MICROPARTICULATE DELIVERY SYSTEMS FOR
PROTEIN AND VACCINE THERAPY

by

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The first time I read an acknowledgements section, it was as a naïve 8 year-old reading one of her first novels. I always imagined that it must be such fun and excitement to be so famous that people would actually read each line of what you pen down. Little did I realize the amount of hard work that went into the background work and "research" in writing a document. And here I am today; at a point when I am struggling with the order of gratefulness that I must list all of my well-wishers and amazing people that have made a contribution in this journey of mine.

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Abstract

BERNADETTE D'SOUZA
MICROPARTICULATE DELIVERY SYSTEMS FOR PROTEIN AND VACCINE THERAPY
(Under the direction of Dr. Martin D'Souza, PhD)

The goal of this research was to investigate a novel drug delivery system for use in the biopharmaceutical industry. Currently, there are many promising biotechnology products which have high potency but are very expensive due to their need for specialized delivery requirements and stability issues. We have proposed the use of microparticles as a drug carrier to address various issues such as; 1) providing sustained release at the administration site, 2) preventing degradation of drug in the presence of enzymes, 3) increasing drug uptake into cells, 4) addition of drug-targeting ligands within the delivery carrier and 5) evaluating the possibility of avoiding adjuvant-use for vaccine antigens.

We carried out formulation studies with two protein and one polysaccharide biomolecule. We used the drug in a polymer matrix microparticle formed by a one-step process. For the cancer vaccine, melanoma whole cell antigens were selected that would specifically be
taken up by the antigen presenting cells (APCs) in the body. We observed that, both *in vitro* as well as *in vivo*, the presentation of the melanoma antigens in a particulate system provided an enhancement in uptake and increase in immune response. Also, we added a targeting ligand in the microparticle which further enhanced uptake after oral administration.

The next research project was to enhance a poorly immunogenic antigen by incorporation into a protein-based microparticle system. Polysaccharide antigens are less immunogenic than proteins. We wanted to evaluate the potentiation of immune response towards pneumonia polysaccharide antigens when presented in association with a protein-based microparticle. An added advantage of the particle delivery system is the achievement of sustained drug release, thus enhancing the depot effect at administration site. We observed a higher immune response and superior protection from live bacteria after immunization with polysaccharide in microparticles.

Our third objective was to test the efficacy of insulin in a microparticulate delivery system for oral administration. We optimized an enteric-protected, sustained release formulation to achieve this. We carried out *in vitro* dissolution experiments to test the release of insulin from the nano/microparticle in simulated fluids. In correlation, *in vivo* bioactivity studies showed that the insulin in microparticles had the ability to lower blood glucose levels in a diabetic rat-model.
CHAPTER 1
INTRODUCTION

The quest to design a good drug delivery system for biologics has been underway and is continuously evolving to keep pace with the advances in drug discovery, gene mapping, proteomics and even cancer targeting. These new research findings are encouraging scientists to investigate novel formulation designs to enhance drug delivery to the site of action and even afford some degree of drug targeting within the formulation. In the past two decades, a tremendous level of attention has been focused on sustained drug administration. In conventional therapy, a drug is administered at high doses, and the patient has to repeat the same dose several times a day. This method of drug administration may result in adverse effects. As a consequence, increasing attention has been focused on methods for administering drugs in a more controlled fashion. One of these methods involves formation of a particulate delivery system where the drug/s of interest can be incorporated within various types of biodegradable polymer matrices.

Particulate delivery systems using biodegradable polymers have been investigated for sustained release and also for targeted delivery to the site of action, which is also very important in increasing therapeutic effects and reducing side effects. Some of the currently used polymers in research can be broadly divided into being derived from either natural or synthetic materials. Natural
polymers include polypeptides and proteins (e.g. albumin, gelatin and collagen), polysaccharides (e.g. hyaluronic acid, starch, and chitosan), virus envelopes and living cells (e.g. erythrocytes, fibroblasts). The latter includes aliphatic polyesters of hydroxyl acids, like poly (lactic acid) (PLA), poly (lactic-co-glycolic acid) (PLGA), poly (D-caprolactone), poly (orthoesters), poly (alkylcarbonates), poly (amino acids), polyanhydrides, polyacrylamides and poly (alkyl-D-cyanoacrylates). One of our objectives involves the study of appropriate polymers that would be suitable for drugs having different physicochemical properties.

There are many techniques available for production of microparticles. We used the spray drying technique which is an easy one-step method that results in the formation of a dry particle and does not incorporate any organic solvents as shown in Figure 1.

Figure 1: Schematic of the spray drying process for preparation of microparticles.
The main goals of this research can be summarized into three main objectives:

1. Formulation and evaluation of albumin microparticles for cancer (melanoma) treatment using whole cell antigens as the immunogenic drug.

2. Development of an albumin formulation containing polysaccharide antigens towards pneumococcal infectious disease.

3. Formulation and evaluation of an oral particulate delivery system for insulin using cyclodextrin as the polymer matrix.

For cancer vaccines that need to be taken up by the antigen presenting areas of the Peyer's patches, the presentation of these antigens into a particulate system itself provides an enhancement in uptake (Bramwell, V. W., et al 2005). Also, particles can be targeted to the Peyer's patches in the intestine using specific lectins which deliver the drug encapsulated particle to antigen presenting cells (macrophages and dendritic cells) and initiate an immune response (Lai Y.H. and D'Souza M.J. 2007; Brown, W.R., 1996).

Another advantage of the particulate delivery system is delivering drugs intact into the small intestine for better absorption: in case of acid labile drugs like Insulin. For polysaccharide vaccines, which require an adjuvant to elicit a strong immune response, formulating the antigen in association with a protein based particulate system is highly desired (Zinkernagel et al 1997).

The particulate delivery system can be very promising in terms of improving drug uptake, and thus the enhancing efficacy at the site of action. In this project, we have proposed a novel approach which incorporates the drug into a
biodegradable and sustained release polymer matrix containing albumin or cyclodextrin. After formulating these drugs in the microparticle delivery systems, several characterization and evaluation studies of drug in the particle were performed. Also, in vivo studies for the studies were carried out to check for the efficacy of the drug when given in animal models.

In conclusion, we wanted to investigate the particulate delivery system for bioactive molecules including protein and polysaccharide vaccines for cancer and infectious diseases as well as a large polypeptide like insulin. In conclusion, the microparticulate delivery system is a promising technique for sustained drug release and delivery, and for targeting in the treatment of cancer, infectious diseases as well as protein drug therapy.
CHAPTER 2
LITERATURE REVIEW

Microparticulate drug delivery system

Microparticles are generally defined as particles with diameter ranging from 1nm up to 1000 micron. They are a class of formulations where drugs are incorporated into polymer matrices. The application of drug loaded microparticles in clinical treatment range from improving patient compliance, keeping constant therapeutic drug levels in systemic circulation (sustained release), maintaining higher concentration of the drug at the pathogenic site and lower concentration of the in normal tissue (targeting) and reducing adverse effect (less toxicity).

Microparticles can be divided into two categories: the first is called homogenous particles where the dug is dissolved or dispersed throughout the polymer matrix; the second is called encapsulated microcapsules where the drug is surrounded by the polymer matrix in the mononuclear or polynuclear state. This technique for drug delivery has been used in the pharmaceutical industry in the areas of sustained release of drugs, taste-masking of unpalatable drugs, masking of unpleasant odors, stabilization of drugs sensitive to atmospheric conditions, modification of physical properties, altering the solubility of drugs, elimination of incompatibilities between two or more drugs and a multitude of other uses (Madan, 1978). The primary focus of particulate research in the pharmaceutical field has been the sustained release of drugs and drug targeting.
One of the most important objectives of modern drug therapy is the prolonged or sustained delivery of drugs and diagnostic agents to specific targets and organs in the body. The advantages of reducing toxic effects by reducing the dose required to reach the therapeutic concentration and reducing the frequency of dosing have long been established. While the conventional peroral route is the most preferred route, limitations often require the need for parenterals. Microparticles present one of the few systems by which sustained release of a drug can be obtained after parenteral administration (Illum & Davis, 1982). These delivery carriers can also be targeted to various organs of the body and can thus help to target the drug to an organ or diseased site selectively (D'Souza and DeSouza, 1995, D'Souza and DeSouza, 1998, D'Souza, et al. 1999, D'Souza and Pourfarzib, 1999, Shah and D'Souza, 1999, Oettinger and D'Souza, 2003 and Tong, et al. 2003). This unique combination of the properties of microparticles is well suited to the objectives of modern pharmaceutics which is to prevent unwanted side effects, reduce the required dose and frequency of administration, and allow the use of highly active but toxic chemicals without causing significant toxicity to other tissues (Gerber, et al. 1995, D'Souza and DeSouza, 1998).

For particulate delivery carriers that need to be injected into the body, certain criteria must be met. They must be nontoxic to the host and should eventually be cleared from the body, hence they must be biodegradable and the degradation products must also be nontoxic and cleared from the body. Various polymers including lactide and glycolide polymers and copolymers meet these
criteria. Albumin, a protein found in the serum of all higher living beings, is also a good candidate as a polymer for microparticles since it is biodegradable, nontoxic and lends itself to the process of microencapsulation very well. Since the first reports of albumin being used in the preparation of microparticles, many drugs and diagnostic agents have been successfully encapsulated and tested, thus proving albumin microspheres as a versatile tool for sustained release and targeting of drugs (Huang, et al. 2003, D'Souza and DeSouza, 1998). Another interesting polymer that was studied in this research is cyclodextrin which has all the above-mentioned qualities and also affords a sustained release property to the drug once microencapsulated.

Spray Drying

There are many techniques available for production of microparticles. The technique used was the spray drying technique which is an easy one-step method that results in the formation of a dry particle and does not incorporate any organic solvents. Spray drying involves transforming a fluid, pumped medium into a dry-powdered or particulate form in a one-step process. This is achieved by atomizing the fluid into a drying chamber, where the liquid droplets pass through a hot-air stream. The objective is to produce a spray of high surface-to-mass ratio- ideally with droplets of equal size- then to evaporate uniformly and quickly the water. Evaporation keeps the product temperature to a minimum, so little high-temperature deterioration takes place (Mosen K., et al, 2004). The atomization into small droplets increases the surface area of the fluid, and subsequent drying time decreases with smaller diameter of the droplets. When
the droplet diameter is below 100 micron, and evaporation time is less than 1 sec, thermolabile materials can also be dried via spray drying, owing to short exposure time accompanied by surface cooling during the evaporation process.

Producing droplets of specific size and surface area by atomization is a critical step in the spray drying process. The degree of atomization under any set of spray drying conditions controls the drying rate and therefore the required particle residence time in the drying cylinder chamber. All atomizing techniques can give good control over the average particle size, but there can be a variation in the particle size distribution depending on optimization techniques. There are two techniques for atomization of the fluid using a spray dryer-1) Two-fluid nozzles and 2) pressure nozzles (Figure 2). The first operates on the principle of dispersing the liquid feed with the help of a high velocity gaseous medium whereas, the function of the pressure nozzles are to convert the pressurized energy supplied by a pump into kinetic energy in the form of a thin film.
Once the fluid is converted into a dry powder in the drying chamber, it must be separated by dry particulate collectors. In general, cyclone separators, bag filters or electrostatic precipitators can be used as the recovery device stage. Cyclones are the most cost-effective; air-pollution abatement devices for particulate matter removal widely used in large-scale industries. The main benefits include its simple design and absence of moving parts. The separation is based on the inertial deposition of the particles from the gas stream as it flows out of the drying chamber. As the particle loaded air stream enters tangentially into the cyclone, it results in a centrifugal force which creates a downward spiral movement. This causes the particles to get deposited in the bottom of the cyclone separator.

This method has been shown to have advantages over other methods like lower residual solvent, higher loading and higher encapsulation efficiency (Bittner, B., et al, 1998). It has been used successfully to produce human growth

In this project, the spray drying technique has been chosen to prepare polymeric microparticles in light of advantages of this method over the others. Protein and polysaccharide antigens have been incorporated for vaccine formulation and a particulate delivery system for protein drug insulin has been prepared using the spray drying method. The effects of microparticles matrix on the stability of encapsulated proteins was investigated using different physicochemical characterization studies. Bioactivity, immunoactivity and solid stage stability were also evaluated using bioassays and enzyme-linked immunosorbent assay (ELISA).
Figure 3: Schematic of a typical spray dryer; depicting (1) drying air inlet + filtration; (2) heating; (3) desiccation chamber; (4) cyclone; (5) collector for drying powder-microparticles; (6) filtration + air outlet; (A) solution, suspension, emulsion to spray; (B) compressed or nitrogen air and (C) spray nozzle (Kissel, T., et al., 2005).
Polymers

The major composition of a particulate delivery system is the polymer matrix. Polymers serve various functions in delivery systems as a result of their unique properties. Polymers are made up of monomeric units. Polymers are generally categorized into two classes; natural and synthetic. These polymers are finding increasing use and roles in drug delivery systems (Linhardt, R.J., 1989). Polymers used in drug delivery systems, whether natural or synthetic need to have minimal effects on biological systems after administration in the body, and should be bio-degradable, non-toxic and readily excreted from the body.

With the rapid growth in cell biology and gene technology which started in mid 1970s, it was possible to manufacture large scale therapeutic peptides and proteins, such as hormones, cytokines, growth factors, monoclonal antibodies, recombinant insulin and recombinant vaccines (Wang W., 2000). Since 1980s, these protein and peptide drugs have been specially interesting to achieve satisfactory therapeutic effects by using a novel drug delivery system, which use biodegradable polymers as a matrix for sustained release and targeting (Conti et al. 1992; Couvreur & Puisieux 1993; D'Souza & DeSouza 1998; Blanco-Prieto, et al 1998; D'Souza et al. 1999a; D'Souza & DeSouza 1995; Oettinger et al. 1996; Oettinger & D'Souza 2003). Biodegradable polymers have been investigated for sustained release and for targeted delivery to the site of action, which is also very important in increasing therapeutic effects and reducing side effects for at least two decades (Arshady 1990).
Natural polymers are commonly used as most of them are readily available, biodegradable, biocompatible, capable of chemical modification and inexpensive compared to synthetic polymers. The majority of natural polymers are proteins such as albumin, collagen, gelatin and polysaccharides like starch, dextran, and cellulose. These polymers are hydrophilic and mostly water soluble, and hence are ideal for acting as carriers of hydrophilic drugs and protein molecules. The release rate from natural polymer matrix usually is controlled by the degree of crosslinking either by chemical (gluteraldehyde) or thermal (heat) crosslinking (Silva, C.J.S.M., et al., 2004). One drawback of these polymers is the necessity to neutralize the residual of chemical crosslinking agents that is needed when encapsulating protein drugs. Albumin is the main polymer used in two of the research projects, as a matrix for melanoma cancer vaccine and polysaccharide pneumococcal vaccine. β-cyclodextrin has been used for the insulin project where sustained release microparticles are formed using this as the polymer matrix. The advantages of using these polymers were their properties of being biodegradable, biocompatible, non-toxic and relatively inexpensive.

Synthetic polymers such as polylactide glycolide, polyanhydrides, poly-ε-caprolactone, poly(amino acids) and poly(ortho esters) are also widely used as polymer matrices in drug delivery systems. They are insoluble in water and mostly are hydrophobic. Thus, they are commonly used as carriers for hydrophobic drugs. They are purified and do not require any crosslinking nevertheless require organic solvents for solubilization. Recently aqueous polymer dispersions have been introduced as an effort to replace the organic
solvents in the encapsulation process. These aqueous polymer dispersions are categorized according to their methods of development and manufacture. These include anionic emulsion polymers on methacrylate base (Lehman, K., et al., 1973), emulsification of cellulose derivatives in water and direct emulsification of neutral, hydrophilic methacrylates in water (Lehman, K., 1986).

