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The potential of polymeric nanoparticles for oral delivery of insulin

by

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With abundant blessings of ***"Maa Saraswati"***

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This dissertation is dedicated to my parents

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Abstract

The present dissertation was aimed at realizing the potential of polymeric nanoparticles for oral delivery of insulin. Different surfactants and stabilizers were screened for compatibility with insulin using circular dichroism (CD), fluorescence spectroscopy (FS) and ultraviolet visible spectroscopy (UV). Insulin stabilizer co-encapsulated nanoparticles were prepared using double emulsion-diffusion-evaporation technique and had an average size of 150 nm. Nanoparticles were characterized at different insulin loadings and effect of stabilizer type and concentration was also evaluated. Nanoparticles were imaged using transmission electron microscopy (TEM) for their morphology. Stability of insulin in nanoparticles was assessed using Fourier transform infra red spectroscopy (FTIR) whereas *in vitro* release/stability studies were carried out in simulated physiological fluids. To study the potential of nanoparticles in delivering insulin orally, pharmacokinetic studies were carried out in healthy rats at a dose of 20 IU/kg and the results revealed the ability of these polymeric carriers to enhance oral bioavailability of insulin 6 folds over simple insulin solution administered orally at the same dose. Tissue distribution analysis revealed an altered pattern of insulin distribution in various tissues with oral nanoparticles compared to plain insulin solution at similar dose of 20 IU/kg over 3 days. Moreover, the nature of stabilizer had no effect on pharmacokinetics of insulin nanoparticles. To further assess the efficacy of insulin nanoparticles in lowering plasma glucose levels and pathological markers of diabetic complication, pharmacodynamic studies were carried out in streptozotocin (STZ) induced diabetic rat model at three different doses of 20, 60 and 120 IU/kg where nanoparticles demonstrated dose dependent lowering of glucose levels in diabetic rats but glucose levels

were not normalized at 20 and 60 IU/kg doses. Results of the diabetic complication study were not very encouraging though fluctuations were observed in the levels of pathological markers, but results were statistically insignificant. Together, this data clearly indicates the more extensive work is required in terms of formulation development to come up with more realistic approach for insulin delivery which gives a more rapid and sufficient insulin release to counter and normalize hyperglycaemia thereby providing benefit in diabetes and associated complications.

Abbreviations

λ_{ex}: Excitation wavelength; λ_{em}: Emission wavelength; µg: microgram; µl: microlitre; AUC: Area under the curve; CD: Circular dichroism; C_{max}: Maximum plasma concentration; CRP: C-reactive protein; CSII:Continuous subcutaneous insulin infusion; Da: Daltons; DCCT: Diabetes control and complication trial; DMAB: Didoceyldimethylammonium bromide; FDA: Food and Drug administration; FS: Fluorescence spectroscopy; FTIR: Fourier transform infra-red spectroscopy; GALT: Gut associated lymphoid tissue; GLP: Glucagon like peptide; GLUT: Glucose transporter; HDL: High density lipoproteins; i.v.: Intravenous; IL-6: Interleukin-6; IU: International units; MC: Magnesium carbonate; MH: Magnesium hydroxide; mg: milligrams; ml: millilitres; NIDDK: National Institute of diabetes and digestive and kidney diseases; nm: nanometer; nmol: Nanomoles; NP: Nanoparticles; PDI: Polydispersity; PGL: Plasma glucose levels; PIL: Plasma insulin levels; PLGA: Poly(lactide-co-glycolide); PVA: Polyvinyl alcohol; s.c.: Subcutaneous; SD: Sprague Dawley; SD: Standard deviation; SEM: Standard error of mean; SGF: Simulated gastric fluid; SIF: Simulated intestinal fluid; STZ: Streptozotocin; RPM: Rotations per minute; TEM: Transmission electron microscopy; TJ: Tight junctions; TG: Triglycerides; T_{max}: Time to achieve maximum plasma concentration; TNF-α: Tumour necrosis factor-alpha; UN: United Nation; UV: Ultraviolet-visible spectroscopy; VitETPGS: Vitamin E tocopheryl polyethylene glycol 1000 succinate; WHO: World health organization; ZC: Zinc carbonate

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CHAPTER 1

Chapter 1: Introduction and literature review

1. Introduction

With the advent of biotechnology more therapeutic proteins and peptides are now available as drugs for treatment of vast majority of diseases like diabetes, cardiovascular complications, hypertension, cancer, haemophilia, anaemia and many more (Newmark, 1988; Brekke and Sandlie, 2003). Significant research efforts have been made investigating oral protein delivery because of the ease of administration, ability to avoid the pain of injections leading to higher patient compliance, for preventing development of resistance in long term therapy and elimination of side effects like infection and vascular necrosis caused by injections. The oral delivery of protein and peptide drugs has been a challenge primarily due to the enzymatic degradation of proteins inside gastrointestinal tract, first pass metabolism, chemical and biological instability, their high molecular weight leading to low permeability and impeding successful absorption across intestinal mucosa (Sood and Panchagnula, 2001; Wang, 1996; Still, 2002) as a consequence they currently administered as injections via subcutaneous or intravenous route.

Statistics reveal that out of top 100 drugs, 38 are proteins and peptides, however only one of them is available as an oral medication (Sinha and Kumria, 2003). The nature of delivery system by which these protein and peptide drugs are delivered has a significant effect on their efficacy. On the other hand, limited success with existing treatments of chronic diseases, has suggested a growing need for a multidisciplinary approach for the delivery of therapeutic proteins and peptides to targets tissues. This has lead to investigations on new and advanced drug delivery systems (ADDS) that

have ability to alter the pharmacokinetics, pharmacodynamics, minimise non-specific toxicity & immunogenicity, as well as ability to target the tissue/cell of interest. These new strategies are based on interdisciplinary approaches that combine polymer science & engineering, pharmaceutics, bio-conjugate chemistry, and molecular biology. One such promising approach for oral delivery of protein and peptide drugs is the intervention of nanotechnology in drug delivery where polymeric nanoparticles are of special interest due to their ability in loading and delivering proteins via oral route with modified pharmacokinetic and pharmacodynamic profile.

Nanoparticles are solid colloidal drug carriers ranging from 10 to 1000 nm in size and are composed of natural, semi synthetic or synthetic polymers that may or may not be biodegradable (Couvreur *et al*., 2002). The encapsulation of peptides and proteins in colloidal particles was shown to protect them against the harsh environment of the GI tract (Lowe and Temple, 1994), and enhance their transmucosal transport (Mathiowitz, 1997; Tobio *et al*., 1998). In addition to this the use of various polymeric materials enable the modulation of physicochemical characteristics (e.g. hydrophobicity, zeta potential), drug release properties (e.g. delayed, prolonged, triggered), and biological behaviour (e.g. targeting, bioadhesion, improved cellular uptake) of nanoparticles (Galindo-Rodriguez *et al*., 2005) providing flexibility to the pharmaceutical scientists in programming desired behaviour of formulation *in vivo*. The particle surface can be tailored by adsorption or chemical grafting of certain molecules such as poly (ethylene glycol) (PEG) (Torchilin, 2002), poloxamers (Lukowski *et al*., 2004), and bioactive molecules (lectins, invasins) (Ann-Clarka *et al*., 2000) which not only provides them with stealth properties but also institutes stability and targetability for more sustained and specific action. Moreover, their submicron size and their large specific

surface area favour their absorption compared to larger carriers. The oral uptake of nanoparticles has long been a topic of debate but studies demonstrated by Florence and co-workers have provided quantitative proof of the importance of the gut associated lymphoid tissue (GALT) in the processes involved in the uptake of nanoparticles delivered orally, and has confirmed the role of the Peyer's patches in the uptake of particles through the small intestine (Florence *et al*., 1995).

2. The disease: Type 1 diabetes

Type 1 diabetes mellitus is a chronic autoimmune disease with both biochemical and anatomical consequences and is characterized by disordered carbohydrate, protein and fat metabolism leading to high blood glucose levels (hyperglycaemia), appearance of ketone bodies in body fluids, higher circulating levels of hormone glucagon (hyperglucagonemia), excessive thirst and increased fluid intake (polydipsia), excessive urine production (polyuria), abnormal lipid and protein profile, blurred vision, excessive weight loss and lethargy. The disease is primarily caused due to auto destruction of pancreatic β cells triggered by viral infection, drugs, and environmental factors or due to genetic predisposition, resulting in complete loss or impaired secretion of endogenous insulin, the absence of which manifests these symptoms (Pickup and Williams, 1997). In addition to blood glucose concentration, glycosylated haemoglobin (HbA1c) is used to measure progression of disease over a long period of time. Healthy levels are considered to be 70-150 mg/dl for blood glucose and < 7 % for HbA1c. The chronic effects of diabetes contribute to microvascular complications which comprise retinopathy, neuropathy and nephropathy and macrovascular complications like coronary artery disease, peripheral arterial disease and

stroke (Fowler, 2008). The global prevalence of diabetes is increasing at an alarming rate and with the current findings the epidemic has covered up almost every part of the planet and is still spreading in developed countries (Fig. 1.1). Data on diabetes prevalence by age and sex from a limited number of countries were extrapolated to all 191 World Health Organization (WHO) member states and applied to United Nation's (UN) population estimates for 2000 and 2030. Urban and rural populations were considered separately for developing countries. The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030 (Wild *et al*., 2004). The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030. The prevalence of diabetes is higher in men than women, but there are more women with diabetes than men. The urban population in developing countries is projected to double between 2000 and 2030. The most important demographic change to diabetes prevalence across the world appears to be the increase in the proportion of people > 65 years of age. Figures 1.2 & 1.3 describe the projections for estimated number of adults with diabetes by age-group, year, for the developed and developing countries (Wild *et al.*, 2004).

In summary, these data provide an updated quantification of the growing public health burden of diabetes across the world. The human and economic costs of this epidemic are enormous. Mortality from communicable diseases and infant and maternal mortality in less developed countries are declining. In association with increasing diabetes prevalence, this will inevitably result in increasing proportions of deaths from cardiovascular disease in these countries, as well as increased prevalence and associated consequences of other complications of diabetes. A concerted global initiative is required to address the diabetes epidemic and save millions of people around the globe.

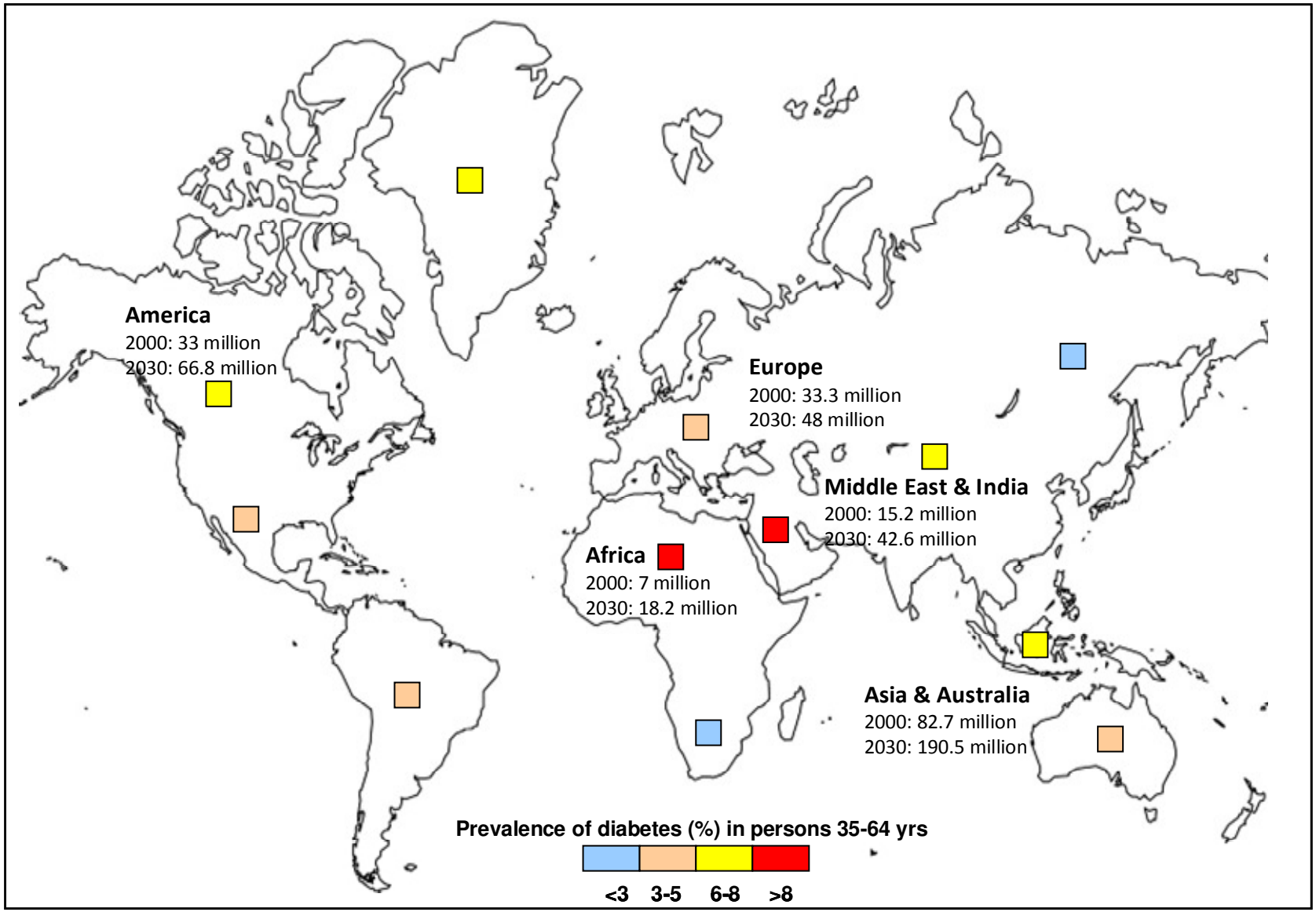

America
2000: 33 million
2030: 66.8 million
Europe
2000: 33.3 million
2030: 48 million
Middle East & India
2000: 15.2 million
2030: 42.6 million
Africa
2000: 7 million
2030: 18.2 million
Asia & Australia
2000: 82.7 million
2030: 190.5 million
Prevalence of diabetes (%) in persons 35-64 yrs
<3
3-5
6-8
>8

Fig. 1.1 Global prevalence of diabetes. Figure modified from Wild *et al*., 2004, Global Prevalence of Diabetes: Estimates for the year 2000 and projections for 2030.

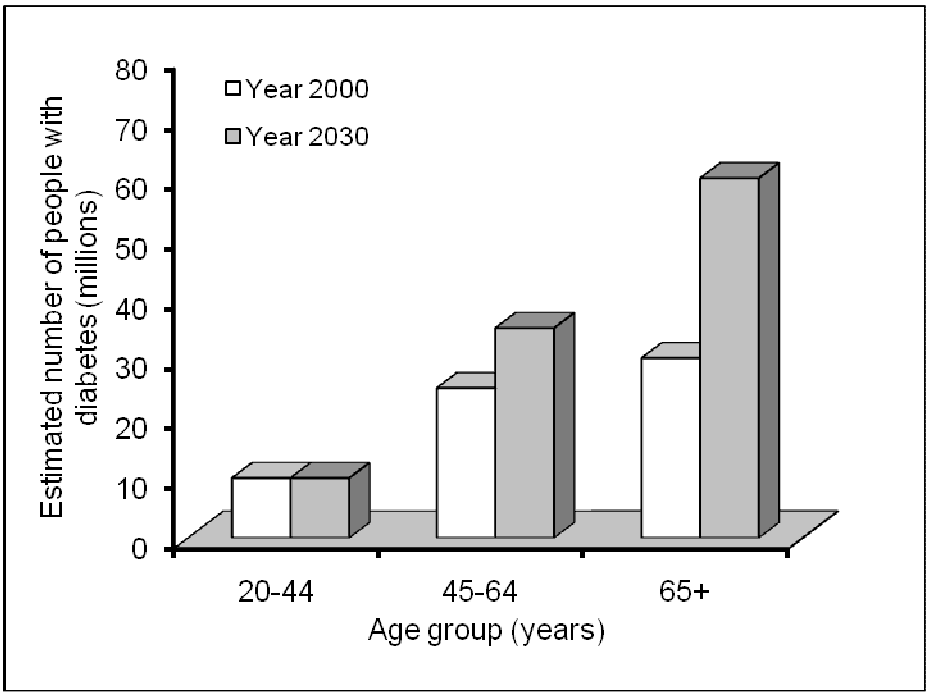


Fig. 1.2 Estimated projections for number of adults suffering from diabetes by age group and year in developed countries. (Modified from Wild *et al.,* 2004)

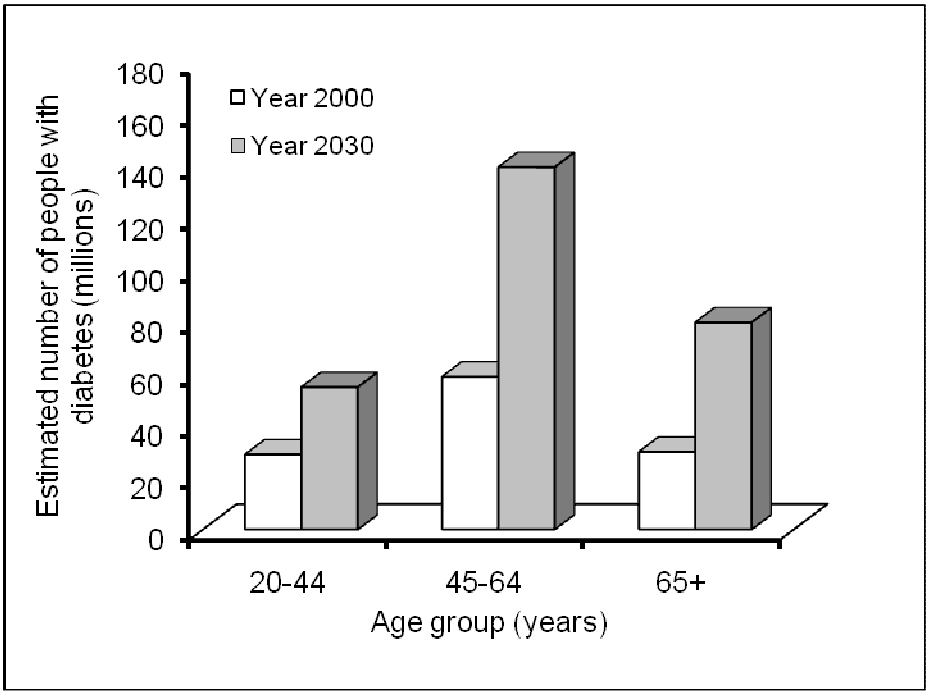


Fig. 1.3 Estimated projection for number of adults suffering from diabetes by age group and year in developing countries. (Modified from Wild *et al.,* 2004)

3. The drug: Insulin

The discovery of insulin is regarded as the hallmark achievement in medical sciences. Insulin is by far the most studied protein in history and it was the first ever naturally produced protein to have its amino acid sequence determined, a feat for which Frederick Sanger won the Nobel Prize in chemistry in 1958 (Sanger and Tuppy, 1951; Sanger and Thompson, 1953). Insulin is a globular hydrophobic protein made up of two polypeptide chains (A and B) held together by 3 disulfide bonds (Fig. 1.4). The hormone contains 51 amino acid residues and weighs ~5800 Da (Blundell *et al*., 1972). The insulin precursor, pro-insulin, is synthesized in the pancreatic β-cells, and subsequently converted to insulin by the enzymatic cleavage of the C peptide (Derewenda *et al*., 1989). Insulin is one of the most thoroughly studied molecules; it is the first protein whose chemical sequence was determined (Ryle *et al*., 1955) and it is the first to be commercially produced by recombinant DNA technology (Johnson, 1983).

The biologically active form of the hormone (which circulates *in vivo* and binds to the receptor) is monomeric, but insulin readily associates into dimers, and with zinc ion three dimers assemble into a hexamer (Zoete *et al*., 2004). The hydrophobic region of the insulin monomer involved in dimer and hexamer formation is the same portion of the molecule which binds to the receptor and is responsible for regulatory function of insulin. The monomer's self-association into dimers and hexamers leads to the burial of hydrophobic moieties, resulting in stable structures with largely polar surfaces. The hexamers crystallize readily and this conformation, which is used for storage in the pancreatic β-cells, has a very low solubility (Brange *et al*., 1987). Detailed description on the history, pharmacology, use and drug delivery aspects of insulin are discussed in the following sections.

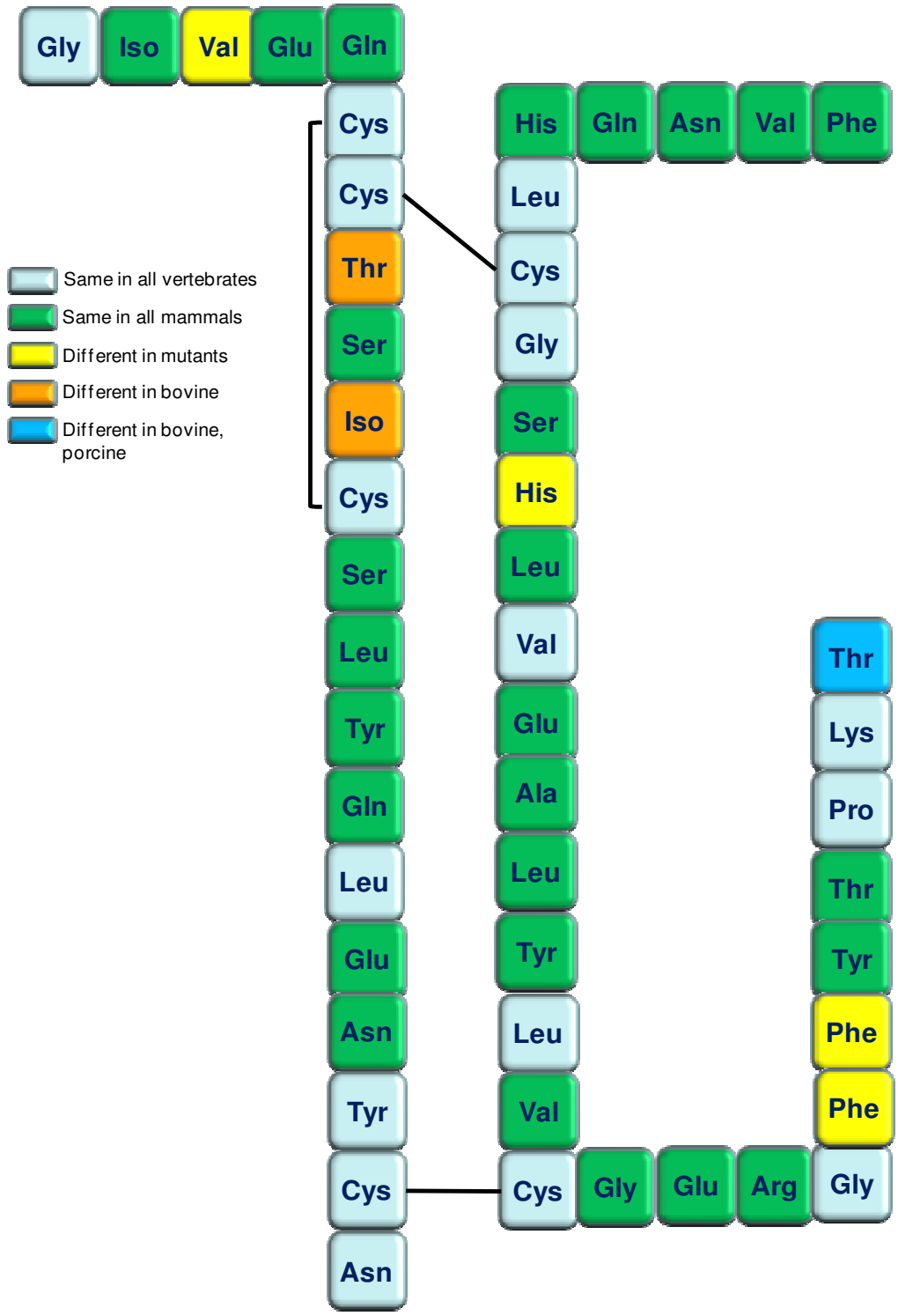


Fig. 1.4 Structure of insulin with amino acid sequence in vertebrates showing presence of 3 disulphide bonds and the changes in amino acids depending upon the vertebrate species.

3.1 Time line of insulin research (University of Toronto, 2010; Wikipedia, 2009)

Table 1.1 Time line of insulin research from discovery till date.

Year	**Event**
1913	The first monograph on diabetes entitled " Diabetes: Its Pathological Physiology" (London: Edward Arnold, 1913) is written by J.J.R. Macleod
1914	Dr. John. G. FitzGerald establishes The Connaught Antitoxin Laboratory in Toronto
1916	F.G. Banting graduates from department of medicine, University of Toronto. J.B. Collip is awarded Ph.D in biochemistry from University of Toronto. C.H. Best enrols at University College (under arts) at University of Toronto.
1918	J.J.R. Macleod is invited to chair the department of physiology in Toronto. Royal College of Physicians awards certificate to F.G. Banting.
1920	On October 31st, F.G. Banting reads "Relation of the Islets of Langerhans to Diabetes with Special Reference to Cases of Pancreatic Lithiasis" by Moses Barron in Surgery, Gynaecology and Obstetrics, Nov. 1920. The article encourages him an idea for experimentation. On 6th November, F.G. Banting meets J.J.R. Macleod who proposes his physiology lab to try out Banting's idea.
1921	J.B. Collip returns to University of Toronto from University of Alberta in Edmonton on Rockefeller travelling fellowship. F.G. Banting with C.H. Best conducts experiments at University of Toronto discovering insulin.
1921	Start of animal experiments: Dog 410 receives extract, followed by dog 408 receiving injection of extract. The extract for the first time is called by the name "isletin". Depancreatized dog 402 receives extract injection and survives for 20 days
1921	J.J.R. Macleod asks F.G. Banting and C.H. Best to repeat the experiment for confirmation of their findings.

1921	The first research article on experimental findings of F.G. Banting and C.H. Best is published in Journal of laboratory and clinical medicine after being presented in journal club, department of physiology at University of Toronto.
1921	Dog 33 named “Marjorie” is depancreatized and longevity experiments starts.
1921	Experiments start in rabbits, conducted jointly by J.B. Collip, F.G. Banting, C.H. Best and J.J.R. Macleod.
1921	Dr. Gilchrist receives first oral administration of extract prepared by Banting and Best with no efficacy.
1921	J.B. Collip demonstrates the efficacy of pancreatic extract to stimulate glycogen synthesis in liver of diabetic dog and to normalize liver functioning.
1921	All four scientists attend annual conference of the American Physiological Society in New Haven. Banting presents "The beneficial influences of certain pancreatic extracts on pancreatic diabetes". The abstract gets published as "The Internal Secretion of the Pancreas" in the American Journal of Physiology, 59 (Feb. 1922).
1922	J.J.R. Macleod includes 3 more researchers in team; J. Hepburn, J. K. Latchford, and E. Clark Noble
1922	First parenteral administration of extract given to a 14 year old hospitalized patient named Leonard Thompson at the Toronto General Hospital. Ed Jeffrey under the supervision of the house physician Dr. Walter R. Campbell and Dr. Duncan Graham, gives the injection which has little effect.
1922	Press coverage starts in Toronto Star Weekly
1922	J.B. Collip’s extract is successfully administered to Leonard Thompson at the Toronto General Hospital.
1922	Connaught Anti-Toxin Laboratories makes agreement for manufacture of insulin.

1922	Banting and Best delivers lecture entitled “The effect of pancreatic extracts on blood sugar and urinary sugar in experimental diabetes" to the Academy of Medicine, Toronto. The paper is published in The Journal of Laboratory and Clinical Medicine, vol. 7, no. 8 (May 1922) as "Pancreatic extracts."
1922	The first official announcement: Discovery of extract that alleviates symptoms of diabetes in humans. This is in form of a research paper entitled “Pancreatic extracts in the treatment of diabetes mellitus" in The Canadian Medical Association Journal and describes the case of Leonard Thompson in detail, authored by Banting, Best, Collip, Dr. Almon. A. Fletcher, and Dr. Campbell
1922	The first major press coverage as “diabetes cure” appears in Toronto Star, Globe, and Telegram.
1922	Production of insulin fails. Best succeeds in producing insulin.
1922	Dr. G.H.A. Clowes, Director of Research at Eli Lilly & Co., makes a formal offer to Macleod about the possibility of collaboration for the large-scale production of insulin, which is declined.
1922	Establishment of a diabetic clinic at Christie Street Veterans Hospital under the direction of Drs. Gilchrist and Banting.
1922	Banting, Best, Collip and Macleod delivers series of papers on insulin at the annual meeting of the Royal Society of Canada.
1922	Macleod presents a paper at the Association of American Physicians annual meeting in Washington, D. C. "The effects produced on diabetes by extracts of pancreas" by Banting, Best, Collip, Campbell, Fletcher, Macleod, and Noble and is published in the Transactions of the Association of American Physicians (1922). First widely publicized announcement of the discovery within the medical profession and the first time the extract was referred to in public as "insulin."
1922	First diabetic patient in U.S. (Jim Haven) receives the extract under Dr. J. R. Williams in Rochester.

1922	Agreement between the Governors of the University of Toronto and Eli Lilly & Co. of Indianapolis for large production of insulin.
1922	J.B. Collip returns to University of Alberta and continues insulin experiments. C.H. Best becomes in charge of the production of insulin for all of Canada at the Connaught Anti-Toxin Labs.
1922	Research on fish as a source of insulin begins in Marine Biological Laboratory in St. Andrews New Brunswick, under J.J.R. Macleod.
1922	Formation of diabetic clinic in Toronto general hospital under Dr. Duncan Graham.
1922	J.J.R. Macleod sends out insulin's formula to Dr. W. D. Sansum in Santa Barbara, California and Dr. Rollin T. Woodyatt in Chicago for small scale production and for clinical use.
1922	Mrs. Charlotte Clark, first patient to receive insulin for surgery at Toronto general hospital. Teddy Ryder, a five-year old diabetic patient from New Jersey, arrives in Toronto for treatment.
1922	Start of clinical trials with insulin at diabetic clinics.
1922	Elizabeth Evans Hughes arrives in Toronto to be treated.
1922	Establishment and first meeting of insulin committee in 1922 at University of Toronto to control the licensing, patenting, and trade marking of insulin as a pharmaceutical product.
1922	Chairman of the insulin committee writes to Banting, Best and Macleod soliciting their accounts of the discovery of insulin and they personally respond with written submission.
1922	A patient (Elsie Needham) revived from coma using insulin.
1922	Production and experimentation with insulin begins in Denmark by August Krogh.
1922	Medical Research council of Great Britain and Ireland gets the patent for insulin production.
1922	Governor of University of Toronto granted the Canadian patent for insulin which is assigned in early 1923.

1922	Britain becomes the first country where commercially manufactured insulin is made available.
1923	Best, Banting and Collip gets USP 1,469,994 for manufacturing insulin.
1923	Ontario Medical Association sets up instruction course for doctors.
1923	Clinical trials on insulin get published for the first time in North America as a special issue in Journal of Metabolic Research highlighting clinical utility of insulin.
1923	Other pharmaceutical team players ventures in insulin production. Termination of exclusive agreement with Eli Lilly & Co. ends.
1923	Insulin is commercially available in U.S. and Canada.
1923	The Nobel Prize in Medicine is awarded to F. G. Banting and J. J. R. Macleod.
1923	Insulin now becomes globally available, marketed and distributed by Pharmaceutical companies of U.K. and United States.
1923	Agreement signed by League of Nations Health Committee for determining potency of insulin.
1923	Insulin becomes patented or trademarked in countries 44 countries world-wide.
1923	J.J.R. Macleod publishes Carbohydrate Metabolism and Insulin. (London: Longmans Green & Co., 1926).
1923	Eli Lilly produces commercial quantities of much purer bovine insulin than Banting *et al* had used
1923	Farbwerke Hoechst, one of the forerunner's of today's Sanofi Aventis, produces commercial quantities of bovine insulin in Germany
1923	Hagedorn founds the Nordisk Insulinlaboratorium in Denmark – forerunner of today's Novo Nordisk
1926	Nordisk receives a Danish charter to produce insulin as a non-profit
1936	Canadians D.M. Scott, A.M. Fisher formulate a zinc insulin mixture and license it to Novo
1936	Hagedorn discovers that adding protamine to insulin prolongs the

	duration of action of insulin
1946	Nordisk formulates Isophane porcine insulin aka Neutral Protamine Hagedorn or NPH insulin
1946	Nordisk crystallizes a protamine and insulin mixture
1950	Nordisk markets NPH insulin
1953	Novo formulates Lente porcine and bovine insulins by adding zinc for longer lasting insulin
1955	Frederick Sanger determines the amino acid sequence of insulin
1966	Synthesized by total synthesis by C.L. Tsou, Wang Yinglai, and co-workers
1969	Dorothy Crowfoot Hodgkin solves the crystal structure of insulin by x-ray crystallography
1973	Purified monocomponent (MC) insulin is introduced
1973	The U.S. officially "standardized" insulin sold for human use in the U.S. to U-100 (100 units per milliliter). Prior to that, insulin was sold in different strengths, including U-80 (80 units per milliliter) and U-40 formulations (40 units per milliliter), so the effort to "standardize" the potency aimed to reduce dosage errors and ease doctors' job of prescribing insulin for patients. Other countries also followed suit.
1978	Genentech produces synthetic 'human' insulin in Escheria coli bacteria using recombinant DNA techniques, licenses to Eli Lilly
1981	Novo Nordisk chemically and enzymatically converts porcine to 'human' insulin
1982	Genentech synthetic 'human' insulin (above) approved
1983	Eli Lilly and Company produces synthetic 'human' insulin with recombinant DNA technology, Humulin
1985	Axel Ullrich sequences a human cell membrane insulin receptor.
1988	Novo Nordisk produces recombinant 'human' insulin
1996	Lilly Humalog "lispro" insulin analogue approved.
2000	Sanofi Aventis Lantus insulin "glargine" analogue approved for clinical

	use in the US and Europe.
2004	Sanofi Aventis Apidra insulin "glulisine" insulin analogue approved for clinical use in the US.
2006	Novo Nordisk Levemir "detemir" insulin analogue approved for clinical use in the US.
2006	Pfizer launches the first inhalation production for Insulin, "Exubera" which was later removed from market due to lack of acceptance.

3.2 The biological genesis of insulin

In a normal person, insulin is synthesized in the β-cells of the pancreas as a 110 amino acid chain called pre-proinsulin (Pickup and Williams, 1997). This polypeptide includes every amino acid of regular insulin. Upon translocation through the endoplasmic reticulum, a 24 peptide sequence at the N-terminus of pre-proinsulin is cleaved and protein becomes pro-insulin. The tertiary structure of pro-insulin folds and forms the three disulfide bonds which are present in insulin (Pickup and Williams, 1997). Pro-insulin is then converted to insulin upon secretion at which point four basic amino acids are individually removed and a 35 peptide sequence known as C-peptide, is cleaved and secreted along with insulin (Fig. 1.5) (Pickup and Williams, 1997). The principal mediator of insulin secretion is the plasma glucose concentration. Insulin release is also promoted by gastrointestinal inhibitory peptide (GIP), glucagon-like peptide 1 (GLP-1), gastrin, secretin, cholecystokinin, vasoactive intestinal peptide, gastrin-releasing peptide, and enteroglucagon. Glucose stimulates insulin secretion by depolarizing the β-cells of the pancreas facilitated by the glucose transporter (GLUT2). The same transporter is responsible for glucose release from hepatocytes when glycogen is brokendown in the liver (Meyes and Bender, 2003) causing opening of Ca^{2+} ion channel which also potentiates insulin secretion.

