} \times 100 = 105\%$$