In this project two aqueous dispersion polymers have been used, namely, Aquacoat®ECD by FMC Biopolymers and Hydroxypropyl methyl cellulose acetate succinate (HPMCAS) by Shin-Etsu. Aquacoat®ECD contains a pseudolatex with 30% solid content and other excipients such as cetyl alcohol and sodium lauryl sulfate. It is generally recognized as safe (GRASP) and is commonly used in the pharmaceutical industry for sustained release, taste masking and also as a moisture barrier. It has also been used in enteric coating of tablets and shown to resist simulated gastric fluid of pH 1.2 and only disintegrated in intestinal fluid. Besides that, it demonstrated very low oral toxicity with LD₅₀>5010mg/kg in rats according to its material safety data sheet. The other polymer used in this research was hydroxypropyl methyl cellulose acetate succinate (HPMCAS) produced by Shin-Etsu. It is insoluble in gastric fluid but shows increasing solubility in intestinal fluid at pH 7.0. This polymer possesses advantages over conventional polymers as encapsulation can be performed in aqueous systems.
**Albumin**

Serum albumin is a natural, water-soluble polymer which consists of a single strand of 580 amino acids wherein 26 amino acids are arginine and 60 amino acids are lysine. It has an isoelectric point of 4.7. The approximate molecular weight of serum albumin is about 67 KD. Serum albumin is a major plasma protein constituent (55% of the total protein in plasma or 35-50 mg/mL). It controls up to 80% of colloid osmotic blood pressure and maintains the blood pH. Albumin as such does not provide any sustained release properties. It has to be crosslinked or stabilized using various methods in order to attain sustained/delayed release properties. The rate of drug release from albumin microspheres and the rate at which the microspheres degrade in the body are dependent on the degree of crosslinking of albumin in the microspheres.

Albumin microparticles have been extensively used as a vehicle for delivery of therapeutics agents since it was first described by Kramer (Kramer, P.A., 1974). This is as a result of biodegradable, biocompatible, non-toxic and non-immunogenic properties of albumin (Addo R.T., et al., 2010; Zolle, I., et al., 1970). Albumin microparticles were first prepared for the detection of abnormalities in the reticuloendothelial system and later for studies on blood circulation (Zolle et al. 1970). Both unlabeled and radio-labeled microparticles were prepared by the method of thermal denaturation using human serum albumin (HSA). Microspheres were obtained in sizes between 12 - 44 μm, with indications that the particle size and rate of degradation could be varied by changes in the method of preparation. These pioneering works outlining the
tremendous potential of albumin microspheres created scientific interest for further examination. This later led to newer methods of manufacture and the discovery of new and innovative applications.

Delivery of drugs from albumin microparticles has been reviewed by Davis et al., Tomlinson and Burger whereas the biodegradation mechanism was reported by Natsume et al., 1990. It was found that proteolytic enzymes such as trypsin and protease resulted in significant weight loss and changes of the topography of microparticles. There are numerous factors that influence the release of drugs from microparticles. Among them are types of matrix used (monolithic or reservoir), the properties of drugs encapsulated including molecular weight, physicochemical properties, drug loading and possible interaction between drugs and polymer matrix. Composition of polymers in the matrix, extent of crosslinking and the environment in the GIT such as pH, and enzymes also impact the release of microparticles. With the advantages of albumin over other polymers, albumin microparticles have been used to deliver wide ranges of therapeutic agents and have attempted for oral delivery (Hori., M., et al., 2005).

In vivo biodistribution studies were performed in different animals showing the highest accumulation in lungs over other organs (Sahin S., 2002; Perkin et al, 1994). Walday, P., et al. also reported the biodistribution of albumin microparticles in pigs and rats. They found that after intravenous administration of air-filled albumin microparticles in rats, nearly 60% of the dose was accumulated in the liver, only 5% in the lungs, and 9% in the spleen and
negligible amounts in the kidney, the heart and the brain. However, over 90% of microparticles were recovered in the pig’s lung.

The synthesis and characterization of albumin microspheres containing a drug along with magnetite was reported (Widder et al., 1983). Their objective was to develop microspheres which could be site delivered by means of an external magnetic field. Kramer reported the entrapment of mercaptopurine into albumin microspheres by the method of Scheffel et al (1972). In vitro studies demonstrated a slow release of mercaptopurine. His work first demonstrated the potential application of albumin microspheres in the chemotherapy of bacterial infections and cancer (Kramer 1974).

Widder et al. 1979 synthesized serum albumin microspheres containing doxorubicin by chemical crosslinking, using formaldehyde and 2, 3-butadione and also by thermal cross-linking. Their results indicated that the microspheres produced by both processes were equally stable but differed in their in vitro drug release characteristics. The microspheres produced by thermal denaturation released the drug at a slower rate compared to the microspheres prepared with a chemical cross-linking agent at concentrations up to 25% v/v. This work gave a new direction to efforts involving the entrapment of thermally labile drugs, since chemical cross-linking usually did not require the application of heat.

In one study, progesterone loaded BSA beads were prepared using a chemical cross-linking method with glutaraldehyde as the cross-linking agent and an oil phase consisting of corn oil and petroleum. They showed that the size of
the beads was dependent on the speed of agitation. They also demonstrated that
the rate of drug release was dependent on the degree of cross-linking of albumin,
which in turn was inversely dependent upon the glutaraldehyde concentration
(Lee et al. 1981). In another report they discussed the entrapment and in vitro
release of morphine, methyl orange, progesterone, insulin and tetanus toxoid
(Royer et al. 1983).

In another study, serum albumin microparticles were prepared using the
method of Zolle et al. 1970, with some modifications, to produce microparticles
with a narrow size distribution of approximately 1 μm, thus demonstrating that the
method of preparation had a strong impact on the microparticles formulation size
(Scheffel et al. 1972). These encouraging results demonstrated the potential for
use of albumin beads as parenteral devices for diagnostic aids, vaccines and the
controlled release of drugs. Through these pioneering efforts, the methods of
albumin microparticles preparation became well established. Consequently, the
focus of research then shifted towards the fine tuning of these methods for
application to individual drugs or drug classes and also towards the
characterization and optimization of the microparticles and identification of
various parameters that control microparticles characteristics.

_Crosslinking of albumin_

It has been reported that albumin microparticles were readily metabolized
in the body, and the extent of metabolism was dependent on the size of the
particles. Since the first reports of albumin being used in the preparation of
microparticles, many drugs and diagnostic agents have been successfully encapsulated and evaluated, thus proving albumin microparticles as a versatile tool for sustained release and targeting of drugs. Furthermore, the physicochemical characteristics of albumin can be easily adapted to accommodate different end users (Tomlinson & Burger 1987). The crosslinking of albumin microparticles has been achieved using either heat (Zolle et al. 1970) or chemical agents like formaldehyde (Arshady 1990), glutaraldehyde (Natsume et al. 1990) and 2, 3 butadione (Burger et al. 1985). Detailed studies have identified various parameters that can be controlled to obtain the required rates of release so that formulation of albumin microparticles has progressed from an art to a science (Sheu & Sokoloski 1986).

Thermal cross-linking of microparticles occurs at temperatures above 70°C, when intermolecular disulfide bridges are formed between free sulfhydryl groups on adjoining protein chains. In this respect, from a polymer science point of view, thermal denaturation of albumin may be considered a curing process and is often referred to in the literature as 'in-liquid curing process'. Gupta et al. (Gupta et al. 1986a; Gupta et al. 1986b) discussed various aspects of adriamycin loaded albumin microparticles prepared by thermal denaturation. They found that depending on the number of washings applied to remove the drug present on the surface, the microparticles exhibited kinetics of drug release that could be described by biexponential first order, bi-phasic zero-order and Higuchi's square-root of time equations. An inverse relation was found between the denaturation temperature and release rate leading to the conclusion that release could be
controlled through the denaturation temperature. Also, the degree of hydration was found to decrease with an increase in denaturation temperature.

Chemical cross-linking of albumin microparticles can be achieved at or below room temperature. When 2, 3-butadione is used for cross-linking, the droplet suspension is transferred into a separate container, the agent added, and the mixture gently stirred (Arshady 1990). When glutaraldehyde is used as the cross-linking agent, it is usually added to the suspension with continued stirring until the desired degree of cross-linking is achieved. Using glutaraldehyde, a variety of linkages may be formed, depending on the molecular (oligomeric) nature of the aldehyde. Formaldehyde is not commonly used for injectable microparticles due to the possibility of toxicity due to unreacted formaldehyde in the microparticles. Burger et al. (Burger et al. 1985) demonstrated that the incorporation of a highly water soluble drug resulted in highly porous albumin microparticles that showed a two fold increase in the degree of swelling compared to blank microparticles. Burgess et al. 1987 have reported in vitro studies demonstrating burst effects of up to 90% of the drug content from albumin microparticles containing water soluble drugs within the first 10 minutes.

Gallo et al. (Gallo et al. 1984) determined the effects of various formulation parameters on albumin microparticles. They found that an increase in denaturation period did not result in a significant change in mean particle size. According to them, a reduction in particle size with an increase in denaturation temperature occurs mainly due to the rate and extent of water evaporation and only partly due to increased cross-linking. There are several methods of
microparticles preparation including, water in-oil (w/o) emulsion, nebulization, coacervation, spray-drying etc. which uses either heat and or chemical agents for albumin cross linking.

One of the most common procedures by which microparticles are prepared is called the suspension crosslinking procedure which involves the formation of small droplets of an aqueous polymer in an immiscible liquid, followed by hardening of these droplets by cross-linking. Another method for preparing albumin microcapsules, multiple emulsion method was also evaluated (Shah et al. 1987). At relatively low temperatures albumin microparticles can be prepared using acetone-heat denaturation methods (Chen et al. 1994). This method is different from the traditional oil in water technique for preparation of albumin microparticles avoiding high temperatures and extended heating times for stabilization. A novel nebulization method (Green et al. 2004) can also be used for preparation of albumin microspheres. Spray drying is one of the latest methods used for preparation of microspheres (Haswani 2005).

In the current study we wanted to develop a microsphere formulation method using albumin as the microsphere matrix, chitosan as the mucoadhesive agent and glutaraldehyde as the crosslinking agent for microencapsulation of proteins, vaccines and amine containing drugs. The procedure involved should not alter the native structure or lose therapeutic activity of the drug candidate and should involve minimal number of processing steps. The method developed should be feasible for scale up and manufacture.
**Chitosan**

Chitosan is a deacetylated derivative of chitin and more specifically when chitin is 50% or more deacetylated, it becomes chitosan (Shepherd et al. 1997). Chitin or poly-β-(1-4)-N-acetyl-D-glucosamine is the second most abundant polysaccharide only next to cellulose, found in nature. It is present, for example, in the exoskeletons of crustaceans such as crab and shrimp. A large quantity of chitin is synthesized and degraded each year in the biosphere, but only negligible amounts are used by humans.

Previous studies have shown that chitosan has good biological adherence properties. In addition, it is a biocompatible compound, it has low toxicity, does not cause erythro-lysis, is biologically degradable by lysozymes and pepsin and its degradation products are also non-toxic. These properties make chitosan an excellent candidate for use in dosage forms such as controlled release drug delivery systems (Felt et al., 1998).

Drugs dispersed in chitosan were found to be released at a slow rate, thus highlighting its potential to produce a sustained-release matrix system. Chitosan has been used in several oral dosage forms, which include:

- Tablet delivery systems (including mini-tablets and buccal tablets) (Asane GS et al. 2011; Songsurang et al, 2011);
- Microspheres, beads, granules and microparticles (Vila et al. 2002);
- Chitosan gels (Säkkinen et al., 2002);
- Chitosan films (Ofori-Kwakye & Fell, 2003)
- Chitosan coated onto delivery devices (Janes et al., 2001).

Chitosan is a copolymer, consists of N-acetyl-D-glucosamine and D-glucosamine where the sugar backbone has a $\beta$-1,4-linkage (Figure 4). The structure is very similar to that of cellulose, except for the acetyl amino group at the C-2 position in place of a hydroxyl group.

![Chemical structure of chitosan (poly-$\beta$-(1-4)-N-acetyl-D-glucosamine)](image)

Figure 4: Chemical structure of chitosan (poly-$\beta$-(1-4)-N-acetyl-D-glucosamine)

Many different grades of chitosan are available and they differ in their degree of N-deacetylation (40-98%) and molecular weight (as high as 2 million Da). Chitosan is a weak base with a pKa value of approximately 6.5 and therefore insoluble at neutral and alkaline pH values. They are soluble in water, and their solubility is dependent upon the degree of deacetylation and the pH of the solution. Chitosans with low degrees of deacetylation ($\leq$ 40%) are more
soluble a neutral pH values, where chitosans with high degrees of deacetylation (≥ 85%) are only soluble up to pH 6.5. (Hejazi & Amiji, 2003).

The viscosity of the chitosan solutions are also dependent on its degree of deacetylation and molecular weight. The chitosan polymer has an extended conformation that is more flexible at higher degrees of deacetylation due to repulsion of the charge. Additionally, the presence of amine and hydroxyl groups allow for more intra- and inter-molecular hydrogen bonding, which also increases viscosity. Chitosan solution viscosity is directly related to concentration where higher concentration of polymer results in higher viscosity. Inversely, higher temperature will decrease the polymer solution viscosity.

**Bioadhesion and Mucoadhesion**

Bioadhesion can be described as the attachment of synthetic or biological macromolecules to the surface of biological tissues. For example, an adhesive bond may form with the epithelial cell layer, the continuous mucus layer or a combination of the two. The term “muco-adhesion” is used specifically when the bond involves the mucus coating and an adhesive polymeric device, while “cyto-adhesion” is used for cell-specific bioadhesion (Vasir et al., 2003).

Singh et al. (1997) have explained the mechanism of bioadhesion between mucin and muco-adhesive polymers as molecular attractive and repulsive forces. In the development of muco-adhesive drug delivery systems, the contact time at the site of absorption of the intended drug molecule is important for effective drug absorption. A muco-adhesive excipient in a
controlled-release dosage form will decrease the rate of clearance and movement of the drug from the mucosal epithelia, such as in the nasal cavity and gastrointestinal tract. This leads to a longer contact time with the absorptive epithelium with improved drug absorption (Shastri et al., 2010).

The good muco/bioadhesive properties of chitosan have been proven by various investigators. Most of these studies were performed with mucin solutions or in cell culture systems (Genta et al., 1997; Thongborisute et al., 2006). Better muco-adhesion was observed for higher-molecular weight chitosan (approximately 1400 kDa) compared to lower-molecular weight chitosan (500-800 kDa) (Dodane et al 1999).

Enteric coating polymers

Oral drug delivery systems with conventional dosage forms like tablets and capsules are the most patient friendly route of drug administration. But, the nature of drugs and their sensitivity towards the conditions in Gl-tract require very sophisticated drug formulations (Degussa 2003c). Many pharmaceutical dosage forms irritate the stomach due to their chemical properties. Others undergo chemical changes in gastric acid and through the action of enzymes, thus becoming less effective (Degussa 2003b).

There has been a tremendous increase in the number of drugs that require site-specific drug release in Gl-tract. Disease of the colon like Crohn's disease, ulcerative colitis and drugs for colon cancer demand a site-specific drug release in colon. Proteins and peptides, and drugs based on biotechnological
route are potential candidates for delivery to the intestine. Some drugs which are poorly soluble in the upper GI-tract need targeted delivery to the colon (Degussa 2003c).

Based on the current requirements, oral drug delivery systems are needed for drug targeting to specific parts of the intestine like colon targeting, for improving bioavailability and increasing solubility, patient friendly dosage forms and reduction in frequency. As we go through the gut, the pH of the GI tract increases gradually. The stomach pH is very acidic in the range of 1.0 – 1.5 but increase to 5.5 – 6.5 in duodenum. In jejunum it increases up to 6.0 – 7.0 and up to 6.5 – 7.0 in ileum. In the proximal part of the intestine, the pH rises up to 7.0 but the range is 5.5 – 7.0 depending on the type of food ingested.