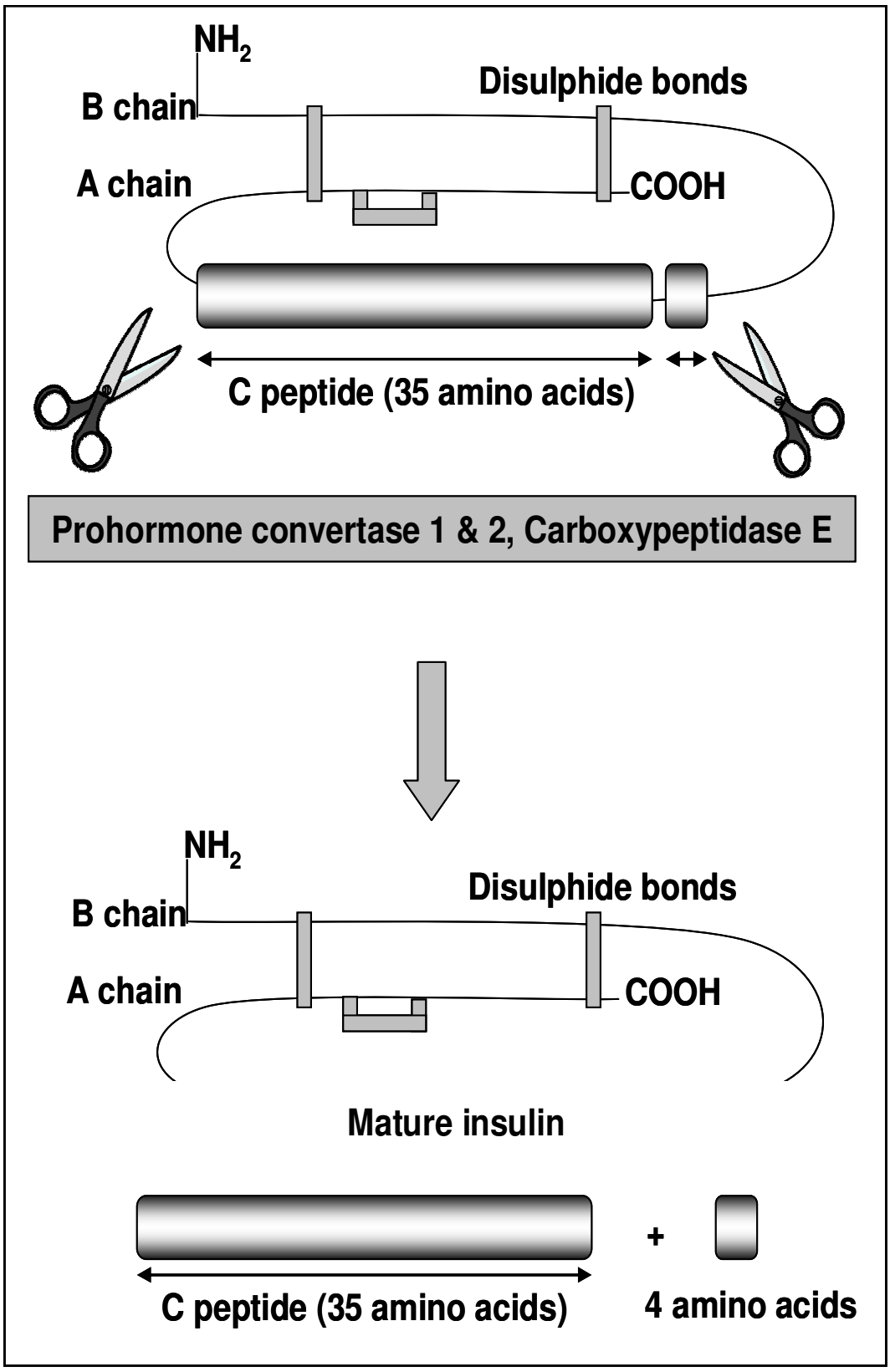


Fig. 1.5 Biogenesis of insulin from pro-insulin (shown on top) by the action of enzymes pro-hormone convertase 1 & 2 and carboxypeptidase E.

3.3 Pharmacology of insulin

Plasma insulin concentrations are often expressed as international units (IU). One international unit of insulin is 6 nmol, equivalent to 34.8 μg (Volund, 1993). For healthy adults, insulin concentrations vary between 5-15 μIU/ml (30-90 pmol/l) for basal levels and 60-90 μIU/ml (360-540 pmol/l) for postprandial levels (Mycek *et al*., 1992). Circulating insulin binds to the insulin receptors that are present in many tissues but primarily in liver, muscle, and adipose tissues. Signals from the insulin receptor initiate the translocation of intracellular vesicles rich with GLUT4 transporters which fuse with the plasma membrane of the cells and are exposed to the extracellular surface. Similarly to the GLUT2 transporter, GLUT4 increases the diffusion of glucose into the cytosol of cells. Upon translocation, glucose can be used by the cells for energy via glycolysis or stored as glycogen or triglycerides. In times of fasting, the breakdown of the glycogen and triglyceride stores maintains cell energy levels.

In addition to glucose transport, insulin has several other significant roles in metabolic homeostasis. It stimulates the uptake of amino acids and synthesis of proteins in muscle tissues, regulates gene transcription, increases glycogen and fatty acid synthesis, and inhibits the breakdown of glycogen, fat, and protein for energy and regulation of hepatic glucose production (Arbit, 2004) as in times of fasting, the liver synthesizes glucose from its glycogen stores through glycogenolysis and from other non-sugar substrates such as pyruvate through gluconeogenesis. The half life ($t_{1/2}$) of insulin in the bloodstream is 5-6 min and its degradation occurs primarily in liver, kidney, and muscle tissues (Duckworth *et al*., 1998). Roughly 50 % of insulin which is released into the portal vein, the route of insulin secretion, is destroyed by the liver and never reaches general circulation.

3.4 Diabetes control and complications trial

In 1993, a monumental clinical study, DCCT (Diabetes control and complication trial) was concluded based on data collected for 10 years on the effects and treatments of Type 1 diabetes (DCCT Research group, 1993). The study was conducted by the National institute of diabetes and digestive and kidney diseases (NIDDK) and incorporated findings from 1,441 volunteers from 29 medical centres in the US and Canada. At the time, the body of work collected from the 10-year study was the most comprehensive on diabetes performed to date and its findings drastically altered the clinical treatment of Type 1 diabetes. The DCCT generated several critical results based on the comparison between conventional insulin therapy consisting of one or two daily injections of regular insulin to intensive insulin therapy requiring smaller, more frequent injections, at least three per day, and frequent monitoring of glucose levels. These results indicated that significant reductions in retinopathy, nephropathy, and neuropathy in patients with Type 1 diabetes were observed in patients who used intensive insulin therapy versus that of conventional therapy (Jeandidier and Boivin, 1999). While some discrepancies were always there in the DCCT, but insulin therapy remains the most rigorous and physiological treatment for diabetes and is the only option for patients suffering from Type 1 diabetes.

3.5 Insulin delivery

Since the discovery of insulin, many non-invasive methods of insulin delivery have been thoroughly investigated. However, to date, no feasible alternative modes of delivery have achieved enough success to replace subcutaneous administration. Current delivery by injection is taxing on the patient who must continuously monitor their blood glucose and self-

administer insulin. Non-compliance to this dosing regimen is common. Also, because it is challenging, treatment with insulin is often seen as a last resort for Type 2 diabetics even though data suggests that early prescription is beneficial. The investigation of alternative routes for insulin delivery is driven by the desire to simplify the administration of insulin and potentially reduce the levels of non-compliance currently seen with insulin therapy.

3.5.1 Pulmonary delivery

Till date, the most successful alternative to subcutaneous injection for insulin is the pulmonary route and in 2006, an inhaled dry powder insulin formulation from Nektar and Pfizer was approved by the FDA for the treatment of diabetes under the name Exubera® (Crotty and Reynolds, 2007). The delivery of insulin to the lungs has several distinct advantages. The lungs have a large surface area (100-140 m^2), have a relatively high permeability, and are highly vascular (Patton *et al*., 2004; Owens *et al*., 2003). Each of these factors contributes significantly to the rapid absorption of insulin from the lungs. Insulin can be absorbed efficiently without the addition of absorption enhancers or enzyme inhibitors, so side effects generally associated with such treatments are nonexistent. The pharmacokinetics of this delivery system shows a more rapid onset of insulin action and more rapid clearance than that of subcutaneous injection of regular human insulin. With the addition of a small amount of chelating agent or absorption enhancer the rate of absorbance and onset is faster even than current short-acting insulin analogues, though toxicity is a concern. When absorbed from the lungs, insulin enters the circulation bypassing the first-pass hepatic clearance, giving it a slightly longer pharmacodynamic profile than endogenous insulin (Brain, 2007). The major drawbacks to

pulmonary insulin delivery are that the pharmacological bioavailability is still only 9-22 % that of subcutaneously injected insulin and that improper inhalation technique can cause large variations in administration, causing administration of higher levels. Because of the lower bioavailability, large quantities of insulin are required when compared to injection, making inhalable insulin significantly more expensive. Due to these drawbacks, heavy criticism of current products for pulmonary insulin delivery has been experienced. The Pfizer announced on October 17, 2007 that it will no longer produce or market Exubera because it failed to gain acceptance among patients and physicians (Simons, 2007). Efforts still continue in the development of an improved inhalable insulin delivery system.

3.5.2 Transdermal delivery

Transdermal insulin delivery would allow similar insulin absorption as current subcutaneous delivery without the pain associated with injection but impermeability of skin to large hydrophilic molecules primarily due to the intracellular lipid layer of the stratum corneum makes transdermal delivery a daunting task. One approach to transdermal delivery is through the use of micro or nanoneedles which permeate the skin in the same fashion as a standard needle with very low levels of associated pain. A significant hypoglycaemic response has been seen in tests with diabetic hairless rats in which the microneedles were inserted to puncture the skin and then an insulin solution was applied at the site of treatment (Martanto *et al*., 2004).

A second approach to transdermal delivery is iontophoresis and sonophoresis which use electrical charge to disrupt the skin tissue and propel insulin into the skin. Low-frequency ultrasound waves, or sonophoresis, has also been explored as a method for transdermal insulin delivery. This

technique uses sound waves to disrupt the stratum corneum thus increasing the permeability of insulin. In a study performed on pigs, a drop in blood glucose was evident with iontophoresis; however it required 60 minutes of treatment, an unreasonably long time for a patient to try to use this method (Kanikkannan *et al*., 1999). Photomechanical waves or laser-generated stress has also been investigated for transdermal insulin delivery (Lee *et al*., 2001; Park *et al*., 2007). All transdermal approaches require extended exposure to topical insulin solutions and the variability in dosing and occurrence of skin irritation limits its standard practice.

3.5.3 Nasal delivery

The delivery of insulin to the nasal cavity has been investigated due to its convenience, large absorptive surface, high vascularity and due to the fact that this route bypasses first pass metabolism (Hinchcliffe and Illum, 1999). However, the low absorption of insulin, mucociliary clearance, and presence of proteolytic enzymes makes this approach challenging. The major approaches for nasal delivery use permeation enhancers, mucoadhesive materials, enzyme inhibitors, or a combination of these methods. Permeation enhancers have a tendency to damage the nasal mucosa and may present a significant challenge for any long-term use of nasally delivered insulin. Nonetheless, studies in which insulin formulations were delivered along with didecanoyl-L-α-phosphatidylcholine (DDPC), an absorption enhancer, had relative bioavailabilities of 8.3-13.2 % compared to intravenous injection (Pontiroli, 1998). The hypoglycaemic effect in these studies had lower variability and faster onset than subcutaneous injection.

Chitosan has also been used for nasal delivery of insulin (Zhang *et al*., 2008; Dyer *et al*., 2002). The study by Illum and co-workers used chitosan in

solution, as a dry powder, and grafted to nanoparticles to deliver insulin. Each approach succeeded in producing a hypoglycaemic effect *in vivo*, with the highest pharmacological bioavailability of 17.0 % for insulin delivered with chitosan powder. Enzyme inhibitors have also been investigated for nasal insulin delivery, though the degree of absorption of insulin does not exceed that of other absorption enhancing methods (Hinchcliffe and Illum, 1999).

3.5.4 Other routes

Ocular (Lee and Yalkowsky, 1999) and vaginal (Degim *et al*., 2005) insulin delivery have achieved only limited absorption levels and severe local adverse reactions indicate that they offer little opportunity for insulin delivery (Lee *et al*., 2002). Buccal or sublingual delivery systems have generated a significant amount of attention due to the ease of administration, low proteolytic activity, and high vascularisation (Senel and Hincal, 2001; Junginger *et al*., 1999; Luo *et al*., 2005; Pozilli *et al*., 2005). The challenges include the thick multilayered structure of the buccal and sublingual mucosa and the constant flow of saliva. A Canadian company specializing in buccal drug delivery, Generex currently produces Oral-Lyn™ which delivers insulin via the buccal route (www.generex.com) and is only intended for those who have pre-diabetes or Type 2 diabetes.

3.5.5 Artificial pancreas

One other major area of research for insulin delivery is the development of an artificial pancreas. The first approach is to have an implantable insulin pump connected to a real-time glucose monitoring system in a feedback control loop (Hanazaki *et al*., 2001). The benefit of this design is that it can use

pump technology currently being used for continuous subcutaneous insulin infusion (CSII) therapy. The biggest challenge to this type of artificial pancreas is maintaining rapid, accurate, and consistent monitoring of the blood glucose levels which can provide continuous or near-continuous glucose determination (Roze *et al*., 2005).

A second type of artificial pancreas referred to as a bio-hybrid pancreas utilizes natural pancreatic β-cells which are encapsulated in some sort of semi-permeable membrane such that glucose and insulin can diffuse through it, but the cells are to be protected from immunological detection and destruction (Lim and Fan, 2007). The premise is that following implantation the natural function of pancreatic β-cells has a more intricate action than what is currently modelled by artificial means. Direct transplantation of pancreatic tissue requires the use of immune suppressive drugs for the remaining lifetime of the individual. However, decreased insulin secretion with time due to cell death and surgical risk of implantation and extrication are major drawbacks (Hou and Bae, 1999). However, there is a delay in the response of the cells due to diffusive restrictions of glucose in the interstitial fluids. Also, over time the fibrous encapsulation of the implant further reduces the flux of glucose and pancreatic secretions into and out of the bio-hybrid pancreas.

A third approach utilizes glucose-responsive biomaterials as the platform of the artificial pancreas. In most cases the biomaterial responds to glucose by biodegradation (Kashyap *et al*., 2007), by swelling changes (Miyata *et al*., 2004; Matsumoto *et al*., 2004) competitive binding of glucose for glycosylated insulin on concanavalin A (Liu *et al*., 1997; Cheng *et al*., 2004) or by sol-gel transition (Kim and Park, 2001) such that insulin trapped in the material is

released. These approaches are promising when performed *in vitro*, but their *in vivo* applications have limited success.

3.6 Oral delivery of insulin

Oral insulin delivery would provide an easy and convenient method of delivery and likely improve patient compliance, a major issue for current parenteral delivery. With an easy administration method, doctors and patients would likely be more inclined to start insulin therapy before the disease becomes advanced or use it for Type 2 diabetics who often resist starting intensive insulin therapy (Campos, 2007). For insulin, however, this delivery route would mimic the physiological pathway of endogenous insulin produced from the pancreas (Owens *et al*, 2003). In the postprandial state, the interaction of insulin and glucose with the liver is the most critical for maintaining euglycaemic levels (Ferrannini *et al*., 1980).

3.6.1 Potential advantages

First of all, in a healthy individual, the majority of ingested glucose is taken up by splanchnic tissue. In order to do so, insulin must be bound to the surface receptors in the liver. Following oral delivery, the first potential site for insulin action is in the liver. Second, the presence of insulin at these receptors is critical to initiate glycogenesis and glycolysis, and inhibit gluconeogenesis and glycogenolysis. Each of these processes is important step in maintaining healthy circulating glucose concentrations. A schematic of the action of insulin on glucose can be seen in Figure 1.6. Third, the sensitivity of the liver is much greater than that of muscle tissues and it responds very quickly to the presence of insulin. Thus, a more rapid hypoglycaemic effect is seen from insulin in the portal vein than from insulin delivered parenterally reducing the risk of postprandial hyperglycaemia.

Fourth, the residence time of insulin on insulin receptors in the liver is significantly less than that of muscle and adipose tissues (Moore *et al.*, 2003). This reduces the risk of sustained hypoglycaemia induced by extended hyperinsulinemic reactivity in peripheral tissues. Finally, one of the most common problems among Type 2 diabetics is insulin resistance in the liver (Lebovitz and Banerji, 2004). These patients require high insulin levels (~100 μIU/ml) in the portal circulation to affect hepatic glucose production (Nathan, 2002). Insulin concentrations at this level can be safely utilized by the liver, but if circulating plasma insulin concentrations were that high in the general circulation, as would be the case for subcutaneously delivered insulin, a severe hypoglycaemic episode would occur. Therefore, orally administered insulin has the potential to treat both Type 1 and Type 2 diabetics in a pharmacokinetic route similar to that of native endogenous insulin production.

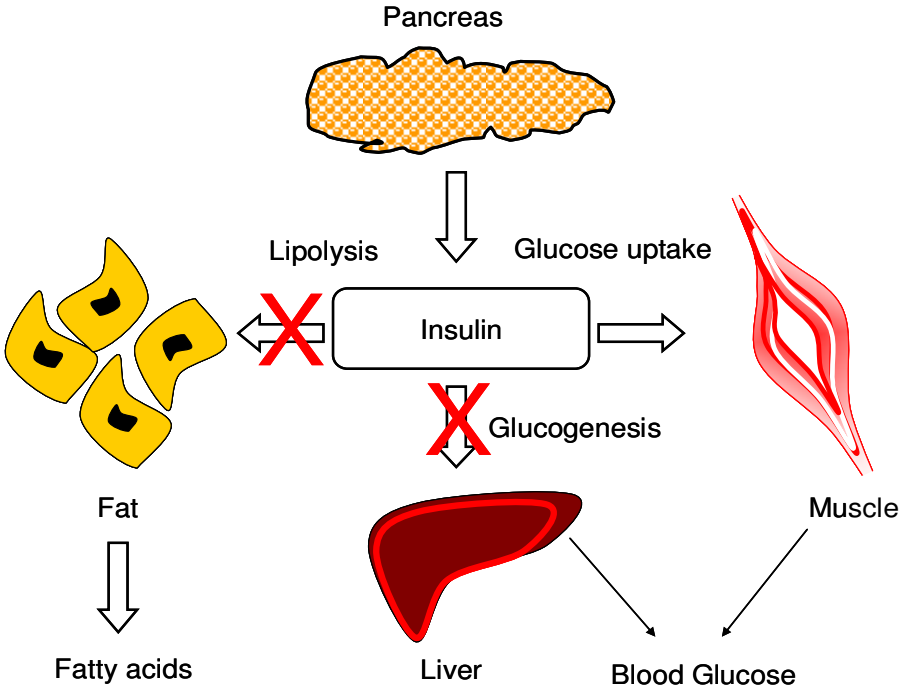


Fig. 1.6 The action of insulin on blood glucose (modified from Stumvoll *et al.*, Type 2 Diabetes, principles of pathogenesis and therapy).

3.6.2 Potential disadvantages

Despite of numerous advantages what oral insulin offers, there are several limitations to oral delivery of insulin for treatment of Type 1 diabetes and these include: absorption of proteins and peptides from different regions of the intestine is not uniform and the cellular morphology of the intestines changes from region to region thereby creating a more individualized requirement for treatment, high proteolytic activity in the gut paralleled with huge variation in pH and ionic conditions leading to insulin degradation and instability when administered orally thereby requiring a high dose which subsequently relates with economic feasibility of the delivery system, the high molecular weight which renders insulin impermeable across gut mucosa, considerable variation in pharmacological response from orally administered insulin due to presence of food and physiological juices and requirement of advanced delivery approaches which not only add on the cost of the delivery system but are often associated with their own inherent disadvantages (approaches and their advantages/disadvantages are covered in the subsequent sections) making oral insulin delivery a daunting task (Gowthamarajan and Kulkarni, 2003).

One other issue is that all the polypeptide and protein delivery platforms developed thus far have relatively low oral bioavailability which is a harbinger of significant inter- and intra subject variability (Arbit and Kidron, 2009). A way to reduce variability is to increase the amount of insulin in the dosage form. Until recently such a proposition was impractical for insulin because of considerations. At the present time, however, the supply of insulin and its price can support such a strategy. Low oral bioavailability also implies that most of the insulin ingested is not absorbed and remains in the gastrointestinal tract. It is most likely that insulin retained in the

gastrointestinal tract will be degraded by peptidases and proteases. Nevertheless, a concern that will need to be addressed in long-term safety studies is whether insulin, a known mitogen implicated in an increased risk of several cancers, including colon cancer, will increase the incidence of cancer when given orally (Giovannucci, 1995; Isha-Shalom *et al.*, 1997; Argiles and Lopez-Soriano, 2001). Finally, while insulin per se may not be toxic, the chemical compounds employed in the various delivery systems as excipients or absorption promoters need to be deemed safe and effective in long-term toxicological and clinical studies.

3.7 Oral insulin delivery: Physiological considerations

The physiology of GI tract plays a significant role in absorption of nutrients and xenobiotics (Layman, 2004) and it's very crucial for oral delivery of peptide drugs like insulin. Absorption of proteins and peptides across the epithelial layer of the GI tract can take place through different pathways like paracellular transport, active transport, receptor-mediated endocytosis, diffusive transport, and pinocytosis (Kunta and Sinko, 2004). Paracellular transport of proteins between epithelial cells occurs most often when the intercellular connection between the two cells is disrupted. Paracellular transport through the tight junctions is the primary pathway for the absorption of hydrophilic proteins from the GI tract which have very low absorption and lack active transport mechanisms, such as insulin. Peptide transporters naturally present in the GI tract provide an alternative pathway for peptide and protein absorption across the epithelial wall of the intestine (Thwaites and Anderson, 2007).

Diffusive transport is another dominant mechanism of protein transport across GI tract but macromolecular proteins such as insulin, however, are

generally too large to easily diffuse across cell membranes and this route of absorption has a low efficiency. The ileum contains a unique concentration of lymphatic tissues concentrated into groups called Peyer's patches which serve as the primary sight for the nanoparticle uptake and hence have been extensively exploited for oral delivery of proteins and peptides via these carrier systems (Florence, 2005).

The biggest concern for oral delivery of proteins is digestion in the stomach and bond cleavage due to very low pH and high content of proteolytic enzymes chiefly pepsin. Proteins delivered orally are digested into smaller peptides or individual amino acids prior to their absorption primarily by pepsin in the stomach and pancreatic enzymes such as trypsin, α-chymotrypsin, and elastase in the small intestine (Langguth *et al*., 1997). The most threatening gastric secretions to orally delivered proteins are HCl and pepsinogen, which is enzymatically converted to pepsin in the presence of HCl. The low pH of the stomach can induce the hydrolytic breakdown of several proteins.

Additionally pepsin, which is most active at low pH levels and deactivated at pH > 5, can cleave the amino bond of phenylalanine, tryptophan, and tyrosine amino acid residues. The next groups of enzymes that are part of the enzymatic barrier to oral insulin delivery are preferentially located in three sub-cellular locations in epithelial cells: the brush-border, the cytoplasm, and the lysosomes (Taylor and Amidon, 1995). The primary degradation of insulin in sub-cellular regions of epithelial cells occurs on their apical surface by brush-border enzymes which include endopeptidases, carboxy-exopeptidases and amino-exopeptidases which cleave insulin at selective regions (Erickson, 1995). There is another group of enzymes called lysosomal proteases which mainly consist of cathepsins, exo and endopeptidases and

they cause protein degradation upon their incorporation into the lysosome (Ciechanover, 2006). The second major challenge to oral insulin delivery is the physical barrier of the intestinal epithelium which covers the luminal side of the intestine. The diffusion rate of large molecules greater than approximately 5000 Da is severely reduced by the mucous and glycocalyx.

3.8 Oral insulin delivery: The state of the art

Oral delivery of insulin has always been an elusive task for pharmaceutical scientists and for diabetic patients and numerous attempts has been made since the discovery of insulin to formulate an effective oral delivery system for it. The approaches that have been utilized to exploit the oral route for insulin delivery can be broadly classified into two categories, classical approaches and advanced delivery systems, which are discussed in depth in the subsequent sections.

3.8.1 Classical approaches

Use of enzyme inhibitors, permeation enhancers, protein pro-drugs and carrier mediated uptake and receptor-mediated transcytosis forms the major part of classical approaches and they have been summarised in Table 1.2 with in depth discussions in the subsequent sections.

Table 1.2 Classical approaches utilized for oral delivery of proteins and peptides.

Approach	*Issues addressed*	*Pros*	*Cons*	**Status**
Enzyme inhibitors	Overcoming enzymatic barrier	Reduced protein loss	High specificity and side effects due to altered digestion	Preclinical
Permeation enhancers	Overcoming physical barrier	Enhancement in protein permeation and reduced protein loss	Irreversible disruption of TJ leads to infections and other diseases	Preclinical
Pro-drugs	Overcoming enzymatic and physical barrier. Alteration of pharmacokinetics	Reduced protein loss, enhanced absorption, increased circulation time, reduced immunogenicity, reduced hepatic clearance	Not applicable to all proteins	Commercialized formulations Granulocyte colony stimulating factor, alpha interfereon 2b, 2a and human growth hormone
Active transport and receptor mediated endocytosis	Overcoming physical barrier	Enhancement in protein absorption especially high molecular weight proteins (>1000 Da)	Natural saturability of transport mechanism.	Preclinical

3.8.1.1 Enzyme inhibitors

The enzymes of the GI tract are well characterized in terms of their physiological action as well as their proteolytic action on proteins and the later has been evaluated both *in vitro* and *in vivo* (Bernkop-Schnurch, 1998). For each protein there are specific enzymes that tend to degrade it and for each specific enzyme a specific inhibitor has been developed till date, the list is long but the significant ones include pepsinostreptin, aprotinin, chymastatin, elastatinal, EDTA, chitosan-EDTA, soybean or basic pancreatic trypsin inhibitor, amastatin, puromycin, bestatin, phosphinic acid dipeptide

analogues, and enterostatin (Hussain *et al*., 2004). The strategy aims at administering protein along with its enzyme inhibitor so as to have the maximum benefit. Plate *et al*., reported delivery of insulin along with pancreatic and soya bean trypsin inhibitor immobilized on to polymers and the delivery system was efficient in maintaining high insulin retention levels even in presence of trypsin, α-chymotrypsin, and elastase (Plate *et al*., 2002). A new methodology that comprised of enzyme inhibition and pro drug approach simultaneously using chitosan-EDTA-protease inhibitor conjugates for oral delivery of peptides and proteins has been developed (Bernkop-Schnurch and Scerbe-Saiko, 1998). Same group reported covalent attachment of enzyme inhibitor with protein thus using a combination of enzyme inhibition and pro-drug strategies to circumvent the enzymatic barrier (Bernkop-Schnurch *et al*., 1998).

3.8.1.2 Permeation enhancers

Paracellular route is one of the most important routes for protein absorption and several small peptide drugs are believed to be absorbed by this route including octreotide, vasopressin analogue desmopressin, and thyrotropin releasing hormone (Pauletti *et al*., 1996) though most proteins, however, are too large and too hydrophilic to be readily absorbed through the tight junctions (TJ) of epithelial cells. Paracellular transport can be enhanced through the use of permeation or absorption enhancing materials which work by chemically disrupting TJ of the epithelial cell layer which constitutes the largest barrier to passive paracellular drug absorption.

Chemically diverse, a large number of permeation enhancers have been used for oral delivery of proteins and these include bile salts like sodium taurocholate and sodium deoxycholate, dimethyl-β-cyclodextrin, chitosan,

hydrochloride, sodium lauryl sulfate, nafamostat, bacitracin, capric acid, taurodeoxycholic acid, glycyrrhizic acid, and zona occludens toxin (Hosny *et al*., 2002; Uchida *et al*., 2001; Shah *et al*., 2007; Radwan and Aboul-Enein, 2001). Use of permeation enhancers along with enzyme inhibitors has been rewarding as shown by Komada *et al*., and Takeyama *et al*., where enzymatic insulin degradation was inhibited by the absorption enhancer nafamostat (Komada *et al*., 1985; Takeyama *et al*., 1991. The covalent attachment of a 7-9 unit length of polyethylene glycol and a hydrophilic hexyl alkyl chain to the lysine B29 residue of insulin resulting in hexyl insulin monoconjugate-2 (HIM2) that increases the enzymatic resistance of insulin along with its permeation across a modelled intestinal epithelium using Caco-2 cells was reported (Clement *et al*., 2002). The only concern with permeation enhancers is their reversible modulation of tight junctions for increased protein absorption as extended opening greatly increases the potential for paracellular transport of other large species such as viruses, bacteria, mycoplasma, and E-coli (Cereijido *et al*., 2007). Enhancement of protein absorption using permeation enhancers via opening tight junctions offers potential for increasing the bioavailability of proteins.

3.8.1.3 Protein pro-drugs

Pro-drugs are modified drugs which are rendered different in their physiological properties by chemical alterations in their molecular structure leading to altered absorption profile with same pharmacological actions (Garnett, 2001). Pro-drugs can also involve the covalent conjugation of a chemical species to the drug which directly alters its absorption, distribution, metabolism, and excretion behaviour (Hou *et al*., 2007). Specifically for oral delivery of insulin, pro-drug strategies generally attempt to address one or

both the enzymatic and physical barriers of the intestinal epithelium. Pro-drugs differ from traditional enzyme inhibitors and permeation enhancers because their action is immediately concentrated in close proximity to the therapeutic protein due to covalent modification. In some cases this modification is reversible such that upon exposure to lumenal, brush-border, or intracellular enzymes the pro-drug is reduced to the native form of the substance.

The general approaches involving pro-drugs for oral delivery include modifying the N-terminus or C-terminus of proteins such that they can't be degraded by amino- or carboxypeptidases, conjugating actively transported compounds, reducing immunogenicity of the protein, or reducing hepatic and renal clearance. Saitoh and Aungst reported increased absorption of platelet fibrinogen receptor antagonist (GP IIb/IIIa) using pro-drug strategy (Saitoh and Aungst, 1997) whereas Weller *et al*., reported enhanced biological action of amidines using amidoximes as pro-drugs (Weller *et al*., 1996). The oral absorption of an ester-type pro-drug GP IIb/IIIa was increased in mice, the efflux was also increased negating the increased absorption rates (Kondo *et al*., 2005). L-α-methyldopa and bisphosphonates have shown to be better absorbed utilizing single amino acid modifications. Sinha and Kumria utilized a pro-drug strategy to target drug delivery to the colon by conjugating with bonds which are specifically cleaved by the microflora of the large intestine, releasing biologically active substrate in the colon (Sinha and Kumria, 2001). PEGylation or conjugation of PEG with proteins is another pro-drug strategy which has shown to reduce enzymatic degradation, increase circulation time, and increases the solubility of therapeutic protein and peptide drugs (Calceti *et al*., 2004). PEGylation has been studied with many drugs such as: adenosine deamidase, collagen,

human growth hormone, nerve growth factor, and insulin (Hinds and Kim, 2002). PEG is highly biocompatible, has been found to be nontoxic, and is approved by the U.S. FDA for use in drugs, foods, and cosmetics and is a readily available polymer species which can be tailored in many different forms including single chains, branched chains, dendrimers and star polymers (Fuertges and Abuchowski, 1990). In recent years a lot of research has been carried out on PEGylated proteins and many of them have been commercialized including granulocyte colony stimulating factor, alpha interferon 2b and 2a, and human growth hormone (Veronese and Harris, 2002).

3.8.1.4 Active transport and receptor mediated endocytosis

The active transport of proteins is not significant for high molecular weight proteins and peptides and is primarily utilized for molecules with low molecular weights (less than 1000 Da). Therefore, absorption by receptor-mediated endocytosis is more often investigated to increase the absorption of per-orally administered proteins (Stoll *et al*., 2000). Receptor mediated endocytic pathway requires addition of a ligand or a spacer with protein moiety which ensures the endocytic uptake of protein and the two most studied entities that have been long associated as ligands in receptor mediated protein uptake are transferrin (Tf) and cobalamin. Many researchers have investigated the potential for oral delivery of Tf-conjugated insulin for systemic targeting (Xia *et al*., 2000; Kavimandan *et al*., 2006) with a view to increase the oral bioavailability of insulin and reported increased transport of insulin across *in vitro* models of the intestinal epithelium using Caco-2 cells.

3. 8.2 Advanced delivery systems

Often times, efforts to modify the absorption of the protein drugs through classical approaches are coupled with advanced delivery systems which maximize the therapeutic benefit. A summary of these drug delivery systems is presented in Table 1.3 with their discussion I subsequent sections.

Table 1.3 Advanced delivery systems for oral delivery of proteins and peptides.

Approach	***Issues addressed***	***Pros***	***Cons***	**Status**
Liposomes and micelles	Overcoming enzymatic barrier by encapsulation	Reduced protein loss, enhanced absorption	Instability and leaky nature	Preclinical
Mucoadhesive delivery	Overcoming physical barrier	Enhancement in protein absorption, enzyme inhibition and permeation enhancement effect in some cases	Long term effects not yet determined, build up in GI tract can cause toxicity	Preclinical
Hydrogels	Overcoming enzymatic barrier by encapsulation	Reduced protein loss, enhanced absorption	Same as mucoadhesive delivery	Preclinical
Particulates (Micro & nano particles)	Overcoming enzymatic and physical barrier	Reduced protein loss, enhanced absorption	Size/surface dependent toxicity concerns. Long term effects from oral route not yet determined.	Preclinical

3.8.2.1 Liposomes and micelles

Liposomes are bilamellar spherical organizations of lipids in which a lipid bilayer forms around an aqueous core (Gregoriadis, 1988). Liposomal delivery of proteins to the GI tract has been investigated by a number of research groups due to their aqueous core and similarity to the cell membrane (Zasadzinski, 1997). Several critical findings were similar throughout these studies. Liposomes are primarily absorbed by the

pinocytosis in the Peyer's patches through absorptive M cells which have high affinity for hydrophobic carriers thus making liposomes an ideal candidate for absorption (Eldridge *et al*., 1990). Moreover, their ease of preparation, versatility of their composition, possibility of using mild conditions when fabricated with proteins and peptides and biocompatibility makes liposomes one of the most suitable carriers for oral delivery of insulin. Kisel *et al*., reported liposomes with phosphatidylethanol as a carrier for oral delivery of insulin and when these liposomes were administered to diabetic rats at a dose of 12 IU/kg orally, hyperinsulinemia was observed along with reduction in plasma glucose levels which was sustained almost for 6 h followed by increase till 24 h but no correlation between insulin level and glucose concentration in the rat blood after oral administration of phosphatidylinositol-containing liposomes was observed (Kisel *et al*., 2001).