There are several drug candidates that have a huge potential for making a large market in the pharmaceutical industry and need to have gastro resistance and delayed release properties for successful oral delivery. Insulin in case of type I diabetes, Calcitonin for Paget’s disease of bone and Epoetin for anemia are some of the examples of protein and peptide based drugs. There are also several non-peptide drugs for example Ibuprofen which is an anti-inflammatory agent, Pseudoephedrine a bronchodilator and 5-Fluorouracil and Doxorubicin which are anti-cancer drugs (Degussa 2003a).

For the formulation to be gastro resistant and specifically release the drug in the intestines, the unique environmental conditions of the intestines unlike the stomach should be considered and taken advantage. Enzymatic activity and pH,
in particular, have been used successfully as stimuli for intestinal specific polymer degradation for drug release. Several enzymes useful for polymeric degradation are found in the large intestine. With the right selection of the polymers, drugs can be targeted for release to a specific part of the intestine like proximal or distal part of the small intestine or the colon etc based on the pH conditions (Siewert et al. 2003).

In one of the earlier studies for oral delivery of insulin and vasopressin, a copolymer of styrene and hydroxymethacrylate as a membrane was used targeting the large intestine. This copolymer was found to be stable at the low pH found in the stomach, and it successfully protects the sensitive peptide drugs from the action of digestive enzymes. When the copolymer reaches the large intestine, however, the azo cross-links are reduced by bacterial enzymatic activity. This reduces the sheet-like membrane to a network of fibers between which diffusion, and thus drug release, may occur (Saffran et al. 1986).

In a 1993 study, Dressman and coworkers proposed using calcium pectinate as a matrix for drug delivery. Calcium pectinate is insoluble because of the cross-link like complex formed between Ca$^{2+}$ and the adjacent strands of pectin. It is stable until it reaches the large intestine, where bacterial polysaccharidase hydrolyzes the glycosidic linkages between monosaccharides. As the matrix degrades, the drug within it is made available to the body. Degradation may also be induced by changes in the pH (Siewert et al. 2003).
In one study, amoxicillin was delivered using hydroxypropyl methylcellulose acetate succinate (HPMCAS) as a matrix polymer. HPMCAS consists of cellulose backbones to which are attached methyl groups, hydroxypropyl succinate, and hydroxymethyl acetate. An advantage of HPMCAS is that the ratio of these side groups affects the extent to which the polymer becomes soluble in the intestine, which in turn determines the rate of drug release. Methyl ethers are unaffected by the high pH; the succinate and acetate groups, however, may be deprotonated. The rate of release is thus inversely related to the extent of methylation of the cellulose backbone. In the HPMCAS system, the rate of drug release varies directly with the drug to polymer ratio thereby demonstrating the control possible over drug delivery rates (Hilton & Deasy 1993).

Specific Eudragit acrylic polymers have been developed for peroral dosage forms with step-wise release of active ingredients in the digestive tract based on the pH conditions. The Eudragit - grades for enteric coatings are based on anionic polymers of methacrylic acid and methacrylates. They contain – COOH as a functional group. The films are insoluble below pH 5 and thus resistant to gastric fluid. By salt formation in the neutral to weakly alkaline medium of intestinal fluid, the films dissolve step-wise at pH values above 5.5 (Degussa 2003b).

Anionic Eudragit L and Eudragit S grades permit the development of pH-dependent systems to achieve linear release profiles or to balance pH-dependent drug solubility. As the coating thickness increases, the typical properties of the
polymer exert an ever-greater influence on the dosage form. Eudragit polymer systems are suitable for the manufacture of pellets, micro tablets, solid granules, compact crystals and various sustained-release dosage forms. If the active ingredient is to be released in dissolved form, this is usually affected by diffusion through polymer structures. With disintegrating dosage forms, release of the active ingredient is accelerated by an enlarged surface area. In the case of poorly soluble active ingredients, the release rate is frequently determined by the disintegration pattern of the dosage form.

If the active ingredient is coated by a largely pore-free, only slightly permeable membrane, its delayed release can be controlled very effectively. However, it is not the degree of permeability of the coating membrane alone which determines the drug diffusion; the solubility of the drug in the buffer solutions and its molecular weight or molar volume in solvated form also play a part. If the active ingredients are present as salts, much thought has to be given to their solubility as a function of pH and the variation of their properties depending on whether they are neutral molecules or ions.

Polymer films form diffusion cells. High concentrations of active ingredient in the core often lead to the formation of saturated solutions in these cells. Drug release then initially occurs linearly via zero-order kinetics, i.e. a constant amount of active ingredient is set free per unit of time. As the core is gradually depleted of active ingredient, the straight line changes to a curve that asymptotically approaches the final value of complete drug release. Each active ingredient and drug formulation requires a special release profile, which must be established by
optimization of the applied coating of a combination of Eudragit grades (Degussa 2003c).

Eudragit matrices provide dosage forms of good mechanical strength and control the diffusion of embedded active ingredients through pores and channels. Release of the active ingredients from matrix structures often occurs proportionally to $t^{1/2}$, i.e. an initial steep rise is followed by a gentler slope. In any case, it is the type and quantity of polymethacrylate used which dictates the release pattern of the final dosage form (Degussa 2003b).

Several studies have been conducted using different grades of Eudragit for drug delivery systems. In one study PLGA microparticles were stabilized using Eudragit and carboxymethylcellulose for oral delivery of vaccines. The formulations with stabilizers showed greater percentage amount of antigen protection compared to the formulations without stabilizers when incubated in a solution of pepsin at pH 1.2. They also found from in vivo studies that the approach has a good potential in increasing the efficacy of microparticulate systems for the oral administration of vaccines (Delgado et al. 2007).

Spray dried Eudragit microparticles were studied as the encapsulation devices for oral delivery of Vitamin-C as a candidate for associated therapy of the treatment of colorectal cancer (Cortesi et al., 2007; Esposito et al. 2002).

Ketoprofen loaded gastroresistant microparticles were prepared by spray drying using Eudragit S and L, cellulose acetate phthalate (CAP), cellulose
acetate trimellitate (CAT) and hydroxypropyl methyl cellulose phthalate (HPMCP) polymers. *In vitro* dissolution studies of the microspheres prepared using these polymers revealed that the acrylic polymers (Eudragit S and L) were more effective compared to the other polymers used (Bonacucina et al, 2006; Palmieri et al. 2002).

**Cyclodextrin**

Dextrins are polymeric carbohydrates formed during the hydrolysis of starch to sugars by heat, acids or enzymes. Dextrin and starch have the general formula, \([-\{C_x(H_2O)_y\}_n\] (\(y = x - 1\)), where in glucose units are joined to one another usually head-to-tail, but dextrin has a smaller and less complex molecule than starch. Dextrins have chemical properties which depend on the extent of starch from which they are derived. They are soluble in water. Some dextrin react with iodine to give a blue color and is soluble in 25% alcohol (called amylodextrin); others a reddish-brown color and soluble in 55% alcohol (called erythrodextrin); and still others yield no color at all with iodine and soluble in 70% alcohol (called achrodextrin). Dextrin forms a strongly adherent paste when mixed with water and it is used as adhesive in the manufacture of gummed tapes, textiles and paper. It is used in producing nutritional products.

Cyclic structure oligomers of glucose (called cyclodextrins) are obtained from the starch digests of bacteria. The individual glucose units are connected by 1,4 bonds. The most abundant cyclodextrins are alpha, beta and gamma cyclodextrin which have 6,7 and 8 glucose units respectively. The interior cavity
is hydrophobic and the outside of the molecule is hydrophilic. The enhanced of characteristics such as stability, aqueous solubility, and reduced volatility, can be modified through mainly propoxylolation reactions of them. Potential applications of cyclodextrins include water-soluble pharmaceuticals, prolonged drug release, tabletting and herbicides and pesticides.

β-cyclodextrins are naturally occurring polymers of cyclic oligosaccharide nature which are capable of inclusion complex formation between the drug and the polymer. β-cyclodextrin is an approved excipient and used in marketed oral drugs; it is non-immunogenic, non-toxic, exhibits high biocompatibility and has been previously studied for its property in forming a self-assembling sustained delivery carrier (Daoud-Mahammed et al. 2008). In this research we have used cyclodextrin as a polymer matrix to encapsulate insulin in its center and deliver it to the intestine which is then absorbed in a slow release fashion.

Figure 5: Cyclic oligomeric ring structure of β-cyclodextrin with of 7 glucose units.
Melanoma Cancer

Melanoma is one of the leading causes of mortality and its rate of incidence is progressively rising worldwide as people are exposed to increasing harmful UV rays which become more significant due to ozone depletion (Slaper et al., 1996). In the initial stages of melanoma, the tumor remains localized to the skin, however, with progression it spreads to the distant organs and causes secondary tumors. Once this tumor metastasizes, the prognosis for survival is as low as 15% with a median survival rate of about 6 months (Morton et al., 1992; Chowdhury et al., 1999). Melanoma accounts for only about 3% of skin cancer cases, but it is the cause of more than 75% skin cancer deaths. The current treatment approved by the FDA includes IFN-γ and Dacarbazine which needs supplementation with other treatment options like surgery and radiation. In an extensive study conducted by Mayo Clinic for determining the efficiency of chemotherapeutic agents, only 10 of 503 melanoma cancer patients achieved complete remission (Ahmann et al., 1989).

Malignant melanoma is not only one of the most aggressive and lethal type of neoplasm, but its incidence is also increasing at an alarming rate (Tsukamoto et al., 2000). It appears that the exposure to ultraviolet radiation (UV radiation) is a major, but not the only etiologic factor involved in the development of melanomas (Sober et al., 2007, Maclennan et al., 2003). Psychological, occupational, environmental, dietary, genetic or systemic factors may also play an important role in the development of melanoma (Damodaran et al., 2008, MacKie RM., 2010). A single unifying hypothesis for the etiology of melanoma...
remains to be determined. The early subtle signs of melanoma on the skin include tumor areas with irregular or angular border, and/or variegated color shades ranging from pink to white and also blue in some cases (Cabrera et al., 2009, Barnhill et al., 1991). The classic clinical signs of more advanced melanoma include alterations in the color of the tumor lesion, recent enlargement in its height or width, development of nodularity, pruritus, ulceration and bleeding.

Melanoma may spread by lymphatic channels or by blood to common sites like liver, lung, bone and brain. Various proposed mechanisms for the tumor cell invasion include mechanical pressure by rapidly growing cells, reduced adhesiveness of cells, cell motility, degradative enzyme production by cells, and several host factors that may promote or inhibit cell migration (Poon et al, 2011). Collagenase IV, a collagen degrading enzyme (a major structural protein of the basement membrane) often helps tumor cells to escape in and out of blood vessels.

Melanomas arise from melanocytes, the dendritic pigmented cells often found within the epithelial layer of skin. Melanocytes are specialized cells capable of synthesizing the pigment melanin in melanosomes using an enzyme called tyrosinase. The conversion of melanocytes into tumor cells often results in several biochemical and cellular changes. Tumor cells often show an increase in the number of melanosomes (DeVita et al., 1993). An important feature of metastatic melanoma cells is their ability to metastasize to distant organs other than the original tumor site.
The success of prophylactic vaccines against infectious diseases has increased interest of researchers to explore the feasibility of using vaccines against cancer. The John Wayne Cancer Institute (Santa Clara, CA) developed a vaccination regimen in the form of a living whole cell melanoma vaccine called CancerVax. This vaccine includes a mixture of irradiated melanoma associated antigens (MAA) derived from allogenic tumor cell lines to treat melanoma skin cancer (Morton et al 1996). Results from this vaccination study showed that the median survival rate of melanoma patients was significantly higher when compared to patients receiving other forms of treatment.

The induction of humoral antibodies and the development of cell mediated responses are directly related to the duration of survival (Morton et al, 1992). Cancer Vaccines, though attractive, are not very effective in inducing levels of immunity that can protect the individual from tumor growth. This is due to their inability to trigger the appropriate cells that initiate the primary immune response. To surmount this shortcoming, we devised an oral vaccine formulation which would target specific regions in the small intestine that are generally responsible for pathogenic entry and initiation of immune responses. Many established pharmaceutical companies have also shown similar interest in immunotherapy for cancer and currently 300 related products are in various phases of clinical trials (Doyle et al, 2010).

With this information in mind, an oral melanoma vaccine was attempted in this project to evaluate the feasibility and ability of polymeric microparticles as an oral delivery system.
Pneumococcal capsular polysaccharide antigens

Microparticles are actively investigated for their potential application in targeted delivery of therapeutics, antibodies and antigens. While, several investigators including our group have demonstrated the targeted delivery of chemotherapy agents to cancer cells \emph{in vivo} and immune-enhancement to vaccine antigens [1, 2], little is known on the cellular and molecular basis for the microparticle mediated therapeutic benefits. We propose to investigate the biological mechanism of microparticles with a polysaccharide antigen which, if successful will protect the individual against an infectious disease like pneumonia caused by the bacterial agent \emph{Streptococcus pneumoniae}.

\emph{Streptococcus pneumoniae} is a commensal bacteria found in the upper respiratory tract of most people. On the basis of polysaccharide capsular chemistry and serological reactions, 91 pneumococcal serotypes have been identified so far [3]. Most healthy children and adults carry multiple pneumococcal serotypes from time to time, and this is a major source for the horizontal spread of pneumococcus within the community. In fact, more than half of the children in industrialized countries are colonized with \emph{S. pneumoniae} at least once by the age of 1 year, and the carriage rate has been reported to be 2-3 times higher amongst children in developing countries. Pneumococcal colonization is mostly asymptomatic, and is often the primary step in the pathogenic route towards acute otitis media and invasive disease. Depending on the immune status of the host and/or other predisposing factors, including primary viral infections, pneumococcal carriage can lead to a wide range of
localized and systemic diseases such as otitis media, sinusitis, conjunctivitis, pneumonia, septicemia, and meningitis.

Two vaccines, a 7-valent conjugate vaccine (Prevnar™, PCV7, Wyeth, for children <5 years of age) and the 23-valent non-conjugated polysaccharide vaccine (Pneumovax™-23, Merck, for adults) are available to address the global pneumococcal disease burden. These vaccines are designed to elicit antibodies to capsular polysaccharides (Ps) of 7 or 23 Pnc serotypes respectively; however they are only effective in reducing invasive disease caused by those Pnc serotypes. Further, Ps in non-conjugated form are not immunogenic in children under 2 years of age [4] and do not confer a T-cell-dependent immune response [5]; to become immunogenic among infants, they require conjugation with a protein carrier, thereby complicating the production process and escalating the cost.

As it is very clear, the capsular pneumococcal polysaccharide antigens in their native form do not offer lasting immunity. In this research we hypothesize that the incorporation of these pneumococcal capsular polysaccharides into a particulate delivery system will result in a stronger and possibly T-dependent immune response by the host, thus leading to a more efficient vaccine.
Insulin

Diabetes mellitus is a chronic metabolic disease and is one of the primary causes of mortality in well developed countries (King et al. 1998). The current treatment options add a large burden to the health care systems in both direct as well as indirect costs (National Diabetes Fact sheet 2011). The major factors causing diabetes are either underproduction of insulin due to accelerated β-Langerhan cells destruction in the pancreas or the unresponsiveness of body cells to the synthesized insulin (Pillay and Makgoba, 1991; Hamilton-Shield, 2007). Hence, efficient glucose management becomes the ultimate goal of health providers for type 2 diabetes, where either insulin replacement or the administration of drugs that decrease the blood glucose level are the major approach to prevent the life-threatening complications of this disease. In case of Type 1 diabetes, the only other therapy is insulin replacement through surgical implantation of pancreatic islets cells which is not patient compliant owing to their side effects (Korsgren and Nilsson, 2009). Therefore providing an easy, patient compliant route for insulin becomes essential in the overall treatment of diabetic patients.

Pancreatic beta cells are responsible for the secretion of insulin, primarily, in response to an increase in blood glucose levels. The actions of insulin have been known for quite some time (Ip C et al 1976). Insulin is involved in membrane transport of glucose, amino acids and certain ions; increased storage of glycogen; formation of triglycerides; stimulation of DNA, RNA and protein
synthesis. It regulates glucose levels in the blood by suppressing its production by the liver and by directing it towards muscle and adipose tissue. Here, the glucose is converted into complex carbohydrates, protein and fat and subsequently stored. Insulin is currently used as a treatment strategy for diabetes mellitus (DM), which is characterized by hyperglycemia and altered metabolism of lipids, carbohydrates and proteins.

Patients suffering from diabetes mellitus can be clinically categorized as having insulin dependent diabetes mellitus (IDDM or type 1 diabetes) or non-insulin dependent diabetes mellitus (NIDDM or Type 2 diabetes). Nevertheless, all types of DM results from low circulating levels of insulin (insulin deficiency) and a decline in the response to insulin by the peripheral tissues (insulin resistance). In the treatment of type 1 diabetes, it is important to provide for constant, 24-hour-a-day basal insulin dose and also to mimic acute insulin release in response to food consumption. Insulin cannot be administered orally, because it undergoes extensive gastric degradation by stomach proteases and therefore only parenteral route is available for its administration. Currently, insulin is available only as a subcutaneous injection that causes pain and is not convenient.

Recently, alternative routes for insulin delivery like the pulmonary route have been investigated. Exubera was marketed by Pfizer (received FDA approval in January, 2006) which was an inhalation form of insulin and though it was very novel in its technique it suffered from high costs and patient compliance issues due to which it was recalled from the market a year later. Thus it is
imperative for researchers to look at delivery options which will not only be as effective as the subcutaneous form but also have enough cost effectiveness and patient compliance to substitute the currently administrated parenteral insulin preparations.

Oral delivery is the most popular method for drug delivery. However two major problems arise in oral delivery of protein molecules, the first is the inactivation of insulin by digestive enzymes in the gastro-intestinal system (mainly the stomach and the proximal regions of the small intestines). This can be overcome by designing carriers that can protect insulin from the harsh environment of the stomach before releasing it into the more favorable regions of the GIT. Additionally, addition of a protease inhibitor in the drug formulation may help to prevent insulin degradation by the proteolytic enzymes.

The other major barrier is the slow transport of insulin across the lining of the colon into the blood stream. An attempt to overcome this can be made by the use of absorption enhancers which facilitate transport of macromolecules across the GI tract. An effective carrier becomes one in which the release rates of drug must be significantly higher in neutral or basic conditions than in acidic conditions. One significant parameter in evaluating the viability of a particular microsphere vehicle for oral delivery is the ratio of the diffusion co-efficient of insulin in the carrier in both, the stomach (acidic environment) as well as in the intestine (neutral environment).
The oral route could provide an attractive option for insulin delivery and since it would provide a noninvasive means to administer sustained levels of insulin in a patient compliant extended release capsules. In recent years, the research involved in using sustained delivery systems for oral administration has increased due to advances in formulation technologies (e.g.: liposomes, microparticles, etc) and also due to expiration on existing patents for products. In this research, we have tested the efficacy of insulin after being incorporated into a microparticulate delivery system and administered orally.
CHAPTER 3

ORAL MICROPARTICULATE VACCINE FOR MELANOMA USING M-CELL TARGETING

Abstract

Purpose

Cancer vaccines are limited in their use, because of their inability to mount a robust anti-tumor immune response. Thus, targeting M-cells in the small intestine, which are responsible for entry of many pathogens, will be an attractive way to elicit a strong immune response towards particulate antigens. Therefore, in the present investigation, we demonstrated that efficient oral vaccination against melanoma antigens could be accomplished by incorporating the antigens in an albumin based microparticle with a ligand AAL (Aleuria Aurantia Lectin) targeted specifically to M-cells.

Methods

The microparticles containing the melanoma antigens were characterized for size, zeta potential and surface morphology. The total protein content in the antigens were evaluated using a Bio-rad DC protein assay. A 10 week immunization schedules was designed and melanoma-specific mice were selected and vaccinated. Blood samples were collected over the immunization time line to check the increase in antibody levels. Once the immunization was complete, the animals were injected with live melanoma tumor cells to check if the immunization could help protect the animals from tumor growth.
Results:

The oral microparticulate vaccine effectively protected the mice from subcutaneous challenge with tumor cells in prophylactic settings. The animals were vaccinated with antigen microparticles having a size range of around 1-1.25 µm where 1 prime and 4 booster doses were administered every 14 days over 10 weeks duration, followed by challenge with live tumor cells which showed complete tumor protection after oral vaccination. With the inclusion of ligand in the microparticles, we observed significantly higher IgG titers (1565 µg/ml) as compared to the microparticle formulations without AAL (872 µg/ml).

Conclusion

The results indicate that the delivery of melanoma antigens using a microparticulate delivery system with a targeting ligand helped to increase the immune response when given via the oral route. This data suggests that the inclusion of a targeting ligand in microparticles may have the potential to target antigens to M-cells for an efficient oral vaccination.
Introduction

Melanoma is one of the leading causes of mortality and its rate of incidence is progressively rising worldwide as people are exposed to increasing harmful UV rays which become more significant due to ozone depletion (Slaper et al, 1996). In the initial stages of melanoma, the tumor remains localized to the skin, however, with progression it spreads to the distant organs and causes secondary tumors. Once this tumor metastasizes, the prognosis for survival is as low as 15% with a median survival rate of about 6 months (Morton et al, 1992; Chowdhury et al, 1999). Melanoma accounts for only about 3% of skin cancer cases, but it is the cause of more than 75% skin cancer deaths. The current treatment approved by the FDA includes IFN-γ and Dacarbazine which needs supplementation with other treatment options like surgery and radiation. In an extensive study conducted by Mayo Clinic for determining the efficiency of chemotherapeutic agents, only 10 of 503 melanoma cancer patients achieved complete remission (Ahmann et al, 1989).

The success of prophylactic vaccines against infectious diseases has increased interest of researchers to explore the feasibility of using vaccines against cancer. The John Wayne Cancer Institute (Santa Clara, CA) developed a vaccination regimen in the form of a living whole cell melanoma vaccine called CancerVax. This vaccine includes a mixture of irradiated melanoma associated antigens (MAA) derived from allogenic tumor cell lines to treat melanoma skin cancer (Morton et al 1996). Results from this vaccination study showed that the median survival rate of melanoma patients was significantly higher when
compared to patients receiving other forms of treatment. This in turn has led to interest in the development of a vaccine which would trigger the immune system to stimulate the cytotoxic cells and inhibit the tumor growth. The induction of humoral antibodies and the development of cell mediated responses are directly related to the duration of survival (Morton et al, 1992). Cancer Vaccines, though attractive, are not very effective in inducing levels of immunity that can protect the individual from tumor growth. This is due to their inability to trigger the appropriate cells that initiate the primary immune response. To surmount this shortcoming, we devised an oral vaccine formulation which would target specific regions in the small intestine that are generally responsible for pathogenic entry and initiation of immune responses. Many established pharmaceutical companies have also shown similar interest in immunotherapy for cancer and currently 300 related products are in various phases of clinical trials (Doyle et al, 2010).

Oral vaccines are currently being investigated for their efficacy in stimulating the mucosal as well as systemic immunity. The mucosal route of entry and initiation of primary immune response is well established where pathogens and other invasive microbes enter the host system via regions in the small intestine. These regions are called the Peyer’s patches, and have pockets containing specialized lymphoid follicles which recognize and orchestrate the stimulation of the immune system. The Peyer’s patches usually comprise of microfold cells (M-cells) which can be used as a target site for antigenic entry. In this study, we use a ligand that will bind selectively to M-cells in the Peyer’s patches of the small intestine where the antigen of interest can be made
available for processing and presentation to the immune cells of the mucosal associated lymphoid tissue (MALT). The ligand *Aleuria aurantia lectin* (AAL) which has alpha fucose specificity (Franziska, et al 2005, Crennell et al, 1994) is structurally similar to neuraminidase factor. It is present in most pathogenic microorganisms and helps to enhance their entry into the M-cells. The microparticle formulation containing the antigen when associated with the ligand targets the M-cell. Thereafter, it is taken up preferentially and transported across the cell where it encounters the underlying antigen presenting cells (APCs) and other immunopotent cells (Bockman, D. E., et al 1973; Roth-Walter F., et al 2005) These antigens are processed and presented either through the endogenous (MHC Class I) or exogenous (MHC Class II) pathway (Kuby, 2000).

The microparticle delivery system is polymer based and behaves like a synthetic adjuvant system owing to the antigen presentation in a particulate form which is more immunogenic than its solution form (Bumgarner, PhD thesis 2002; Bramwell et al, 2005). This preferentially leads to an adjuvant-free formulation that can overcome some of the problems with adjuvant-associated vaccines. Some of the current adjuvants in the marketed vaccines have the potential to cause serious health and safety hazards to the vaccinated individual. One advantage of this microparticulate vaccine is the absence of alum (aluminum hydroxide gel) which has been shown to be associated with Alzheimer's disease (Campbell and Bondy 2000).

In order to design a successful vaccine, not only does the physical form and foreignness of the antigen play a role but also the manner in which the
antigen gets processed and presented to the immune cells. For this vaccination model, we used whole cell antigens obtained from S-91 cell line (Cloudman murine melanoma). Single target tumor antigen approach suffers from the limitations of both the chosen antigen and the MHC type of the patient (Jaffee E.M., 1999). Polyvalent whole cell based vaccine can overcome the complications by providing a wide coverage of potential tumor antigens (Copier et al, 2006). Thus we developed a microparticulate whole cell antigen vaccine which would provide a robust immune response towards all the potential melanoma tumor antigens.

Materials and Methods

Materials

Bovine Serum Albumin Fraction V [(BSA), Product Number 9048-46-8)], sterile deionized water and phosphate buffered saline (1 X PBS) pH 7.4 were purchased from Thermo Fisher (Waltham, MA). The ligand Aleuria Aurantia Lectin (AAL) was purchased from Vector Laboratories, INC, CA. Ethylcellulose (EC, Aquacoat ECD) and hydroxypropyl methylcellulose acetate succinate (HPMCAS, AQOAT) were purchased from FMC Biopolymers, PA. The ELISA kit specific for IgG antibodies was purchased from Bethyl Laboratories, TX. The BioRad DC (detergent compatible) protein assay kit was obtained from BIO-RAD, CA. The S-91 Cloudman murine melanoma cells were obtained from ATCC, VA. For in vitro cell culture, RPMI-1640, Fetal Bovine Serum (FBS), glutamine and penicillin streptomycin was purchased from Atlanta Biologicals, GA. For the in
vivo immunization studies, 4-6 weeks old female DBA-2/J mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA)

Equipment

The Buchi mini spray dryer (B-191) was purchased from Buchi Corporation (New Castle, DE). The Malvern Zetasizer Nano ZS (for particle surface potential and size analysis) was obtained from Malvern Instruments (Worcs, UK). The Biotek plate reader used for ELISA was obtained from Biotek Instruments, VT. The JEOL JSM 5800L scanning electron microscope (for surface morphology analysis) was obtained from JEOL USA (Peabody, MA).

Isolation of melanoma cancer antigens

The S-91 cell lines were grown in RPMI-1640 media supplemented with fetal bovine serum (10%) and glutamine (1%) as the growth supplement and Penicillin-Streptomycin (1%) as an antibiotic. The cells were grown in 75cm² tissue culture flasks in an incubator with atmosphere of 95% air and 5% CO2 at 37°C until they were confluent. The cells were washed with phosphate buffered saline PBS pH 7.4. The cell pellet was homogenized in a hypotonic buffer and centrifuged for 5 min at 1200 rpm to remove the cell debris. The supernatant was collected as the whole cell lysate (WCL) antigen and used for oral delivery.

Determination of total protein content in the melanoma vaccine:

The protein content in the antigen lysate was quantified using the Bio-Rad DC Protein Assay® according to the manufacturer's protocol. A pure albumin
standard was used to obtain the standard curve (Figure 6). The concentrated antigen lysate were diluted and the assay was performed in triplicate. The drug loading and subsequent dosing of the mice were based on this total protein content.

Formulation of the vaccine in albumin microparticles

Microparticles of the melanoma vaccine were prepared by a spray drying technique developed in our laboratory (U.S. patent, filed October 2009). The whole cell lysate antigen microparticles were prepared using a matrix in the ratio of 14:3:1 of albumin, Ethylcellulose (EC) and HPMCAS. Briefly, HPMCAS was dissolved in PBS pH 7.4 under continuous stirring. Separately, the albumin was solubilized in deionized water followed by the addition of EC (0.75% w/v). The HPMCAS solution was then added to the albumin-EC solution with stirring. The ligand AAL (0.25% w/w) was added to the aqueous polymer solution and finally the whole cell lysate was added to the polymer solution before spray drying using the Buchi Mini Spray dryer. The parameters used for spray dryer were optimized in our laboratory (Gayakwad et al 2009). In this spray drying process, the solution is passed through an atomizer to disperse the liquid in a controlled drop size spray. The small size of the drops - owing to the large surface area and a transient exposure to high temperature, dries up quickly to form the vaccine microparticles.
Characterization of the Particulate vaccine

*Scanning Electron Microscopy (SEM) of antigen microparticles:* The surface morphology, size and uniformity of the microparticles were determined by the JEOL scanning electron microscopy. The formulations were evenly spread on metal stubs and coated with gold and dried. These gold-coated microparticles were vacuum dried and then imaged under a nitrogen airflow stream.

*Particle Size Distribution:* The uptake of the vaccine particles into the microfold cells (M-cells) is determined by the size of the microparticles. Therefore, the size of the antigen-loaded microparticles was measured using laser diffraction particle sizer (Malvern Zetasizer ZEN1600). The microparticles were suspended in 2 ml of citrate buffer (pH 3.8) and evaluated for its size distribution.

*Determination of zeta potential:* Zeta potential is a physical property exhibited by any particle in suspension which measures its ability to remain a dispersed system without agglomeration. For antigen vaccine microparticles that are administered as an oral suspension using citrate buffer, the particles need to retain stability in this vehicle before it is delivered. If the microparticles aggregate in the suspension, it may lead to slower drug uptake. Hence, zeta potential of the particle needs to be studied to predict the vaccine stability during administration; and it is also seen that a particle with a positive surface charge tends to be preferentially taken up by the Peyer’s patch regions. The vaccine as well as blank formulations were suspended in deionized water and measured by the Malvern Zetasizer as described earlier (Bhowmik et al, 2011). A low
polydispersity index (PI) is indicative of a good particle diameter distribution range correlation.

*In vitro cytotoxicity study*

In this study, approximately 2 $\times$ $10^4$ of macrophage cells (RAW 246.7) were cultured in 96-well sterile NUNC plates. The murine macrophages were then exposed to 100 µl aliquots of the microparticle formulation in different concentrations ranging from 10-1000µg/ml. All batches of the antigen microparticles were sterilized under UV hood for 30 mins. After 12 hours of incubation at 37 °C, 20 µl of the Alamar blue dye (diluted 1:10 with 1X PBS) was added to evaluate the cytotoxicity of these microparticles. The plate was incubated for 4 hours (at 37°C in a humidified 5% CO₂ atmosphere) and then read at 490nm using a microplate reader (Bio Tek model No. ELx800). The alamarBlue® assay is based on the principle of oxidative/reductive reaction dynamics of a cell indicating its metabolic activity. Following continued growth of cells, the REDOX indicator in the dye changes from an oxidized (nonfluorescent, blue) form to a reduced (fluorescent, red) form. If the microparticle formulation is cytotoxic to the RAW cells, the dye will change its fluorescence which is read by the BioTek plate reader.