Recently, Dwivedi et al., reported silica coated liposomes for oral delivery of insulin. *In vivo* evaluation of formulations in healthy Wistar rats revealed their glucose lowering ability at a dose of 28 IU/kg compared to subcutaneous insulin administration at a dose of 4 IU/kg. The glucose reduction profile with liposomes showed biphasic behaviour where first glucose reduction occurred at 0.5 h followed by next glucose reduction at 2 h which was sustained till 4 h in group administered with oral liposomes whereas in group administered with subcutaneous insulin, hyperglycaemic levels were returned by 4 h (Dwivedi *et al*., 2010). The instability of liposomes in the GI tract due to aggregation in concentrated solutions and their leakiness due to transient nature of lipid layer along with action of lipases and bile salts making them to ooze out the drug content render them questionable in oral delivery of proteins (Degim *et al*., 2006).

3.8.2.2 Mucoadhesive delivery

Mucoadhesion phenomenon has been widely investigated for the trans-epithelial transport of insulin across the mucosa of GI tract leading to increased oral bioavailability of insulin (Takeuchi *et al*., 2001) because these systems bind to the mucus layer covering the epithelial and brings the protein within close proximity to the epithelial layer. In addition to this, these delivery systems are also characterized by increased residence time in the body paralleled with generation of high drug pay load at the sight of absorption. The most commonly investigated mucoadhesive polymers include chitosan, poly(acrylic acid), poly(methacrylic acid), sodium alginate, and cellulose derivatives (Krauland *et al*., 2004). Whitehead *et al*., utilized unidirectional release from mucoadhesive patches to significantly improve the relative bioavailability (compared to subcutaneously administered insulin) of insulin when placed *in situ* in the intestine of rats (Whitehead *et al*., 2004). The polymers which provide enzyme inhibition and/or permeation enhancement through indirect modulation of the immediate surroundings leading to increase in protein uptake were also tried (Lee *et al*., 1999) but still most of these strategies are in preclinical stages.

3.8.2.3 Hydrogels

Hydrogels are three dimensional water-swollen, cross-linked polymeric networks produced by the reaction of one or more monomers and are highly biocompatible as they resemble living tissue due to their high water content and soft texture like cartilage and tendons making them ideal systems for delivery of bioactives (Gupta *et al*., 2002). Hydrogels are finding increasing applications in field of drug delivery and biomedical sciences for delivery of proteins like insulin and glucose sensitive hydrogels have been

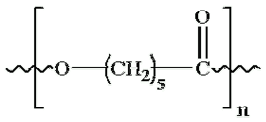
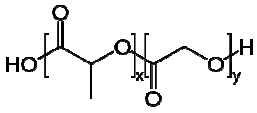
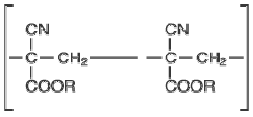
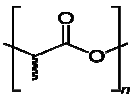
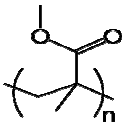
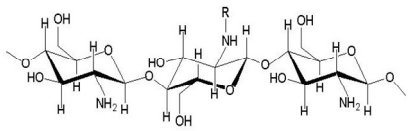
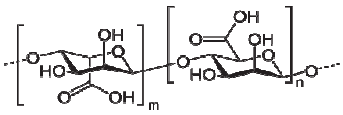
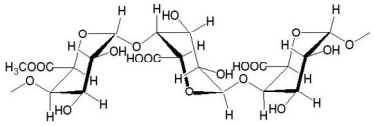
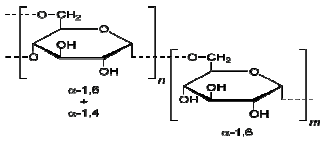
experimented for stimuli based insulin delivery in particular (Peppas *et al*., 2000; Qiu and Park, 2001; Kashyap *et al*., 2007). Several different pH responsive hydrogels have been investigated for oral insulin delivery (Podual *et al*., 2000; Makham, 2005) with limited success.

3.8.2.4 Particulate

3.8.2.4.1 Nanoparticles

Current era belongs to particulate technology based on polymeric materials for oral delivery of proteins in form of microspheres and nanoparticles which differ only in their size. Furthermore, these particulates can be sub classified as matrix (dispersed) systems and reservoir (capsule) systems based on location of drug inside these polymeric systems. Matrix systems are based on the homogeneous distribution of drug molecules throughout the polymer matrix resulting in pin ball structures whereas reservoir systems are characterized by a body and shell design in which the drug is contained within the centre of a particle which is surrounded by a polymeric shell to form a drug reservoir system (Gupta and Kompella, 2006). Many polymers and non polymeric excipients with different formulation processes have been tried out to entrap or encapsulate insulin in biodegradable and non biodegradable nanoparticles in the past with an aim to allow oral delivery of insulin feasible (Table 1.4). Individual nanoparticulate systems based on different polymeric and non polymeric materials are discussed in the subsequent sections.

Table 1.4 **Synthetic and natural polymers used for preparing insulin nanoparticles with their structures, related physicochemical properties and approval status.**

Synthetic polymers	
	PCL: Biodegradable polyester, Tg -60 °C, slow degradation, FDA approved
	PLGA: Biodegradable polyester, Tg 40-60 °C, variable degradation depending on PLA and PGA ratio and molecular weight, FDA approved
	PACA: Biodegradable polyesters, Tg 45-47 °C, slow degradation depending on molecular weight, ethyl, n butyl and octyl substituted versions are FDA approved
	PLA: Biodegradable polyesters, Tg 50-80 °C, molecular weight dependent degradation rate, FDA approved
	PMMA: Non biodegradable acrylic polymer, Tg > 225 °C, molecular weight dependent degradation rate, FDA approved
Natural polymers	
	CS: Biodegradable cationic polyaminosaccharide, Tg 203 °C, molecular weight and deacetylation dependent degradation rate, FDA approved
	AA: Biodegradable anionic polysaccharide, Tg 110 °C, molecular weight dependent degradation, FDA approved
	PC: Biodegradable hetero-polysaccharide, Tg 37 °C, molecular weight dependent degradation, FDA approved
	DX: Biodegradable polysaccharide, Tg 222.7 °C, concentration and molecular weight dependent degradation, FDA approved

PACA: Poly(alkylcyanoacrylate); PCL: polycaprolactone; PLGA: poly(lactide-co-glycolide); PLA: polylactic acid; PMMA: poly(methylmethacrylate); CS: chitosan; AA: alginic acid; PC: pectin; DK: dextran.

3.8.2.4.1.1Cyanoacrylate and microemulsion based systems

Insulin associated to polymeric nanoparticles remains biologically active after oral administration in diabetic or healthy animals in contrast to free insulin, which has no significant effect. The protection of insulin against proteolytic enzymes was first investigated *in vitro* by incubating insulin-loaded poly(alkylcyanoacrylate) (PACA) nanocapsules in the presence of gastric (pepsin) and pancreatic (trypsin, chymotrypsin and pancreatin) enzymes (Damge *et al*., 1997). It was demonstrated that nanocapsules preserved 75% of insulin, but free insulin in solution was largely degraded. Research investigating the efficacy of insulin nanocarriers in experimental model of diabetes was first time demonstrated by Damge *et al*., using insulin-loaded poly(isobutylcyanoacrylate) (PIBCA) nanocapsules which were administered orally to fasted streptozotocin (STZ) induced diabetic rats and showed normalization of hyperglycaemia from 300 to 125 mg/dl (Damge *et al*., 1988). Further, dose dependent response was reported where 12.5 IU/kg insulin dose was efficacious for 6 days, 25 IU/kg dose for 9 days and 50 IU/kg for 20 days in lowering hyperglycaemia (Damge *et al*., 1990). The effect of nanocapsules was more pronounced in fasted animals compared to feed animals and plain insulin administered as a solution failed to elicit any glucose lowering response and this finding was in agreement with physiological absorption of insulin which never exceeds 0.5 % as reported in literature (Peppas and Kavimandan, 2006). This was the first proof of concept that insulin encapsulated in polymeric nanocapsules retained its pharmacological activity and was therapeutically efficacious in reducing the elevated glucose levels when administered orally in diabetic rats and moreover, the intensity of glucose reduction was also a function of site of administration in gastrointestinal tract as ileum had most pronounced

absorption (Michel *et al*., 1991). A similar approach was carried out in diabetic dogs at 100 IU/kg insulin dose and maximal glucose reduction was obtained at the 9th day of dose administration along with reduction in glycosuria, blood glucagon and somatostatin (Damge *et al*., 1995). Sai *et al*., reported reduced incidence of diabetes and lymphocytic inflammation of endogenous islets when insulin nanocapsules were fed to genetically autoimmune type 1 diabetic mice at a dose of 100 IU/kg using polymeric colloidal particles (<300 nm) consisting of oily core surrounded by a polymeric coat prepared using poloxamer-188 as surfactant (Sai *et al*., 1996). When these nanocapsules, dispersed in oily medium containing poloxamer and deoxycholic acid were administered orally in STZ induced diabetes rats, a prolonged anti-hyperglycaemic effect from 2nd h till 13th day was observed indicating potential role of oil and surfactants in protecting insulin from harsh GI conditions (Damge *et al*., 1997).

The role of absorption enhancers in modulating the *in vivo* efficacy of insulin loaded poly(ethylcyanoacrylate) (PECA) nanospheres was first time reported where sodium cholate was used as absorption enhancer and glucose reduction lasted 8 h after oral administration (Radwan, 2001). In another study glycyrrhizic acid and capric acid were used as absorption enhancers and biological activity up to 12 days in 50% of diabetic rats was observed (Radwan and Aboul-Enein, 2002).

Graf and co-workers prepared and characterized poly(alkylcyanoacrylate) nanoparticles based on microemulsions with different structure-types and containing insulin as a model protein. A phase diagram of the pseudoternary system isopropyl myristate, caprylocaproyl macrogolglycerides, polyglycerol oleate and water was established. The size distributions of the resulting nanoparticles prepared by interfacial polymerisation from selected

microemulsions using ethyl-2 cyanoacrylate and butyl-2 cyanoacrylate were unimodal but template- and monomer-dependent and ranged from 160 to 400 nm. Entrapment ranged from 11.5 to 20.9% and a near zero-order release was observed after an initial burst. Release of insulin was monitored for 6h. Insulin-loaded nanoparticles were 320-350 nm in size. The microemulsion-structure was retained during the polymerisation process as determined by NMR. This study showed that these microemulsions with flexible formulation possibilities for the solubilisation of peptides and proteins depending on their microstructure could serve well as a platform for designing encapsulation processes for oral delivery of insulin (Graf *et al*., 2008). The study showed that microemulsions can be used as nanoparticle templates for the incorporation of insulin, or peptide and protein delivery in general, with a simple one-step preparation by interfacial polymerisation. The resulting nanoparticles were spherical but only showed modest entrapment efficiency due to insulin molecules rapidly partitioning in the aqueous pseudo-phase and thus escaping the relatively slowly-forming polymer network. The low entrapment of insulin in the nanoparticles, combined with the possible influence of monomer concentration on the entrapment efficiency and the possibility that insulin interferes with the polymerisation will have implications for the release of drug from these carriers and this warrants their further examination. Based on their previous findings where microemulsions served as a template for obtaining insulin nanoparticles Graf and co-workers investigated microemulsions containing isopropyl myristate, caprylocaproyl macrogolglycerides, polyglyceryl oleate with an aim to enhance the entrappment efficiency of insulin in microemulsion formulations and to investigate the *in vitro* release and bioactivity of insulin in nanoparticles dispersed in the microemulsion

templates. The prepared nanoparticles were spherical in size (200-400 nm) without significant difference between different microemulsion templates, types and amounts of monomer. Entrapment efficiency increased (from 16-32% for PECA nanoparticles produced from o/w template, from 11.5-52.3% for PBCA nanoparticles prepared from o/w template, from 20.9-42% for PECA nanoparticles prepared using bi-continuous template and from 17.5-43.6% for PBCA nanoparticles from w/o template) significantly with increasing monomer concentration from 200 to 1200 mg but decreased with increasing aqueous fraction in the microemulsion template. Insulin loading however, showed an opposite trend. *In vitro* release profiles of insulin from the nanoparticles dispersed in the microemulsion templates were controlled by the monomer concentration only and in all the nanoparticles (PECA and PBCA, prepared using different templates), a burst release of insulin was observed around 15 min after which a very inconsistent release was observed. The release of insulin from nanoparticles almost followed zero order profile after 60-120 min and almost in all the cases complete release of entrapped insulin was seen at the end of 6 h. Intra-gastric administration of nanoparticles in diabetic rats showed an increase in plasma glucose levels for first 3 h thereafter decrease in glucose levels was observed. The rise in glucose levels with administration of nanoparticles was attributed to the physiological stress response to intra-gastric administration by rats. Insulin entrapped in PBCA nanoparticles and dispersed in the o/w microemulsion template was not able to significantly reduce blood glucose over controls at any time. Insulin-loaded PECA nanoparticles dispersed in the o/w microemulsion template caused a consistent reduction in blood glucose. A reduction to 68% of the initial blood glucose value was achieved 9 h after intra-gastric administration. Blood glucose values decreased further by 5%

over the next 3h. The reduction in blood glucose of 30–40% of the original value was maintained for at least 36h. No significant serum insulin levels were detected in the study. As regards the lack of insulin detected in the rat serum, it remained unclear whether this was simply due to insulin being quickly transported out of the blood into the peripheral tissues or due to an ineffective/delayed release from the formulations which resulted in concentrations too low to be detected, or a combination of both (Graf *et al*., 2009).

McDowell and co-workers reported insulin loaded PECA nanoparticles for oral delivery in brushtail possum. These nanoparticles were prepared using interfacial polymerization and had uniform size of 200-300 nm with 78% insulin entrappment efficiency. *In vitro* release of insulin-loaded PECA nanoparticles in phosphate buffer (0.067 M, pH 7.4) at 37°C was triphasic and showed controlled release of insulin for more than 6 h with more than 30% of insulin still entrapped inside the nanoparticles. Following *in vitro* incubation of nanoparticles with enzyme solutions prepared from the GIT of brushtail possum, lumen enzymes were more aggressive towards insulin compared to mucosal enzymes and the hindgut lumen was the GIT region with the lowest degradation. For the first time in a marsupial species, the *in vivo* pharmacokinetics of insulin-loaded, PECA nanoparticles were investigated following i.v. and intra-caecal administration and measured by radioimmunoassay. The low cross reactivity of human and endogenous brushtail possum insulin suggested that brushtail possum was a suitable non-diabetic model to study pharmacokinetics of insulin. The i.v. pharmacokinetics of insulin solution and insulin-loaded nanoparticles were similar. On intracaecal dosing, co-administration of a permeation enhancer (EDTA) resulted in a small increase in plasma insulin concentration

compared to insulin-loaded nanoparticles alone but the absolute bioavailability (compared with intravenous insulin injection) of insulin from PECA nanoparticles was less than 1 %. The study demonstrated that transit time to the caecum of brushtail possum following oral delivery was 12 h for fluid and particulate formulations < 1 mm diameter and was independent of the time of day the dose was given. Brushtail possum is a potential non-diabetic model for the study of insulin pharmacokinetics (McDowell *et al.*, 2009).

3.8.2.4.1.2 Methacrylic acid and acrylic acid based systems

Methacrylic acid and acrylic acid based polymers have also been evaluated as potential materials for oral delivery of insulin. Foss and co-workers reported nanoparticles made of methacrylic acid (MA) and acrylic acids (AA) grafted with PEG to form P (AA-g-PEG) and methacrylic acid grafted with PEG which resulted in P (MA-g-PEG) configuration. Insulin was loaded onto polymers by adsorption process where polymers were incubated with concentrated insulin solution for a particular time after which pH was lowered to complex the polymers to trap insulin inside their matrix. The prepared systems displayed pH dependent stability and particle size varied from 200 nm at pH 2.0 to 2000 nm at pH 6.0 for both the types of nanoparticles. *In vitro* release was carried out in USP II dissolution apparatus and pH of the media was changed after 1 h from acidic (pH 3.0) to mildly basic (pH 7.0) so as to reciprocate the physiological conditions. It was observed that in nanoparticles prepared using P (AA-g-PEG), 20% of the entrapped insulin was released in gastric condition till 60 min and when pH was changed to 7.0, cumulatively 80% insulin was released and by the end of 4 h complete insulin was released from the nanoparticles. Whereas in

nanoparticles prepared using P (MAA-g-PEG), almost 90% of the entrapped insulin was released in first 60 min (under acidic pH) and release was completed by 4 h. In both the cases, the release was controlled though the release rate order was not determined. When these nanoparticles were administered in diabetic Wistar rats, a considerable change in elevated glucose levels was observed in comparison to control group. The insulin-loaded particles had a substantial effect on glucose in 30 min. Insulin-loaded P(AA-g-PEG) nanoparticles caused a significant serum glucose reduction, nearly 50%, while the insulin-loaded P(MAA-g-PEG) nanoparticles had a larger experimental error. The study was based on incorporating pH sensitive and calcium binding properties in the system by utilizing methacrylic acid and acrylic acid and for stabilizing insulin in the system using PEG (Foss *et al.,* 2004)

Agarwal *et al*, reported poly(methacrylate) based microparticles for oral delivery of insulin and characterized microparticles prepared by the co-precipitation process by size exclusion chromatography, differential scanning calorimetry, fourier-transform IR spectroscopy, and powder X-ray diffractometry, and studied the solid state conformation of insulin before and after entrapment in the polymeric carrier. Microparticles were prepared by dissolving insulin in 0.01 N HCI and alcohol USP to get a final concentration of 32% v/v. Eudragit L100, a representative poly(methyacrylate) polymer, was then dissolved in this solution which was transferred to a beaker containing cold water with homogenization to obtain microparticles. Insulin powder, microcapsules, and a physical mixture of insulin and Eudragit L100 were then analyzed by SEC-HPLC, DSC, FTIR, and XRD to observe changes in protein conformation as result of the manufacturing process. While DSC, XRD and FTIR results were of limited value due to limits of instrument

sensitivity and not much information could be obtained using these techniques, on the other hand size exclusion chromatography data indicated that higher order aggregates were not formed during microcapsule formation indicating the stability of insulin was significantly preserved during the particle preparation process. It was concluded that formulating insulin into microparticles by the co-precipitation process is an attractive and stable method for protein delivery and might be suitable for oral delivery of insulin (Agarwal *et al*., 2008). The *in vivo* behaviour of insulin microparticles in lowering elevated glucose levels in model of diabetes was not evaluated in the study and it becomes difficult to conclude whether the insulin entrapped in microparticles was pharmacologically active or not though the results of *in vitro* studies indicated a significant preservation of insulin structure as revealed by results of size exclusion chromatography.

Deutel *et al*., reported a novel insulin-thiomer complex nanoparticulate delivery system based on poly (acrylic acid) intended for oral administration. Insulin loaded nanoparticles were obtained by the formation of hydrogen bonds between poly (vinyl pyrrolidone) (PVP) and poly (acrylic acid)-cysteine (PAA-Cys) or poly (acrylic acid) (PAA), respectively, in the presence of insulin. The particles prepared had average size from 250-400 nm with low polydispersity values. Transmission electron microscopy indicated that there was no aggregation between particles, spherical morphology and overall sizes were in submicron ranges. To facilitate administration of nanoparticles via oral route and to mimic the conventional dosage form, nanoparticles were formulated into enteric coated tablets and suspensions. When administered *in vivo* in diabetic rats, the particles exhibited 22% reduction in blood glucose levels within 30 min of formulation administration and the anti-hyperglycaemic activity lasted till 4 h. The pharmacokinetics of

nanoparticles exhibited a short T_{max} about 30 min and C_{max} about 1.8 μg/l for nanoparticles made using PAA-Cys PVP and around 1.25 μg/l for nanoparticles prepared using PAA and PVP. Furthermore a 2.3-fold improvement of the AUC of insulin could be achieved due to the use of thiolated PAA instead of unmodified PAA (Deutel *et al*., 2008). This study showed the benefits of using mucoadhesive polymers for oral delivery of insulin where cysteine conjugation with poly (acrylic acid) lead to significant improvement in AUC compared with systems without cysteine conjugation.

One of the studies aimed at evaluation of nanoparticles in protecting insulin from harsh gut conditions was carried out by Perera and co-workers. They used thiolated poly (acrylic acid) nanoparticles as a valuable matrix to protect insulin from degradation by serinproteases of the intestine and characterized the *in vitro* behaviour of nanoparticles in presence of serine proteases chiefly elastase, trypsin and chymotrypsin. Within *in vitro* degradation studies with trypsin, alpha-chymotrypsin, and elastase it could be demonstrated that the obtained nanoparticles are capable of protecting 44.47% of the initial insulin amount from tryptic degradation, 21.33% from chymotryptic degradation, and 45.01% from degradation by elastase compared to insulin solutions (Perera *et al*., 2009). This study once again provided proof of concept that insulin encapsulated or entrapped in nanoparticles is protected from the harsh gut environment especially enzymatic degradation and extremely low pH conditions, which further assures its stability and pharmacological action *in vivo*.

3.8.2.4.1.3 Chitosan based systems

Initial studies involving use of chitosan were primarily based on tight junction opening properties of chitosan and in one of the studies by Pan and

co-workers, chitosan were used as a coating material and as a stabilizer for PLGA nanoparticles. PLGA nanoparticles entrapping insulin were prepared using the double emulsion method and chitosan was used as the surfactant for stabilizing the formulation. The formed nanoparticles were spherical in shape, unimodal with a particle size range from 200-400 nm and positive zeta potential values. Uncoated PLGA nanoparticles had lower entrappment efficiencies and showed burst release *in vitro* whereas chitosan coating not only improved the entrappment efficiency but also retarded the burst release of insulin from nanoparticles. When administered via intra-gastric route in diabetic rats, chitosan coated PLGA nanoparticles demonstrated significant lowering of plasma glucose levels 14-16 h post administration compared to uncoated nanoparticles given at the same dose from the same route. Moreover, chitosan coated nanoparticles displayed an increase in relative pharmacological bioavailability by 15% (compared with oral administration of uncoated nanoparticles at same dose). Overall results from this study demonstrated the potential of chitosan in modulating entrappment efficiency of insulin in PLGA nanoparticles, retarding burst release of insulin and gave a flexibility of altering the zeta potential of nanoparticles which plays a significant role in mucoadhesion and absorption as positively charged chitosan coated PLGA nanoparticles are more liable to adhere with negatively charged gastric mucosa (Pan *et al*., 2003). Chitosan has also been used as a carrier matrix for preparing insulin nanoparticles both in its native form and in modified form. The latter approach has gained a considerable interest in recent years because alteration of chemical structure of chitosan with different groups modifies its physicochemical properties. Quian and co-workers reported several novel functionalized graft copolymer nanoparticles consisting of chitosan (CS) and the monomer methyl methacrylate (MMA),

N-dimethylaminoethyl methacrylate hydrochloride (DMAEMC), and N-trimethylaminoethyl methacrylate chloride (TMAEMC). The modification of chitosan was carried out in order to improve its solubility in broad pH ranges. The nanoparticles were prepared using free radical polymerization and they had an average size of 150-280 nm with low polydispersity values as revealed by results of dynamic light scattering and transmission electron microscopy analysis. Insulin loaded nanoparticles were near higher size range and had almost 100% entrappment efficiency. Stability of insulin inside nanoparticles was analyzed using FTIR which showed significant preservation of secondary structure of insulin inside nanoparticles. *In vitro* release showed that these nanoparticles provided an initial burst release followed by a slowly sustained release for more than 24 h. These graft copolymer nanoparticles enhanced the absorption and improved the bioavailability of insulin via the gastrointestinal (GI) tract of normal male Sprague-Dawley (SD) strain rats to a greater extent than that of the phosphate buffer solution (PBS) of insulin but pharmacodynamic glucose lowering ability of modified chitosan insulin nanoparticles was not evaluated (Quian *et al.,* 2006).

Chitosan has also been investigated as an absorption enhancing excipients in nanoparticle formulations. In one study gold reduced chitosan nanoparticles entrapping insulin were reported for oral delivery owing to its electronegative property where it can be used as a reducing agent to obtain the gold nanoparticles, and due to its polyelectrolyte nature which would also act to stabilize the formulation. It was envisaged that this strategy would render dual advantage by providing sufficient charge through the amino groups which will aid in the subsequent attachment of the biomolecules as well render optimum stability and subsequently help to improve the uptake

of the nanoparticles. The prepared nanoparticles were under 100 nm in size with broad size distribution. Varying concentrations of chitosan used for the synthesis of gold nanoparticles demonstrated that the nanoparticles obtained at higher chitosan concentrations (>0.1% w/v) were stable showing no signs of aggregation. The fluorescence spectra of native insulin and insulin entrapped inside nanoparticles was reported to be similar though careful examination of overlay reveals the difference indicating some degree of secondary structure alteration in insulin. The nanoparticles also showed long term stability in terms of aggregation for about 6 months. Insulin loading of 53% was obtained and found to be stable after loading. Pharmacodynamic studies were conducted in diabetic Wistar rats. Blood glucose lowering at the end of 2 h following administration of insulin loaded gold nanoparticles to diabetic rats was found to be 30.41 and 20.27% for oral (50 IU/kg) and nasal (10 IU/kg), respectively.

Bayat and co-workers reported another series of modified chitosans for insulin nanoparticles intended for oral delivery. They synthesized triethyl chitosan (TEC) and dimethyl ethyl chitosan (DMEC) by partial quaternization of chitosan and degree of quaternization of about 52 and 51% were kept for DMEC and TEC, respectively, which has been shown to be optimum for the permeation enhancing effect. Based on these polymers, nanoparticles were prepared using polyelectrolyte complexation technique using insulin solution. The obtained nanoparticles had sizes from 171-257 nm with entrapment efficiencies around 85%. The *in vitro* release profiles of insulin from chitosan and its derivatives nanoparticles was studied at pH 6.8. Insulin released from CS, TEC and DMEC nanoparticles after 5 h in pH 6.8 phosphate buffer solutions were 33.4, 44.3 and 47.3%, respectively. These results indicated that these systems had a small burst effect and were capable

of sustaining insulin release for 5 h, indicating a good insulin incorporation Complexation inside nanoparticles. Insulin release from TEC and DMEC nanoparticles was higher than CS nanoparticles because of more solubility of TEC and DMEC polymers in neutral and alkaline media. Ex vivo study in rat colon indicated that after 120 min at 37 °C, the amount of insulin transported across the intestinal barriers with TEC and DMEC nanoparticles was significantly higher than free insulin, and presence of free polymers enhanced the transport. Consequently, on the basis of these results, it was hypothesized that insulin was not able to transport across membrane and nanoparticulate systems facilitated the transport of insulin through the intestinal barriers and presence of quaternized derivatives of chitosan enhanced the transport. Pharmacodynamic study in diabetic Wistar rats indicated the failure of free insulin in eliciting a glucose lowering response when injected directly in the colon of rats whereas all the nanoparticle formulation showed significant lowering of blood glucose levels when injected in the colon with TEC and DMEC nanoparticles showing better performance than CS indicating the role of chemical modification in enhancing absorption of nanoparticles. Though the results of the study showed the potential of quaternized derivatives of chitosan in lowering the elevated glucose levels in diabetes, but the mode of formulation administration by passing the stomach does not rule out the possibility of formulation failure if given via oral route as chitosan and its derivatives are al prone to dissolution in the acidic environment, so the real picture of formulation action is still missing (Bayat *et al*., 2008).

In another study involving use of modified chitosan nanoparticles for oral delivery of insulin a basal insulin formulation was designed and its structural characteristics were investigated *in vitro* and biological activities in

type 1 diabetic rats. Zinc-crystallized insulin was physically loaded into hydrophobically modified glycol chitosan (HGC) nanoparticles by a dialysis method. Glycol chitosan is a novel chitosan derivative and carrier of drugs because of its solubility in water at all pH and biocompatibility. The series of insulin-HGC formulations were prepared with different feed weight ratio of insulin to HGC from 0.5:1 to 4:1. The blood glucose lowering response of nanoparticles via oral administration were investigated in streptozotocin-induced diabetic rats after single subcutaneous injection of regular insulin and insulin-HGCs. The highest loading efficiency and content were obtained in insulin-HGC when a 1:1 feed weight ratio of insulin to HGC was employed. The hydrodynamic diameter of insulin-HGC nanoparticles were in the range of 200 to 500 nm with narrow size distribution. Insulin-HGC effectively sustained insulin release up to 40% within 12 hours followed by a slower controlled release. Insulin-HGC showed an extended blood glucose lowering effect up to 24 h and provided normal blood glucose levels after oral glucose (1.5 g/kg) load at 24 hours post-injection while regular insulin showed severe hypoglycemia. The prolonged time action profiles and low variability of insulin-HGC formulation resulted in improved blood glucose control in diabetic rats and fulfilled a pattern desirable of a basal insulin (Jo *et al*., 2008).

Reis *et al.*, reported polyelectrolyte based nanocarriers for oral delivery of insulin. They prepared dextran alginate nanoparticles coated with chitosan and albumin to provide mucoadhesive, biodegradable, biocompatible, and acid-protective properties to the nanoparticles with high insulin entrapment efficiencies of 90, 82, and 66% for uncoated, chitosan-coated, and albumin-chitosan-coated alginate nanospheres, respectively. The particles were prepared using nanoemulsion dispersion followed by triggered in situ gel

complexation method and had an average size around 300 nm. Formulations with smaller size and high payload of albumin coating were orally administered to diabetic Wistar rats and physiological response following oral delivery showed that insulin albumin-chitosan-coated alginate nanospheres reduced glycemia approximately 72% of basal values (Reis *et al*., 2008). Pharmacokinetic studies were not conducted. The study indicated that the choice of coating polymer had significant influence on the insulin release profile and was crucial to prevent peptic digestion. The use of additional protein albumin is first time reported and the formulation benefits from it as albumin serves as an important enteric coating providing acid and protease protection enabling uptake of active drug following oral dosage.

The modification of chitosan is generally carried out in order to render it more mucoadhesive and to change its physicochemical properties to suit drug delivery applications. One of the study conducted by Mi and co-workers utilized modification of chitosan by N-trimethyl groups as native chitosan is insoluble at physiological pH and mucoadhesive properties are executed when it is solubilised in acid in protonated form. They synthesized N-trimehyl chitosan (TMC) polymers with different degrees of quarternization so as to study the effect of degree of quarternization on mucoadhesion. Nanoparticles (NPs) self-assembled by the synthesized TMC and poly (gamma-glutamic acid) (gamma-PGA, TMC/gamma-PGA NPs) were prepared for oral delivery of insulin. The loading efficiency and loading content of insulin in TMC/gamma-PGA NPs were 73.8 % and 23.5 %, respectively. TMC/gamma-PGA NPs had superior stability in a broader pH range to CS/gamma-PGA NPs; the *in vitro* release profiles of insulin from both test NPs were significantly affected by their stability at distinct pH environments. At pH 7.0, CS/gamma-PGA NPs became disintegrated,

resulting in a rapid release of insulin, which failed to provide an adequate retention of loaded insulin, while the cumulative amount of insulin released from TMC/gamma-PGA NPs was significantly reduced. At pH 7.4, TMC/gamma-PGA NPs were significantly swelled and a sustained release profile of insulin was observed. Confocal microscopy confirmed that TMC/gamma-PGA NPs opened the tight junctions of Caco-2 cells to allow the transport of insulin along the paracellular pathway. Transepithelial-electrical-resistance measurements and transport studies implied that CS/gamma-PGA NPs can be effective as an insulin carrier only in a limited area of the intestinal lumen where the pH values are close to the pKa of CS. In contrast, TMC/gamma-PGA NPs may be a suitable carrier for transmucosal delivery of insulin within the entire intestinal tract. One of the major objectives of formulating insulin into nanoparticles is to assure its safe transit from gastric acid and proteases. Polymers like chitosan and chitosan derivatives which are acid sensitive need to be analyzed for drug release in acidic environment in order to assure that no drug is released from nanoparticles during their transit in stomach. In addition to this, the current study did examined the potential of modified chitosan nanoparticles to translocate by opening tight junctions in Caco-2 cell lines, but systematic pharmacokinetic and pharmacodynamic studies in animal model were not conducted to evaluate the fate of particles when give via oral route. The study again provided proof of concept that modulation of chemical structure of chitosan alters its mucoadhesive properties and also render it soluble at physiological pH (Mi *et al.*, 2008).

However, TMC exhibits decreased intrinsic mucoadhesivity than chitosan salts, which might be attributed to strong interactions between fixed positive charges on the quaternary amino groups that lead to decreased

chain flexibility and inhibited interpenetration into the mucus layer. Thiolated polymers (thiomers) have been developed as a category of mucoadhesive polymers with reactive thiol groups immobilized on the polymeric structure. They can tightly adhere to the intestinal mucus layer for a prolonged time through covalent bonding with mucin glycoproteins via thiol-disulfide exchange reactions, hence providing a steep drug concentration gradient at the absorption sites and exerting an additional permeation enhancing effect. Besides, the permeation enhancing effect of thiomers is also ascribed to the inhibition of protein tyrosine phosphatase and intestinal P-glycoprotein. Thiolated chitosan, however, is also insoluble at physiological pH, which restricts its effective application. Therefore Yin and co-workers prepared, TMC-cysteine conjugate (TMC-Cys) which was expected to combine the mucoadhesion and permeation enhancing effects of TMC and thiomers that were related to different mechanisms. TMC-Cys/insulin nanoparticles (TMC-Cys NP) were prepared through self-assembly, and were characterized for their physicochemical properties. TMC-Cys nanoparticles were prepared in various molecular weights from 30, 200, and 500 kDa. All nanoparticles possessed sizes of 100–200 nm, positive surface charges, and insulin EE of approximately 90% (except TMC-Cys 500 kDa). *In vitro* release showed that higher degree of quarternization of TMC-Cys led to slower insulin release from TMC-Cys NP in 0.2 M PBS (pH 7.4). A faster and more complete release was observed for TMC-Cys(30 kDa) NP, while similar profiles with slower release rates were obtained for TMC-Cys(200 kDa) NP and TMC-Cys(500 kDa) NP. *In vitro* mucoadhesion studies revealed that TMC-Cys NP exhibited an about 2-fold increase in mucin adsorption compared to TMC NP. Besides, an increase in the molecular weight and degree of quarternization, led to a slightly stronger

affinity towards mucin. In the DSC thermograms, the endothermic peak at about 230 °C for mucin that characterized its melting disappeared after incubation with TMC-Cys at pH 7.4 for 8 h, which evidenced that nearly the entire amount of mucin had been conjugated onto TMC-Cys through disulfide bonding. Permeation studies in Caco-2 cell lines showed that As compared to insulin solution and TMC NP, TMC-Cys NP led to enhanced P_{app} values of insulin through rat ileum by 3.3–11.7 and 1.7–2.6 folds, promoted Caco-2 cell internalization by 7.5–12.7 and 1.7–3.0 folds, and augmented uptake by Peyer's patches by 14.7–20.9 and 1.7–5.0 folds, respectively. Biocompatibility evaluation demonstrated that thiolation exerted unappreciable influence on the cytotoxicity of TMC ($p > 0.05$). Polymer (30 and 200 kDa) solutions were confirmed non-toxic at the test concentration and incubation time, while polymer (500 kDa) solutions induced appreciable intestinal damage despite insignificant difference to the negative control in the LDH assay ($p > 0.05$). Besides, all the TMC-Cys NP demonstrated absence of toxicity, including polymers of 500 kDa. *In vivo* pharmacodynamic studies in diabetic rats showed oral and ileal administration of TMC-Cys(200,30) NP led to notable hypoglycaemic effects as compared to insulin solution, which lasted until 8 h and 7 h post-administration, respectively, with the maximum blood glucose depression of 35% and 70%, respectively. Besides, a more potent as well as prolonged hypoglycaemic effect was observed when compared to TMC(200,30) NP, which was in agreement with the mucoadhesion and permeation enhancement results. The results of study demonstrated that through thiolation of TMC, the advantages of TMC and thiomers for oral delivery of protein drugs were combined, including mucoadhesion and permeation enhancing effects that were related to different mechanisms and these two

different strategies can be combined for oral delivery of insulin (Yin *et al*., 2009).