*In vivo evaluation of the particulate vaccine*

Determination of immune response after bi-weekly immunization: The immunogenicity of the microparticulate antigen and vaccine efficiency was evaluated using 4-6 weeks old female DBA-2/J mice obtained from Jackson
Laboratories (Bar Harbor, Maine, USA). Animal care and experimentation were conducted according to the protocol approved by Mercer University's Institutional Animal Care and Use Committee (IACUC). The mice were immunized over a 8-week vaccination regimen. The DBA mice were vaccinated by oral route where a blank microparticle group served as the experimental control. A specialized blunt feeding needle was used to administer the microparticles by oral gavage using Harvard apparatus (or via the oral route with a specialized blunt feeding needle). A prime dose of 250 µg of the antigen was administered with subsequent booster doses administered every alternate week upto 8 weeks. Serum samples were collected at weeks 0, 2, 4, 6, 8, 10. Blank microparticles were given to a group of mice as specificity control. A lectin control of oral microparticles without AAL was included to compare it with the oral group administered with AAL tagged microparticles.

**ELISA analysis of IgG antibody response:** The antibody titer evaluation was carried out in a 96-well NUNC microplate using enzyme linked immunosorbent assay (ELISA). An indirect ELISA was carried out where the plates were coated overnight with 0.625 mg/ml of the WCL antigen. The plates were then blocked for non-specific binding with 1% BSA. The serum samples containing the IgG were then incubated for 2 hours and further polyclonal anti-mouse; HRP-conjugated, IgG-specific, secondary antibody was added to it. The plates were then read at 570 nm for absorbance of color by the substrate. A standard curve was plotted using a purified mouse IgG at different concentrations to obtain a linear curve
and corresponding unknown antibody levels were calculated. Statistical analysis was performed by One-way ANOVA Tukey test using GraphPad Prism software.

**Oral vaccine efficacy and tumor-challenge study:** At the end of 10-weeks, once serum samples were collected and analyzed, the vaccinated mice were challenged with live S-91 melanoma cells. This study consisted of groups, namely the oral microparticle group (treatment) with AAL, oral microparticle group (treatment) without AAL and blank group. A group of non-vaccinated mice which served as the experimental untreated controls were also challenged with the same tumor cells in order to compare the tumorigenicity of the tumor cells. Specifically, $1 \times 10^6$ S-91 melanoma cells, were suspended and injected subcutaneously in the neck region or upper back of the mice (n=6 for each study group). Briefly, the skin was gently lifted and a 1.0 ml syringe with a 27-gauge needle, containing the tumor cell suspension in 100µl was inserted subcutaneously and administered. The study was continued until the average tumor volume in any of the groups reached 1000 mm$^3$. The tumor size was measured every alternate day with the aid of Vernier calipers beginning on day seven when the initial tumor development was observed. The tumor volume was calculated using the formula Tumor Volume = $\frac{1}{2} [L \times (W)^2]$ where length (L) is the longer diameter and the width (W) is the shorter diameter (Benencia et al, 2006).

**Statistical Analysis:** One-way ANOVA Tukey test was used to test the significance of differences between vaccinated and untreated group. Data were analyzed using GraphPad Prism software. A P-value <0.05 was considered significant.
Results

Determination of total protein content in the melanoma vaccine:

A 1% drug loading was used for the microparticle formulation and a dose of 25 mg of the microparticles containing the 250 μg of the antigen vaccine was administered to the mice on the basis of this quantification of vaccine antigen.

![Standard curve for protein quantification](image)

Figure 6: The total protein content of the melanoma antigens was estimated using a Biorad DC protein assay. The concentration after extrapolation of the absorbance to concentration was found to be around 27 mg/ml.
Product yield after spray drying

The product yield was calculated to determine the amount of microparticles obtained after the spray drying process. The percent yield was calculated as follows:

\[
\text{Product Yield} = \frac{\text{weight of microparticles (mg)}}{\text{weight of BSA (mg)} + \text{weight of Drug (mg)}} \times 100\%
\]

A yield of around 77% was obtained for the antigen loaded microparticle batches that were produced using the B-191 Buchi-mini spray dryer (Table 1).

Surface charge, size and morphological characterization of antigen microparticles:

The zeta potential of the microparticles was in the range of 5.75 ± 0.5 mV (Table 1). The zeta potential of the microparticles in citrate buffer (used for administration) provides valuable information with regard to its in-vivo stability after suspension.

The Malvern instrument showed a representative size of the microparticle formulation which was from 0.989 μm - 1.25 μm in diameter (Table 1). Earlier studies have indicated that microspheres between 1-5 μm are phagocytosed efficiently by antigen presenting cells, such as macrophages (Ahsan et al, 2002) and dendritic cells (Foged et al, 2005).
After preparation of the particles by the spray dryer, characterization of its morphological features such as shape, size and uniformity in size distribution were analyzed using scanning electron microscopy. This property is important to understand the integrity of the embedded drug when it is introduced \textit{in vivo}. Majority of the microparticles had a uniformly spherical surface morphology, with a small fraction exhibiting doughnut shape (Figure 7).

<table>
<thead>
<tr>
<th>In vitro characterization</th>
<th>Melanoma-antigen microparticles</th>
<th>Blank microparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product yield</td>
<td>77 ± 7.5%</td>
<td>82 ± 5%</td>
</tr>
<tr>
<td>Mean particle size(μm)</td>
<td>1.25</td>
<td>0.989</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>5.75 ± 0.5</td>
<td>5.02 ± 0.25</td>
</tr>
<tr>
<td>Total protein content</td>
<td>27 mg/ml</td>
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Table 1: \textit{In vitro} physical characterization representing microparticle melanoma antigen and blank formulations; mean values are reported with standard deviation for a sample size of n=3.
Figure 7: Surface Morphology of microparticles showed that the particles had a spherical surface with a size distribution of around $1.2 \pm 0.5 \mu m$. 
In vitro cytotoxicity study

In vitro cytotoxicity was performed to determine the possible toxicity of the microparticles when exposed to murine macrophage cell line. A positive control for maximum cytotoxicity and a negative control of only cells in media were used as experimental controls measured using the alamarBlue assay (Figure 8). It indicated that these antigen-loaded microparticles exert no acute toxicity in murine macrophages after 12 hours of incubation.

![Figure 8: In vitro cytotoxicity assay of melanoma loaded microparticles.](image)

Figure 8 represents the cytotoxic evaluation of the melanoma-antigen microparticles in an in vitro cell culture model. The data shows more than 80% cell viability measured as an average of n=3 (+ SE) after 24 hours of incubation with the microparticles. The microparticle formulations were added at doses of
0.05, 0.1, 0.25, 0.5 and 1 mg/mL in 0.2 mL of complete cell culture of media. Cells alone in media was used as the negative control whereas atropine sulphate (cytotoxic agent) as the positive control which resulted in complete cell death.

*In vivo antibody response study*

Our study suggested that the oral administration of melanoma WCL antigen microparticles was able to significantly increase the antibody titers in the mice for groups containing the lectin ligand in comparison to the other groups involved in the study. After 8 weeks of vaccination, a significant level of antibody response was seen in the oral melanoma group containing AAL (Figure 9). The serum IgG response of orally immunized mice with AAL ligand in the vaccine was compared with the oral group without AAL and showed a significant increase in serum IgG titer, starting from 6th week onwards ($p<0.05$). The figure shows the levels of IgG at weeks 0, 2, 4, 6, 8, 10 in the vaccinated v/s the control mice. The mice immunized with blank microparticles showed a rise in the levels of IgG as well, however, the levels were not as pronounced as the group containing the vaccine antigen.
Figure 9: Pre-bleed serum IgG titer levels as an average (+SE) in female DBA-2/J mice during vaccination (1 prime and 4 booster).

The IgG levels were significantly higher in the oral vaccination group with and without AAL than the blank microparticle and untreated groups after week 6 (*p<0.01). The animals dosed with blank microparticles as well as the untreated animals had basal antibody levels throughout the vaccination. The animals were administered 250 μg/mouse of vaccine antigen in each dose.
**Tumor challenge study**

Following the tumor challenge study, the group vaccinated with AAL associated microparticles showed the highest level of protection against tumor development (Figure 10), when monitored for 4 weeks after tumor induction. The control animals showed a palpable tumor development after approximately 7 days of injection with the live tumor cells.

![Mean tumor volume (mm³)](image)

*Figure 10: Mean Tumor volume (+ SD) measurement after vaccination for 10 weeks.*

DBA-2/J mice challenged with 1*10⁶ melanoma cells and the tumor growth was measured from the 7th day after challenge. All the study animals from the control group, oral blank and oral antigen without AAL developed tumors; however, the tumor volume in the oral antigen group was much smaller
than the control and untreated groups. The animals in the oral antigen group that received microparticles with the lectin AAL showed either no tumor or a palpable tumor during 4 weeks of observation. The oral microparticle group with and without AAL was able to protect the mice from tumor growth (* p<0.01) as seen during the measurement period compared to the untreated group which showed tumor starting from the first week which clearly exhibited the protection of the animals after vaccination. In the oral blank microparticle group the tumor growth over four weeks is slower than the untreated group possibly due to non-specific immune responses towards the albumin matrix.
Discussion

The major challenge in designing a successful vaccine is the delivery of antigens to the part of the immune system where maximum stimulation and proliferation of the professional immunopotent cells can be achieved. In a previous study with model antigens, it was shown that microparticles are capable of exhibiting potent serum antibody responses to entrapped antigens, following oral administration (O'Hagan et al, 1998). Further, lectins which are naturally occurring proteins with an affinity for sugar residues have been incorporated to bind specifically the surface receptors on microfold cells (M-cells) in the intestine (Clark et al, 2000) e.g. UEA coated liposomes target murine M-cells (Clark et al 2001, Chen et al 1996). One promising ligand is Aleuria Aurantia Lectin (AAL) which has an α-fucose specificity and shows structural similarity to neuraminidases which are expressed by most pathogens that enter the host via M-cells. The M-cells are specialized cells found in areas of the small intestine called the Peyer's patches that effectively bind, transport and deliver macromolecules and microorganisms to the cells of the underlying antigen presenting cells of the mucosal immune system (Bye et al, 1984).

We generated a microparticle-based oral vaccine containing the ligand AAL not only for proof of concept studies in murine animal model but also for achieving relevancy to human vaccination. When incorporated in a particulate biodegradable matrix, AAL targets these particles containing antigen to the M-cells (Peter et al, 2001). Further, the antigen is transported within the particle to the macrophages and dendritic cells (Banchereau et al 1998) underlying the
Peyer's patches to produce antigen specific serum IgG response and to protect the animals after injection with tumor cells for the post vaccination challenge study.

When formulating an oral vaccine, it is important to note that a successful immunization by an antigen often correlates to its uptake into inductive tissues, e.g. Peyer's patches in the small intestine (Brown et al, 1996). For the oral route these mucosal immuno-competent sites are populated with M-cells which are the main portals of entry for particulate antigens. In fact, a number of pathogens including Salmonella and reovirus (Wu et al, 2001; Weinstein et al, 1998; Wolf et al 1981) target these cells to gain access to the host system. Thus the targeting of antigens to M-cells can be an effective method for enhancing immunity.

Previously, we carried out optimization studies with different M-cell specific ligands to study preferential microparticle uptake in an in vitro M-cell model (Janet et al, 2010). Higher particle trafficking into M-cells was observed in the presence of the AAL ligand. This directed us to incorporate AAL into our microparticulate formulation and carry out an in vivo test to study increase in uptake efficiency of melanoma antigen in the overall development of an improved cancer therapy. In literature, very few studies have been reported where AAL was used as the targeting agent to deliver cancer antigens selectively to the M-cells and enhance the immune response. As hypothesized, the in vivo data suggested that we could deliver the antigens more selectively to M-cells since the AAL experimental group showed a higher serum antibody levels as compared to the group that did not receive AAL in the formulation. This study
confirms our previous findings that AAL ligand increases M-cell uptake which can be used to target antigens when designing marketed oral vaccines.

Antigen presentation is a critical feature of any immunization strategy and the key lies in manipulation of the way these antigens can be presented (Kuby, 2000). The antigen is often degraded and or can cause oral tolerance when given in the solution form. Hence we employed our spray drying technique to incorporate antigen and lectin into the matrix of the microparticles. The size obtained for the vaccine particles is in the range of 0.989-1.25 μm which is within the optimal size for phagocytosis (Tabata and Ikada 1988). In our laboratory we have seen that microparticles containing different drugs are readily taken up by phagocytic macrophages (D'Souza and DeSouza, 1995; D'Souza and Pourfarzib, 1999). Using this platform technology, we could administer antigens and other protein molecules for successful vaccination therapy (Uddin AN and D'Souza MJ, 2009). In comparison to soluble antigen which can develop oral tolerance, the microparticle delivery system ensures targeting of the antigen specifically to the M-cells avoiding enterocyte uptake and bypassing T-cell clonal anergy (Malik, et al, 2010). Furthermore, we have made major advances in the formulation technology, allowing us to produce microparticles using the spray dryer in a "one-step process". We addressed high production costs and scale-up issues for manufacturing of vaccine on a commercial basis by employing a continuous, organic solvent-free formulation technique. This method avoids toxicity issues as well as prevents the loss of targeting ligand from the microparticle surface.
Chitosan was used as a cationic polymer which imparted a positive surface charge to the microparticles which was used to our advantage (Genta et al 1999). The particles remained in contact with the negative mucosal lining of the intestine for a prolonged period of time. We also incorporated excipients like ethylcellulose and HPMCAS as enteric coating polymer to protect the particles from the acidic environment of the stomach. The excipients used in the formulation are non-toxic and generally regarded as safe (GRAS) accepted excipients.

To test the inherent toxicity of the microparticles we carried out a 12 hr cytotoxicity assay using alamarBlue assay. The viability of RAW 246.7 cells after incubation with antigen loaded microparticles was reported by comparing with cells alone which should be completely viable. There was no significant difference observed between cell viability when compared amongst different concentrations of microparticle formulations. All batches of microparticles showed greater than 80% viability after 12 hours of incubation measured using the alamarBlue Redox assay. The above results gave us a good understanding of the optimum concentration of microparticles that would not cause any cytotoxic effect after oral delivery. Taking into consideration the gastric milieu, our microparticles around the concentration range of 2-5 mg/animal would be sufficiently diluted in the gastro-intestinal tract to remain non-cytotoxic.

Vaccines are frequently used for protection against viral, bacterial and parasitic infections. We used a homogenized mixture of the whole cell lysate of S-91 tumor cells which contain melanoma specific antigens. Previously, in our
laboratory, the extracellular antigens (ECA) from B16 melanoma cells were studied for immune response in mice models (Yin et al, 2007). We extended this melanoma study to a different melanoma cell line, the Cloudman S-91 cell line which is a superficial solid tumor. The S-91 cells were thus studied for its similar response in immunogenicity and tumor protection as the B-16 when the antigen is administered as an oral particulate vaccine. Also, this cell line has been studied for its translatability to human melanoma tumor models (Peter et al, 2001).

To study the efficacy of vaccine, the mice were challenged with the live S-91 tumor cells after completion of immunization regimen. The oral vaccine microparticles with AAL gave protection to the animals during the 4 weeks after challenge. A significant difference was observed in the animals vaccinated with the antigen as compared to the untreated animals. The blank microparticles showed a higher degree of protection as compared to the untreated animals which can be attributed to the immune response against bovine serum albumin. As previously observed in our laboratory, bovine serum albumin microparticles were shown to elicit non-specific immunogenicity (Bumgarner, PhD dissertation, 2002). It has also been established that induction of the immune system depends upon antigen reaching and being available in lymphoid organs (Zinkernagel et al., 1997) which was achieved by formulating an M-cell targeted delivery system accessible to the mesenteric lymph nodes which utilizes the Peyer's patches. Also the use of a protein biodegradable adjuvant such as albumin in this antigen formulation helps to increase uptake into phagocytic
macrophages and other antigen presenting cells that are available underlying the follicle-associated epithelium of the Peyer's patches in the small intestine. The vaccine delivery by the formulation demonstrated in the present investigation here opens up new avenues for vaccine administration with the use of more patient convenient routes for cancer therapy. The site-specific targeting with a ligand allowed for avoidance of a synthetic adjuvant to boost immune response.
CHAPTER 4

CHARACTERIZATION OF A MICROPARTICLE FORMULATION FOR A PNEUMOCOCCAL CAPSULAR POLYSACCHARIDE ANTIGEN USING A PROTEIN MATRIX DELIVERY SYSTEM

Abstract

Purpose

The objective of this research was to enhance the immune response of a capsular polysaccharide antigen using a microparticulate formulation towards an infectious bacterial agent like *Streptococcus pneumoniae*.

Methods

In this study, microparticles were used as a delivery carrier to administer a polysaccharide vaccine for pneumonia. Several serotypes of the polysaccharide antigen (including 6B, 19F and 23F) were chosen for the immunization study, each of which was formulated separately into a microparticle. The Luminex bioassay was used as a quantification tool for the polysaccharides to determine the encapsulation efficiency. *In vivo* immunogenicity studies were performed where the capsular polysaccharide antigen in the microparticles were administered sub-cutaneously as the vaccine. Bleeds were done and samples were analyzed for circulating IgG to the specific polysaccharides antigens. We also wanted to understand how the immune system generates the response and
if there is any enhancement in the functional antibody levels after immunization with the microparticles. So, after immunization, we challenged the animals with live strains of the bacteria and 3-5 days later, nasal wash samples were collected and the bacterial colonization in the nasal tract was measured.

**Results**

We obtained a formulation with an average size of around 2.5 μm in diameter having a zeta potential of 6.05 ± 1.56 mV. After quantifying the polysaccharide antigens to have a bioactive content of around 80%, we used this as our dosing correction and immunized different sets of animals to check the immune response after injection of these polysaccharides in a protein-based microparticle. All our studies showed a consistent increase in the immune response obtained from polysaccharides in the microparticles as compared to the solution form of the polysaccharides. The functional antibody levels were also higher in the groups that received the microparticle form of the polysaccharide antigen. After a nasal challenge with one serotype of live bacteria, we saw a higher protection in the study group that had been immunized with the microparticles as compared to the solution group.

**Conclusion**

These studies confirm our hypothesis of using a protein-based microparticulate delivery system to increase the immunogenicity of a polysaccharide antigen. We can further check if these can be used as a replacement for chemical – conjugation of polysaccharides.
Introduction

*Streptococcus pneumoniae* is a commensal bacteria found in the upper respiratory tract of most humans. When infected with a virulent form of the bacteria, the individual's lungs fill with pus and fluid accumulation which makes it difficult for the transfer of oxygen in the respiratory tract. This is a disease that can be treated but if neglected can be life threatening (Lee C.J., 1987). There are currently 3 pneumonia vaccines i.e. a 7-valent conjugate vaccine Prevnar™ (PCV7, Wyeth, for children <5 years of age), the 13-valent conjugate vaccine Prevnar13 (PCV13, Wyeth, for children <5 years of age) and the 23-valent non-conjugated polysaccharide vaccine Pneumovax™-23 (Merck, for children >2 years and adults < 55 years of age); available to address the global pneumococcal disease burden. The pneumococcal capsular polysaccharide antigens in these vaccines do not have a very immunogenic structure, and thus, are not very effective in their native form (Flanagan M.P., et al 1999; O'Brien K.L., et al 2009). Also this vaccine does not confer a T-cell-dependent immune response; and to be immunogenic among infants, they require conjugation with a protein carrier (Seo J., et al 2002), thereby complicating the production process and escalating the cost.

Our hypothesis is to use a microparticulate vaccine for these polysaccharide antigens contained in a protein matrix that will help to potentiate its immune response after subcutaneous vaccination. Increasing the size of the Ps antigenic moiety by anchoring to an inert carrier and/or extended exposure (eg-sustained release from the matrix) to the specific lymphoid cells/organs may
augment T-dependent immune response (Flanary S., et al 2009). Pre-cross linked albumin microparticles have been shown to deliver antigens to targeted lymphoid tissues (Kwon Y.J., et al 2005). These microparticles are biodegradable and inert. The process to make these microparticles is a one-step spray drying process that gives a dry vaccine which is very stable and has shown to retain 80% of the polysaccharide's bioactivity. The current research looks at the enhanced immune response in mice immunized with subcutaneous pneumonia capsular polysaccharide antigen of different serotypes, namely 6B, 19F and 23F; delivered through the microparticulate delivery system and the effectiveness of challenging the immunized animals with live bacteria to check for functional protection from development of the disease.

Materials and Equipment

Murine Serum Albumin [(MSA), Product Number 9048-46-8)], sterile deionized water and phosphate buffered saline (PBS) pH 7.4 were purchased from Thermo Fisher (Waltham, MA). The pneumococcal capsular polysaccharide antigens were purchased from American Type Culture Collection (ATCC), VA. The Luminex assay plates were obtained from Millipore, MA. For \textit{in vitro} assays, human lymphocyte cells were obtained from Centers for Disease Control and Prevention, GA. (CDC) RPMI-1640, Fetal Bovine Serum (FBS), glutamine and penicillin streptomycin was purchased from Fisher Scientific, PA. For the \textit{in vivo} immunization studies, 4-6 weeks old female Swiss-webster mice were obtained from Charles River, MA.
The Buchi mini spray dryer (B-191) was purchased from Buchi Corporation (New Castle, DE). The Malvern Zetasizer Nano ZS (for particle surface potential and size analysis) was obtained from Malvern Instruments (Worcs, UK). The Luminex reader used for all Luminex assays was obtained from Luminexcorp, TX. The JEOL JSM 5800L scanning electron microscope (for surface morphology analysis) was obtained from JEOL USA (Peabody, MA). The *in vitro* viable cell counter was obtained from Beckman Coulter, Inc. CA.

**Methods**

*Formulation of polysaccharide antigens using albumin as the polymer*

The lyophilized pneumococcal polysaccharide antigen/s of interest was solubilized to make a stock solution of 2mg/ml using de-ionized water with overnight agitation. Microparticles containing the pneumococcal polysaccharide antigen/s were prepared by a spray drying technique developed in our laboratory (U.S. patent, filed October 2009). Briefly, the microparticles were prepared using a protein matrix where mouse serum albumin (MSA) was used as the polymer which was cross linked for 8 hours. The polysaccharide antigens were then added to the aqueous polymer solution before spray drying using the Buchi Mini Spray dryer. The parameters used for spray dryer were optimized in our laboratory (Gayakwad et al 2009). In this spray drying process, the solution is passed through an atomizer to disperse the liquid in a controlled drop size spray. The small size of the drops - owing to the large surface area and a transient exposure to high temperature, dries up quickly to form the antigen microparticles.
Content analysis

The microparticles containing the pneumococcal polysaccharide antigens were evaluated to determine the content of the incorporated antigen. A specific bioactive technique called the Luminex assay was used for the same. Briefly fluorescent beads were coated with the polysaccharide antigen of interest, followed by detection of the antigen recovered after extraction from the microparticles. The extraction process was carried out by using surfactants and proteinase K to break down the albumin and then determine the antigen content via LUMINEX analysis (Dunbar S., et al 2007; Pickering J.W., et al 2002).

Characterization of the Particulate vaccine

The uptake of the polysaccharide microparticles into immune cells largely depends on certain physical properties such as size, surface charge and morphology. We therefore determined the size and surface charge of these particles containing the antigen with the use of a Malvern instrument.

Particle Size Distribution. Uptake of the vaccine particles into antigen presenting cells (APCs) such as macrophages, dendritic cells, etc, is determined by the size of the microparticles. Therefore, the size of the antigen-loaded microparticles was measured using laser diffraction particle sizer (Malvern Zetasizer ZEN1600). The microparticles were suspended in 2 ml of citrate buffer (pH 3.8) and evaluated for its size distribution.
**Measurement of zeta potential.** Zeta potential is a physical property exhibited by any particle in suspension which measures its ability to remain a dispersed system without aggregating. For antigen vaccine microparticles that are administered as a suspension, it is important that they remain dispersed for the duration of administration. The antigen-loaded as well as blank microparticulate formulations were suspended in deionized water and measured by the Malvern Zetasizer as described earlier (Bhowmik et al, 2011). A low polydispersity index (PI) is indicative of a good particle diameter distribution range correlation.

**Scanning Electron Microscopy of polysaccharide antigen microparticles.** The surface morphology, size and uniformity of the microparticles were determined by the JEOL scanning electron microscopy. The formulations were evenly spread on metal stubs and coated with gold and dried. These gold-coated microparticles were vacuum dried and then imaged under a nitrogen airflow stream.

**In vivo evaluation of the particulate vaccine**

**Luminex analysis of polysaccharide specific IgG antibodies.** Serum samples were analyzed for circulating IgG to serotype specific polysaccharides using Luminex technique. Briefly, the luminex plates were pre-wet with 200 uL Phosphate buffer saline (PBS). The fluorescent luminex beads coated with 19F, 23F and 6B serotypes of polysaccharide antigens were added to the plates at 5000 beads/well/bead (19F+23F+6B). Before addition, the beads were removed.
from 4 deg and washed 2X with PBS. 25 uL of PBS containing beads were then added to each plate. To this, 25 uL of the undiluted serum samples were added to the wells. Simultaneously, 7 dilutions of an in-house standard antibody were added to the wells. This was incubated at 37 deg for 20 mins under shaking conditions. After this, the wells were washed 3X with PBS. A secondary anti-mouse antibody containing Phycoerythrin fluorescent dye was added to the samples. Another incubation for 20 minutes with shaking at 7 deg followed by 3X washing with PBS. Finally, 130 uL of Luminex sheath fluid was added and the plates were read in the reader for the 3 different bead regions.

Study I: Determination of immune response after a single dose of antigen.

The immunogenicity of the microparticulate polysaccharide antigen and vaccine efficiency was evaluated using 4-6 weeks old female Swiss-webster mice obtained from Charles River, MA. Animal care and experiments were conducted according to the protocol approved by the Centers for Disease Control and Prevention's Institutional Animal Care and Use Committee (IACUC). The mice were immunized over a 5-week vaccination regimen. The Swiss-webster mice quarantined for a period of 10 days before any experiments were initiated. The animals were immunized via the subcutaneous route where a blank microparticle group served as the experimental control. A 27-gauge needle was used to inject the microparticles. A prime dose of 50 µg of the antigen was administered with two booster doses administered every alternate week upto 5 weeks. Serum samples were collected at weeks 1, 3 and 5.
Study II: Immune response generated from combination of two polysaccharide antigens in solution versus microparticulate form. Swiss-Webster mice were used and 6 animals were allotted to each group and divided as as-1) naïve animals not receiving any immunization, 2) Combination of 19F and 23F polysaccharides in solution form, 3) Combination of 19F and 23F polysaccharides in microparticles form. A prime dose of 50 µg per polysaccharide per mouse was given followed by 2 boosters at intervals of 2 weeks. At the end of the study, a max bleed was taken after the animals euthanized and their serum was tested for polysaccharide specific IgG antibody immune response.

Study III: Comparison of immune response after immunization with microparticles of marketed Pneumovax® with its solution form. For this study, Swiss-Webster mice were used in groups of 10 animals per group and divided into 4 groups as-1) naïve animals not receiving any immunization, 2) Pneumovax® given in its marketed solution form, 3) Pneumovax® in microparticles and 4) Blank microparticles for background immune response. A prime dose of 5 µg per polysaccharide per mouse was given followed by 2 boosters at intervals of 2 weeks. At the end of the study, a max bleed was taken after the animals euthanized and their serum was tested for polysaccharide specific IgG antibody immune response.

Study IV: Mechanistic Studies for functional antibodies. Functional antibodies will be measured by the opsonophagocytic killing assay (OPK) (Romero-Steiner S., et al 2006). This is a functional assay to detect the level of antibodies that will be effective in killing the pneumococcal bacteria. The serum
samples (10μL) of the vaccinated and control groups are incubated with the 20 μL of the specific bacterial strain (same as the vaccinated antigen polysaccharide used) having the complementary polysaccharide antigen to begin the pre-opsonization process. Following this, 10 μL of baby rabbit complement will be added to the mix. Following this, phagocytic neutrophils cells differentiated from HL-60 leukocytes will be added at a 400:1 ratio of effector neutrophil cells to target bacterial cells. On completion of incubation, bacterial cocktail on Todd Hewitt with 0.5% yeast extract plates @ 37°C overnight and bacterial density enumerated. Percentage killing of bacteria with the serum is used to calculate the OPK titer.

Study V: Vaccine efficacy and nasal-challenge study. A week after the last booster, at the end of the immunization with 19F polysaccharide antigens, the vaccinated animals were intranasally challenged with live bacterial strain of *S. pneumoniae*. This study consisted of 3 groups, namely the microparticle group (treatment) containing the pneumococcal polysaccharide antigen 6B, the solution group containing the pneumococcal polysaccharide antigen 6B and blank microparticle group. A group of non-vaccinated mice which served as the untreated control were also challenged with the same bacteria. Three to five days post-challenge, each mouse will be euthanized and the nasal wash will be enumerated for *S. pneumoniae*.

Statistical Analysis: One-way ANOVA Tukey test was used and a P-value <0.05 was considered significant.
Results

In vitro characterization of the polysaccharide antigens in microparticles

The product yield was calculated to determine the amount of microparticles obtained after the spray drying process. A yield of around 81% was obtained for the antigen loaded microparticle batches that were produced using the B-191 Buchi-mini spray dryer.

The zeta potential of the microparticles was in the range of 5.75 ± 0.5 mV. The zeta potential of the microparticles in citrate buffer (used for administration) provides valuable information with regard to its in-vivo stability after suspension.

The Malvern instrument shows a representative size analysis of the microparticle formulation which was from 0.989 μm -1.25 μm in diameter. Earlier studies have indicated that microspheres between 1-5 μm are phagocytosed efficiently by antigen presenting cells, such as macrophages (Ahsan et al, 2002) and dendritic cells (Foged et al, 2005). After preparation of the particles by the spray dryer, characterization of its morphological features such as shape, size and uniformity in size distribution were analyzed using scanning electron microscopy. This property is important to understand the integrity of the embedded drug when it is introduced in vivo. Majority of the microparticles had a uniformly spherical surface morphology, with a small fraction exhibiting doughnut shape (Figure 11).
Figure 11: SEM image of the polysaccharide antigen when encapsulated in a cross linked albumin and was found to be variable from 1 μM to 2.5 μM
<table>
<thead>
<tr>
<th><strong>In vitro characterization</strong></th>
<th><strong>Blank MPs</strong></th>
<th><strong>Vaccine MPSs</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Product yield</td>
<td>81 + 7.5%</td>
<td>82 + 5%</td>
</tr>
<tr>
<td>Size (μM)</td>
<td>3.96 + 0.34</td>
<td>2.03 + 0.32</td>
</tr>
<tr>
<td>Zeta Potential (mV)</td>
<td>5.54 + 2.89</td>
<td>6.10 + 3.35</td>
</tr>
<tr>
<td>Content (Bioactive)</td>
<td>-</td>
<td>80%</td>
</tr>
</tbody>
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Table 2: *In vitro* characterization of the polysaccharide antigen microparticles after spray drying.

Size of the particles matched that of the SEM images whereas the particles had a slightly positive charge due to presence of excipients. The content analysis which was carried out using a bioactive Luminex assay was 80% of the initial loaded antigen. Mean values are reported with standard deviation for a sample size of n=3.
In vivo antibody response study

Study I: Immune response after a single dose of 19F polysaccharide antigen.