It is established by various groups that the medium chain fatty acids are capable of enhancing paracellular drug absorption. It is reported that sodium salts of medium chain fatty acids such as caprylate (C 8), caprate (C 10) and laurate (C 12) are able to enhance the paracellular permeability of hydrophilic compounds. Based on this Rekha and Sharma modified chitosan using lauryl succinyl groups to prepare lauryl succinyl chitosan (LSC). Nanoparticles and microparticles were prepared using LSC and tripolyphosphate adopting ionic gelation method. The particles were negatively charged with size ranging from 315 nm to 1.090 μm. The mucoadhesive capacity was established ex vivo using the jejunum of rat intestine. Confocal microscopy studies showed the tight junction permeability in Caco 2 cells and *in vivo* uptake of the FITC-insulin from loaded nanoparticles by the rat intestinal epithelium. Cytotoxicity studies in Caco-2 cell lines showed 100% cell viability with LSC as the polymeric matrix for the formulations. The results demonstrated that the modified chitosan with both hydrophilic (succinyl) and hydrophobic (lauryl) moieties had improved the release characteristics, mucoadhesivity as well as the permeability of the insulin compared to the native chitosan particles. The novel lauryl succinyl chitosan nanoparticles had insulin loading of about 48.1±1.3 IU/100 mg particle with a very low release at pH 1.2. The particles, however demonstrated a controlled delivery of insulin at pH 7.4. The low release at pH 1.2 at the gastric pH minimizes the loss of insulin that could happen at stomach. In addition the particles exhibited calcium binding capacity, which could be an added advantage in protecting the loaded insulin from the enzymatic degradation. The particles showed size difference

with degree of derivatization and had a negative charge. These lauryl succinyl chitosan particles were found to be highly mucoadhesive which could be due to the hydrophobic interaction of the lauryl groups to the hydrophobic domains of mucosa as well as its negative zeta potential. Pharmacodynamic studies were conducted in diabetic Wistar rats at a dose of 60 IU/kg and the results indicated 34% reduction in blood glucose levels of diabetic rats 3 h post administration of nanoparticles and was sustained up till 6 h. The study highlighted the possibility of a chitosan derivative with both hydrophilic and hydrophobic groups linked via amino group substitution to function as an oral peptide delivery system and again added to the mucoadhesive potential of chitosan for oral delivery of insulin (Rekha and Sharma, 2009).

A multitasking nanoparticulate strategy for oral insulin delivery involving chitosan was reported by Reis and co-workers. The design of nanoparticle was based on mucoadhesive, biocompatible polymers with enhanced insulin stability inside the formulations. Core-shell nanoparticles were prepared using dextran-alginate as the core complexed with chitosan-polyethylene glycol-albumin as the shell. The obtained nanoparticles had average size > 200 nm. Pharmacodynamic studies conducted in diabetic Wistar rats revealed dose dependent glucose lowering action of nanoparticles when administered orally at a dose of 25, 50 and 100 IU/kg. Glucose lowering effect of nanoparticles lasted for more than 24 h with peak effect at 14 h and after 4 days of oral nanoparticle formulation administration at a dose of 50 IU/kg, the metabolic status of diabetic rats improved with a reduction in water intake, urine excretion and proteinuria. Ex vivo studies were conducted in isolated intestinal loop of rats and the results revealed that FITC-insulin-loaded nanoparticles administered to an isolated intestinal loop were taken

up by the intestinal mucosa. They strongly adhered to villus apical enterocytes and markedly labeled Peyer's patches as seen in the results of confocal microscopy. The overall uptake and efficacy of these formulations was explained by a protective effect against proteolytic enzymes by the albumin coating, by the mucoadhesive properties of chitosan-polyethylene glycol, and by the possibility of chitosan reversibly altering tight junctions leading to an improved absorption of insulin It was concluded that nanoparticles preserved insulin and exert an antidiabetic effect after oral administration. The significant advantage of core-shell nanoparticles lies in the fact that since the size and material properties of both the core and the shell can be manipulated at will, a vast range of particles can be made with myriad properties, Because of their small size, these particles have a very high geometric surface area which is easily accessible. This can be exploited by introducing a bio-recognition or receptor (biological or synthetic) function to the surface. The particles can then act as nano-sensors for use in assay technology as well.

Strategies to design effective and safe colloidal carriers for biopharmaceuticals have evolved through applying the knowledge gained in nanotechnology to medicine. Designing a colloidal carrier to serve as a protein delivery device requires an understanding of the effect of different materials on the physicochemical, physiological and toxicological parameters for clinical application. Woitiski and co-workers evaluated the influence of formulation components on the physicochemical factors and biological function involved in the development and optimization of newly designed nanoparticles for orally dosed insulin using nanoparticulate strategy. Nanoparticles were prepared using ion gelation and complexation method where biomaterials were combined forming an alginate, dextran sulfate

and poloxamer hydrogel containing insulin, stabilized in nanoparticles with chitosan and poly (ethyleneglycol) and coated with albumin. Nanoparticles ranged in size from 200 to 500 nm with 70–90% insulin entrapment efficiency, and electrostatic stabilization was suggested by zeta potential values lower than −30 mV. This combination of formulation components was selected for insulin protection against harsh gastric pH and proteolytic conditions, and to improve insulin absorption through intestinal mucosa by combining nanoparticle uptake and insulin release at the site of absorption. *In vitro* release from nanoparticles in enzyme-free simulated digestive fluids showed that depending on the formulation, between 12% and 67% of the initial amount of insulin was released under gastric conditions. In the first 5 min, no release was observed in nanoparticles prepared with 0.22% calcium chloride or 0.25% albumin coating solution. Retention of insulin within nanoparticles was sustained for longer periods in the formulation with 0.25% albumin. However, higher levels of albumin coating resulted in reduced retention, probably due to a weakening of the interaction between insulin and the alginate nuclei. This explanation is consistent with the observation that higher concentrations of albumin coating also resulted in lower levels of entrapment efficiency. The highest overall retention of insulin under gastric conditions was observed in formulations coated with 0.25% and 0.50% albumin, and the highest release was observed for nanoparticles formulated without poloxamer 188. Other formulations providing high insulin retention were 0.03% followed by 0.04% dextran sulfate solution, which may reduce the alginate nuclei porosity in a synergistic way with higher concentrations of calcium chloride. After gastric simulation, nanoparticles were transferred into simulated intestinal fluid where

additional release of insulin occurred for a 30 min period for all formulations, eventually reaching equilibrium. At this point, no further release of insulin was observed, and up to 35% of insulin was retained within nanoparticles depending on the formulation composition. The mechanism of insulin release in intestinal simulation is probably related to dissolution of the alginate nucleus as a result of calcium loss, and also by electrostatic repulsion between alginate and insulin, which are both negatively charged in neutral pH. Therefore, the amount of insulin retained within nanoparticles under intestinal simulation may be tightly bound to the alginate nucleus, requiring a more extensive dissolution for additional release. The cumulative release in simulated intestinal fluid was lowest for nanoparticles formulated with PEG, considering that insulin may be retained within the PEG domains, in comparison to nanoparticles with no PEG in the formulation. *In vitro* assessment of bioactivity of insulin released from different nanoparticle formulations was determined by stimulation of rat L6 myoblasts in cell culture, and then Western blot immune-detection of the phosphorylation status of cellular PKB. The results indicated that insulin retained 90% of biological activity for the two tested formulations. The formulations differed by the presence of PEG and were used due to similar insulin entrapment efficiency, different insulin *in vitro* release profiles and different particle sizes-all of which can affect insulin structural stability and biological activity. The results of FTIR analysis showed that spectra of designed nanoparticles did not present significant variations from the spectrum of the standard formulation and insulin, suggesting the presence of the described biomaterials in all studied formulations and supporting the results presented in the results of *in vitro* bioactivity characterization of insulin as described above indicating that secondary structure of insulin was significantly

preserved in the nanoparticles. Pharmacokinetic and pharmacodynamic assessment of formulations in animal model of diabetes was not carried out and based on the results of *in vitro* characterization, this strategy represents a suitable methodology for designing insulin nanoparticles for oral delivery but additional studies based on *in vivo* evaluation of these formulations will present a better picture regarding formulation efficacy (Woitiski *et al.*, 2009). Later on same group designed nanoparticles integrating biomaterials that govern the functional behavior of orally dosed insulin is focused on improving insulin stability and absorption by facilitating its uptake and translocation throughout the intestinal membrane, while providing protection from acidic and enzymatic degradation in the gastrointestinal tract. Nanoparticles were prepared by ionotropic pregelation of alginate/dextran sulfate solution containing poloxamer 188 and insulin with divalent calcium ions, followed by polyelectrolyte complexation with oppositely charged chitosan and albumin. Box-Behnken experimental design was performed to determine the relationship between calcium chloride, chitosan and albumin on response variables for the optimization of nanoparticle formulation for orally dosed insulin. It was observed that nanoparticle mean diameter depended upon concentration of calcium chloride and chitosan, where the minimum mean size (394 nm) corresponded to the lowest calcium chloride (0.20%) and chitosan (0.04%) concentrations. Moreover, particle size decreased with increasing albumin concentration. At low calcium concentrations, intramolecular crosslinking of individual alginate chains led to a pregel state and compact coiled structure. However, in situations of calcium saturation at higher concentrations, lateral association of a number of alginate chains led to larger structures. Chitosan complexation then contributed to the size of the alginate nucleus in

proportion to its initial diameter, since larger nuclei had more carboxylic groups to interact with amino groups of chitosan. Moreover, the effect of albumin concentration on decreasing nanoparticle diameter was possibly related to reduction in the electrical repulsion among biopolymers, where modifications in the electrical state caused the particle to swell or to shrink, depending on whether the electrical repulsions increased or decreased, respectively. Entrapment efficiency of insulin was over 85% and was influenced by calcium ions and albumin concentration. High insulin entrapment with values above 90% was obtained with higher concentrations of calcium chloride, while maintaining the other factors at constant levels. Insulin release in enzyme-free simulated digestive fluids was carried out to determine if insulin is retained within nanoparticles for protection against acidic degradation, and if insulin is released at neutral pH for absorption through the intestinal mucosa. Insulin was retained within nanoparticles in enzyme-free simulated gastric fluid after 120 min, except for batches with highest concentration of chitosan (0.11%) or albumin (0.75%), where insulin release was up to 33% depending on these factors at the highest level. Higher concentration of chitosan influenced insulin release due to nanoparticle swelling based on chitosan properties at low pH and then the diffusion of ions present in the simulated gastric fluid inside the nanoparticles would have broken the ionic interaction between insulin and the alginate/dextran nucleus. High concentration of albumin resulted in higher insulin release likely due to a weakening of the electrostatic interaction between insulin and the alginate/dextran nuclei reliant on the pH conditions. After gastric simulation, nanoparticles were transferred to enzyme free simulated intestinal fluid. High levels of cumulative insulin release (greater than 80%) were observed after 180 min in the simulated intestinal fluid. Thus, it

appeared that pH triggered insulin release, potentially as nanoparticles passed from the acidic gastric medium, into a neutral intestinal medium. Based on the experimental responses and the criteria of desirability defined by constraints, solutions of 0.20% calcium chloride, 0.04% chitosan and 0.47% albumin constitute the optimum formulation of nanoparticles for orally dosed insulin nanoparticles. The results presented in this study highlights the importance of study design and optimization based on statistical methodology. Use of Box-Behnken design resulted in optimizing the best excipients concentrations required for formulation design and *in vitro* release and further utilization of these results in predicting the *in vivo* behaviour of nanoparticles can lead to more comprehensive formulations in terms of efficacy (Woitiski *et al*., 2009).

Other attempts addressed to modulate physiochemical properties of chitosan include the synthesis and utilization of N,O-carboxymethyl chitosan (NOCC) in preparing insulin loaded nanoparticles intended for oral delivery. NOCC is a chitosan derivative having carboxymethyl substituents on some of both the amino and primary hydroxyl sites of the glucosamine units of the chitosan structure. It was reported that NOCC is non-toxic, either *in vitro* in fibroblast culture assays or *in vivo* in testing with intra-peritoneal, oral, or subcutaneous treatments. Additionally, NOCC is suitable as an excipient in ophthalmic formations to improve the retention and bioavailability of drugs. Insulin loaded NOCC nanoparticles were prepared using ionic gelation method where NOCC was controllably gelled with tripolyphosphate in presence of insulin solution. NOCC nanoparticles were formed at conditions of 2 mg/ml of NOCC and 1 mg/ml of TPP. It was found that the encapsulation efficiency and process yield decreased with increasing NOCC to TPP weight ratio. Furthermore, the cumulative release of insulin from

insulin-loaded NOCC nanoparticles decreased with decreasing NOCC-to-TPP weight ratio, but it increased with decreasing the initial concentration of insulin. The higher the pH of the phosphate buffered saline, the greater the amount of cumulative release of insulin-loaded NOCC nanoparticles, and thus they could protect insulin from acid. The study reported the feasibility of using NOCC as a carrier matrix for oral insulin delivery but additional pharmacokinetic and pharmacodynamic studies are required to present a better picture of formulation *in vivo*. In addition to this, the biocompatibility and cytotoxicity evaluation of this substituted pH sensitive chitosan derivative should be carried out to endorse its acceptability for oral delivery of proteins (Lin and Lin, 2009).

Recently Sandri and co-workers evaluated the penetration enhancement properties of nanoparticles (NP) based on N-trimethyl chitosan (TMC 35% quaternization degree) loaded with insulin. The permeation performances of TMC NP were compared with those of chitosan (CS) NP and also with TMC and CS solutions. CS and TMC NP were prepared by ionotropic gelation of TMC and CS (polycations) with TPP polyanion. The ionotropic gelation took place when the positively charged amino groups of TMC and CS interacted with the negatively charged TPP. Insulin-loaded CS and TMC NP had dimensions of about 250 nm and had high yield and high encapsulation efficiencies from 75-94%. *In vitro* cytotoxicity analysis in Caco-2 cell lines revealed good biocompatibility after both 1 and 2 h of contact time. This indicates that trimethylation (35%) did not change the biocompatibility of CS also in nanoparticulate form. *In vitro* permeability study in Caco-2 cell lines showed that TEER%profile remains almost close to 100% value during the experiment time to indicate that insulin as free molecule did not impair the permeability barrier of the Caco-2 monolayer *in vitro* and the paracellular

pathway. All the polymeric samples caused a decrease of TEER profiles conceivably due to the capability of chitosan and its derivative to widen the paracellular route by interacting and interfering with junctional proteins of tight junctions. CS solution allowed a significant decrease of TEER value after a time as short as 30 min to indicate a ready action while TMC took longer time to induce the same effect. Not only polymer solutions but also nanoparticulate systems were able to determine a decrease of TEER values suggesting a modulation of the cell junction integrity. CS and TMC as nanoparticles and as polymeric solutions determined P_{app} (cm/sec) value higher than that of insulin solution ($p<0.05$) to indicate that all the polymeric samples enhanced insulin permeation. CS NP showed highest P_{app} value followed by TMC and CS as polymeric solutions and TMC NP, even if no significant differences could be evidenced between polymeric samples. The values of ER confirmed P_{app} trend and evidenced that the system based on CS as nanoparticles was able to increase insulin permeation up to twofold. Ex vivo permeation experiments in excised rat duodenum, jejunum and ileum showed that All the polymeric samples, both as solution and as NP, were characterized by P_{app} values of labelled insulin much higher than that of labelled insulin solution towards rat duodenum and jejunum ($p<0.05$), allowing enhanced permeation of insulin across these intestine tracts. The study demonstrated the ability of TMC to enhance insulin permeation both as NP and as solution more than native chitosan and its biocompatibility which suggests that TMC could be a potential polymeric material for developing mucoadhesive permeation enhancing nanoparticles for oral insulin delivery. Pharmacokinetic and pharmacodynamic evaluation of TMC based formulations are further required to define its *in vivo* efficacy in lowering blood glucose levels in diabetic animals (Sandri, *et al.,* 2010).

More recently, Sonaje and co-workers reported pH-sensitive nanoparticle (NP) system composed of chitosan and poly(g-glutamic acid) for the oral delivery of insulin. Insulin-loaded NPs were prepared by an ionic-gelation method. Briefly, insulin solution was premixed with an aqueous g-PGA and subsequently, sodium tripolyphosphate and magnesium sulfate were blended into the insulin/g-PGA mixture and thoroughly stirred. The mixed solution was then added, by flush mixing into an aqueous chitosan solution at pH 6.0 to form NPs which had a mean particle size of 232.9 nm with a positive zeta potential of 26.34 mV and their insulin entrappment efficiency and loading capacity were 70.62% and 18.3%, respectively. The prepared nanoparticles were freeze dried for additional stability of insulin in formulations and no significant changes were observed in nanoparticle characteristics after the freeze drying process. Biodistribution studies were carried out by administering insulin loaded nanoparticles prepared using radioactively tagged chitosan (chitosan labelled with ^{99m}Tc). A significant amount of radioactivity was observed in the stomach even at 24 h after dosing, indicating that there were some 99mTc-labeled NPs remaining in the stomach over the time. Additionally, a certain amount of the encapsulated insulin might release from NPs while in the stomach, due to the pH instability of test NPs. To overcome these problems, we lyophilized NPs and filled the dried NPs in powder form in a gelatin capsule with an enteric coating polymer, Eudragit. The small angle X-ray scattering (SAXS) profiles indicated that the freeze-drying process did not significantly disrupt the internal structure of NPs; additionally, their pHsensitivity was preserved and the insulin release was pH-dependent. The results obtained in the native PAGE analysis indicated that the released insulin molecules were neither fragmented nor aggregated. *In vitro* release of insulin demonstrated that

freeze dried nanoparticles released insulin at pH 2.5, 6.6 and 7.0; at pH 7.4, test NPs became unstable and disintegrated, resulting in a fairly fast release of insulin. When formulations were administered via oral route in diabetic rats, no hypoglycaemic effect was observed after oral administration of the capsule filled with the free-form insulin, indicating the poor oral absorption of insulin in the absence of a suitable delivery system. On the other hand, oral administration of the Eudragit L100-55-coated capsule filled with the freeze-dried NPs and SC injection of the free-form insulin solution produced a significant hypoglycaemic effect. SC injection of the insulin solution produced a sharp decrease in blood glucose levels (75% in 2 h), which gradually returned to the basal levels at 10 h, whereas the hypoglycaemia after oral administration of the Eudragit L100-55-coated capsule produced a slower but prolonged reduction in blood glucose levels. The hypoglycaemia after oral administration of the EudragitS100-coated capsule was not as significant as the one produced by the Eudragit L100-55-coated capsule, which could be attributed to the delayed release of NPs from the Eudragit S100- coated capsule. The study showed that the enteric-coated capsule could prevent the loaded NPs from contacting the highly acidic medium in the stomach, but liberated them rapidly in the proximal segment of the small intestine. The results obtained in the *in vivo* study indicated that oral administration of the enteric-coated capsule could significantly enhance the intestinal absorption of insulin and provide a prolonged hypoglycaemic effect (Sonaje *et al.,* 2010).

3.8.2.4.1.4 Polycaprolactone based systems

Polycaprolactone (PCL) is a biodegradable polyester with a low melting point of around 60°C and a glass transition temperature of about −60°C. PCL

is degraded by hydrolysis of its ester linkages in physiological conditions (such as in the human body) and has therefore received a great deal of attention for use as an implantable biomaterial. In particular it is especially interesting for the preparation of long term implantable devices, owing to its degradation which is even slower than that of polylactide. PCL is an Food and Drug Administration (FDA) approved material that is used in the human body as (for example) a drug delivery device, suture (sold under the brand name Monocryl or generically), or adhesion barrier. It is being investigated as a scaffold for tissue repair via tissue engineering, GBR membrane. It has been used as the hydrophobic block of amphiphilic synthetic block copolymers used to form the vesicle membrane of polymersomes. A variety of drugs have been encapsulated within PCL beads for controlled release and targeted drug delivery. Polycaprolactone (PCL) has also been investigated as a potential biodegradable polymer for oral delivery of insulin. Nanoparticles prepared with a blend of a biodegradable polyester (poly(-ε-caprolactone)) and a polycationic non-biodegradable acrylic polymer (Eudragit® RS) as a drug carrier for oral administration of insulin were reported by Damge and co-workers. The nanoparticles were prepared by multiple emulsion technique and were characterized both *in vitro* and *in vivo*. The prepared nanoparticles had mean size of 370 nm, an overall positive charge and a high encapsulation efficiency of insulin (96.3%). *In vivo* studies were carried out in diabetic Wistar rats at a dose of 25, 50 and 100 IU/kg. The results showed that insulin nanoparticles administered orally by force-feeding in diabetic overnight fasted rats, decreased glycaemia in a dose-dependent manner by comparison with rats treated with empty nanoparticles. Whereas the lowest concentration of insulin loaded nanoparticles (25 IU/kg) was ineffective, the 100 IU/kg insulin

nanoparticles significantly reduced glycaemia by 41% ($p < 0.01$) 4 h after the administration, this effect being maintained at least up to 8 h (39%, $p < 0.05$). A similar profile was observed with 50 IU/kg insulin nanoparticles but the decrease of glycaemia was less pronounced. Because these rats remained fasted over the whole experiment, a strong fall in glycaemia was observed 24 h after nanoparticles administration. Nevertheless, a slight reduction of glycaemia (12%, $p < 0.05$) was still noted with the concentration of 100 IU/kg insulin but not with the others. Finally, calculation of the areas under the curves (trapezoidal method) over a 24 h period showed that 50 and 100 IU/kg insulin nanoparticles reduced the areas by 23% ($p < 0.05$) and 38% ($p < 0.01$) respectively compared to the area obtained with unloaded nanoparticles. An aqueous insulin solution given by gavage to diabetic rats did not affect glycaemia, whatever the dose administered (25, 50 and 100 IU/kg). When insulin nanoparticles were administered orally, plasma insulin level also increased but the peak value was lower (+ 90%, $p < 0.01$) and observed only between 4 and 6 h post administration. Then, basal values were reached again at 24 h. The relative bioavailability of insulin, calculated from the areas under the curves and the doses administered orally and subcutaneously was 13.21% (compared with subcutaneous administration of insulin solution). In the same study, fluorescein labelled insulin was entrapped in the nanoparticles in order to understand the uptake phenomenon using these carriers via oral route. When insulin-loaded nanoparticles were administered in the lumen of the isolated intestinal loop, they were found, from 5 min onwards, as strongly fluorescent particles in the lumen of the intestine, next to the apical pole of the villi. This labelling remained obvious during the whole experiment whereas small fluorescent points appeared in the domes of the Peyer's patches after 30 min; however, they were not found in the

enterocytes situated all along the axis of the villi. Furthermore, focused fluorescent points could be observed in the connective axis of the villi representing probably labelled insulin inside vascular structures. This study clearly indicated that encapsulation of insulin into nanoparticles (prepared with a blend of a biodegradable polymer, poly(ε-caprolactone) and a polycationic non biodegradable polymer of a polyacrylic nature, Eudragit® RS), allowed the preservation of its biological activity leading to its prolongation of action and its oral absorption in a diabetic rat model (Damge *et al*., 2007). The two important concerns using polycaprolactone as the carrier material for oral use are; firstly the medical grade of the polymer is contaminated with toluene and tin which opens the discussion about suitability of this polymer for human use and secondly its degradation profile and rate which are even much slower than poly (lactide-co-glycolide) based polymers.

Recently Socha and co-workers reported blend nanoparticles as reported by Damge and co-workers based on the blend of biodegradable polyester (poly-epsilon-caprolactone) and a polycationic non-biodegradable acrylic polymer (Eudragit RS). The particles were prepared by emulsion evaporation diffusion method and had an average size around 350 nm with unimodal distribution as evident from the dynamic light scattering results. Two commercial insulin formulations were used for preparing nanoparticles, Actrapid and Novorapid. The zeta potential was positive whenever Eudragit RS was part of the nanoparticles matrix. The encapsulation efficiency was ~ 96% except for Novorapid-loaded particles of poly-epsilon-caprolactone (only 35%). *In vitro* release studies revealed a burst release from nanoparticles, which may be of interest for oral delivery. Novorapid-loaded nanoparticles were orally administered to diabetic rats and allowed the

glycaemia to be decreased when compared with free nanoparticles but results were not very encouraging (Socha *et al.,* 2009). Extensive *in vivo* studies including oral uptake of nanoparticles, pharmacokinetic behaviour and pharmacodynamic efficacy studies were not carried out and the study was primarily based upon characterizing the formulations in terms of size and zeta potential with respect to blend composition. More recently, Damge and co-workers prepared insulin nanoparticles using a blend of a biodegradable polyester poly (epsilon-caprolactone) and a polycationic nonbiodegradable acrylic polymer (Eudragit RS) for oral administration of a short-acting insulin analogue, aspart-insulin. Nanoparticles were prepared using multiple emulsion technique and had average size around 700 nm, unimodal with 95% entrappment efficiency of insulin. *In vitro* release studies showed that more than 30% of entrapped insulin was not released in a neutral release media over 24 h and there was a strong burst release observed initially. When administered orally to diabetic rats, insulin-loaded nanoparticles (50 IU/kg) decreased fasted glycaemia for a prolonged period of time and improved the glycaemic response to glucose in a time-dependent manner, with a maximal effect between 12 and 24 h after their administration. In parallel, plasma insulin levels increased. However, higher (100 IU/kg) and lower (25 IU/kg) doses of insulin did not exert any biological effect. It was concluded that polymeric nanoparticles composed of poly (epsilon-caprolactone)/Eudragit RS were able to preserve the biological activity of the insulin analogue aspart-insulin; however, the postprandial peak suppression was prolonged more than 24 h by comparison with regular insulin working only 6-8 h. This effect was possibly explained by the monomeric configuration of aspart-insulin, which was probably better taken up by the intestinal mucosa than regular insulin (Damge *et al.,* 2010).

3.8.2.4.1.5 Lipid based systems

Solid lipid nanoparticles (SLN) represents another interesting group of colloidal carriers that have been investigated for oral delivery of insulin because of their good tolerability, biodegradation, possibility of production on large industrial scale and finally due to the fact that protein and peptides formulated as SLN formulation experience improved bioavailability, prolonged blood residence time and modified tissue distribution (Garcia-Fuentes *et al*., 2005; Muller *et al*., 2006). Common disadvantages of SLN include their aggregation behaviour, unpredictable gelation tendency, their unexpected dynamics of polymorphic transitions and inherent low incorporation rate due to the crystalline structure of the solid lipid, drug expulsion from system due to crystal formation and poor physicochemical stability due to high aqueous and lipid content (Freitas and Muller, 1999) makes them less preferred colloidal carriers for oral delivery of proteins.

Zhang and co-workers reported insulin loaded solid lipid nanoparticles modified with wheat germ agglutinin-*N*-glutaryl-phosphatidylethanolamine. The reason for modifying the nanoparticles with wheat germ agglutinin lies in the fact that wheat germ agglutinin (WGA) binds to *N*-acetyl-D-glucosamine and sialic acid residues present in the mucous lining of the gastrointestinal tract, exhibiting a molecular weight of 36 kDa. As compared with other plant lectins with different carbohydrate specificity, the WGA binding rate to intestinal cell lines of human origin, human colonocytes and prostate cancer cells was highest as reported in literature (Gabor *et al*., 2001). Moreover, the WGA not only binds to the cell membrane, but it is also taken up into the cytoplasm of enterocyte-like Caco-2 cells and in addition to this it is quite resistant to acidic pH and enzymes and is suppose to provide stability to the insulin inside nanoparticles. The formulations had average

particle size of 65 nm and when given orally in healthy rats at a dose of 50 IU/kg, both solid lipid nanoparticles and wheat germ agglutinin modified solid lipid nanoparticles reduced plasma glucose levels at 1 h and 6 h respectively and the relative bioavailability of insulin (compared with subcutaneous administration of insulin solution) attained was 4.99 and 7.11% respectively. Systems with wheat germ agglutinin prolonged the release of insulin and its intensity of action was comparatively lower than unmodified nanoparticles at the same dose (Zhang *et al*., 2006). Sarmento and co-workers prepare cetyl plamitate based solid lipid nanoparticles intended for oral delivery of insulin. Nanoparticles were prepared by w/o/w double emulsion method and had an average size around 360 nm with zeta potential values around -3.4mV. Transmission electron microscopy analysis showed that these particles exhibit a spherical shape, and a dense lipid matrix without aggregation. The presence of insulin on the surface of SLN was not responsible for significant agglomeration, predicting absence of aggregation after oral administration. The entrappment efficiency of insulin in cetyl palmitate SLN was 43%. This result meant that approx. 57% of protein was in the dispersion medium, i.e., solubilised by the surfactant molecules. It is noteworthy that being a hydrophilic molecule a much lower entrappment efficiency of insulin within the lipid matrix of SLN was expected. Nonetheless, the modified w/o/w double emulsion method was shown to be a suitable production procedure to achieve relatively high encapsulation for insulin. Insulin loaded solid lipid nanoparticles were evaluated for their glucose lowering potential in overnight fasted diabetic rats. Insulin-loaded SLN decreased glycaemia by comparison with rats treated with oral insulin solution and empty nanoparticles. This hypoglycaemic effect was observed to occur in a biphasic way, with an initial peak between 4 and 8 h with a

decrease of 25% of the initial glucose level and later after 12 h of assay prolonged for up to 24 h. This may be related with the typical biphasic drug release pattern with an initial burst and prolonged release from SLN, characterized by a microcrystal matrix structure. Such biphasic pattern can originate an immediate release of insulin but also retain a significant fraction of the drug entrapped into the lipid matrix during prolonged time. Insulin released from SLN in the intestinal lumen was able to be directly internalized as discussed above, being the first responsible for the physiological effect. Then, after arrival to the appropriate sites for nanoparticle uptake in the posterior ileum, SLN were able to be absorbed, resulting in significant hypoglycaemic effect compared with oral insulin solution administration. Afterwards, it could be postulated that SLN undergo physiological degradation and the insulin enter into the blood circulation. It is generally accepted that nanoparticles with hydrophobic surfaces such as SLN, are taken up more extensively by the intestinal epithelium than those with hydrophilic surfaces. Thus, the uptake of nanoparticles with lipid matrix is potentially facilitated. Also, the bioadhesive properties of lipids can lead to a gradient diffusion of insulin from the high concentrations in the SLN matrix towards the intestinal cells. The association of both mechanisms has been related with the prolonged physiologic affect of proteins after oral administration (Sarmento *et al*., 2007).

In another study solid lipid nanoparticles of insulin were prepared and were modified with cell penetrating peptide, octaarginine (R8). Cell penetrating peptides, alone or coupled with various cargo molecules, can cross biological membrane barriers without significantly damaging the membranes and with little cytotoxicity to the cells. Of the cell penetrating peptides used, octaarginine (R8), a member of the synthetic

peptide family of arginine-rich peptides, carries various cargos for intracellular delivery. Arginine-rich peptides share efficient cellular uptake and an abundance of basic arginine amino acids with the most notable cell penetrating peptide, human immunodeficiency virus (HIV)-1 TAT-derived peptide. It has been suggested that the internalization mechanisms of arginine-rich peptides may depend on various factors, such as the type of peptide, the nature of the cargo, and the linker between them. SLNs loaded with insulin and R8 (Ra-Ins-SLNs) were prepared using the spontaneous emulsion solvent diffusion method. The nanoparticles had average size of 150 nm, zeta potential of 32.65 mV, insulin's entrappment efficiency of 62.29 % and octaarginine's entrappment efficiency of 58%. The *in vitro* release of insulin or R8 from R8-Ins-SLNs was characterized by an initial rapid release and subsequent sustained release in pH 6.8 dissolution media. *In vivo* absorption experiments provided a relative pharmacological bioavailability value of R8-Ins-SLN of 10.39 % (compared to subcutaneous insulin administration). The study demonstrated the ability of octaarginine modified solid lipid nanoparticles for oral delivery of insulin (Zhang *et al*., 2009). *In vitro* studies pertaining to stability of insulin with octaarginine were not carried out and subsequent cytotoxicity and safety studies in rats were also not reported as despite the highly efficient intracellular delivery of arginine-rich peptides, their immune responses when injected into the human body must be fully understood because they are derived primarily from non-human proteins, and very few studies have focused on the activity of immune cells after the uptake of these peptides (Trehin and Merkel, 2004).

Thompson and co-workers prepared solid lipid nanoparticles entrapping insulin using nano-complex formation approach. Initially, novel amphiphilic polyallylamine (PAA) were previously synthesised by randomly grafting

palmitoyl pendant groups and subsequent quaternising with methyl iodide. These self assembled polymers when incubated with insulin at pH 7.4, spontaneously formed nanocomplexes. The transmission electron microscopy images showed that non-quaternised polymer complexes appeared to form vesicular structures at low polymer:insulin concentrations. However, at higher concentrations they formed solid dense nanoparticles. The presence of quaternary ammonium moieties resulted in insulin complexing on the surface of aggregates. All polymers exhibited high insulin complexation efficiency between 78 and 93%. The hydrodynamic size of all complexes exceeded 1μm at lower polymer concentrations and declined with increasing polymer concentration. At polymer to insulin mass ratio of 2:1, all polymers were able to spontaneously form complexes in the range of 100-200 nm. *In vitro* characterization of nanocomplexes using differential scanning calorimetry revealed that there was insulin-polymer interaction which depended on polymer-insulin ratio and this led to change in secondary structure of insulin as indicated in DSC analysis. The cytotoxicity of amphiphiles and PAA were assessed using IC_{50} values which determine the polymer concentration required to reduce the cell survival by 50%. Overall, all modified polymers had higher IC_{50} values compared to the unmodified PAA after 24 h incubation with Caco-2 cells, indicating they were less cytotoxic than the parent polymer leading to the conclusion that palmitoylation of PAA reduced the cytotoxicity up to 2-3 fold. *In vitro* enzymatic degradation studies in trypsin revealed that 24% of free insulin was degraded after 4 h while all complexes were able to protect insulin from trypsin degradation whereas reverse trend was observed in degradation studies using α-chymotrypisn where significant insulin degradation was observed in nanocomplexes. This work showed that these novel comb

shaped amphiphilic polymers were able to spontaneously form nano-complexes with insulin in pH 7.4 buffer at room temperature. This mild fabrication process offers an alternative formulation to micro/nanoparticles, which the conventional encapsulation technique might cause protein degradation. However, the different nanocomplexes prepared in the study depending upon polymer insulin ratio, had variable degree of protection against enzymatic degradation of insulin. Further investigations are required in these systems both *in vitro* and *in vivo* to evaluate their efficacy in protecting insulin against enzymatic degradation. Results of the study were quite fascinating but all the polymers exhibited certain degree of cytotoxicity (Thompson *et al*., 2009).