Figure 12: The antibody response after a single dose of 19F polysaccharide injection.

The immune response in the microparticle group showed a higher level of stimulation after a single dose of subcutaneous administration. The polysaccharide antigen in solution was significantly less than the microparticle group (*p<0.01).
Study II: Immune response from combination of 19F and 23F polysaccharide antigens.

![Graph showing immune response to polysaccharide antigens 19F and 23F](image)

**Figure 13:** Immune response after administration of polysaccharide antigens 19F and 23F.

Combination of two polysaccharides 19F and 23F were administered subcutaneously. Two formulation were tested - combination of polysaccharides in solution form (CSoln) and combination of polysaccharides in microparticles (CMPs). 19F and 23F specific serum IgG levels were analyzed. The animals vaccinated with the microparticles of the polysaccharide antigens showed a higher immune response than those that were administered the solution form of the antigens.
Study III: Immune response after immunization with microparticles of marketed Pneumovax®.

Figure 14: Immune response after administration of Pneumovax containing the polysaccharide antigens.

The immune response to Pneumovax from the animals that were administered microparticles showed a higher response in polysaccharide 19F but did not have any effect on 6B and 23F polysaccharides after one prime and two booster doses.
Study IV: Mechanistic study for functional antibodies

Figure 15: Schematic of the opsonization assay that helps detect levels of functional antibodies involved in killing the live bacteria.
Along with a good immune response, we expect the generation of functional antibodies that will be responsible in invoking effector cells of the body to kill the live bacteria. For this purpose, we looked at the effect of opsonizing bacteria present in the serum samples. What we saw (Figure 16), is the presence of functional antibodies that lead to selective killing of the bacteria. The animals injected with microparticles of the polysaccharide antigen showed a lower number of viable bacteria after the incubation with serum samples.
Study V: Bacterial nasal challenge study

In order to determine the effect of the immunization of the polysaccharide antigens in the microparticulate formulations, a challenge study was performed after the end of immunization period was complete. Live strains of Streptococcus pneumoniae bacteria containing capsular polysaccharides with serotypes 19F were challenged by an intranasal administration. The animals were allowed to inoculate the bacterial strains for 3-5 days in their nasal tract. They were then euthanized and the nasal wash samples were collected and analyzed for presence of pneumonia bacterial colonies (Figure 17).

![Reduction in bacterial growth after challenge with S. pneumoniae](image)

Figure 17: Nasal challenge study after infection with live bacterial strains. The polysaccharide 6B in solution form was able to reduce bacterial colonization only by 60% whereas the microparticle immunized group showed a 95% reduction in bacterial colonization.
Discussion

The physico-chemical characterization studies has provided us with invaluable information on the formulation factors and conditions that need to be manipulated to obtain protein-polymer based microparticles with specific size, shape and zeta potential. With this information, we were able to successfully customize microparticles to specific antigens and achieve their delivery after subcutaneous administration. The IgG specific immune response after the pilot study run where one dose of antigen was given and the generation of immune response tracked over a duration of 10 days, helped us assess the best fit formulation for the particular antigen in a protein matrix.

The next series of experiments helped us to understand different aspects of how the microparticles can enhance the polysaccharide response when the antigen could be localized to the lymph nodes and spleen after subcutaneous administration. (Per Artursson P., et al 2006). The immunogenicity studies provided important information on the rate and efficiency of immune response with microparticulate antigens. Further, mechanistic studies were carried out and indicated the progress of immune-response in mice in terms of affinity maturation and functionalization of the antibodies. We saw, as expected, higher opsonophagocytic activity levels in animals immunized with microparticulate polysaccharide antigens. With these immunization studies, we can expect to obtain a high antibody response which has significant increase in functional antibodies that can help us protect the animals after a possible infection by the bacteria. These results will also demonstrate whether survival after vaccination
correlates with certain T-cell responses which will be analyzed by flow cytometric analysis. It will be interesting to examine the pneumococcal polysaccharide antigen primed cells for Th2 specific immune response. Successful T-dependant immune response to pneumococcal Ps will be vital for cost-effective customization of multivalent polysaccharide vaccines.

Once the animals were immunized, a challenge study was carried out where the animals were intranasally administered a dose of live bacterial strain of \textit{S. pneumoniae} containing the polysaccharide specific capsules. We expect stronger memory responses in the particulate vaccinated group than the antigen alone which we saw by the decrease in bacterial growth in the nasal tract. We expect the microparticle to contribute their biological effect through adjuvant and depot effect. Based on the data we obtained and the inert nature of the matrix polymers, we do not expect strong co-stimulatory role to be played by the microparticulate polysaccharide antigens.

The use of microparticulate encapsulated delivery system is being actively pursued by many researchers for a variety of applications. Most of the research is skewed towards targeted therapeutics and immune-therapeutics. Hence, the success of this project has opened up a new avenue for the application of this unique technique. Polysaccharides without a protein carrier are poorly immunogenic. In children, they only stimulate T-independent immune response. This poses a stiff challenge in a disease caused by a multi-faceted pathogen such as \textit{S. pneumoniae}. By virtue of its capsular heterogeneity, 92 pneumococcal serotypes have been identified so far and although conjugating
the pneumococcal polysaccharides to a protein carrier assures a T-dependent immune response in children and protection as evidenced with PCV7, it involves high costs. But expanding the number of serotypes in the already available conjugate vaccine is difficult due to prohibitory costs and technicalities. Since our studies have successfully demonstrated a functional antibody immune response and protection after nasal challenge to the microparticulate pneumococcal polysaccharides, this can revolutionize the concept of multimeric vaccination.

While it assures immediate commercial benefits with the possibility of manufacturing T-dependent vaccine with relative ease, it offers flexibility to formulate different combination of capsular polysaccharide attenuated to geographical needs. Supported by this information, we can incorporate co-stimulatory cytokines into our formulation that can target dendritic cells (DCs) which have receptors for both IL-12 and IL-2 [17,18] and have the capacity to present exogenous antigens to both class I (cross-presentation) and class II pathways (Seo J., et al 2002), we expect that both T-cell subsets will be activated by vaccination.

The data gained in this study will be useful to further the possibility of an oral vaccination which will prove vital to increase patience compliance and coverage and in turn the herd immunity. This is highly relevant to infants and children who already have extremely busy immunization schedules. Based on the optimism generated with our studies, we are confident that the successful outcome of this project will help to initiate clinical scale application of a
microparticulate vaccine using these pneumococcal capsular polysaccharide antigens.
CHAPTER 5

DEVELOPMENT OF B-CYCLODEXTRIN BASED SUSTAINED RELEASE MICROPARTICLES FOR ORAL INSULIN DELIVERY

Abstract

Purpose

Polymeric microparticles have been previously demonstrated to deliver various therapeutic agents efficiently to targeted regions by protecting the drug from harsh gastric milieu of the gastrointestinal tract. Here we investigated the hypoglycemic effect of β-cyclodextrin polymeric insulin microparticles in diabetic rats via the oral route of administration.

Methods and results

Beta-cyclodextrin microparticles were prepared by a unique one-step spray drying technique and stabilized by incorporating enteric retardant polymers in the formulation. The insulin loaded microparticles had a mean size 0.8 ± 0.25 μm with a zeta potential of 3.57 ± 0.62 mV. As seen with the chromatographic analysis, the drug content in the microparticles was determined to be 94.9 ± 2.77%. The microparticles showed greater than 80% viability after 24 hours of incubation with RAW cells. For the in-vitro release study, the microparticles were able to protect the insulin in gastric fluid where no significant release was detected, followed by only 50% release in intestinal fluid for the first 8 hrs of the study. This was seen
to correlate with the *in-vivo* data where 50% glucose inhibition was seen after 8 hrs of oral administration in diabetic rats.

**Conclusion**

This data suggests that the insulin microparticles were able to reduce glucose levels in diabetic animals. These result help to motivate towards further research since having an oral route of insulin delivery would be very desirable due to its requirement for chronic use and cost-effectiveness.

**Introduction**

Diabetes mellitus is a chronic metabolic disease and is one of the primary causes of mortality in well developed countries (King et al. 1998). The current treatment options add a large burden to the health care systems in both direct as well as indirect costs (National Diabetes Fact sheet 2011). The major factors causing diabetes are either underproduction of insulin due to accelerated β-Langerhan cells destruction in the pancreas or the unresponsiveness of body cells to the synthesized insulin (Pillay and Makgoba, 1991; Hamilton-Shield, 2007). Hence, efficient glucose management becomes the ultimate goal of health providers for type II diabetes, where either insulin replacement or the administration of drugs that decrease the blood glucose level are the major approach to prevent the life-threatening complications of this disease. In case of Type I diabetes, the only other therapy is insulin replacement through surgical implantation of pancreatic islets cells which is not patient compliant owing to their
side effects (Korsgren and Nilsson, 2009). Therefore providing an easy, patient
compliant route for insulin becomes essential in the overall treatment of diabetic
patients.

Insulin is a peptide of around 6000 Da that is essential for glucose
utilization and is used for treatment of diabetes. Insulin being a peptide drug is
unstable and currently needs to be administered via the subcutaneous or
intramuscular route which can be extremely painful. Over the last few decades,
novel strategies towards developing alternate routes of insulin delivery
(Heinemann, 2010; Klonoff, 2003) and using modified insulin for treatment are
being investigated in clinical trials (Clement et al. 2004; Hinds et al. 2005;
Hoffman and Qadri, 2008; Krishnankutty et al. 2009). Amongst these, the
pulmonary and oral routes have gained most interest (Henkin, 2010; Ma et al.
2006; Geho et al. 2009; Klingler et al. 2009) in the pursuit of developing a novel
drug delivery system. Insulin being used to treat a chronic disease like diabetes
needs frequent administration and thus a more patient compliant, inexpensive
route like oral administration is preferred (Roques M., et al 1992). A major
challenge for oral delivery of protein drugs is attributed to several barriers such
as the acidic environment of the gastrointestinal tract (GIT), enzymatic
degradation and permeability issues through the intestinal mucosa (Fix, 1996;
Lee, 2002; Hamman et al. 2005). Thus we designed an oral delivery system that
will increase the bioavailability of oral insulin by protecting it from the
biological/intestinal milieu and also involving a one-step economical
manufacturing technique.
Our approach towards improving the delivery of insulin is based on the formulation of microparticles which will protect it from degradation by the proteases in the stomach as well as enhance its uptake into the intestinal lining. The particulate delivery carrier will; 1) include the insulin within itself so as to protect it from the proteolytic enzymes; and 2) help to deliver it to the small intestine as a particle that can be processed and taken up into the cells of the intestine. Also, from our previous studies, microparticles have shown a sustained release of drugs thus enhancing its overall efficacy (Vanbever et al. 1999). Over the years, microparticles have gained interest in their ability to deliver drugs in a site-specific manner. Particularly in our lab we have focused on the microparticulate delivery of various therapeutic small molecules as well as protein drugs (Jones et al. 2010; Uddin et al. 2009). Microparticles when formulated with enteric retardant polymers, improve bioavailability of the drug by improving its stability in the GIT (Delie and Blanco-Prieto, 2005). An ideal microparticle formulation should have reasonably high protein encapsulation efficiency, loading capacity and sustained release of the loaded protein with retained bioactivity (Ye et al. 2010; Jain, 2000). In order to achieve the following, we have used a β-cyclodextrin particle-based system as the matrix for the formation of insulin microparticles.

Microparticles composed of biopolymers are promising delivery systems for oral route of administration. The concept of polymeric carrier systems offers advantages of delivering drugs/antigens to a specific target site. Currently some of the biopolymers being investigated for Insulin delivery are methacrylate, poly
(esters) or cellulose based. However, some studies have shown that PLGA-based carrier systems exhibit a high burst effect of insulin after administration leading to undesirable hypoglycemic effects and harmful immune response owing to their uncontrolled insulin release pattern (Hinds et al. 2005). Thus a polymer which can release insulin in a controlled manner without appreciable initial burst would be considerable. β-cyclodextrins are naturally occurring polymers of cyclic oligosaccharide nature which are capable of inclusion complex formation between the drug and the polymer. β-cyclodextrin is an approved excipient and used in marketed oral drugs; it is non-immunogenic, non-toxic, exhibits high biocompatibility and has been previously studied for its property in forming a self assembling sustained delivery carrier (Timmy S.A., et al 2002; Sajeesh S., et al 2006; Daoud-Mahammed et al. 2008). Our unique patented (US Patent No.6, 555,110; Patent No.7, 105,158; Patent No.7, 425,543) one-step spray drying technique conferred a sustained release property to the β-cyclodextrin matrix. Spray drying has been used efficiently for making microparticles of insulin (Hamishehkar et al. 2010; Maltesen et al. 2008; Qian et al. 2009).
Materials and Methods

Materials

β-cyclodextrin [Cavamax® W7], ISP Technologies, Inc. CAS#7585-39-9, sterile deionized water was used in the formulation. Ethyl cellulose (EC, Aquacoat ECD) and hydroxypropyl methylcellulose acetate succinate (HPMCAS, AQUACOAT) were purchased from FMC Biopolymers, PA as enteric-retarding agents. The HPLC grade reagents acetonitrile, methanol and O-phosphoric acid were purchased from Sigma Aldrich. The RAW 264.7 cells (Cat. No. TIB-71) were obtained from ATCC, VA. For in vitro cell culture, RPMI-1640, Fetal Bovine Serum, Glutamine and Penicillin Streptomycin was purchased from Atlanta Biologicals, GA. Alamar Blue dye for cytotoxicity assay was purchased from Invitrogen. QuantiChrom™ Glucose Assay kit (DIGL-200) was obtained from BioAssay systems, CA. Size 9 capsules were obtained from Capsugel, NJ.

Apparatus

The Buchi mini spray dryer (B-191) was purchased from Buchi Corporation (New Castle, DE). Particle size distribution and zeta potential analysis was carried out using the Malvern Zetasizer Nano ZS, obtained from Malvern Instruments (Worcs, UK). Scanning electron microscopy images were obtained using a JEOL JSM 5800L microscope (for surface morphology analysis) from JEOL, USA (Peabody, MA). For HPLC analysis, a reverse phase Cyano column (UltraspHERE) was used to quantify the insulin. An USP Dissolution apparatus type I (basket apparatus) was obtained from Distek, Inc. NJ. Harvard apparatus for feeding capsules to rats was used.
Animals

Sprague-Dawley male rats weighing 100-150 g (4-6 week old) were obtained from Charles River Laboratories, Wilmington, MA, USA and housed in the Mercer university vivarium. All animals were quarantined for one week before studies commenced. The animal studies were conducted as per the protocol approved by Institutional Animal Care and Use Committee (IACUC) at Mercer University.

Preparation of Insulin Microparticles

Microparticles of the insulin were prepared by a spray drying process developed in our laboratory (U.S. patent No. 7,425,543; September 16th, 2008). The formulation comprised of a β-cyclodextrin matrix containing 1% of insulin. Briefly, HPMCAS was dissolved in phosphate buffered saline (PBS) pH 7.4 under continuous stirring. Separately, the β-cyclodextrin was solubilized in deionized water followed by the addition of ethyl cellulose (0.75% w/v). An aqueous solution of the β-cyclodextrin with ethyl cellulose and HPMCAS was made and the pH was adjusted to 3.1 using 0.1 M HCl followed by addition of the insulin and spray dried using the Buchi -191 mini spay dryer. The parameters used for spray dryer were optimized in our laboratory.