El Sayed and co-workers reported a different lipidic system for insulin, intended for oral delivery. Nanoparticles were prepared by complexing low molecular weight chitosan and insulin solution in tris buffer. In order to obtain a nanoparticle system, insulin–chitosan polyelectrolyte complex was mixed with an oily phase and surfactant system. The optimal component concentration range to obtain a transparent system was obtained by the construction of a phase diagram. For constructing the ternary phase diagram, oleic acid was used as the oil phase and labrasol and plurol oleique combination was used as the surfactant mix. The pseudo- ternary phase diagram was constructed by titration of homogeneous liquid mixtures of oil and surfactants with deionized water at room temperature. The weight ratios of oleic acid/Smix used were 9/1, 8/2, 7/3, 6/4, 5/5, 4/6, 3/7, 2/8 and 1/9. Samples were vortexed for 5 min and then diluted with water in an incremental method, and mixed using vortexer for 3 min to accelerate their equilibration. Following the addition of aliquot of water, the mixture was visually examined for transparency. After water titration, titration of oil

surfactant mixtures with chitosan insulin polyelectrolyte complex was performed in the same manner. Stability of insulin inside this oily dispersion was monitored using its melting temperature analyzed by dynamic light scattering and was found to be stable. The particle size of insulin chitosan polyelectrolyte complex was around 2 μm but after dispersing in the oily phase, the particle size was 108 nm, highlighting the importance of ternary phase composition in yielding nano size droplets. The nanoparticles in oily dispersion were unimodal. The chemical stability as indicated by RP-HPLC chromatograms demonstrated that insulin was not degraded or aggregated after formulation. The results of ELISA for estimating immunological reactivity of insulin in dispersion also gave similar stability profile for insulin in formulations. The stability of insulin in dispersion was attributed to the formation of polyelectrolyte complex which prevented free insulin from interacting directly with interfaces. When formulations were evaluated for their pharmacodynamic efficacy, free insulin oral solution showed no hypoglycaemic effect. The blood glucose levels of the rats decreased remarkably after oral administration of chitosan nanoparticles, achieving a significant decrease after 3 h compared to the control group ($P < 0.05$). More interestingly, the hypoglycaemic effect was maintained without recovery to the baseline for the first 12 h. In the same experiment, another group of STZ-diabetic rats was given insulin-loaded nanoparticle preparation which was stored at 4 °C for 30 days to assess the pharmacological activity of the incorporated insulin after storage and no significant difference ($P < 0.05$) between the groups taking the fresh and stored nanoparticle preparation was observed at all time intervals indicating that the biological stability of insulin was maintained at 4 °C. The study explored the possibility of formulating an

oral insulin delivery system by combining the advantages of nanoencapsulation and the use of an oily vehicle (El Sayed *et al.*, 2009).

Wang *et al*., described a novel method for the preparation of hydrophilic peptide-containing oily formulations involving the freeze-drying of water-in-oil emulsions (FWE) for oral delivery of insulin. Anhydrous reverse micelles (ARM) were prepared by freeze drying w/o emulsions (FEW). The w/o emulsion were prepared using insulin solution as the aqueous phase, soya bean phosphatidylcholine (SPC) as the surfactant and a mixture of cyclohexane-dichloromethane-diethyl ether (4:1:1) as the organic solvent. Different mass ratios of SPC-to-insulin (from 30:1 to 3:1) were investigated to determine the minimum amount of SPC required to solubilize the drug, and so form an ARM system. Results of dynamic light scattering experiments revealed that particle size of the ARMs was below 30 nm with the mean diameter of the insulin-containing ARMs around 23 nm. *In vitro* characterization using differential scanning calorimetry proved that a part of insulin was still in crystalline form as evident from a strong sharp endotherm peak around 233 °C. *In vitro* release of insulin from ARM showed that after 24 h at pH 2, only 12% of total insulin was released from the ARM formulation. However, in a neutral medium (pH 6.8), the drug release was even slower than in an acidic medium, and only 6.5% of insulin was released after 24 h. Pharmacodynamic evaluation of formulations in diabetic rats showed that oral administration of 20 IU/kg insulin-containing ARMs reduced the plasma glucose level of the fasting rats to 71.3% within the first 4 h and this continued for 12 h and then returned to 80.7% after 24 h. For the group given insulin solution, the glucose levels were reduced to 93% within the first 4 h and this continued only up to 4 h. For the group given MCT oil containing SPC, the glucose levels were not reduced but increased slightly to

105% within the first 8 h and then returned to 100% after 16 h. The study demonstrated a simple procedure for the preparation of ARMs involving lyophilization of water-in-oil emulsions. It can be easily used to produce stable dry products or ARMs as a carrier for hydrophilic agents, such as peptides or proteins, which are usually unstable in aqueous solutions (Wang *et al.,* 2010).

3.8.2.4.1.6 Calcium phosphate based systems

Morcol *et al*., designed calcium phosphate-PEG-insulin-casein nanoparticles which were 600 nm in size and were characterized by hydrophobic core of calcium phosphate-PEG-insulin and hydrophilic coat of casein, the latter layer was intended to protect the insulin from harsh acidic environment of the stomach. When these nanoparticles were evaluated for their pharmacodynamic action at a dose of 100 IU/kg in fasted non-obese diabetic mice, they lowered glucose levels by 80% within 1 h of administration and their action was sustained till 12 h. The same formulation when administered in fed mice at same dose displayed a glucose reduction of 50% after 3 h and the effect was maintained till 5 h. This study showed the effect of fasted and fed state of the diabetic animal had a significant effect on the pharmacokinetic and pharmacodynamic performance of nanoparticulate formulations (Morcol *et al*., 2004). Paul and Sharma reported tricalcium phosphate microparticles for oral delivery of insulin where an attempt was made to load insulin in tricalcium phosphate (TCP) microspheres and coat with pH sensitive polymer of methacrylate derivative to render microsperes pH sensitive. Microspheres were prepared by first obtaining fine calcium phosphate powder by reaction between calcium nitrate and ammonium phosphate in paraffin oil followed by subsequent stirring. Insulin and

eudragit were loaded onto microspheres by adsorption process where blank microspheres were incubated in insulin and eudragit solution respectively. Microspheres were uniform in size as seen by transmission electron microscopy and were 106-200 μm in size with 8 IU/100 mg for porcine insulin and 23 IU/100 mg for human insulin as the entrappment efficiencies. The release of insulin from the microspheres was carried out in simulated gastric (SGF, pH1.2) and intestinal (SIF, pH 6.8) fluids, and also in PB (pH 7.4) at 30 °C. The formulation was stable in SGF and the release of insulin was less than 10%, even for 8 h. This release was attributed to the dissolution of insulin that was adhered onto the microspheres during the polymer coating procedure. Insulin was released into the SIF in a sustained manner for over 8 h. However, in PB at pH 7.4 the release was instant with complete release in over 1-2 h. CD and spectral deconvolution analysis of entrapped insulin demonstrated substantial retention of native α-helix content. Insulin released in the intestinal fluid (SIF) maintained the native conformation without showing any aggregation. Biological activity of the formulation was studied on induced diabetic rats. A dose dependent lowering of BGL in diabetic rats was observed with 3 and 5 IU of porcine insulin-loaded TCP microspheres. The percentage reduction in BGL was about 50% for 5 IU. With 6 IU of human insulin loaded TCP microspheres also, a similar reduction was observed. However, the action of insulin was slower indicating the variation in action with different species of insulin. It has been established from this preliminary study that insulin loaded in to TCP microspheres is highly compatible with no degradation or loss of biological activity of loaded insulin (Paul and Sharma, 2008).

More recently, pegylated calcium phosphate nanoparticles (CaP) were developed for oral delivery of insulin. The particles were prepared by

controlled precipitation of calcium chloride solution in presence of sodium citrate with sodium pyrophosphate. Polyethylene glycol was conjugated with the calcium phosphate nanoparticles after activating the phosphate group by carbodiimide chemistry. Insulin was loaded onto nanoparticles by diffusion filling method. The prepared particles had an average size of 47.9 nm but the particle size varied between 22 and 500 nm with a significantly larger number of particles (84%) falling below 100 nm. The insulin loading was found to be 129.74 6 4.66 IU/ 100 mg for CaP and 262.74 6 5.75 IU/100 mg for CaPPEG. PEG conjugation significantly increased the loading, by almost 100 percent. The loading efficiency was also increased from 21.6 to 43.3%. The PEG conjugation made the particles more hydrophilic thereby increased the insulin loading. Transmission electron microscopy revealed some aggregation in particles and irregular particles with sharp edges in the size range 50-100 nm were observed. The cytotoxicity of these nanoparticles was evaluated using L929 fibroblast cells by indirect contact method and cell viability was quantitated by MTT assay and the results showed that after incubation with the extract of CaP-PEG nanoparticles the cells were viable exhibiting its noncytotoxic character of nanoparticles. The nanoparticles prepared were crystalline and confirmed to be calcium phosphate from the XRD spectral analysis. *In vitro* release of insulin from nanoparticles in SGF and SIF showed that he insulin release in SGF was negligible for both CaP and CaP-PEG (0.5% for CaP and 9.8% for CaP-PEG). It seemed that nearly 90% of the drug was released in the 8 h of the study (in PBS, pH 7.4) for CaP-PEG, and almost completely for CaP. The insulin released at the 24th hour was similar to the value at the 8th h (11.7 IU/mL for CaP and 23.2 IU/mL for CaPPEG), also indicating total release within 8 h. Protection of insulin from the gastric environment was achieved by coating the nanoparticles with a pH

sensitive polymer that dissolved in the mildly alkaline pH environment of the intestine. The conformation of the released insulin, studied using circular dichroism, was unaltered when compared with native insulin. The released insulin was also stable as it was studied using dynamic light scattering. Radioimmunoassay was performed and the immunoreactivity of the released insulin was found to be intact (Ramachandran *et al*., 2009). *In vivo* pharmacokinetic and pharmacodynamic studies were not conducted in the present investigation. Moreover, the broad size range particles excecuted could be one of the limiting factors in their efficacy and might result in variable glucose lowering response *in vivo* due to variable absorption.

3.8.2.4.1.7 Poly (lactide-co-glycolide) based systems

Insulin loaded PLGA nanoparticles are one of the most successful polymeric systems fabricated till date for safe and effective delivery of insulin orally. PLGA has been the choice of matrix material for drug delivery due to its biodegradability, biocompatibility and non toxic nature. Carino and co-workers reported insulin loaded nanospheres based on PLGA, fumaric anhydride oligomer (FAO) and iron oxide. The nanoparticles were prepared using phase inversion method and particle size of formulations was 5 μm. Coulter sizing of the specific insulin containing formulation made of 1.6% insulin in FAO: PLGA with 10%iron oxide added showed that 80% of the spheres were smaller than 1 μm. However, when considered on a volume percent basis, 25% of the volume was made of spheres less than 4 μm in size and 10% of spheres were less than 1.6 μm. *In vitro* release in phosphate buffer saline at physiological temperature showed that FAO:PLGA, 10%iron oxide formulation released approximately 70% of the theoretically loaded insulin by 6 h and slowly continued to release while the formulation

without iron oxide only released about 35% within this time period. Both formulations released a large amount of their insulin in the first hour of release, but a smaller percentage of that observed in the nanospheres made solely of PLGA. These nanoparticles when administered orally in diabetic rats at a dose of 64 IU/kg showed a glucose reduction between 4 and 6 h and in addition to these they completely blocked the increased glycaemic response to a subcutaneous glucose tolerance test. The maximal glucose reduction obtained with these nanoparticles was around 11.4% compared to control animals where same dose was administered inraperitoneally (Carino *et al*., 2000).

Pan and co-workers prepared insulin loaded PLGA nanoparticles adopting double-emulsion solvent evaporation method. The prepared particles had an average size of 149.6 nm with unimodal particle size distribution and low polydispersity values around 0.09. Nanoparticles were prepared using poloxamer 188 as the surfactant. Insulin entrappment efficiency was not high and of given load 42.8% was entrapped inside nanoparticles. Analysis of insulin entrappment revealed that maximum amount of entrapped insulin was surface bound with PLGA nanoparticles due to hydrophobic interactions between hydrophobic domains of insulin and PLGA. The release behaviour *in vitro* showed an initial burst effect followed by a slower rate stage which lasted more than 8 h. Pharmacodynamic assessment of formulations was carried out in diabetic rats after intragastric administration of nanoparticles at a dose of 10 IU/kg. The results of *in vivo* studies demonstrated that after gastric administration of nanoparticles at a dose of 10 IU/kg, the plasma glucose level decreased significantly after 4 h ($P < 0.05$), 10 h later the glucose level decreased to the lowest (52.4%, $P < 0.01$) and the relative pharmacological bioavailability was calculated to be 10.3%

(compared with subcutaneously administered insulin solution). This study demonstrated the potential of PLGA loaded insulin nanoparticles in lowering the elevated glucose levels in an animal model of diabetes. Moreover, the glucose lowering ability of nanoparticles was shown to be of sustained nature and 52% glucose lowering 10 h was observed which indicated a significant benefit from the formulations. *In vitro* characterization of insulin's stability before and after loading on to nanoparticles was not carried out (Pan *et al*., 2002).

Insulin is a hydrophilic protein and often hydrophilic proteins and peptides show lower entrappment efficiencies in PLGA matrix due to inherent physiochemical nature. One of the studies done by Cui and co-workers adopted insulin modification as a measure to increase its entrappment inside PLGA nanoparticles. The strategy adopted involved modification of insulin with soya bean phosphatidylcholine (phospholipid) by complex formation to render it more hydrophobic and soluble in organic solvents with a view to increase its entrappment inside hydrophobic PLGA matrix. Insulin-phospholipid complex was prepared by an anhydrous co-solvent lyophilization method where insulin powder and phospholipid were co-dissolved in dimethyl sulfoxide containing 5% glacial acetic acid accompanied by gentle agitation until formation of a clear mixture which was then freeze-dried overnight. Insulin was formulated into nanoparticles by a reverse micelle–solvent evaporation method using dichloromethane as the organic solvent and polyvinyl alcohol as the surfactant. The prepared nanoparticles had varied size and entrappment efficiency which depended upon the ratio of PLGA and phospholipid content of the complex. Nanoparticles with polymer/phospholipid ratio of 1:1 displayed particle size around 172 nm with entrappment efficiencies

around 51.5% whereas formulations prepared with polymer/phospholipid ratio of 5:1 displayed size around 428 nm with 90% entrappment efficiency of insulin. Effect of nature of organic phase and molecular weight of PLGA on nanoparticle characteristics was also studied and the results showed that out of different solvents used, a mixture of dichloromethane and acetone in 1:3 gave smallest particle size and highest entrappment efficiency compared to others and use of PLGA with medium molecular weight gave smallest particle size and appreciably good entrappment efficiency. *In vitro* studies in dissolution medium of pH 6.8 showed that the amount of phospholipid combined insulin released from all of the nanoparticles was not more than 15% over 24h, whereas that of free insulin exceeded 60%. Simultaneously, almost 60-70% of phospholipid was released after 12 indicating that insulin-phospholipid complex suffered dissociation and most of drugs were released as free form. *In vivo* administration of formulations in diabetic rats showed glucose reduction between control and insuli-phospholipid nanoparticle group at all the times ($p < 0.05$), especially 6 h after administration ($p < 0.001$). Oral administration of 20 IU/kg insuli-phospholipid nanoparticles reduced the fasting plasma glucose level to 57.4% within the first 8 h and this continued for 12 h (Cui *et al.,* 2006).

Other strategies to enhance entrappment efficiency of insulin and prevent its burst release from PLGA nanoparticles included incorporation of hydroypropylmethyl cellulose (HPMC) in PLGA matrix. Insulin loaded nanoparticles with and without HPMC were prepared using modified emulsion solvent diffusion method. The prepared particles had an average size of 150 nm for systems without HPMC and 169 nm for particles prepared using HPMC. The use of HPMC helped in increasing the entrappment efficiency of insulin inside PLGA nanoparticles (2.55 vs. 3.17% using HPMC).

In vitro release demonstrated 50.46% and 19.77% insulin release from nanoparticles over 1 h from nanoparticles without HPMC and with HPMC respectively. In SIF, the insulin release from nanoparticles without HPMC and with HPMC over 1 h was 65.64% and 61.16%, respectively. These results showed that the initial release of insulin from HPMC modified nanoparticles in SGF was markedly reduced compared with PLGA nanoparticles without HPMC. Pharmacokinetic studies were conducted adopting oral administration of nanoparticles both in healthy animals and in diabetic rats. The results demonstrated that there was no significant difference in the pharmacokinetic behaviour of formulations in healthy and diseased states and the T_{max} of nanoparticles with and without HPMC was 4 and 6 h, respectively with serum insulin levels around 40 μIU/ml and compared with subcutaneous insulin the relative bio availabilities were 3.68% and 6.27% respectively. Nanoparticles demonstrated slow absorption and sustained elimination pattern. Pharmacodynamic evaluation of formulations in diabetic rats revealed their glucose lowering ability where almost 60% and 40% glucose reduction was reduction was observed with nanoparticles with and without HPMC around 12 h post administration and glucose lowering was observed till 16 h after which the glucose profiles showed a return to diabetic levels not before 24 h. The study revealed that presence of HPMC could retard the burst release of insulin from formulations and provide higher insulin entrappment efficiency in nanoparticles. Pharmacodynamic and pharmacokinetic performance of formulations displayed the potential of PLGA nanoparticles in delivering insulin orally (Cui *et al*., 2007).

Recently Satander-Ortega and co-workers carried out extensive studies with an aim to stabilize insulin in PLGA nanoparticles and to study the colloidal stability and degradation of PLGA based blends in simulated physiological

fluids in order to predict the stability of insulin in nanoparticles formulated using these blends. For this two different polyoxyethylene derivatives that differ in their hydrophobic/philic character, namely, a poloxamer (Pluronic F68®, HLB=29) and a poloxamine (Tetronic 904®, HLB=14.5), were used to obtain PLGA-PF68 and PLGAT904 blend formulations, respectively which were further used as the polymeric matrix for preparing insulin loaded nanoparticles. PLGA nanoparticles were prepared by a modified emulsion-solvent diffusion technique employing w/o/w double emulsion method. Nanoparticles with PLGA:poloxamer and PLGA:poloxamine ratios of 50:50 were also prepared by a modified emulsion-solvent diffusion technique in an identical way. The nanoparticles had average size of 126 nm for blank PLGA nanoparticles, 126 nm for blank PLGA:poloxamer blend, 128 nm for blank PLGA:poloxamine blend, 126 nm for PLGA:poloxamer blend at insulin pH 12, 255 nm for PLGA:poloxamer blend at insulin pH 2, 128 nm for PLGA:poloxamine blend at insulin pH 12 and 174.9 nm for PLGA:poloxamine blend at insulin pH 2. The entrappment efficiencies insulin were 34.7% and 40.6% in PLGA:poloxamer blend at insulin pH 12 and 2 respectively and 9.6% and 11% in PLGA:poloxamine blend at insulin pH 12 and 2 respectively. Blank systems were evaluated for their aggregation and stability in simulated physiological fluids which demonstrated that pure PLGA nanoparticles were unstable whereas nanoparticles prepared from blend polymers were stable in simulated gastric fluid and showed no signs of aggregation as monitored by change in particle size of formulations. Steric and electrostatic repulsions by poloxamers and poloxamine contents were held responsible for providing stability to the PLGA nanoparticles. Similar stability profile was observed in simulated intestinal fluid with polymer blends exhibiting enhanced stability. The second set of experiments was

carried out with enzymes dissolved in the simulated gastrointestinal fluids. In order to quantify the particle degradation, the mean size of the nanoparticles and the percentage of PLGA converted in lactate by the action of the enzymes were measured as a function of time. It was observed that presence of poloxamer and poloxamine in PLGA matrix severely retarded the generation of lactate in the enzymatic medium due to their protein repellent properties.

The other goal of the study was to understand the effect of insulin's charge on nanoparticle characteristics. The encapsulation of positively charged insulin (referred to as ins-pH 2) was carried out dissolving this protein in HCl 0.01 M during the particle synthesis. Likewise, insulin with negative charge (ins-pH 12) was incorporated to the particles using a NaOH 0.01 M solution. The mean size of both formulations showed a clear dependence with the net charge of the encapsulated insulin. The size was much lower when both PLGA and insulin were negatively charged (*ins-pH12*), independently on the surfactant nature. On the contrary, when PLGA and insulin were oppositely charged (ins-pH 2), the charge cancellation during the synthesis favored the aggregation of small growing particles, yielding bigger nanospheres that were finally stabilized by the poloxamer or poloxamine action. *In vitro* release of insulin from nanospheres in enzyme free simulated gastric fluid showed an initial burst effect, around 20%, followed by a close to zero release velocity but a delayed release of insulin was observed in PLGA:poloxamine blend nanoparticles and this was attributed to that the presence of poloxamines with low HLB values in PLGA nanoparticles which decreased the degradation of the PLGA matrix slowing down the pore formation. The insulin released at long time was higher in the PLGA-PF68 samples than in the PLGA-T904 ones with 60-80% insulin release

over 7 days. Release profiles in enzyme free intestinal fluid were similar for both blend formulations, as they were practically independent on the surfactant nature and on the insulin charge during its encapsulation. The similitude between the PLGA-PF68 and PLGA-T904 formulations was due to a lower degradation of the polyester matrix at pH close to 7. This lowered PLGA degradation at neutral pH masked the effect of the surfactant in the degradation of the systems, and consequently, in the release of the protein. The results obtained in this study with regard to use of different polyoxyethylene derivatives as blend with PLGA for preparing insulin nanoparticles demonstrated that The use of Pluronic-F68® for encapsulating insulin in PLGA based nanocarriers generates stable particles that were able to maintain their integrity in simulated gastrointestinal fluids. The release of the insulin could be speeded up or slowed down just by tuning the encapsulation pH. Nevertheless, it will be necessary to perform *in vivo* studies to corroborate the findings of the *in vitro* results (Satander-Ortega *et al*., 2008).

In spite of the encouraging potential of oral applications of biodegradable polymeric nanoparticles, there has been a serious problem with the particulate system that it was too hard for water-insoluble polymers to encapsulate hydrophilic insulin sufficiently. Recently Sun *et al*., reported a new method for increasing the entrappment efficiency of insulin in PLGA nanoparticles. Insulin was rendered more hydrophobic by ion pairing it with anionic surfactant sodium oleate to form insulin-sodium oleate complex (Ins-S.O). At acidic pH insulin remains positively charged due to protonation of amino groups and in such a situation sodium oleate, which is an anionic surfactant ion pair with insulin to form insulin-sodium oleate complex which is more hydrophobic than the native insulin molecule due to presence of

oleate groups. Insulin-sodium oleate complex was prepared by hydrophobic ionic pairing technique where insulin in solution was precipitated by sodium oleate and the precipitated insulin-sodium oleate was collected, washed to remove unreacted insulin and sodium oleate and freeze dried. The complex efficiency was 96% and formation of complex was verified by zeta potential measurements and X-ray diffraction technique. The zeta potential of the Ins-S.O complex passed from 25.4 mV through 0 and reached a level of -62 mV as the molar ratio of S.O to insulin changed from 1.85 to 9.23. The net surface charge on the Ins-S.O complex nearly became 0 as the result of the complete complexation when the molar ratio of S.O to insulin got to 6 : 1. When the molar ratio of S.O to insulin was higher than 6 : 1, the zeta potential remained negative, owing to increased S.O content. PLGA nanoparticles loaded with Ins-S.O complex were prepared using an emulsion solvent diffusion method and Ins-S.O complex was dissolved in the organic phase itself with PLGA and PVA was used as the surfactant. Following nanoparticle preparation, optimization studies were carried out and effect of PVA concentration, effect of PLGA concentration and effect of insulin loading was studied to elucidate their effects on nanoparticle characteristics. Results indicated a significant decrease in particle size and entrappment efficiency on by increasing the concentration of PVA in the aqueous phase due to higher stabilization and portioning of complex in the aqueous phase. Particle size of nanoparticles and insulin's entrappment efficiency increased with increase in PLGA concentration due to increase in viscosity of the system and decrease in diffusion of water molecules. Ins-S.O complex had practically no effect on particle size of nanoparticles but on increasing the complex, entrappment efficiency decreased and this was attributed to increase the concentration gradient of insulin between the oil phase and the

external water phase, thereby promoting drug diffusion into aqueous phase. Formulations were evaluated *in vivo* using subcutaneous and oral route. It was found that subcutaneous administration in rats reduced the plasma glucose level to almost the same percentage for free insulin, insulin in the complex and in the complex loaded in PLGA nanoparticles. The plasma glucose levels for free insulin, insulin in the complex and in the complex loaded PLGA nanoparticles were decreased to 59.0%, 56.0% and 57.8%, respectively. In order to confirm the potential use of Ins-S.O complex-loaded PLGA nanoparticles for oral delivery, the pharmacological effects were evaluated in diabetic rats. It was observed that oral administration of the saline (control group) and free insulin group (20 IU/kg) did not appreciably lower the blood glucose levels of the animals. Conversely, oral administration of Ins-S.O complex loaded nanoparticles (20 IU/kg) produced significant plasma glucose reduction. Oral administration of Ins-S.O complex-loaded PLGA nanoparticles reduced the fasting plasma glucose level to 23.85% within the first 12 h and this continued for 24 h. The pharmacological relative bioactivity to the subcutaneous formulation was around 11.5%. This study demonstrated the potential of hydrophobic ion pairing in increasing the entrappment of insulin in PLGA nanoparticles and that formed particles were active both via subcutaneous and oral route. The results of *in vivo* study clearly indicated the potential of Ins-S.O loaded PLGA nanoparticles in lowering the elevated glucose levels by 23.85% within first 12 h and sustaining the pharmacological action of formulation till 24 h, via oral route (Sun *et al*., 2010).

Inspite of different developments in dsigning PLGA loaded nanoparticles for oral delivery of insulin, there are certains drawbacks associated with PLGA that threaten the stability of insulin inside these systems. However, it is well

know that proteins and peptides undergo loss of activity after encapsulation or entrapment in PLGA based systems (Tobio *et al*., 1999, Sanchez *et al*., 1999; Wada *et al*., 1989). Moreover, during the release, hydration of insulin, reduction of pH deriving from polymer degradation (Taluja *et al*., 2007) and the presence of hydrophobic surfaces, are all potential sources of insulin inactivation (Lin *et al*., 1994; Domb *et al*., 1994) which necessisiates use of additional stabilizers in formulating these systems.

3.8.2.4.2 Limitations of polymeric carriers for nanoparticles

Poly (alkylcyanoacrylate) based nanoparticles represents one of the strategies for oral delivery of insulin where protection of insulin from harsh gut environment is afforded but their clinical effectiveness is limited due to their inherent toxicity profile, incomplete release of entrapped insulin and low pharmacological bioavailability of the dosage form (McDowell *et al*., 2009). In one study, poly(alkylcyanoacrylate) nanoparticles based on -methyl, -ethyl, butyl and -isobutyl substitution were assayed for their toxicity on peritoneal resident and thioglycolate-elicited macrophages and cellular viability was assessed by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, oxidative burst by nitroblue tetrazolium (NBT) reduction and NO production by nitrite evaluation. The nanoparticles tested led to cellular morphological modifications and induced toxicity in both types of macrophages in culture. The polyalkylcyanoacrylate nanoparticles uptake by peritoneal macrophages caused an increase in respiratory burst, as assessed by the NBT reduction assay, and induced the release of soluble toxic factors to the culture medium. The association of LPS (lipopolysaccharide) with nanoparticles significantly stimulated the production of nitric oxide (NO) by resident macrophages with –butyl substituted polymer showing the least toxicity (Cruz *et al*., 2004).

The use of microemulsions based on isopropyl myristate, caprylocaproyl macrogolglycerides, polyglyceryl oleate for oral delivery of insulin is interesting but there exist a broad size distribution of nanoparticles from 160-400 nm that causes variable absorption in addition to sustained hypoglycaemic episode till 36 h without detectable serum insulin levels (Graf *et al.,* 2009). Nanoparticles prepared using acrylic acid and methacrylic acid show pH dependent sizes with 200 nm at pH 2 to 2 μm at pH 6. This drastic change in size with change in pH questions their absorption and pharmacological variability as orally administered nanoparticles undergoing almost a 10 fold increase in size from gastric to intestinal segment as this would affect drug release and absorption of nanoparticles *in vivo* (Foss et al., 2004).

Exogenous insulin requirement in Type 1 diabetic patients is based on individual glucose insulin homeostasis and the required dose of insulin is prescribed after titration of glucose lowering. Insulin loaded vesicular nanoparticles based on polylactic acid-pluronic-polylactic acid (PLA-Pluronic-PLA) copolymers displayed significant lowering of plasma glucose levels (71%) at a dose of 50 IU/kg but failed to elicit dose response relationship when administered at 100 IU/kg in same diabetic model (Xiong *et al*., 2007). On the other hand, dextran sulphate chitosan nanoparticles at a very high dose of 100 IU/kg were able to lower the plasma glucose levels by 27%, 14 h post administration (Sarmento *et al*., 2007). Chitosan has been one of the most exploited biopolymer for oral delivery of insulin due to its biocompatibility, non toxicity and biodegradable nature. Chitosan based insulin nanoparticles have been investigated as oral delivery system for insulin and quite fascinating results have been obtained in the *in vivo* studies and chitosan have provided with one of the best *in vivo* paharmcodynamic

profile both in terms of glucose lowering and time of action but the feasibility of using chitosan has been limited due to various reasons. Firstly it's a natural polymer and hence purity, source and polydispersity affects its performance in nanoparticle fabrication and generally chitosan tends to form nanoparticles in higher size ranges. Secondly, commercially available chitosan has a large molecular weight and requires to be dissolved in acetic acid at approximately pH 4 during the preparation of dosage form and this pH could destabilize insulin (Sung et al., 2008). Thirdly, chitosan nanoparticles display pH sensitivity and at gastric pH they tend to swell and dissolve whereas at intestinal pH of 6.8, chitosan based nanoparticles tend to shrink. This causes anomaly in particle size which subsequently affects insulin release in gastric environment and incomplete release in intestinal conditions. Studies indicates that the blood glucose level of diabetic rats decreases at about 2-4 h following oral administration of nanoparticles and the blood glucose lowering effect sustains over 8-10 h of administration unlike insulin administered by subcutaneous route of which its blood glucose lowering effect tapers off after 3 hours of injection. Finally, chitosan has been approved by FDA as food additive (Illum, 1998) and for wound dressing but its status as polymer for drug delivery has yet not been approved by FDA because of its safety profile which is influenced by its degree of deacetylation, molecular weight, salt formation and degree of substitution on the molecule (Kean and Thanou, 2010).

Lipid based systems investigated for oral delivery of insulin chiefly comprise of liposomes, microemulsions and solid lipid nanoparticles. Liposomes suffer from considerable instability in physiological environment and hence they tend to aggregate leading to variable pharmacological effect as seen in case of insulin. Moreover, since they are composed of lipids, their membranes

become leaky over time and subsequently the drug load decreases and hence they are classified under thermodynamically unstable systems (Degim *et al*., 2006). Similarly, solid lipid nanoparticles show aggregation behaviour, unpredictable gelation, polymorphism and crystallization which cause issues with their physicochemical stability (Freitas and Muller, 1999). Microemulsions on the other hand represent thermodynamically stable systems but the quantities of surfactant and co-surfactants (> 10% w/v) required for stabilizing microemulsions limits their use. Moreover, need for pharmaceutically acceptable ingredients limits the choice of microemulsion components leading to difficulties in formulation (Bagwe *et al*., 2001). Studies carried out with calcium phosphate loaded insulin nanoparticles have revealed their potential as carriers for oral delivery of insulin but their large size (600 nm), high dose (100 IU/kg) required to carry out 80% reduction in blood glucose levels after 1 h with sustained hypoglycaemia lasting till 12 h (Morcol *et al*., 2004) and their solubility at low pH (4 and below) questions their stability in gastric environment (Epple *et al*., 2010).

Research on PLGA based nanoparticles for oral delivery of insulin has gained impetus since last few decades. Among all the classes of polymers for drug delivery applications with most acceptable regulatory profile, PLGA has few disadvantages as the carrier material for fabricating protein/peptide based nanoparticles. Several potential sources of irreversible inactivation of proteins encapsulated in PLGAs have been identified in literature (Zhu *et al*., 1999), these include: (1) elevated levels of moisture, providing sufficient protein mobility for reactivity; (2) an acidic microclimate induced by acidic degradation products and carboxylic acid end groups of PLGA; and (3) adsorption of the protein to the polymer surface, which may catalyze protein unfolding and aggregation. Apart from this the slow degradation profile of

PLGA which subsequently causes delayed insulin release is a parameter of concern for its use as a polymer in oral insulin nanoparticulate formulations.

Biocompatibility of nanoparticles which in turn refers to biocompatibility of polymer is the host response to them. Colloidal carriers are generally captured by macrophages or phagocytes in the body. Larger particles are known to be readily taken up by the reticuloendothelial system (RES) cells (cells of liver and spleen) in the body. RES recognize the particles after adsorption of proteins and opsonins (mainly complement factors and immunoglobulins) on the surface of the particles. This process seems to be influenced by the surface curvature of the carrier systems, where smaller carriers lead to reduced adsorption of proteins and opsonins and therefore, are less susceptible to be attacked by RES. Assessment of tissue compatibility of nanoparticles is essential because they are phagocytosed by the cells followed by inflammatory and tissue response. Histocompatibility is vital in oral, transmucosal, and regional administration. The morphology of the cells gives a clear picture about the inflammatory condition after administration of polymeric colloidal carriers which in fact reflects the biocompatibility of polymeric carrier. Mittal *et al*., investigated the effect of PLGA (50:50) molecular weight (14,500, 45,000, 85,000, 137,000 and 213,000 Da) and copolymer composition; PLGA (50:50; mol wt. 85,000 Da, PLGA (65:35; mol wt. 97,000 Da), and PLGA (85:15; mol wt. 87,000 Da) on release behaviour *in vitro* and *in vivo* from estradiaol loaded PLGA nanoparticles. The particles were administered orally to healthy male Sprague Dawley rats that were sacrificed after 11 days of administration. Histopathological analysis of different tissues (liver, spleen and intestinal segments) revealed absence of toxicity or any cellular change occurring inside the organs thereby confirming suitability of PLGA for oral administration (Mittal *et al.,* 2007).

Recently Semete *et al.*, demonstrated biodistribution and safety of PLGA 50:50 (mol wt. 45,000–75,000 Da) nanoparticles prepared using modified double emulsion solvent evaporation technique. An *in vitro* cytotoxicity study was conducted to assess the cell viability following exposure to PLGA nanoparticles. Viability was determined by means of a WST assay, wherein cell viability of greater than 75% was observed for PLGA nanoparticles. *In vivo* toxicity assays were performed via histopathological evaluation, and no specific anatomical pathological changes or tissue damage was observed in the tissues of Balb/C mice. The extent of tissue distribution and retention following oral administration of PLGA particles was analyzed for 7 days. After 7 days, the particles remained detectable in the brain, heart, kidney, liver, lungs, and spleen. The results show that mean percentages (40.04%) of the particles were localized in the liver, 25.97% in the kidney, and 12.86% in the brain. The lowest percentage was observed in the spleen (Semete *et al*., 2010).

Of all the different nanoparticulate systems that have been investigated for oral delivery of insulin, the use of PLGA as the polymeric matrix has been most beneficial due to its biocompatibility, biodegradability and approval by FDA for human use. PLGA degrade in the body through the hydrolytic cleavage of the ester linkage to lactic acid and glycolic acid which are easily metabolized by the Kreb's cycle and eliminated as carbon dioxide and water. The biodegradation of PLGA occurs at slow rate and therefore do not affect the normal cell function (Jain *et al.*, 2000). Furthermore, PLGA has been extensively tested for safety and toxicity in various animal species and currently used in humans for reabsorbable sutures, bone implants, contraceptive implants, as a graft material for artificial organs and as supporting scaffold in tissue engineering research. Long term

biocompatibility of PLGA was demonstrated by the absence of any untoward effect on intravascular administration of nanoparticles formulated using PLGA to the arterial tissue in pig and rat models of restenosis (Mooney *et al*., 1997). There are number of marketed formulations based on PLGA polymer currently in clinical use (Table 1.5) where toxicity of PLGA in microparticulate form has been well evaluated but long term toxicity of PLGA in nanoparticulate form is still under evaluation and except for short term PLGA based nanoparticle toxicity evaluation studies (Mittal *et al*., 2007; Semete *et al*., 2010), nothing else is available in literature at the moment.

Table **1.5** FDA approved medicinal products containing PLGA polymer

Product	**Polymer**	**Active**	**Indication**	**Route**	**Status**
Nutropin Depot®	PLGA	Human growth hormone	Growth defeciencies	sc/im	Marketed
Sandostatin LAR®	PLGA-glucose	Octreotide acetate	Acromegaly	sc/im	Marketed
TrelstarTM Depot	PLGA	Triptorelin pamoate	Prostate cancer	im	Marketed
Decapeptyl®	PLGA	Triptorelin pamoate	Prostate cancer	im	Marketed
Pamorelin	PLGA	Leuprolide acetate	Prostate cancer	sc	Marketed
Atridox®	PLGA	Doxycycline hyclate 10%	Periodontitis	tp	Marketed

sc: subcutaneous route; im: intramuscular route; tp: topical route

Based on current literature review where among different polymeric materials, PLGA stands out to be the polymer of choice in fabricating inert and non toxic nanoparticles for oral delivery of proteins and peptides, herein we used PLGA for designing and characterising insulin loaded polymeric nanoparticles for oral delivery. The PLGA polymer selected for fabricating insulin nanoparticles was PLGA 50:50 with average molecular weight of 45 K Da. PLGA is available in many copolymer composition ratios (ratio of lactide content to glycolide content) and in many molecular weight ranges. The reasons for selecting PLGA 50:50 with average molecular weight are as follows; firstly PLGA polymers containing 50:50 ratio of lactic and glycolic acids are hydrolyzed much faster than those containing higher proportion of either of the two monomers (Jain *et al*., 2000; Kitchell and Wise, 1985), secondly the molecular weight of the polymer has opposite effects on nanoparticle size and encapsulation efficiency as smaller sized nanoparticles can be prepared with lower molecular weight polymer, however, at the expense of reduced drug encapsulation efficiency whereas on the other hand, an increase in polymer molecular weight increases encapsulation efficiency and the size of the nanoparticles (Blanco and Alonso, 1997; Song *et al*., 1997) and hence to strike a balance between optimum particle size and encapsulation efficiency of insulin, average molecular weight PLGA was selected. The reason for selecting PLGA 50:50 with medium molecular weight range was to have an optimum ratio of hydrophobicity and hydrophilicity in the polymer matrix along with optimum degradation rate as excess of either of the two properties of the polymer (copolymer composition and molecular weight) can alter nanoparticle characteristics both *in vitro* and *in vivo*.

Hypothesis

Therefore, realizing the potential of the nanoparticles for oral administration of insulin and having identified the potential pitfalls of the existing formulations, the dissertation is geared to address the following questions.

1. Biodegradable polymers in particular, medium range molecular weight (~40 kDa) PLGA have been extensively studied in drug delivery, however the micro environmental changes on hydrolysis and ageing of polymer on storage are shown to have deleterious effects on encapsulated proteins. Can stabilizer co-encapsulation improve stability of the encapsulated insulin?
2. Does the coencapsulated stabilizer and the nature of stabilizer influences the insulin loading and pharmacokinetics?
3. Can oral insulin nanoparticles strictly control hyperglycaemia and prevent specific associated complications in rat model of insulin dependent diabetes?

CHAPTER 2

Chapter 2: Compatibility studies

Aim: To assess the compatibility of insulin with surfactants/stabilizers used in nanoparticle preparation by circular dichroism, fluorescence spectroscopy and ultraviolet spectroscopy

1. Introduction

Drug dosage forms are complex systems consisting of multiple components in addition to the active pharmaceutical ingredient (API) and these components are pharmaceutically labelled as excipients. Excipients facilitate formulation design and perform a wide range of functions to obtain desired properties for the finished drug product. Their function ranges from surfactants and stabilizers to solubilisers and processing aids that impart a specific functional property to the final product e.g. modifying drug release. In addition to their functional performance, ideally, excipients should be chemically stable, nonreactive with the drug and other excipients, inert in the human body, have low equipment and process sensitivity, have pleasing organoleptic properties, and should be well characterized and accepted by the industry and regulatory agencies (Brittain, 2007).

Considering these aspects, protein-excipients preformulation studies play a crucial role in anticipating protein-excipients incompatibilities and identifying logical path in designing robust dosage forms. Protein-excipients compatibility studies play a pivotal role in protein formulation development especially when excipients are surface active agents (surfactants) as these excipients are known to cause protein denaturation, aggregation and folding that can result in loss of protein activity (Bollag and Edelstein, 1991).

It is well known that surfactants bind strongly to protein, thereby inducing conformational changes in them that often results in changes of polarity and

alteration of protein's stability. In addition to this, formation of aqueous solution/organic solvent or solid protein/organic solvent interfaces during microparticles/nanoparticles preparation process is a commonly encountered destabilization mechanism for proteins resulting in adsorption, protein unfolding, and subsequent aggregation. Proteins, being amphipathic in nature, tend to occupy these interfaces resulting in denaturation. The presence of water makes the protein flexible and more reactive. Many methods have been used to investigate these changes, such as calorimetry for thermal stability (Kelley and McClements, 2003), turbidimetry for protein aggregation (Valstar *et al*., 2000), fluorimetry for polarity (Ray and Chakrabarti, 2003) and NMR for structure of complex (McCoy and Wyss, 2002). Over the past few years, circular dichroism (CD), fluorescence spectroscopy (FS) and ultraviolet-visible spectroscopy (UV) have emerged as better techniques for understanding secondary and tertiary structural changes in proteins and peptides (Kelly and Price, 2000; Cowgill, 1964).

CD and FS have been used extensively to give useful information about protein structure, the extent and rate of structural changes and ligand binding. In the field of protein design and manipulation, CD and FS have been used to assess the structure and stability of the designed protein fragments. Studies of protein folding make extensive use of CD to examine the folding pathway; the technique has been especially important in characterising "molten globule" intermediates which may be involved in the folding process. CD is an extremely useful technique for assessing the structural integrity of membrane proteins during extraction and characterisation procedures. CD is particularly good for determining whether a protein is folded, and if so characterizing its secondary structure, tertiary structure, and the structural family to which it belongs, comparing

the structures of a protein obtained from different sources (e.g. species or expression systems) or comparing structures for different mutants of the same protein, demonstrating comparability of solution conformation after changes in manufacturing processes or formulation, studying the conformational stability of a protein under stress, thermal stability, pH stability, and stability to denaturants and how this stability is altered by buffer composition or addition of stabilizers and excipients and determining whether protein-protein interactions alter the conformation of protein (Venyaminov and Yang, 1996). CD relies on the differential absorption of left and right circularly polarised radiation by chromophores (Woody, 1996) which either possess intrinsic chirality or are placed in chiral environments (Fig 2.1). Proteins possess a number of chromophores which can give rise to CD signals. In the far UV region (180-240 nm), which corresponds to peptide bond absorption, the CD spectrum can be analysed to give the content of regular secondary structural features such as α-helix and β-sheet. The CD spectrum in the near UV region (250-320 nm) reflects the environments of the aromatic amino acid side chains and thus gives information about the tertiary structure of the protein. Figure 2.2 demonstrates CD spectra observed for proteins that differ in their structural configurations.

FS is a technique that gives both structural and dynamic information about proteins and is widely used for investigating protein structure and function and offers several advantages over conventional techniques. Its intrinsic sensitivity, suitable time scale, non invasive nature, ability to incorporate fluorophores in a site specific manner and minimum perturbation to protein, makes FS one of the most extensively exploited technique (Chattopadhyay and Raghuraman, 2004). The intrinsic fluorescence of tyrosine residues which is sensitive to perturbations is used as a means of monitoring

unfolding/refolding transitions in insulin as tryptophan residues, which are the major cause of fluorescence in proteins are absent in insulin (Royer, 1995). UV remains a fast, accurate, quantitative, and non destructive technique with much to offer in studies of protein folding and structural integrity (Pelton and McLean, 2000; Mach *et al*., 1995). Proteins and peptides absorb UV wavelength from 190 to 300 nm due to presence of specific structural motifs. Peptide back bone in proteins absorbs around 190-200 nm due to π-π^* transitions and change in absorption around this wavelength is correlated with change in secondary structure of protein. The presence of aromatic amino acids also causes π-π^* transitions that are associated with higher wavelengths absorption (250-280 nm) and subsequent changes in the absorption around this wavelength can be correlated with changes in protein structure and function.

Polyvinyl alcohol (PVA), Vitamin E tocopheryl polyethylene glycol 1000 succinate (VitETPGS) and Didodecyldimethylammonium bromide (DMAB) are most commonly used surfactants in fabricating nanoparticles for drug delivery applications (Bhardwaj *et al*., 2005). PVA is the most widely used non ionic surfactant employed in fabricating PLGA and PLA based nanoparticles and it confers no charge and act by stabilizing the system with it long polymeric chains conferring steric hindrance. VitETPGS is also a non ionic surfactant that confers a negative charge on the nanoparticles thereby stabilizing the system. Listed in FDA (Food and Drug Administration) GRAS (generally regarded as safe) list, VitETPGS find applications as surfactant for increasing the oral bioavailability of poor bioavailable drug molecules. DMAB is a quaternary ammonium salt but its congeners like Cetyltrimethylammonium bromide (CTAB) have been used since decades as antiseptics and disinfectants. Due to higher toxicity of CTAB and other

quaternary ammonium salts, their use is restricted in drug delivery but DMAB finds numerous applications as surfactant for oral delivery of nanoparticles due to its inherent safety profile and non toxic nature at the concentrations used in the nanoparticles preparation (Bhardwaj *et al*., 2009). The stabilizers employed in nanoparticle preparation, $Mg(OH)_2$ (MH), $ZnCO_3$ (ZC) and $MgCO_3$ (MC) are inert, non toxic inorganic salts which are listed in US FDA GRAS list and are routinely employed as stabilizers and adjuvants in oral stabilizer formulations (suspensions and chewable tablets) safe for human use. The present chapter investigates the compatibility between insulin and formulation excipients surfactants (DMAB, PVA and VitETPGS) and stabilizers (MH, ZC and MC) at different concentrations utilizing CD, FS and UV.

2. Materials

Bovine serum insulin (nominal strength~ 27 units/mg) was purchased from Sigma (St. Louis MO, US). PVA (cold water soluble with molecular weight (30-70 K Da) was purchased from Sigma (St. Louis MO, US). VitETPGS (NF Grade) was a gratis sample from Eastman Company (Kingsport, UK). DMAB was purchased from Sigma (St. Louis MO, US). All the stabilizers (MH, ZC and MC) were purchased from Fischer (UK). Distilled water was prepared in house and all the reagents used were of analytical grade.

3. Methods

3.1 CD spectroscopy

To verify the integrity of the secondary structure of insulin when formulated into nanoparticles using surfactants and stabilizers, CD studies were carried out. Briefly, an equal volume of insulin solution in 0.05 N acetic acid was

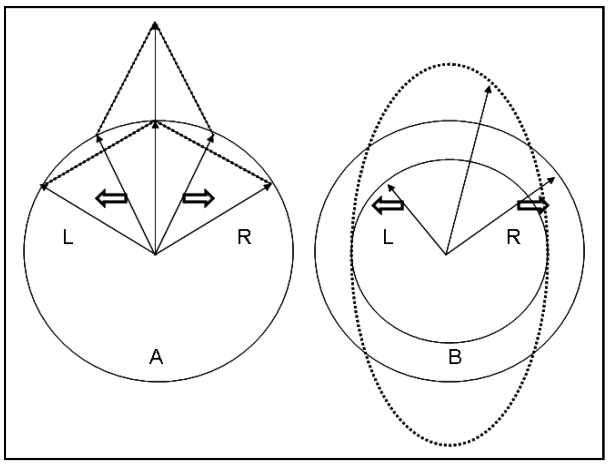


Fig. 2.1 Basics of CD. (A) The two wavelengths have the same amplitude and combined generate plane polarised radiation; (B) The wavelengths are of different magnitude and generate elliptically polarised radiation.

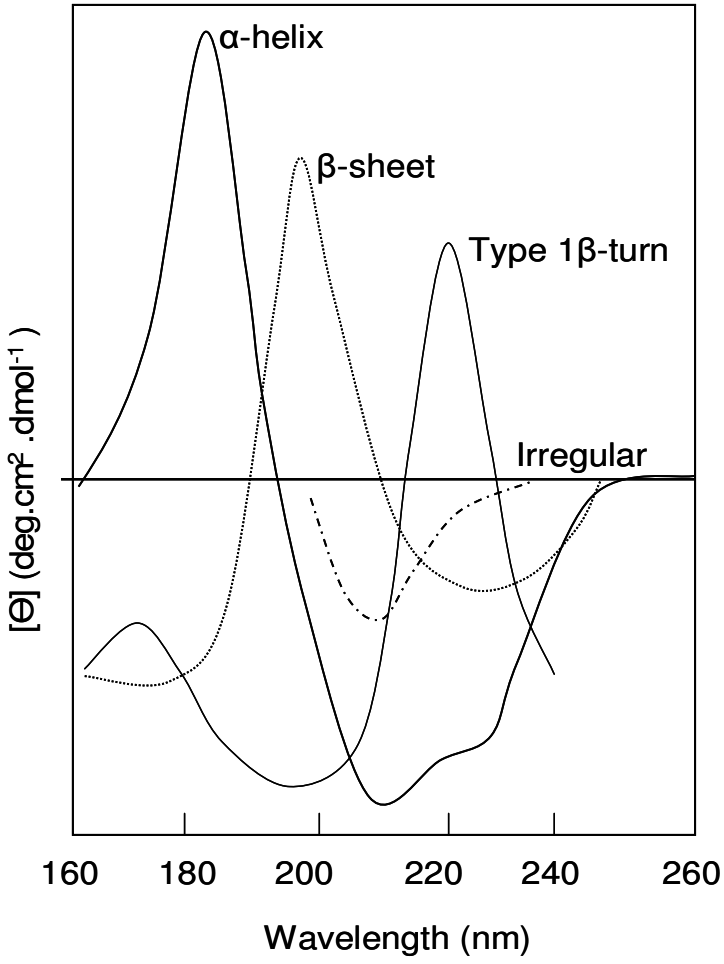


Fig. 2.2 Far UV CD spectra associated with different types of secondary structures.

mixed with three different concentrations of surfactants: DMAB, Vitamin ETPGS and PVA in such a way that final mixtures contained 100 μg/ml of insulin and 10, 100 and 1000 μg/ml of individual surfactants. The mixtures were stirred at 25°C for 30 min at 1000 rpm. For evaluating the compatibility of insulin with stabilizers used in nanoparticulate formulations (MH, ZC and MC), native insulin solution was prepared in 0.05 N acetic acid and was mixed with stabilizer suspension in such a way that final insulin concentration was 100 μg/ml and stabilizer strength was at 0.5, 1 and 2% w/v. The mixtures were stirred at 25°C for 30 min at 1000 rpm after which they were centrifuged and supernatants were analyzed by CD. Centrifugation of mixtures to obtain clear supernatants was important as a mandatory pre-requisite for CD analysis as highly scattering particles present in the sample lead to large spectral noise in the scan (Kelly and Price, 2000). CD spectra were acquired at 25°C using a Chirascan Spectropolarimeter (Applied Photophysics, Surrey, UK) in the far UV region (190-250 nm) in a 0.01 cm path length cell using a step size of 1 nm. The lamp housing purged with nitrogen and an average of 3 scans was obtained. CD spectra of the appropriate blank reference were recorded and subtracted from the insulin spectra, to eliminate contributions from the aqueous phase. The mean residual molar ellipticity was calculated from the raw CD signal using a mean residue weight of 116 Da, and insulin concentration calculated from the corrected absorbance of the insulin solution at 280 nm.

3.2 Fluorescence spectroscopy

Intrinsic fluorescence spectroscopic experiments were carried out to assess any change in the three dimensional structure (the local environment of aromatic residues) of insulin when in contact with surfactants and stabilizers

used in particle preparation process. Native insulin solution (2 μg/ml in 0.05 N acetic acid) was mixed with surfactant solutions (0.05, 0.5 and 5% w/v concentration) and mixture was stirred at 25°C for 30 min at 1000 rpm after which the solutions were analyzed for fluorescence. In another experiment native insulin solution (prepared as above) was mixed with stabilizer suspension in such a way that final insulin concentration was 2 μg/ml and stabilizer strength was at 0.5, 1 and 2% w/v. The mixtures were stirred at 25°C for 30 min at 1000 rpm after which they were centrifuged and supernatants were analyzed for fluorescence. Measurements were carried out at 25°C in 1 cm path length quartz cuvettes using a fluorescence spectrophotometer (Cary Eclipse, Varian Ltd, UK). Excitation was set at 280 nm, and intrinsic fluorescence emission was measured in the range 290–350 nm. The excitation and emission slits were set at both 5.0 nm and the scan rate was kept at 600 nm/min with photomultiplier tube detector voltage between 600-800 V. The fluorescence spectrum of native insulin was compared with insulin in combination with surfactant/stabilizer at same concentration to study any changes in the local tyrosine environment. An average of 3 scans was performed for each sample in triplicate.

3.3 UV spectroscopy

UV studies were carried out on a Unicam UV 300 spectrophotometer (Thermoelectron, UK) at room temperature in a 1-cm quartz cuvette. The UV scans were obtained from 200-350 nm with a step size of 1 nm with 0.05 N acetic acid as blank. Briefly, insulin solution in 0.05 N acetic acid was mixed with surfactant solutions and stabilizer suspensions at different concentration in such a way that final mixture contained 2 μg/ml insulin and individual surfactants and stabilizers were maintained at 0.5, 1 and 2% w/v.

The mixtures were stirred at 25°C for 30 min at 1000 rpm, centrifuged and the supernatants were subjected to UV analysis. The UV spectrum of native insulin solution was compared with UV spectra of insulin at same concentration in combination with surfactants and stabilizers at different concentrations. An average of 3 scans was performed for each sample in triplicate.

4. Results and discussion

4.1 CD spectroscopy

Figure 2.3 and 2.4 shows the CD spectrum of native insulin compared with CD spectrum of insulin in presence of DMAB, PVA and VitETPGS respectively. The CD spectra of insulin in combination with stabilizers are shown in figure 2.5 for MH and ZC and in figure 2.6 for MC. The CD spectrum of control insulin solution in the far UV region (190-250 nm) showed extrema at 196, 209 and 222 nm with the ellipticity values becoming negative $\underset{\sim}{}$ 204 nm. The control CD spectrum (native insulin) was in close agreement with the CD spectrum of insulin reported in literature obtained previously by other investigators (Ettinger and Timasheff, 1971; Sadhale and Shah, 1999). All spectra (insulin in combination with surfactants and stabilizers) nearly overlapped and showed clear extrema of similar magnitude at 209 and 222 nm, which are indicative of the known α-helical and beta pleated sheet conformations respectively (Wu and Yang, 1981). For all insulin surfactants and insulin antacids combinations, CD spectra were very similar to the insulin standard solution, suggesting minor, if any, changes of insulin secondary structure thus, these excipients seem to be compatible to formulate insulin nanoparticles, while preserving structure and thus, potentially, also maintaining insulin activity. CD spectroscopy is a

valuable tool in understanding the secondary and tertiary of insulin as in insulin monomer, the A chain represents two sections of α-helix whereas B chain contains a larger section of α-helix, together these helices represent the active region of the molecules responsible for pharmacological activity and are visible as extremeas at 209 and 222 nm in CD spectrum of insulin (Huang *et al*., 2004) and loss of CD signal at 209 and 222 nm is strongly corroborated with disappearance of α-helical configuration and loss of signal at 196 nm with β-sheet confirmation which indicates significant changes in the secondary structure of insulin resulting in inactivation/denaturation of the molecule (Sadhale and Shah, 1999). Overall, the used concentrations of surfactants and stabilizers under investigation retained the structural integrity of insulin without causing any change in its secondary structure as demonstrated by CD spectroscopy.

4.2 Fluorescence spectroscopy

FS was carried out to ascertain any changes in secondary and tertiary structure of insulin in combination with surfactants and stabilizers. It was observed that all the surfactants exhibited some degree of interaction with insulin and the phenomenon was dependent upon the concentration of surfactant used. When DMAB was used as the surfactant at 0.05 % and 0.5 % w/v concentration, a slight decrease in fluorescence intensity was observed. At 5 % w/v concentration, fluorescence quenching was observed and tyrosine emission peak shifted towards lower wavelength (blue shift) (Fig. 2.7). At lower concentrations of 0.05% and 0.5% w/v of DMAB, the concentration dependent slight decrease in fluorescence intensity can be possibly attributed to the adsorption of hydrophobic chains of DMAB with hydrophobic domain in insulin. DMAB is a cationic surfactant with two long dodecyl chains in its

structure that forms the hydrophobic part of the surfactant and causes it to hydrophobicaly bind with hydrophobic domains in insulin. The fluorescence quenching and blue shift observed at 5% w/v DMAB concentration was attributed to the embedding of tyrosine residues in vesicles/micelles formed by DMAB at high concentration as the majority of tyrosine residues in insulin are exposed to solvent and only one tyrosine residue is buried inside the molecule (Inada, 1960). Previous studies conducted by Caputo and London regarding addition of a surfactant to a protein which contains tryptophan/tyrosine residues which are exposed to the aqueous solvent causes a blue shift in protein emission spectrum if the tryptophan/tyrosine residues gets embedded in the surfactant vesicles or micelles (Caputo and London, 2003) further verified the possibility. The efficacy of a surfactant in stabilizing an emulsion is quite dependent upon the concentration of surfactant and usually higher concentration of surfactants results in better stabilization of emulsion and attainment of smaller particle size. Keeping this in mind 1% DMAB w/v concentration was selected which was well below the concentration at which DMAB interacted significantly with insulin and at the same time the selected concentration of DMAB was sufficient enough to stabilize nanoparticle formulation.

With PVA as surfactant, no changes in fluorescence emission spectrum of insulin were recorded at 0.05% and 0.5% w/v but at 5% w/v PVA concentration, a strong fluorescence quenching was observed without any blue shift phenomenon (Fig. 2.7).

PVA at lower concentrations of 0.05% and 0.5% w/v did not influenced the tyrosine microenvironment in insulin possibly due to lack of charge and lack of micelle formation as PVA is a non micelle forming surfactant and its use a surfactant for nanoparticles is primarily due to its long hydroxyl chains

which causes steric repulsion as a mechanism for stabilizing nanoparticles (Song *et al.,* 2006). Whereas at high PVA concentration (5% w/v), the hydroxyl groups interact with tyrosine and cause a local change in its microenvironment which results in fluorescence quenching. Insulin contains tyrosine residues positions 14 and 19, which fluorescence upon excitation at 280 nm; the emission maximum and intensity being dependent on the local environment since water causes a red shift and quenching of fluorescence (Gok and Ates, 2001). Other possible reasons that might have caused fluorescence quenching at high PVA concentration include; denaturation of insulin by PVA and alteration of solubility of insulin causing it to precipitate out of the solution. The later reason can be ruled out as no visual precipitation was observed in

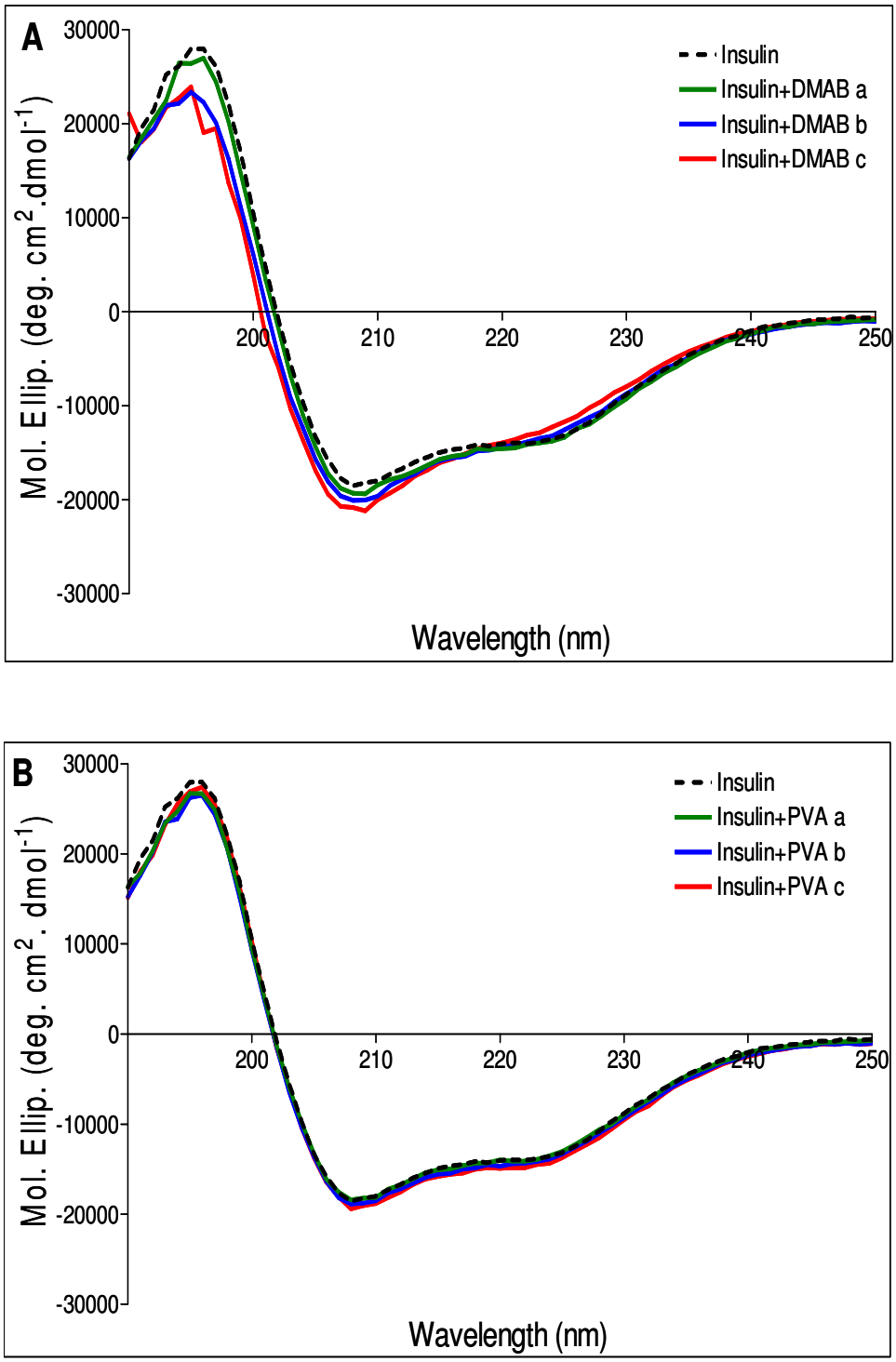


Fig. 2.3 Far UV (190-250 nm) CD spectra of insulin and insulin surfactant combinations in 0.05N acetic acid. (A) DMAB (B) PVA. DMAB and PVA concentrations are represented as alphabetical keys: a=10, b=100 and c=1000 µg/ml. All insulin solutions were 100 µg/ml and temperature was 25°C. All samples were analyzed in triplicate.

PVA (5% w/v) insulin systems. Based on results, insulin's stability with PVA and on requirement of optimum surfactant concentration for stabilizing nanoparticle systems, 1% w/v PVA was selected as the surfactant.

For insulin in combination with different VitETPGS concentrations, a significant increase in fluorescence intensity was observed from 0.05 to 0.5% w/v after which the fluorescence intensity decreased but was still higher than 0.05% w/v concentrations (Fig. 2.8). The possible explanation of this phenomenon could be that tyrosine residues at 14^{th} and 19^{th} position are able to interact with ligands in native insulin molecule. As a ligand, the non ionic surfactant VitETPGS with lower concentration primarily acts on these tyrosine residues through the aromatic ring stacking. With the increase of VitETPGS concentration from 0.05 to 0.5% w/v, more and more tyrosine residues interact with VitETPGS due to the unfolding of insulin. When VitETPGS reaches its second critical micellar concentration (CMC) and above, insulin is refolded and tyrosine residues are no more exposed to VitETPGS, and accordingly the aromatic ring stacking between VitETPGS and the tyrosine residues disappears which explains the decrease in fluorescence at 5% w/v concentration of VitETPGS (Sun *et al.,* 2005). Since interaction of insulin with VitETPGS involves unfolding of insulin at lower concentrations with intensity of interaction increasing with surfactant concentration, VitETPGS was not selected as the stabilizer for fabricating insulin loaded nanoparticles. Other possible reasons counting for interaction of VitETPGS with insulin include; the negative surface charge on VitETPGS holds strong affinity for the positively surface charged insulin as in acidic solutions insulin exists as a protonated species and VitETPGS is known to furnish negative charge in solution as indicated by negative zeta potential of nanoparticles formulated using VitETPGS (Zhang and Feng, 2006) and the

possible tendency of VitETPGS to strongly adsorb on insulin due to ionic and hydrophobic interactions as molecule of VitETPGS possess bulky shape and a large surface area with tocopherol succinate as the hydrophobic portion (Mu and Feng, 2003). With stabilizers (Fig. 2.9 for MH and ZC and Fig. 2.10 for MC) no change in fluorescence emission of insulin was observed from insulin samples incubated with stabilizers at different concentrations and the obtained emission spectra overlapped with spectrum of native insulin solution indicating conformational stability of insulin in presence of stabilizers. The used stabilizers are inorganic carbonates of magnesium and zinc with very low intrinsic solubility. In addition to this their weakly basic nature, their inability to cause change in microenvironment of tyrosine residues and absence of hydrophobic domains which might interact with hydrophobic domains of insulin, render them pharmaceutically compatible with insulin. Moreover, the possibility of insulin stabilization by the weakly basic stabilizers cannot be overruled though low because stabilizers used had very low solubilities which questions generation of their ionic forms which is reactive and especially in the case of ZC as zinc ions are known to cause formation of insulin dimers, tetramers and hexamers which are pharmacologically inactive species and don't contribute in the therapeutic action of insulin but contribute towards insulin's stabilization (Dunn, 2005). Formation of these high ordered structures of insulin impart stability to insulin and physiologically insulin is stored in pancreas in form of insulin hexamers in co-ordination with zinc ions. Once released the hexamers dissociate into insulin monomers which are pharmacologically responsible for insulin action. The overall results obtained from fluorescence measurements suggested that DMAB and PVA below 5% w/v and MH, ZC

and MC in the studied concentration range were compatible and preserved secondary structure of insulin.

4.3 UV spectroscopy

UV was not a particularly useful technique in this case because the information it yielded for insulin surfactant and insulin stabilizer combinations was mostly concentrated around 200 nm, a region characterised by strong absorption from peptide backbone. In addition to this, most of the organic molecules display a strong tendency to absorb around this wavelength. Moreover, a very weak absorbance was observed around 260-280 nm with all insulin surfactant and insulin stabilizer combinations indicating a small contribution in absorbance from tyrosine and cysteine residues in insulin which are shown as representative examples in figures 2.11 and 2.12 for insulin DMAB and insulin MH combination respectively. Hence no conclusive information regarding the interaction of surfactants and stabilizers with insulin could be interpreted using UV. Proteins in solution absorb ultraviolet light with absorbance maxima at 280 and 200 nm. At 280 nm, the absorbance of protein is mainly due to the amino acids tryptophan, tyrosine and cysteine (Kahn, 1979). Peptide bonds are primarily responsible for the peak at 200 nm (Venyaminov and Yang, 1996). Secondary, tertiary, and quaternary structure all affect absorbance, therefore factors such as pH, ionic strength, etc. can alter the absorbance spectrum of the proteins. Absence of strong absorbance around 260-280 nm was attributed to the tryptophan deficient structure of insulin and therefore the absorption spectra obtained could not be reliably interpreted for assessing information regarding conformational stability of insulin in presence of surfactants and stabilizers.

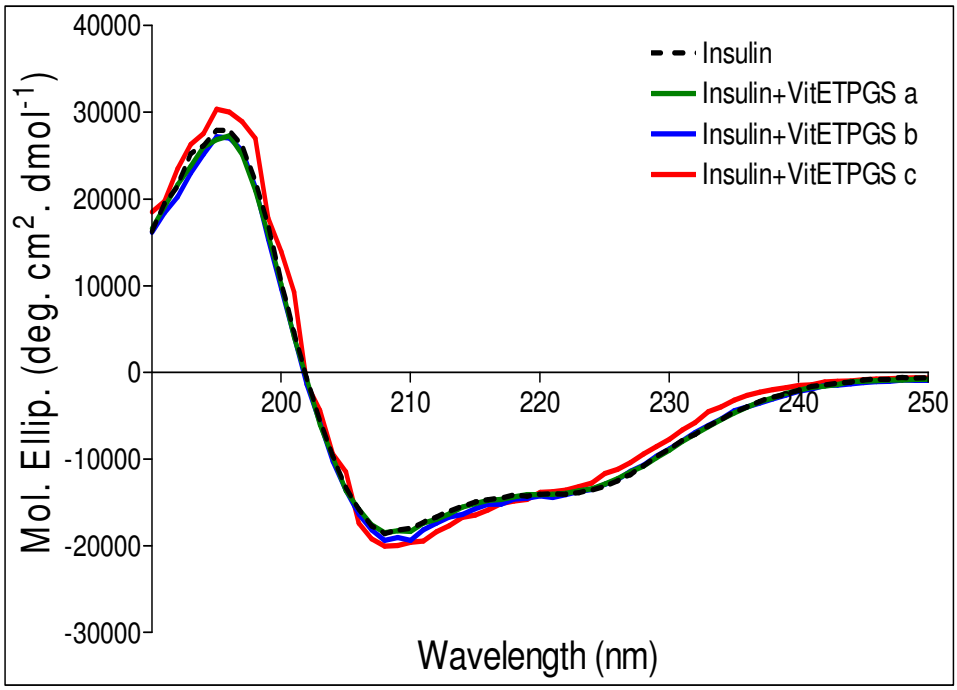


Fig. 2.4 Far UV (190-250 nm) CD spectra of insulin and insulin VitETPGS combinations in 0.05N acetic acid. VitETPGS concentrations are represented as alphabetical keys: a=10, b=100 and c=1000 µg/ml. All insulin solutions were 100 µg/ml and temperature was 25°C. All samples were analyzed in triplicates.