Determination of Particle Morphology and Zeta Potential

The surface morphology, size and uniformity of the microparticles were determined by scanning electron microscopy [SEM] (JEOL, JSM5200, and
Tokyo, Japan). The formulations were evenly spread on metal stubs and coated with gold and dried with the help of an ion-sputtering device. These gold-coated microparticles were vacuum dried and then imaged under a nitrogen airflow stream. The size of the microparticles was confirmed using Malvern Zetasizer (ZEN1600, Malvern Instrument Limited, UK). A low polydispersity index (PI) is a test for a good particle diameter distribution range correlation. The PI and particle size of microparticles were measured simultaneously by the M3-PALS method. To measure zeta potential, the insulin and blank β-cyclodextrin formulations were suspended in citrate buffer pH 4.0 at a concentration of 2 mg/ml and measured by the Malvern Zetasizer using the M3-PALS technology.

Chromatographic analysis of Insulin in β-cyclodextrin particles

To carry out the content analysis of Insulin in the formulation, the spray dried microparticles were crushed using a mortar and pestle method and then analyzed for total insulin content using an HPLC technique for insulin detection. Reverse phase HPLC analysis was carried out using a Cyano column which gave an insulin retention peak at 8 minutes. The degassed mobile phase consisted of 0.05 M potassium phosphate monobasic pH 2.4 (pH adjusted with 85% o-phosphoric acid) and acetonitrile in the ratio of 75:25. The injection volume was 100 µl, flow rate 0.5 ml/min and detection wavelength was 250 nm. A standard assay for Insulin measurement was carried out using concentrations ranging from 0.781 µg/ml to 500 µg/ml and 0.1 N HCl was used as the sample diluent. The insulin content from the microparticles was run in triplicates and
extrapolated using the standard curve. The developed HPLC assay was used for all further analysis of Insulin samples.

Cytotoxic analysis of Insulin formulation

In this study, approximately $2 \times 10^4$ of macrophage cells (RAW 264.7) were cultured in 96-well sterile NUNC plates. The macrophages were then exposed to 100 µL aliquots of the microparticle formulation in different concentrations ranging from 10-1000 µg/ml. All batches of insulin microparticles were sterilized under UV hood for 30 minutes. After 24 hours of incubation at 37°C, 20 µl of the Alamar blue staining dye (diluted 1:10 with 1X PBS) was added to evaluate the cytotoxicity of these microparticles. The plate was incubated for 4 hours (at 37°C in a humidified 5% CO₂ atmosphere) and then read at 490 nm using a microplate reader (Bio Tek model No. ELx800).

The alamarBlue® assay is based on the principle of oxidative/reductive reaction dynamics of a cell indicating its metabolic activity. Following continued growth of cells, the REDOX indicator in the dye changes from an oxidized (non-fluorescent, blue) form to a reduced (fluorescent, red) form. If the microparticle formulation is cytotoxic to the RAW cells, the dye will change its fluorescence which is read by the BioTek plate reader. (Jayanthan et al. 2011).
In vitro Insulin release at pH 1.2 and 6.8 from microparticles

The in vitro release studies of the insulin loaded microparticles were carried out at 37±0.1°C using simulated gastric fluid (SGF) pH 1.2 for the first 2 hrs followed by simulated intestinal fluid (SIF) pH 6.8 for the next 24 hours. A USP type I (basket) dissolution apparatus was used with 70 ml fluid volume. Insulin microparticles (25 mgs) were added to the basket and immersed in the dissolution vessel. The apparatus was then set to 100 rpm and samples were taken at predetermined time intervals. Sink conditions were maintained throughout the experiment. Each experiment was carried out in triplicate and the results were in agreement within ±5% standard error.

In vivo assay of glucose inhibition post-insulin oral administration

Insulin loaded β-cyclodextrin microparticles were used for in vivo oral studies. Diabetes was induced in Sprague-Dawley rats by injecting streptozotocin (55mg/kg bodyweight) dissolved in saline (0.9% NaCl solution in distilled water) intra-peritoneally and their blood glucose levels were checked after 72 hours. Animals with blood glucose level above 300 mg/dl were chosen for in vivo studies. Rats were divided into four study groups a) oral insulin microparticles, b) oral insulin microparticles in an enteric coated capsules (size 9), (c) oral blank microparticles (control); and d) subcutaneous insulin control. These rats were fasted overnight prior to the drug administration. Following the dosing, the blood glucose was monitored for a period of 12 hrs.
For analysis, Quantichrom Glucose assay (Schmidt, Hocherl et al. 2007) was used to develop a standard curve using different glucose concentration was made and the serum glucose concentration was calculated colorimetrically as

\[
\frac{OD_{\text{sample}} - OD_{\text{blank}}}{\text{slope}}
\]

Statistical analysis

One-way ANOVA Tukey test was used to test the significance of differences between transdermal, subcutaneous and untreated vaccine group. Data was analyzed using GraphPad Prism software. A P-value <0.05 was considered significant.

Results

Particle Morphology and Zeta Potential

As observed from the SEM studies and the Malvern Zetasizer, the size of insulin microparticles ranged from 0.5 -1.2 μm in diameter with a mean size of 0.8+ 0.25 μm (Table 3). Earlier studies have indicated that microparticles between 1-5 μm are taken up efficiently in specialized endothelial cells. Majority of the microparticles had a uniform surface morphology, exhibiting an irregular shape. Figure 18 shows a representative analysis for surface morphology of the microparticle formulation.
The zeta potential of the microparticles had a charge of $3.57 \pm 0.62$ mV when measured in citrate buffer. Zeta potential is the electro kinetic potential of a colloidal system and is the surface charge of a particle that helps in preventing flocculation during administration.

<table>
<thead>
<tr>
<th>In vitro parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (µm)</td>
<td>0.5-1.2 µm</td>
</tr>
<tr>
<td>Zeta Potential (mV)</td>
<td>$3.57 \pm 0.62$</td>
</tr>
<tr>
<td>Microparticle Yield</td>
<td>$67 \pm 4.5%$</td>
</tr>
<tr>
<td>Insulin Content</td>
<td>$94.9 \pm 2.77%$</td>
</tr>
</tbody>
</table>

Table 3: Physical parameters and characterization of the spray-dried β-cyclodextrin insulin microparticles
Figure 18: Scanning Electron microscope image of the cyclodextrin-insulin microparticles show non-spherical morphology with a size of ~ 0.5 μm-1.2 μm in size at 5.00 KX and 20.00 KX magnifications.
Content analysis of Insulin in β-cyclodextrin particles

β-cyclodextrin insulin microparticles, when prepared by the spray drying technique gave a 100% loading efficiency. The drug content in the microparticles determined as per our HPLC technique was 94.9 ± 2.77%. This content analysis was taken into consideration in dose calculation for the animals.

Figure 19: Retention peak of Insulin for detection of content from microparticles after spray drying.
Cytotoxicity of Insulin formulation

In vitro cytotoxicity was performed to determine the possible toxicity of the insulin microparticles when exposed to macrophages. The microparticles showed greater than 80% viability after 24 hours of incubation with Alamar Blue dye when evaluated in concentrations ranging from 0.05 to 1 mg/ml (Figure 20). A negative control of only cells in media and a positive control (showing 95% cytotoxicity) were used as experimental controls. It indicated that these insulin-loaded microparticles exert no acute toxicity in murine macrophages after 24 hours of incubation.
Figure 20: *In vitro* cell cytotoxicity of the insulin microparticles in different concentrations up to 1 mg/ml.

The cells exhibited more than 80% viability in the presence of the β-cyclodextrin insulin microparticles (n=3/group) after 24 hours of incubation at concentrations ranging from 0.05 – 1 mg/ml.
**In vitro Insulin release at pH 1.2 and 6.8 from microparticles**

In SGF pH 1.2, no significant release of insulin was detected over 2 hours. Once the media was changed to SIF pH 6.8, a sustained release was detected over a period of 26 hours where the release half-life was observed around 8 hours (Figure 21). The data represents an average value of 3 insulin microparticle samples. At 28 hours, 98% cumulative insulin release was seen from the microparticles.

![Insulin release profile](image)

**Figure 21:** *In vitro* dissolution study of insulin microparticles.

The insulin in Cyclodextrin nanoparticles showed a sustained release pattern with 50% of the drug being released at 8.2 hours.
Hypoglycemic effect following oral Insulin microparticles administration on fasted diabetic rats

The rats were administered 4 IU/kg/day of insulin in microparticles orally. Either the microparticles in suspension were given using an oral gavage needle or by inserting the microparticle-loaded capsule in the esophagus of rats using a capsule feeding needle. A subcutaneous control was included which received insulin solution at a dose of 0.4 IU/kg/day. The glucose inhibition profile after treatment with the β-cyclodextrin insulin microparticles was studied over a period of 12 hours, as shown in Fig 4.

Inhibition in blood glucose levels of 30% within the first two hours was observed in the oral microparticles given in suspension as compared to around 5% observed in the blank animal group. After 12 hours of the dose the rats that received oral microparticles in suspension showed a 77% glucose inhibition which was comparable to the subcutaneous control group. The insulin microparticles in the enteric coated capsules showed around 60% glucose inhibition (Figure 22).
Figure 22: *In vivo* biological effect of insulin microparticles on glucose levels in the blood.

The hypoglycemic effect observed following oral administration of β-cyclodextrin insulin microparticles in suspension and capsules, a control of blank oral microparticles and a subcutaneous treatment control. (Mean ± S.E, n=6). The dose of insulin administered was 4IU/kg/day in the oral groups. Statistically significant differences were observed from oral blank microparticles (p<0.05).
Discussion

The main goal of this research was to demonstrate the potential of using sustained release $\beta$-cyclodextrin microparticles to deliver insulin via the oral route. Providing a sustained release insulin formulation helps to reduce the frequency of dosing and adds more feasibility to treat chronic hyperglycemia. Numerous routes for innovative insulin delivery have been previously tried including the marketed Exubera where insulin is given as a dry powder for inhalation. This product was recalled due to its inability to attain enough patient compliance and due to the high cost of the device for administration of insulin (Heinemann, 2010). Thus, the oral delivery systems for drugs still remains the most cost-effective and patient compliant.

Oral delivery of insulin is limited by its low bioavailability and is normally insufficient to provide a sufficient therapeutic effect. So to overcome the challenges of an effective oral delivery system of insulin, we have proposed to use a microparticulate system employing a one-step spray drying process that is inexpensive and results in the formulation of solid microparticles which can be administered using a capsule. The highlight of our delivery system was the inclusion of $\beta$-cyclodextrin as the core matrix. Here, we described the physicochemical properties (size and zeta potential) of these $\beta$-cyclodextrin microparticles and how it influences the release pattern of the insulin using an HPLC analysis to detect the free insulin (Yilmaz B., et al 2010). In addition we provided information about the in vitro safety profile of these microparticles and
also the efficiency of these particles in glucose inhibition in vivo (Reis C.P., et al. 2007).

Particle size is a major factor in determining the uptake of insulin microparticles in the intestinal cells and thus getting access to the blood stream. The mechanism of insulin absorption from microparticles seems to be associated with insulin internalization, probably through vesicular structures in enterocytes and Peyer's patches (Sarmento et al. 2007). For this, particles ranging from 1-10 μm are ideal (Eldridge et al. 1989), and we have optimized our formulation to obtain a size of around 1 μm. For an oral delivery in the form of a suspension, it is desirable to have the particles with good suspendability. Zeta potential is a good indicator of the stability and aggregation of microparticles (Yan P., et al. 2002). Also, in a clinical setting, after the enteric coated capsules break down in the intestine, the microparticles should remain a homogenous suspension for efficient uptake into epithelial cells along the mucosal lining.

The modified spray drying technique that was used gave a microparticle yield of about 70-75% the initial weight. In this process, the solution is passed through an atomizer to disperse the liquid in a controlled drop size spray. The small size of the drops - owing to the large surface area and a transient exposure to high temperature, dries up quickly to form the insulin microparticles where insulin is incorporated into the β-cyclodextrin polymer matrix. The loading efficiency of the microparticles as determined by the content analysis was found to be significantly high thus exhibiting the integrity of the insulin through the spray
drying process. The excipients used in the formulation were biodegradable and biocompatible and no cytotoxicity was seen in *in-vitro* studies.

Cyclodextrins and their derivatives are known to address the issues of poor loading efficiency and affect the overall release pattern of drugs from its polymeric matrix (Sajeesh et al. 2010). As seen from the *in-vitro* release study, the absence of insulin in the dissolution medium for the first two hours (SGF) demonstrates the robustness of the microparticulate formulation in harsh conditions of GIT. The nearly complete release of insulin over the course of 26 hrs suggests that microparticles helped to stabilize the insulin. The release of insulin from microparticles showed a sustained trend where 50% of the drug was released around 8.2 hours. The transit time of a drug through the absorptive area of the gastrointestinal tract is between 9 to 12 hours. Thus we carried out the *in-vitro* release study over a period of 12 hrs where the first two hours were simulated to stomach conditions followed by a higher pH to represent the intestinal environment.

To understand the influence of β-cyclodextrin insulin microparticles, we tested the formulation in a diabetic rat model to monitor its bioactivity on glucose utilization in cells. On a molecular level, insulin promotes the energy dependent translocation of glucose transporters (Glut 4/Glut1) from the intracellular domain to the plasma membrane (Jerry N., et al 2001; Ito Y., et al 2007). Following insulin association with its transcellular receptor, the activation of the glucose transporter occurs resulting in maximum transport of glucose within the cell (Pessin and Saltiel, 2000; Petersen and Shulman 2002). The observed glucose
inhibition from the *in-vivo* study can be attributed to the enhanced uptake of the microparticles followed by an increased absorption of insulin. From the results, we observed an *in-vitro in-vivo* correlation where the animals showed a 50% glucose inhibition after 8 hrs of administration. A maximum effect of 80% glucose inhibition was observed with the insulin microparticles in the oral suspension group and was comparable to the glucose inhibition in the subcutaneous group. For the insulin microparticles in capsules, a lag phase, where no glucose inhibition occurred was observed possibly due to time taken for the dissolution of the capsule and the release of the insulin microparticles into the intestinal lumen. The blank microparticles did not show any significant glucose inhibition following its administration.
CHAPTER 6
SUMMARY AND CONCLUSION

This research has demonstrated the feasibility of using a microparticulate delivery system for both protein and polysaccharide drug candidates. We have seen efficacy in both in vitro as well as in vivo condition for each of the three research projects. The particulate delivery system can be very promising in terms of improving drug uptake, and thus enhancing efficacy at the site of action.

In the cancer study, the immunization with melanoma antigens in a microparticulate formulation was seen to confer immune recognition to the animals which lead to tumor protection capacity after challenge with live tumor cells. Antigen presentation is a critical feature of any immunization strategy and the key lies in manipulation of the way these antigens can be presented. Using the spray drying technology it is possible to incorporate multifunctional ligands for targeting which in our study helped increase uptake into immune cells after oral administration and thus generated a higher immune response.

Polysaccharide antigens without a protein carrier are poorly immunogenic. In our pneumococcal polysaccharide antigen studies, our hypothesis of using a protein-based microparticulate vaccine for the poorly immunogenic antigens did show a positive outcome where the immune response after vaccination was better than the polysaccharide antigens in solution form. In all the in vivo experiments, the groups receiving the microparticulate form of the antigen
showed a higher response and also showed functional antibody levels that were not as significant in the solution groups. Hence, the success of this project has opened up a new avenue for the application of this unique technique. Since our studies have successfully demonstrated a functional antibody immune response and protection after nasal challenge to the microparticulate pneumococcal polysaccharides, this can revolutionize the concept of independent vaccination.

For our oral insulin study, we wanted to demonstrate the feasibility of using the spray drying technique to achieve an oral formulation of insulin. Using the inexpensive, one-step spray drying process we could incorporate enteric coating polymers and other excipients that would result in a sustained release, stable formulation of insulin. Here, the β-cyclodextrin served as a very good polymer matrix which showed very promising in vitro as well as in vivo results. The hypoglycemic effects due to the insulin microparticles were significantly higher than those obtained from the blank microparticles and had comparable activity similar to the subcutaneous injection of insulin. This concept opens a large domain of perspectives for different drugs for enhancing their release and culminating into a sustained delivery formulation.
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