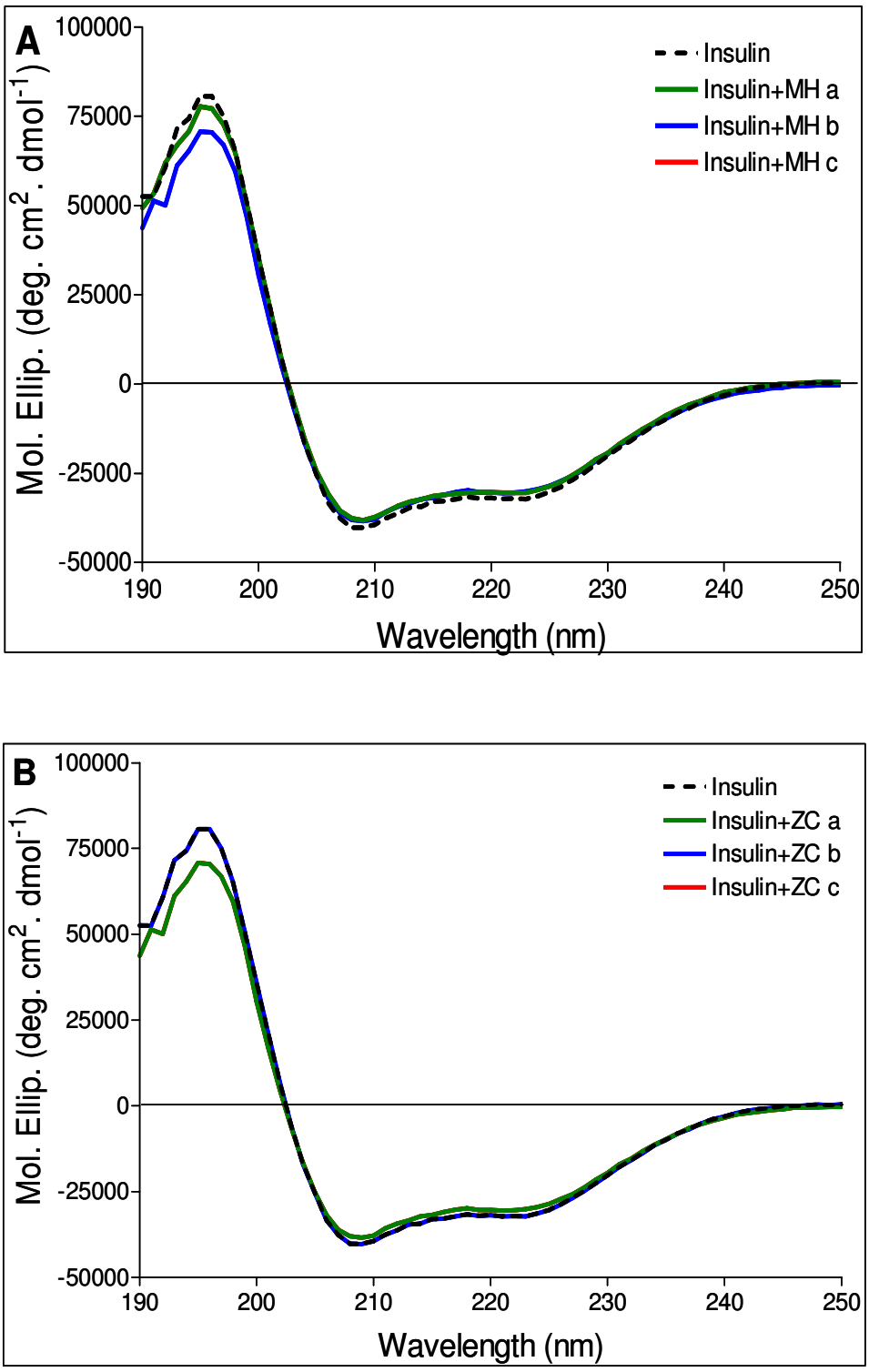


Fig. 2.5 Far UV (190-250 nm) CD spectra of insulin and insulin stabilizer combinations in 0.05N acetic acid. (A) MH (B) ZC. MH and ZC concentrations are represented as alphabetical keys: a=0.5%, b=1% and c=2% w/v. All insulin solutions were 100 μg/ml and temperature was 25°C. All samples were analyzed in triplicates.

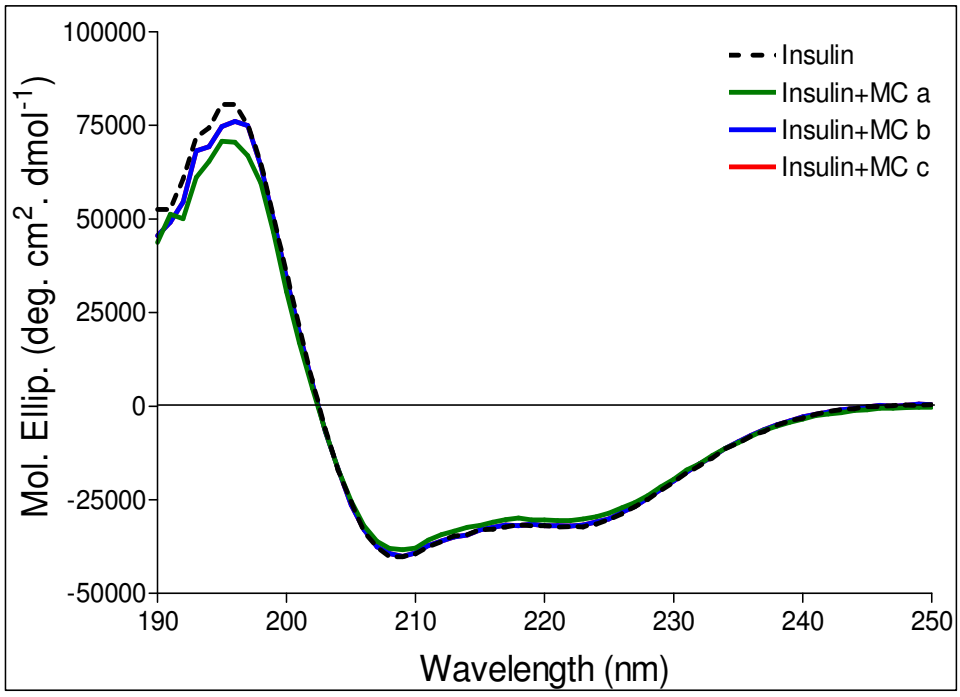


Fig. 2.6 Far UV (190-250 nm) CD spectra of insulin and insulin MC combinations in 0.05N acetic acid. MC concentrations are represented as alphabetical keys: a=0.5%, b=1% and c=2% w/v. All insulin solutions were 100 μg/ml and temperature was 25°C. All samples were analyzed in triplicates.

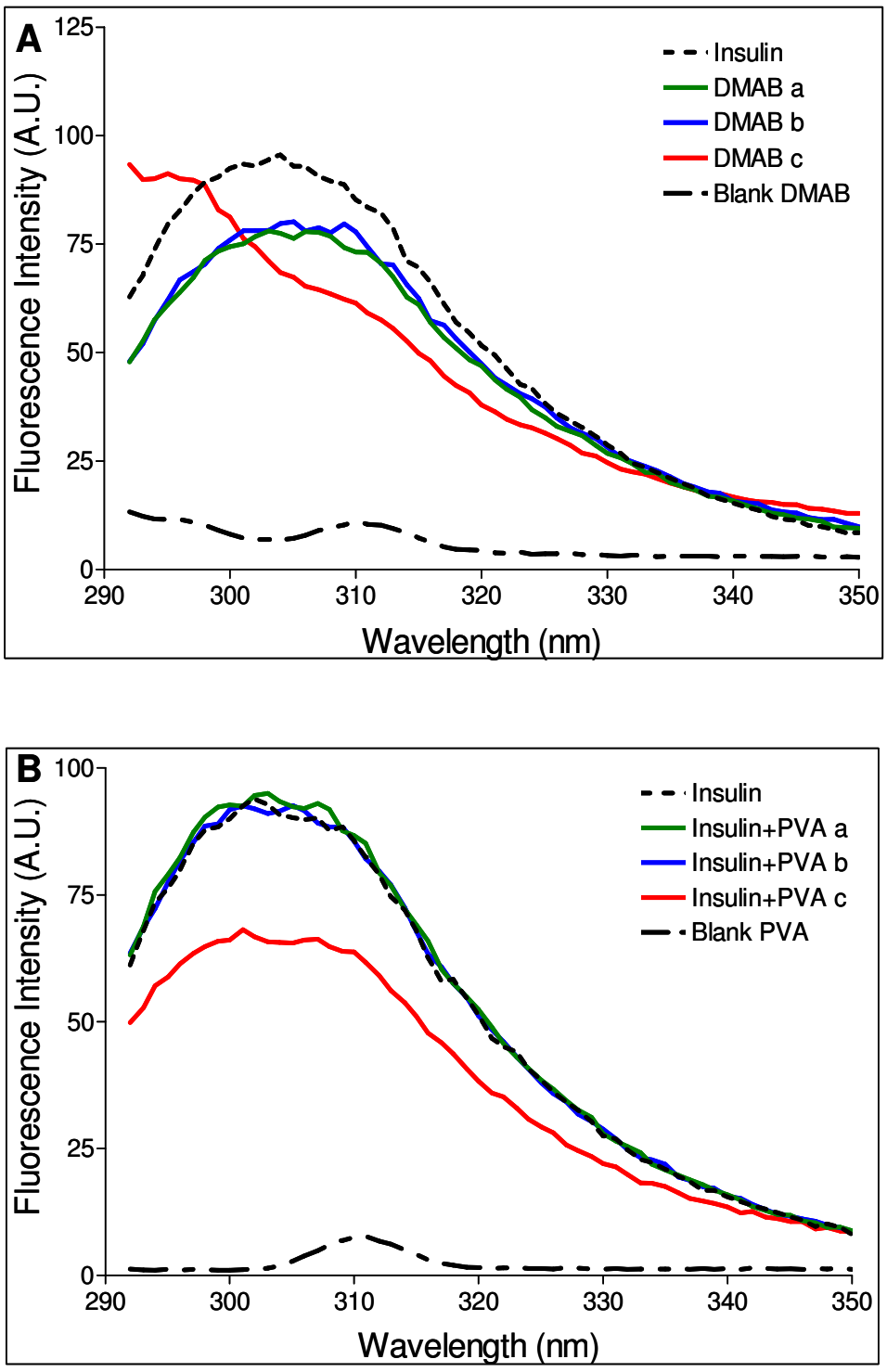


Fig. 2.7 Fluorescence emission (290-350 nm) scans of insulin (2µg/ml) in 0.05 N acetic acid and in combination with DMAB and PVA at 25°C after exciting at 280 nm. (A) DMAB (B) PVA. Alphabetical keys in the legend indicate concentrations of DMAB and PVA used: a=0.05, b=0.5 and c=5% w/v. The blank surfactant solutions were maintained at 0.2 µg/ml. All samples were analyzed in triplicates.

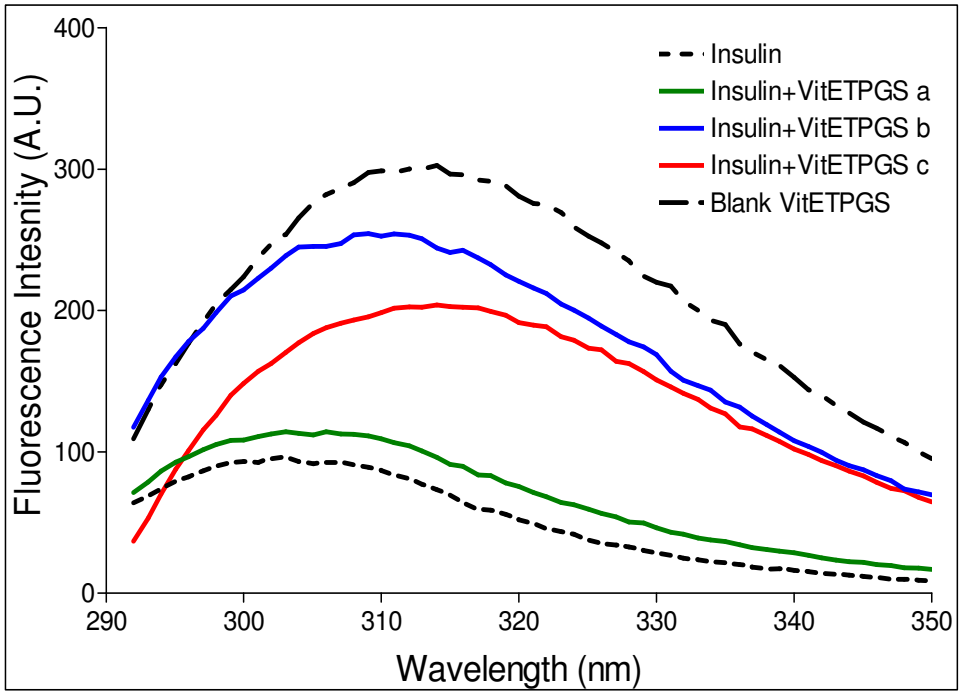


Fig. 2.8 Fluorescence emission (290-350 nm) scans of insulin (2µg/ml) in 0.05 N acetic acid and in combination with VitETPGS at 25°C after exciting at 280 nm. Alphabetical keys in the legend indicate concentrations of VitETPGS used: a=0.05, b=0.5 and c=5% w/v. The blank surfactant solution was maintained at 0.2 µg/ml. All samples were analyzed in triplicates

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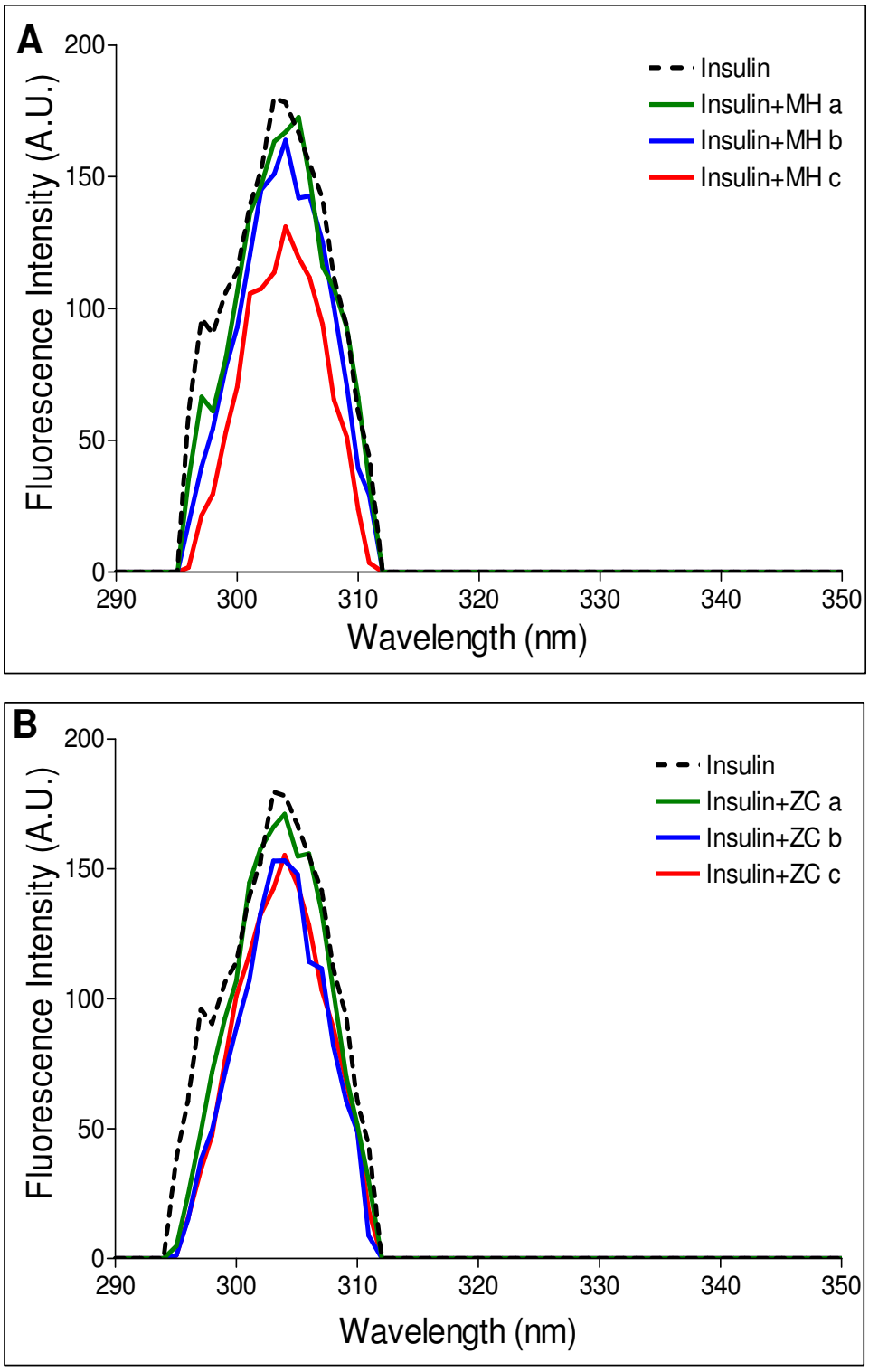


Fig. 2.9 Fluorescence emission (290-350 nm) scans of insulin (2µg/ml) in 0.05 N acetic acid and in combination with MH and ZC at 25°C after exciting at 280 nm. (A) MH (B) ZC. MH and ZC concentrations are represented as alphabetical keys: a=0.5%, b=1% and c=2% w/v. All samples were analyzed in triplicates.

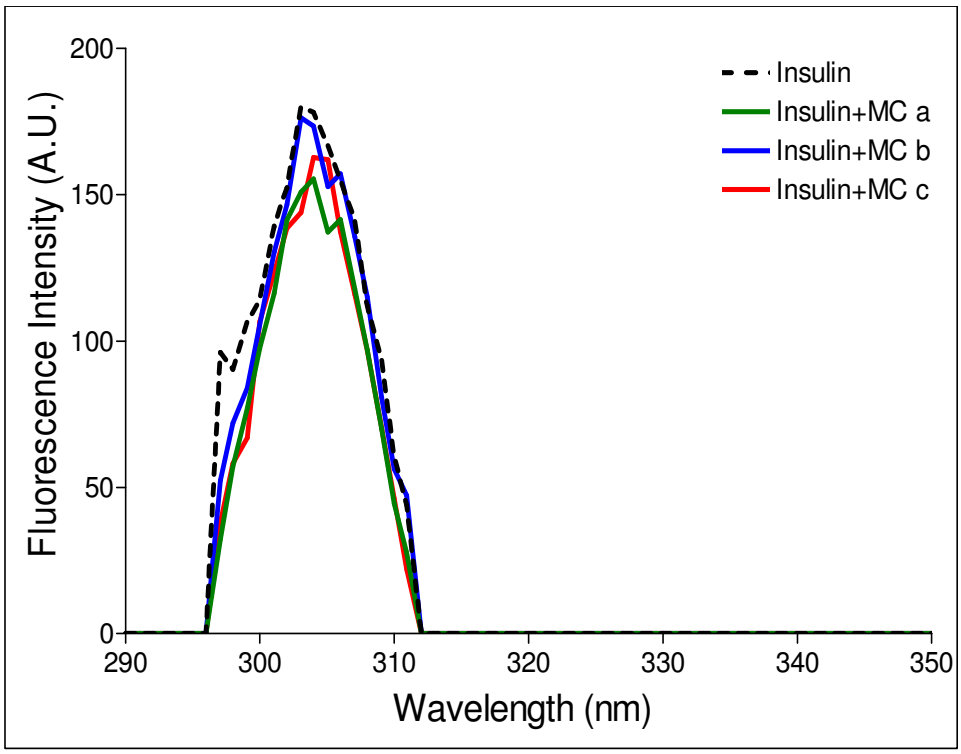


Fig. 2.10 Fluorescence emission (290-350 nm) scans of insulin (2µg/ml) in 0.05 N acetic acid and in combination with MC at 25°C after exciting at 280 nm. MC concentrations are represented as alphabetical keys: a=0.5%, b=1% and c=2% w/v. All samples were analyzed in triplicates.

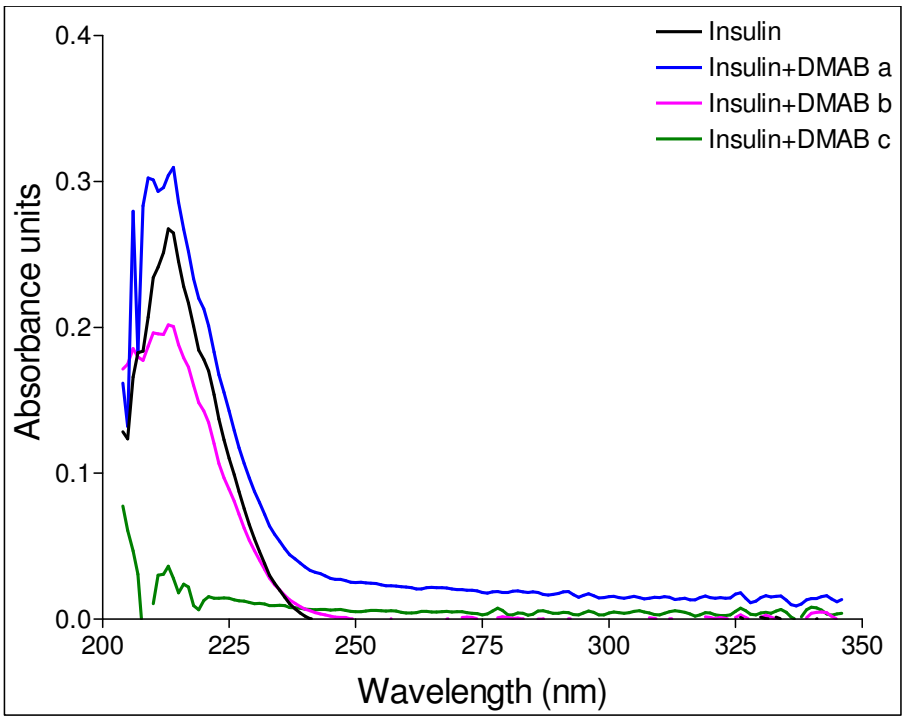


Fig. 2.11 UV scans of insulin solution (2µg/ml) in 0.05 N acetic acid and in combination with DMAB at 25°C. Alphabetical keys in the legend indicate concentrations of DMAB used: a= 0.5, b=1 and c=2% w/v. All samples were analyzed in triplicates.

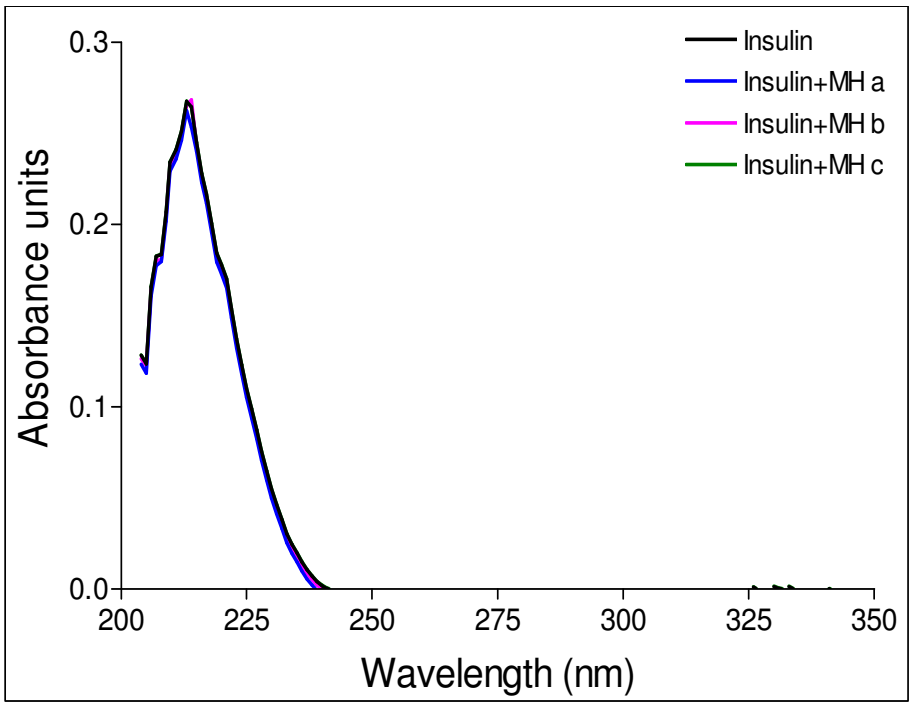


Fig. 2.12 UV scans of insulin solution (2μg/ml) in 0.05 N acetic acid and in combination with MH at 25°C. Alphabetical keys in the legend indicate concentrations of MH used: a=0.5, b=1 and c=2% w/v. All samples were analyzed in triplicates.

5. Conclusions

The results obtained from present investigation pertaining to assess the compatibility of insulin with formulation surfactants and stabilizers lead us to following deductions.

1. CD revealed no significant changes in the extrema of insulin at 196, 209 and 222 nm with surfactants and stabilizers indicating compatibility between insulin and excipients. DMAB, PVA and VitETPGS were compatible with insulin in the studied concentration range (10, 100 and 1000 μg/ml). All the stabilizers (MH, ZC and MC) were found to be equally compatible with insulin in the studied concentration range (0.5-2% w/v) as they did not perturbed the secondary structure of insulin as revealed my similar extrema values.
2. FS gave a better picture about the interactions between insulin and formulation excipients. DMAB and PVA were found to be compatible with insulin below 5% w/v concentration whereas VitETPGS was incompatible with insulin in the studied concentration range (0.05-5% w/v) and even the lowest concentration of 0.05% showed interaction with insulin which was postulated due to combination of aromatic ring stacking, ionic and hydrophobic interactions. DMAB and PVA at 5% w/v concentration interacted with insulin due to micelle formation and hydroxyl group interaction with tyrosine respectively. Stabilizers investigated were shown to be compatible with insulin in the studied range (0.5-2% w/v).
3. UV provided no additional conformational data as the absorbances obtained were mostly concentrated in near 200 nm region and absence of absorbance around 260-280 nm could not be reliably interpreted to assess the interaction between insulin and excipients.

4. The interaction between insulin and VitETPGS was attributed to the following reasons; (i) the negative surface charge on VitETPGS holds strong affinity for the positively surface charged insulin as in acidic solutions insulin exists as a protonated species, (ii) tendency of VitETPGS to strongly adsorb on insulin due to ionic and hydrophobic interactions, (iii) a possible role of VitETPGS acting as a ligand and causing aromatic ring stacking resulting in insulin unfolding and refolding depending upon concentration of surfactant.
5. Based on the results of compatibility screening where DMAB and PVA below 5% w/v concentration did not perturbed the secondary structure of insulin and on the requirement of optimum (more the better) amount of surfactant concentration for stabilizing the formulations (0.5 vs 5% w/v), DMAB and PVA were selected at 1% w/v concentration for formulating insulin nanoparticles. VitETPGS was excluded from the study as even at lower concentrations of 0.5% w/v, its interaction with insulin was observed.

CHAPTER 3

Chapter 3: Formulation development and characterization

Aim: Preparation & characterization of insulin encapsulated nanoparticles

1. Introduction

PLGA based drug delivery systems (microparticles/nanoparticles) have been extensively investigated as controlled release dosage forms for insulin (Takenaga *et al*., 2001; Jain *et al*., 2005; Pinto *et al*., 2007). PLGA has been the most investigated biodegradable polymer, in part due to its approval in drug formulations and medical devices by the FDA and partly due to its versatility in terms of availability of different molecular weights, compositions and variable degradation rates acting as a drug depot.

However, it is well know that proteins and peptides undergo loss of activity after encapsulation or entrapment in these systems (Tobio *et al*., 1999, Sanchez *et al*., 1999; Wada *et al*., 1989). Moreover, during the release, hydration of the protein, reduction of pH (Fig. 3.1) deriving from polymer degradation (Taluja *et al*., 2007) and the presence of hydrophobic surfaces, are all potential sources of protein inactivation (Lin *et al*., 1994; Domb *et al*., 1994) thereby necessitating use of stabilizers in formulating these systems. Uchida *et al*., reported insulin loaded PLGA microparticles prepared using oil/oil method that resulted in degradation of insulin into an unidentified product during the release period (Uchida *et al*., 1996). The same group later reported PLGA microparticles of insulin prepared using water/oil/water method that preserved entrapped insulin from degradation up to 20 days of release (Uchida *et al*., 1997). The different stability exhibited from insulin in the different type of microspheres was ascribed to processing parameters such as preparation method, rate of organic solvent removal and additives used.

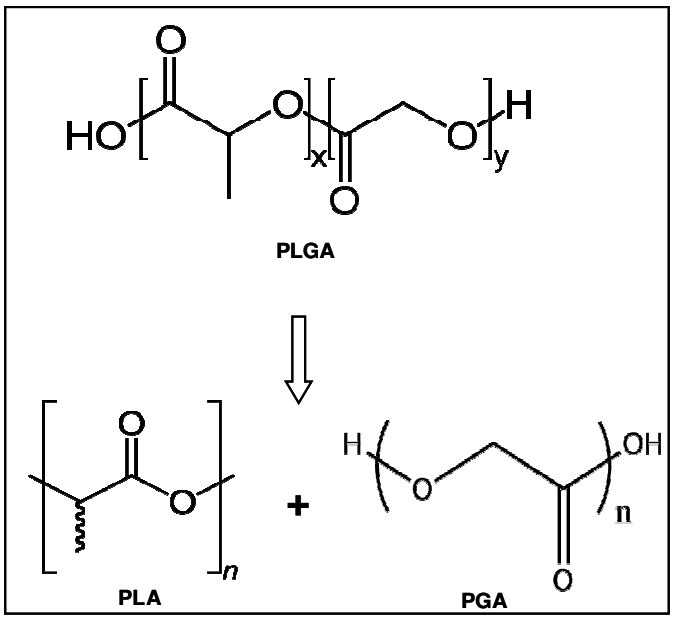


Fig. 3.1 Chemical structure of PLGA and its degradation products polylactic acid (PLA) and polyglycolic acid (PGA) and 'n' refer to the relative amounts of lactide and glycolide units respectively in a specific PLGA copolymer. (Modified from Taluja *et al*., 2007)

Shao and Bailey reported insulin PLGA microspheres prepared using double-emulsion-solvent evaporation and emulsion-solvent evaporation techniques and subjected microspheres to accelerated stability studies at 40°C and 75% relative humidity. Insulin was found to degrade in all microsphere formulations with an average of <50% of the initial loading amount remaining intact at the end of 4 weeks. *In vitro* release studies in phosphate buffered saline at 37°C showed very slow and incomplete (<30% in 30 days) release and analysis of the unreleased insulin within the microspheres revealed that an average of ~11% of the encapsulated insulin remained intact. The degradation of insulin within biodegradable polyester microspheres during stability and release studies was attributed to the gradual decrease in the pH within the microspheres due to progressive

polymer hydrolysis resulting in the production of dl-lactic and glycolic acids (Shao and Bailey, 2000).

Many strategies have been attempted to stabilize insulin inside PLGA microspheres. Trotta *et al*., reported the use of lactose, albumin and lactose-albumin mixtures as stabilizers for preparing insulin encapsulated PLGA microsphere using water diffusion extraction method and obtained entrapment efficiencies of 60% (with albumin) and 75% (albumin-lactose mixture). The water diffusion extraction procedure along with use of stabilizers prevented chemical modification of insulin and retained its stability inside the microspheres (Trotta *et al*., 2004). Rosa *et al*., reported insulin loaded PLGA microspheres prepared using multiple emulsion-solvent evaporation technique in which three non-ionic surfactants: poloxamer 188 (Pluronic F68), polysorbate 20 and sorbitan monooleate 80, at different concentrations (1.5 and 3. 0% w/v) were used separately as stabilizers. Insulin stability within microspheres after processing, storage for 6 months and after release was evaluated and it was observed that polysorbate 20 at 3% concentration was most effective in stabilizing insulin against acidic microclimate of microspheres (Rosa *et al*., 2000). Zhu *et al*., reported moisture and acidic pH (<3) triggered unfolding of bovine serum albumin in PLGA milli-cylinders and microspheres, resulting in peptide bond hydrolysis and noncovalent aggregation. They further modified the polymeric matrix by introducing stabilizer MH that increased microclimate pH and prevented bovine serum albumin's structural losses and aggregation for over one month and this strategy for stabilizing protein was extrapolated for angiogenic basic fibroblast growth factor and bone-regenerating bone morphogenetic protein-2 (Zhu *et al*., 1999). Han *et al*., prepared insulin PLGA microspheres using solid/oil/oil method and to further increase the

entrapment efficiency, modified iso-electric point precipitation method was adopted that lead to high entrapment efficiencies (90-100%). Study about the secondary structure of insulin by Fourier transform infrared spectroscopy (FTIR) indicated high insulin structural integrity in the formulations (Han *et al*., 2008).

Since the pioneering work by Damge and co-workers who reported ester based insulin nanocapsules, a number of researchers have developed PLGA based nanoparticles for insulin (Table 3.1). Barichello *et al*., reported Nanoprecipitation-solvent displacement method for preparing insulin PLGA nanoparticles (Barichello *et al*., 1999). The same group also developed pluronic based gels encapsulating insulin PLGA nanoparticles as a means for further controlling the release of insulin from the system (Barichello *et al*., 1999). Pan *et al*., reported insulin PLGA nanoparticles prepared using double emulsion method employing poloxamer 188 as emulsifier but no additional excipients were used in the study to stabilize insulin in polymeric matrix (Pan *et al*., 2002). In another study, insulin PLGA nanoparticles were prepared using double emulsion employing chitosan coating to increase the encapsulation efficiency of insulin, reduce the initial burst and improve the release behaviour of nanoparticles (Pan *et al*., 2003). Insulin nanoparticles based on different polymeric blends were prepared using PLGA: poloxamer and PLGA: poloxamine blend matrices. The particles were prepared employing modified solvent diffusion technique and insulin encapsulation efficiency was shown to be dependent on the composition of the nanoparticles, those containing hydrophilic poloxamer derivatives being the most effective in entrapping the drug molecules. The formation of the blend system displayed positive effects on the release characteristics of the nanoparticles. Nanoparticles exhibited a reduced initial burst and a nearly

linear, constant release rate over a time period of two weeks (Csaba *et al*., 2004). In order to increase the entrapment efficiency of insulin in nanoparticles prepared using hydrophobic polymers like PLGA, Cui *et al*., prepared liposolube complex of insulin by reacting insulin with soya bean phosphatidylcholine resulting in insulin-lipid complex that gave insulin entrapment of 90% in the nanoparticles (Cui *et al*., 2006). Cui *et al*., also reported pH responsive insulin PLGA nanoparticles by complexing the pH responsive hydroxypropylmethyl cellulose (HPMC) with PLGA. The particles were prepared employing modified emulsion solvent diffusion method in water (Cui *et al*., 2007). Kumar *et al*., reported insulin PLGA nanoparticles using double emulsion method employing different stabilizers such as pluronic F68, trehalose, and sodium bicarbonate with a view to maintain insulin's structural integrity during microencapsulation and subsequent release. The presence of stabilizers in the nanoparticles resulted in an increase in the particle size but a reduction of encapsulation efficiency. Insulin release rate was comparatively higher for the batches containing stabilizers when compared with controls without stabilizers and also the presence of stabilizers resulted in sustained release of insulin. This was the first reported study where a stabilizer sodium bicarbonate was utilized for providing near neutral pH in insulin PLGA nanoparticles but *in vitro* studies confirming the preservation of secondary structure of insulin during process development were not reported (Kumar *et al*., 2007).

In order to further sustain the release of insulin from PLGA nanoparticles, Liu *et al*., reported insulin PLGA nanoparticles encapsulated in cross-linked PVA gels. *In vitro* release further indicated that entrapment of the nanoparticles into the PVA hydrogels reduced the release rate and the total amount of insulin released, suggesting that PLGA nanoparticles entrapped

into the PVA hydrogels showed more suitable controlled release kinetics for protein delivery (Liu *et al*., 2007). In another experiment, insulin was ion paired with sodium dodecyl sulphate (SDS) in order to improve its entrapment efficiency in PLGA nanoparticles that were prepared using spontaneous emulsion solvent diffusion method. The formed hydrophobic complex (insulin-SDS) improved the affinity of insulin for PLGA resulting in higher entrapment efficiencies (89.6%). Further studies in animals revealed the anti-hyperglycaemic activity of nanoparticles confirming *in vivo* activity of the hydrophobic complex (Shi *et al*., 2008). Santander-Ortega *et al*., recently reported insulin nanoparticles based on PLGA:poloxamer (Pluronic F68) and PLGA:poloxamine (Tetronic T904) blends using modified solvent diffusion technique. The formulations were evaluated for stability in simulated gastric and intestinal fluids where a strong interaction between the pure PLGA nanoparticles and the digestive enzymes was observed. However, this interaction was considerably reduced in the blend formulations, although the PLGA:poloxamine systems became colloidally unstable in the simulated gastric fluid (Satander-Ortega *et al*., 2009). Recently, Sun *et al*., reported insulin-sodium oleate complex for enhancing insulin entrapment efficiency in PLGA nanoparticles using emulsion solvent diffusion as nanoparticle preparation method and PVA as the surfactant (Sun *et al*., 2010). This concept was based on the previous work of Shi *et al*., who utilized SDS as the ion pairing agent for insulin.

To best of our knowledge, till date there has been only one formulation strategy (Kumar *et al*., 2007) reported in literature that has utilized stabilizer sodium bicarbonate for stabilizing insulin against acidic microclimate change in PLGA nanoparticles due to hydrolysis, but *in vitro* confirmation of insulin's stability has not been reported. The use of stabilizer excipients,

Table 3.1 Summary of strategies that have been attempted for oral insulin delivery using PLGA based nanoparticles.

Nanoparticles	*Preparation method*	*Size (nm)*	*Stability evaluation*	*In vivo studies (oral)*	*Ref.*
PLGA	NP-SD	160-170	-	Normal rats	Barichello *et al*., 1999
PLGA	DE, poloxamer 188 as emulsifier	149.6	-	Diabetic rats	Pan *et al*., 2002
PLGA	DE, CS as emulsifier	-	-	Diabetic rats	Pan *et al*., 2003
PLGA, insulin-PL	RMSE	200	-	Diabetic rats	Cui *et al.*, 2006
PLGA-HPMC	MESD	169	-	Diabetic rats	Cui *et al.*, 2007
PLGA	DE, poloxamer, trehalose and $NaHCO_3$ as stabilizers	-	-	Diabetic rats	Kumar *et al., 2007*
PLGA+poloxamer, PLGA+poloxamine	MSD	-	Blank nanoparticles in SPF	-	Satander-ortega *et al*., 2009
PLGA, insulin-SO	ESD	160	-	Diabetic rats	Sun *et al*., 2010

CS: Chitosan, DE: Double emulsion, ESD: Evaporation solvent diffusion, insulin-PL: Insulin phospholipid complex, insulin-SO: Insulin sodium oleate complex, MESD: Modified emulsion solvent diffusion, MSD: Modified solvent diffusion, NP-SD: Nanoprecipitation solvent diffusion, RMSE: Reverse micelle solvent evaporation, SPF: Simulated physiological fluids.

sugars and proteins like albumin is well reported for microparticulate systems based on proteins encapsulated in PLGA microparticles. We hypothesized that co-entrapment of an stabilizer along with insulin would certainly prevent generation of acidity in PLGA nanoparticles and would assist in preserving secondary structure of insulin intact and to further confirm our hypothesis, FTIR (Fourier transform infra-red) spectroscopy and stability studies of nanoparticles in simulated physiological fluids were carried out.

2. Materials

Bovine serum insulin (nominal strength 27 units/mg) was purchased from Sigma (St. Louis MO, US). PVA (cold water soluble with molecular weight 30-70 K Da) was purchased from Sigma (St. Louis MO, US). DMAB was purchased from Sigma (St. Louis MO, US). VitETPGS (NF grade) was a gift sample from Eastman Company, Kingsport (UK). PLGA 50:50 (Molecular weight (35-45 K Da) was purchased from Boehringer Ingelheim (Germany). Ethyl acetate was purchased from Fisher Scientific Ltd. (UK). All the stabilizers (MH, ZC and MC) were purchased from Fischer scientific (UK). Distilled water was prepared in house and all the reagents used were of analytical grade.

3. Methods

3.1 Fluorimetric based analytical method for insulin estimation

Stock solution of insulin was prepared by dissolving 1 mg insulin in smallest volume of 0.05 N acetic acid and then volume was made up using distilled water to 100 ml to give a final concentration of 10 μg/ml. Different volumes of stock solution were diluted up to 5.0 ml with distilled water. The dilutions

prepared were 1, 2, 4, 6, 8 and 10 μg/ml. All the measurements were performed on spectrofluorimeter in the concentration-fluorescence mode. The fluorescence emission values of insulin solutions were measured between 290-350 nm (emission), after exciting at 280 nm. The selectivity of developed analytical method was also checked after spiking insulin solution with 1% DMAB and 1% PVA. The excitation and emission slits were kept at 5 nm with averaging time of 5 seconds. The spectrofluorimeter was set at 0.95 correlation cut off with minimum 50 units and maximum 1000 units as fluorescence intensity and all the solutions were prepared fresh and were assayed in triplicates and subsequently a calibration curve (fluorescence intensity versus concentration of insulin) was prepared.

3.2 Preparation and characterization of insulin nanoparticles

Insulin-MH, insulin-ZC and insulin-MC coencapsulated PLGA nanoparticles were made by double emulsion method (Fig. 3.2). Briefly, 50 mg PLGA was dissolved in 2.5 ml of ethyl acetate to form the organic phase. The aqueous phase comprised of insulin solution in 0.05 N NaOH (100 μl) along with 100 μl of 1 % stabilizer suspension in distilled water. The aqueous phase was added to organic phase and the system was homogenized at 15000 rpm for 1 min to form the primary emulsion. The primary emulsion was then added into 5 ml (1%) surfactant solution (DMAB and PVA separately) with second homogenization step at 15000 rpm for 1 min to form the secondary emulsion. The secondary emulsion was then added to 25 ml of distilled water and was left on stirring overnight for solvent evaporation. Next day, nanosuspensions were centrifuged at 14000 rpm for 30 min to remove unentrapped insulin and free surfactant to collect nanoparticles. The supernatant was used for the estimation of unentrapped insulin and the pellet obtained was washed twice

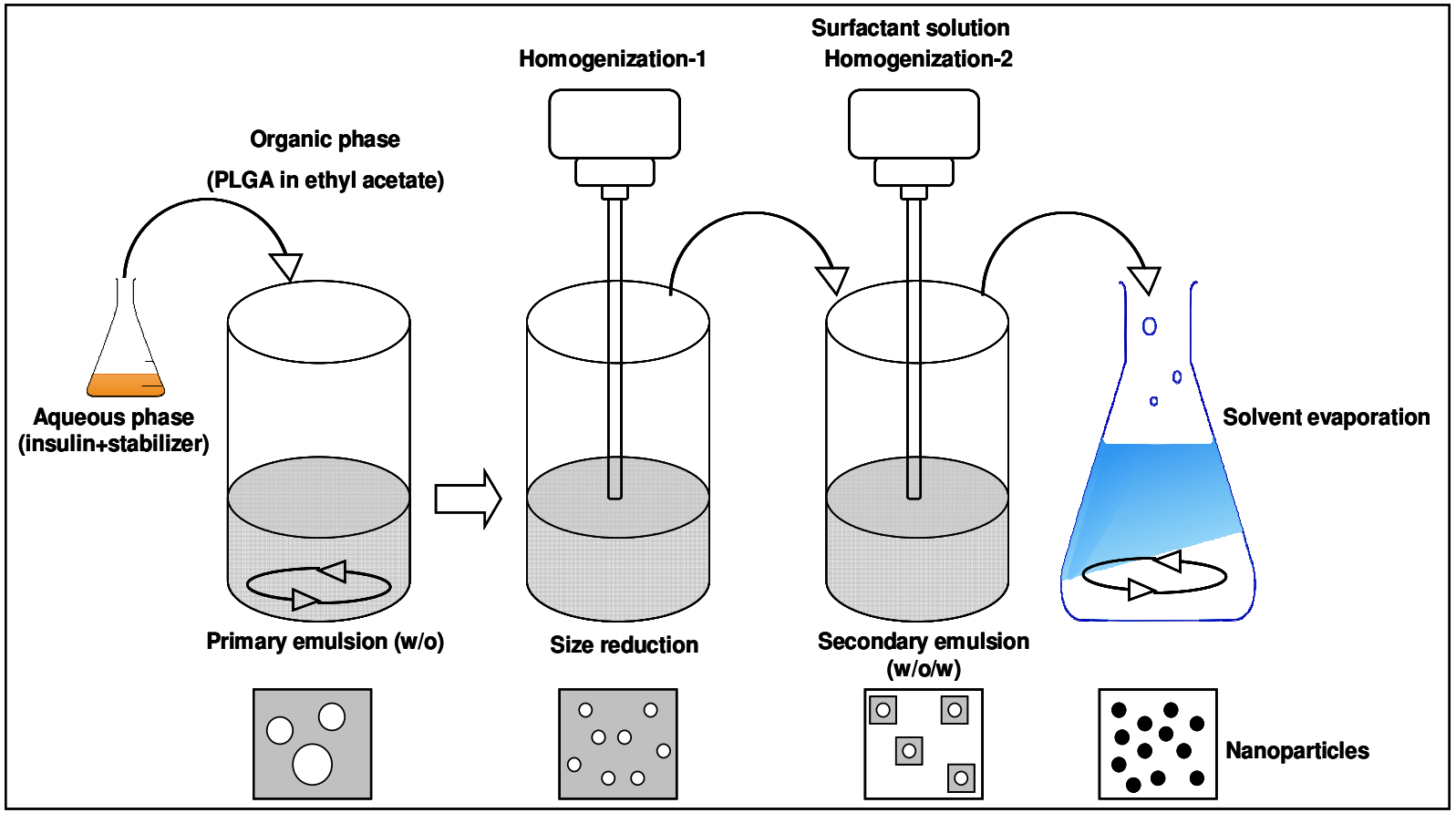


Fig. 3.2 A schematic representation of nanoparticle preparation methodology.

with distilled water to remove any unbound surfactant. The pellet was then dispersed in 1 ml of distilled water using vortex mixer and was freeze dried using trehalose (5% w/v) as the cryoprotectant. The freeze dried nanoparticles were dispersed in 1 ml of distilled water as the diluent and were characterized for their size and polydispersity (PDI) using dynamic light scattering (Nano ZS, Malvern Instruments, Malvern, UK). Dynamic light scattering measurements were obtained in the size versus intensity mode and the data for the particle size was represented as mean of five measurements.

The percentage of drug incorporated into the nanoparticles was determined by centrifuging the drug-loaded nanoparticles and separating the supernatant and estimating the un-entrapped insulin using the developed analytical method. Briefly, 2 ml of nanoparticles suspensions were centrifuged at 14000 rpm for 30 min. The pellet obtained was used further for stabilizer quantification in nanoparticles and the supernatant was used for estimating the amount of un-entrapped insulin. The fluorescence emission values of insulin in supernatant were measured between 290-350 nm (emission), after exciting at 280 nm. The excitation and emission slits were kept at 5 nm with averaging time of 5 seconds. The spectrofluorimeter was set at 0.95 correlation cut off with minimum 50 units and maximum 1000 units as fluorescence intensity and all the solutions were prepared fresh and were analyzed in triplicate. The entrapment efficiency of was calculated using the formula:

$$EE\ (\%) = \frac{Ii - Iu}{Ii} \times 100$$

%EE=Percent entrapment efficiency; Ii=Insulin loaded initially; Iu=Insulin unentrapped estimated from supernatant. For determination of stabilizer

content of nanoparticles, back titration was performed. Briefly, the pellet obtained as above was digested in known excess volume of pre-standardized 0.1 N hydrochloric acid to neutralize all the stabilizer content in the nanoparticles. This neutralized stabilizer solution was back titrated with pre-standardized 0.1 N sodium hydroxide solution using phenolphthalein as indicator to achieve the end point that indicated excess of acid remaining in the solution after neutralization of stabilizer. The amount of excess acid was calculated from normality equation and it was subtracted from the amount of total acid added initially to give the amount of acid consumed in neutralizing the stabilizer in nanoparticles. From standard stoichometric reaction of individual stabilizers (MH, ZC and MC) and acid, the amount of stabilizer present in nanoparticles was calculated. The nanoparticles were made at 2, 4 and 8% w/w of initial insulin load to the polymer to understand the effect of the drug load on nanoparticle characteristics. Similarly, the effect of stabilizer was also studied keeping the insulin concentration same.

3.3 FTIR studies for evaluating insulin's stability in nanoparticles.

One milligram of freeze dried nanoparticles were mixed with 300 mg of micronized KBr powder and compressed into discs at a force of 10 kN for 2 min using a manual tablet press (Perkin-Elmer, Norwalk, USA). IR-spectra were recorded using a Jasco FT/IR-spectrometer-4200 (Easton, MD, U.S.). For each spectrum a 256-scan interferogram was collected with a 2 cm^{-1} resolution in the mid-IR region at room temperature. The scanning speed was kept at 1 mm/sec and 3-Sigma Gaussian was used for apodization. Insulin spectra were obtained according to a double subtraction procedure (Dong *et al*., 1990) and insulin free systems were collected under identical conditions for blank subtraction. For better understanding of insulin in

nanoparticulate formulations second derivative IR was carried out. The spectra were deconvoluted using 100 points and then Savitsky–Golay algorithm was applied using second order derivative and second order polynomial at 25 data points, and the baseline was corrected using a 3-4 point adjustment. In addition, the spectra were area-normalized in the amide regions from 2000 to 1000 cm^{-1} using the spectral manager software (Jasco) and area-overlap was compared to the insulin standard. All samples were run in duplicate.

3.4 Transmission electron microscopy

The morphology of insulin nanoparticles was investigated using transmission electron microscopy (TEM) (LEO 912, Cambridge, UK). Carbon-coated 200 mesh copper grids were glow discharged and specimens in distilled water were dried down to a thin layer onto the hydrophilic support film. 20 µl of 1% aqueous methylamine vanadate (Nanovan, Nanoprobes, NY, US) stain was applied and the mixture dried down immediately with filter paper to remove excess liquid. The dried specimens were imaged with a LEO 912 energy filtering transmission electron microscope at 120kV. Contrast enhanced, zero-loss energy filtered digital images were recorded with a 14 bit/2K Proscan CCD camera.

3.5 In vitro stability/release studies

In vitro stability/release studies were carried out under simulated physiological conditions. Six milligrams of insulin was dispersed in 300 µl distilled water and 100 µl aliquots (corresponding to 2 mg insulin) were mixed with 5 ml of simulated gastric fluid (SGF: 40 mg NaCl + 64 mg pepsin + 1.6 ml of 1 M hydrochloric acid, diluted to 20 ml at pH 1.2) and was stirred

at 100 rpm at 37°C for 2 h after which the mixture was centrifuged at 14000 rpm for 15 min to remove any precipitate and was analyzed for insulin content. In another experiment 6 mg insulin was dispersed in 300 µl distilled water and 100 µl aliquots (corresponding to 2 mg insulin) were mixed with 5 ml of simulated intestinal fluid (SIF: 136 mg KH_2PO_4 + 50 mg pancreatin in 20 ml distilled water at pH 6.8) and was stirred at 100 rpm at 37°C for 6 h after which the mixture was centrifuged at 14000 rpm for 15 min to remove any precipitate and was then analyzed for insulin content. CD spectra of native insulin in simulated fluids were recorded in the far UV region (190-250 nm) in a 0.01 cm path length cell using a step size of 1 nm. The lamp housing purged with nitrogen and an average of 3 scans was obtained. CD spectra of the appropriate blank reference were recorded and subtracted from the insulin spectra, to eliminate contributions from the aqueous phase. The mean residual molar ellipticity was calculated from the raw CD signal using a mean residue weight of 116 Da, and insulin concentration calculated from the corrected absorbance of the insulin solution at 280 nm. Native insulin suspension (equivalent to 2 mg insulin) subjected separately in simulated SGF and SIF was used as the control. For assessing behaviour of nanoparticles under simulated physiological conditions, nanoparticles were centrifuged at 10000 rpm for 30 min and pellet was collected. The pellet (nanoparticles equivalent to 2 mg insulin) was dispersed in 1 ml of distilled water and was transferred into pre-activated dialysis membranes 12 KDa molecular weight cut off. The nanoparticles were suspended in 5 ml of modified SGF (without pepsin) and were stirred at 100 rpm at 37°C for 2 h followed by modified SIF (without pancreatin) for 6 h at same conditions of stirring and temperature. Insulin released from nanoparticles after 2 h in SGF and after 6 h in SIF was quantified using the developed fluorimetric method.

4. Results and discussion

4.1 Fluorimetric based analytical method for insulin estimation

Fluorimetric based analytical method was adopted and validated for insulin estimation in nanoparticles. A linear relationship in fluorescence with respect to insulin concentrations (1-10 μg/ml) was obtained with a good correlation value (R^2= 0.99683 ± 0.00015). The method validation parameters are given in Table 3.2.

In insulin the A-chain has an N-terminal glycine residue and contains 4 residues of cysteic acid, which forms dithio bonds. The B-chain has an N-terminal phenylalanine residue and contains 2 residues of cysteic acid, the two chains together containing a total of 7 residues of phenylalanine and tyrosine that are responsible for fluorescent activity of insulin (Fig. 3.3). Earlier methods developed using fluorimetry utilized bovine insulin solutions at pH 9.3 and after exciting insulin solutions at 278 ± 2 nm; emission was monitored at 305 nm. These methods failed to produce a linear relationship between fluorescence intensity and lower insulin concentrations and 10-200 μg/ml was the reported detection range.

We adopted and modified the method by using insulin solution at pH 3 and were able to reduce the detection range of insulin 10 times lower than the reported method. In addition to this, the developed method was rapid, sensitive, quantitative, qualitative, and specific and additionally stability indicating method for insulin as any change in fluorescence emission was strongly correlated with change in the environment of tyrosine residues in insulin which suggests possible perturbations in secondary structure of the molecule.

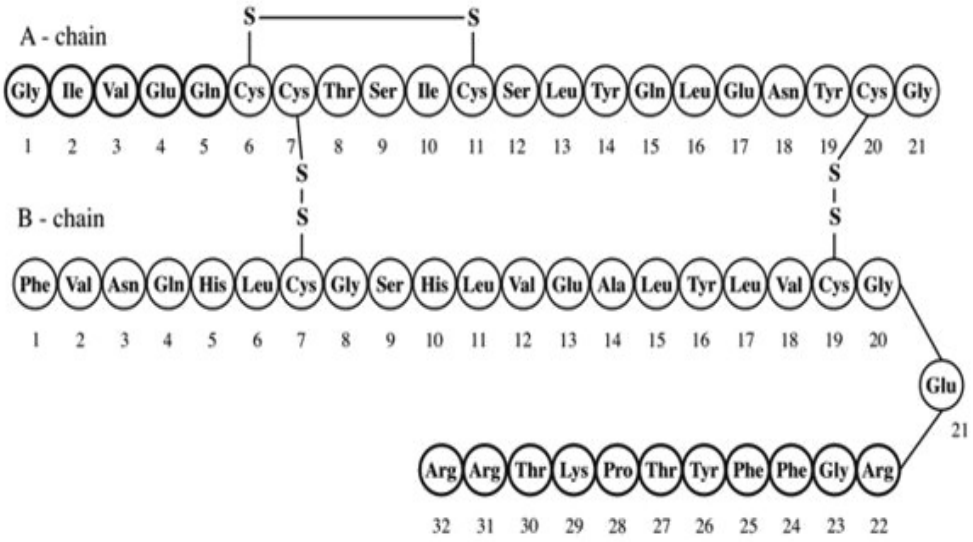


Fig. 3.3 Peptide chains A and B of insulin showing amino acid sequence. The amino acids, phenylalanine and tyrosine residues are responsible for the fluorescent properties of insulin in solution.

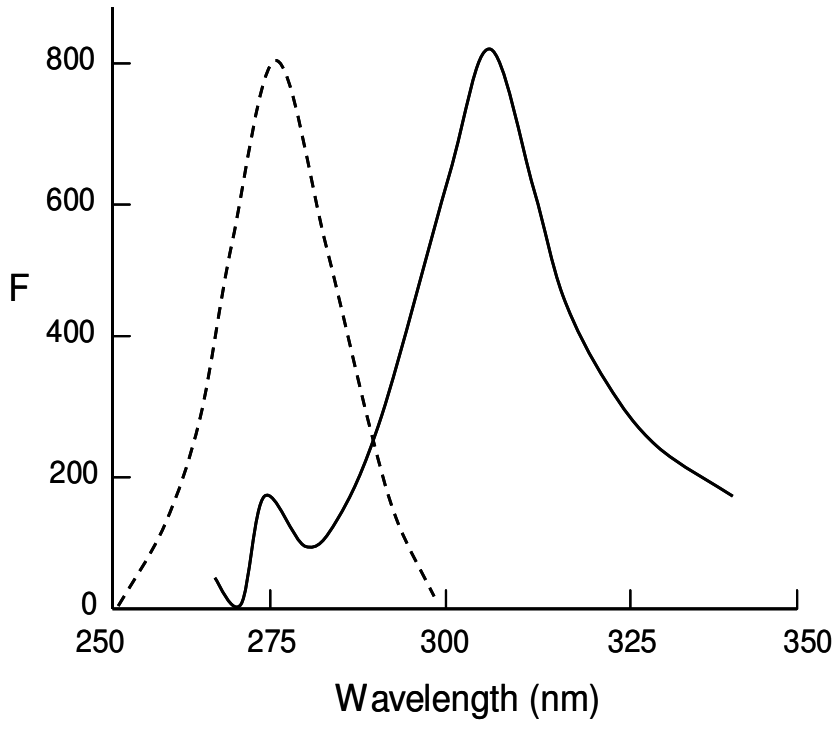


Fig. 3.4 The excitation and emission spectra of 0.4 mg/mL bovine insulin (pH=9.3) solution at constant λem=305nm, λex=282 nm respectively.

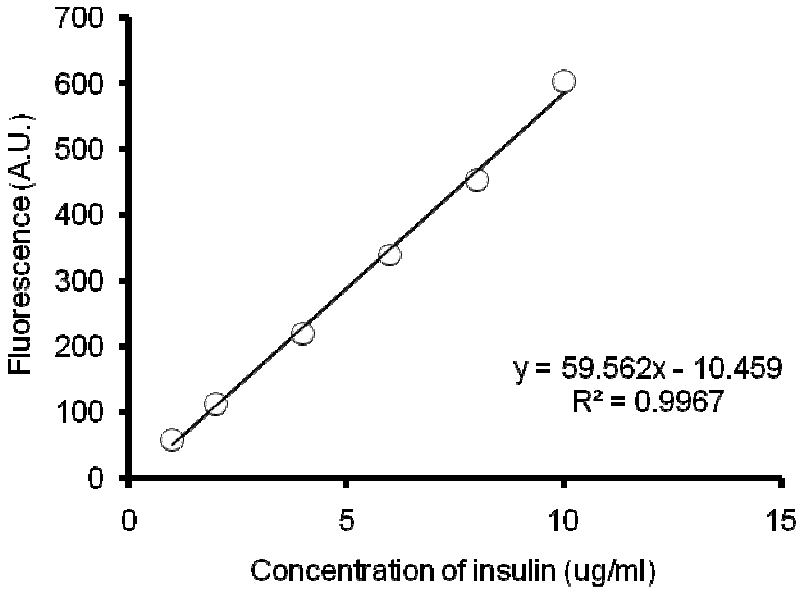


Fig. 3.5 Fluorescence (arbitrary units) vs. concentration of insulin solutions in μg/ml. All the solutions were analyzed in triplicate at room temperature. Excitation wavelength was 280 nm and emission wavelength was monitored from 290-350 nm.

Table 3.2 *In vitro* method validation parameters.

Parameters	***Values***
Linearity range[a]	1-10 μg/ml
R^2	0.99683 ± 0.00015
Slope	59.473 ± 0.078
Intercept	10.06713 ± 0.36515
Excitation wavelength	280 nm
Emission wavelength range	290-350 nm

[a]Linearity established using six concentrations (n=3). Values are Mean ± S.D.

4.2 Preparation and characterization of insulin nanoparticles

Double emulsion evaporation is a well reported method for entrapping hydrophilic molecules like insulin and lysozyme (Bilati *et al*., 2005) in PLGA nanoparticles. Earlier work reported from our lab showed that water soluble drugs like doxorubicin hydrochloride can be successfully entrapped in PLGA nanoparticles with high entrapment efficiencies and small particle size (Kalaria *et al*., 2009). The entrapment of proteins into nanoparticles is in itself a challenging task as each protein is characterized by essential properties like molecular weight, hydrophilicity and stability that might be somehow different with respect to another protein. The choice of a correct formulation strategy is mainly driven by solubility and molecular stability considerations of protein under investigation and in this context, the water-in oil-in water (w1/o/w2) double emulsion method was used for successfully encapsulating insulin in PLGA nanoparticles, mostly because it allows hydrophilic proteins like insulin to be dissolved in an aqueous (w1) phase before the encapsulation process (Bilati *et al.,* 2005). However, insulin might be altered to some extent by interfaces and agitation stress with this method. Formation of aqueous solution/organic solvent or solid protein/organic solvent interfaces is a commonly encountered destabilization mechanism for insulin resulting in adsorption, unfolding, and subsequent aggregation (Kwon *et al*., 2001; Shao and Bailey, 2000; Rosa *et al*., 2000). Insulin being amphipathic in nature, tend to occupy these interfaces resulting in denaturation. The presence of water makes insulin flexible and more reactive. Emulsification of insulin in PLGA in presence of organic solvents generates a large hydrophobic surface responsible for protein denaturation to a variable degree (Sah, 1999; Johansen, 1998). The type of organic solvent may also play a critical role. Efforts to replace more hydrophobic methylene chloride by

less hydrophobic ethyl acetate have yielded mixed results. Ethyl acetate usually induces less emulsification-induced denaturation and hence was used in the current study. However, some researchers have reported poor encapsulation efficiency, poor nanoparticle characteristics, and higher initial burst release as associated drawbacks (Bilati, *et al.,* 2005) of using ethyl acetate as the organic solvent which makes ethyl acetate a less attractive solvent for nanoencapsulation despite having lower toxicity than other routinely used organic solvents.

Figure 3.6 shows the particle size and PDI of insulin nanoparticles prepared using two different surfactants: DMAB and PVA at 2 % drug loading and 1% MH concentration. Figure 3.7 shows the size intensity distribution for the corresponding formulations. Compared to PVA, DMAB lead to smaller particles and PDI and this was due to stronger action of DMAB compared to PVA in lowering the surface tension at oil-water interface.

The stabilization of the emulsion by surfactants mainly depends on their relative ability to lower the interfacial tension between aqueous and organic phases, which in turn is reflective of ability of hydrophobic portion of surfactant to bind to organic phase droplet and hydrophilic portion of surfactant molecule to remain saturated with water phase. It is possible that long hydrophobic chain of DMAB binds to the surface of organic droplet with significantly higher binding constant relative to more hydrophilic PVA, making DMAB more efficient at lowering the interfacial tension and resulting in smaller particles.

Based on these results, DMAB and PVA were selected for further formulation optimization. Using the same particle preparation protocol, 2, 4 and 8 % insulin to the weight of polymer were tried in order to assess the change in particle size and entrapment efficiency. When 1% DMAB was used

as the surfactant, a decrease in particle size and PDI was observed with increase in insulin loading from 2-8% (Fig. 3.8) whereas with 1% PVA as the surfactant, particle size showed an increasing trend with increase in insulin loading from 2-8 % whereas no specific trend was observed for subsequent PDI values (Fig. 3.9). With 1% DMAB as the surfactant, entrapment efficiency of insulin increased from 63.34 to 64.78%; however, no linear trend was observed. The entrapment efficiency of insulin increased with an increase in drug loading when 1% PVA was used as the surfactant (Fig. 3.10). The decrease in particle size of insulin loaded PLGA nanoparticles with increase in drug loading from 2-8 % at fixed concentration of 1 % DMAB can be attributed to increase in particle number so as to increase in overall surface area as the process of drug loading is driven by an equilibrium, which when gets disturbed on increasing the drug load, tends to re-equilibrate itself by phenomenon of size reduction thereby increasing the surface area to accommodate adsorption of extra amounts of drug (Govender *et al*., 1999) which subsequently causes an increase in entrapment efficiency of the system till a threshold value is increased at which the PLGA matrix gets saturated and entrapment efficiency of the system decreases on further increasing drug loading as seen with 1% DMAB systems with not much significant difference between 2 and 8% insulin loading. However, this is not only the valid explanation of this process and detailed studies are required to actually predict the effect of drug loading on particle size and entrapment efficiency as there are multiple factors like free energy of drug in nanoparticles, size entropy, the interaction parameter χ which characterizes the compatibility between a drug and the hydrophobic core and controls the enthalpic contribution to the free energy, Hildebrand-Scatchard solubility parameter δ, internal nanoparticle pressure, laplace pressure and internal

particle forces that work on molecular level together contribute to the effect of loading on nanoparticle size and entrapment efficiency of drug (Kumar and Prudhomme, 2007).

The increase in particle size of insulin loaded PLGA nanoparticles with increase in insulin loading from 2-8 % using 1 % PVA was attributed to the increased adsorption of insulin on the surface of PLGA nanoparticles that subsequently caused an increase in entrapment efficiency of the system with an increase in insulin loading.

The addition of MH had no effect on particle size, PDI and entrapment efficiency of insulin in nanoparticles (Fig. 3.11 & 3.12). Using same nanoparticle preparation protocol, ZC and MC were also evaluated for their effects as potential stabilizers for co-encapsulation with insulin in nanoparticles. The addition of ZC (Fig. 3.13 & 3.14) and MC (Fig. 3.15 & 3.16) had no effect on nanoparticle size, PDI and insulin's entrapment efficiency in the nanoparticles. The entrapment efficiency of stabilizers in the nanoparticles showed almost a linear increasing trend with increase in stabilizer loading except for MC which displayed a reverse trend. With 0.5% loading, the entrapment of MH was found to be 35% and it increased from 58 to 85% as MH's loading was increased from 1 to 2%. A similar trend in entrapment efficiency with respect to stabilizer loading was observed for ZC but the values were lower than MH, the highest being 70.5% with 2% ZC loading. A reverse trend in entrapment efficiency of stabilizer versus stabilizer loading was observed for MC that showed lowest entrapment of 18.35% with 2% loading and highest entrapment of 27% with 0.5% loading (Table 3.3). The amount of stabilizer inside PLGA nanoparticle was critical for insulin's stability against the acid induced damage caused by the degrading PLGA matrix and logically higher stabilizer content related to

higher acid microenvironment neutralizing potential imparting better stability profile to insulin. In addition to this insulin was found to be compatible with high concentrations of stabilizers and there was no significant change observed in particle characteristics at high stabilizer loading which allowed us to use stabilizers at 2% concentration inside nanoparticles. Moreover among the screened stabilizers, MC showed anomalous behaviour with respect to stabilizer loading and stabilizer entrappment whereas MH and ZC showed similar trend in entrappment efficiencies with their respective loadings. Based on above facts; compatibility, no effect on particle characteristics and requirement of higher amount of stabilizer to neutralize acidic microclimate in PLGA nanoparticles, MH and ZC at 2% concentration were selected as the stabilizers for co-entrappment with insulin in PLGA nanoparticles. Figure 3.17-3.19 shows the size vs. intensity distribution results for nanoparticles prepared using 1 % DMAB, 2% MH and 2, 4 and 8% insulin loading respectively whereas figure 3.20 shows the size vs. intensity distribution for nanoparticles prepared using 1% DMAB at 2% insulin loading without using stabilizer MH. Size vs. intensity distribution results for nanoparticles prepared using 1% PVA and 2% MH at different insulin loadings are shown in figure 3.21-3.23. The data demonstrates the promise of DMAB as a surfactant for insulin nanoparticles over traditional PVA that displayed appreciable entrapment efficiency but resulted in bigger particles. Therefore 1% DMAB was used for subsequent studies.

The addition of stabilizers in the primary emulsion was carried out after making their fine suspension in distilled water. Since the stabilizers existed as powders, this exercise was necessary to entrap them along with insulin in PLGA nanoparticles. Though weakly basic, the stabilizers were inert in

nature offering no avenues for any ionic or hydrophobic interactions either with PLGA or with insulin. Therefore with increase in stabilizer concentration for all the three stabilizers (MH, ZC and MC) there was no change observed in particle size, PDI and entrappment efficiency of insulin. The quantitation of stabilizer content in nanoparticles was carried out and the exercise itself assured the entrappment of stabilizers in PLGA nanoparticles. Moreover, particle size reduction of stabilizer suspension so as to assure their entrappment in PLGA nanoparticles was further confirmed by two successive homogenization steps during primary and secondary emulsion formation. The homogenization steps involved sucking up of liquid (primary or secondary emulsion) through a nozzle and then dispersing the emulsion out of fine outlets with tremendous pressure. As the liquid passed through this outlet, most of the applied pressure energy was transferred into kinetic energy. The particle break-up is initiated by a combination of turbulence and laminar shear though occurrence of other processes in causing particle size reduction (e.g. impact between particles and/or the wall, laminar and/or turbulent shear, cavitation and torsion) cannot be overruled (Peters and Muller, 1998). Previous studies reported by Gupta *et al*., used 2% w/v MH as stabilizer for co-entrappment with hepatitis B surface active antigen in PLGA nanoparticles where addition of MH was carried out as a suspension and was followed by ultrasonication to facilitate entrappment in nanoparticles (Gupta *et al*., 2006). In another study insulin loaded PLGA nanoparticles were prepared and sodium bicarbonate which is an insoluble stabilizer was included as a suspension during preparation of nanoparticles adopting double emulsion technique (Kumar *et al*., 2007).

Increase in stabilizer entrappment efficiency with increase in stabilizer loading was possibly attributed to the water insoluble nature of stabilizers

especially MH and ZC, which caused them to preferentially partition to more hydrophobic organic phase containing PLGA then aqueous phase and due to adsorption of stabilizers on PLGA particles. Whereas the unsual trend in entrappment efficiency with respect to loading was observed in basic MC and this was possibly explained by the basic nature of stabilizer which caused it to partition more in aqueous phase than in organic phase (Barichello *et al*., 1999).

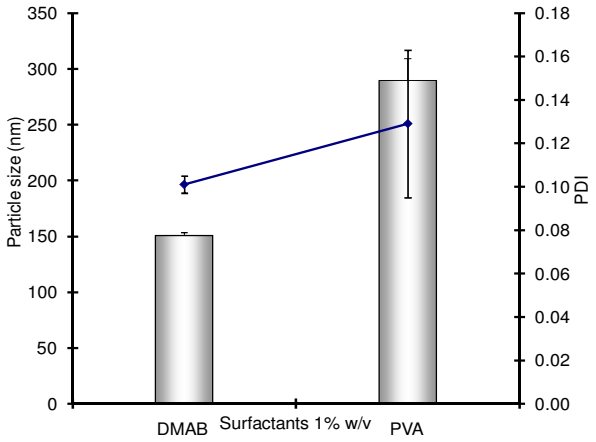


Fig. 3.6 Particle size and PDI of insulin loaded PLGA nanoparticles prepared by double emulsion method using two different surfactants (1% DMAB and 1% PVA). Insulin loading was 2% and 1% MH was used as stabilizer. Data represents mean with standard deviation at n=3.

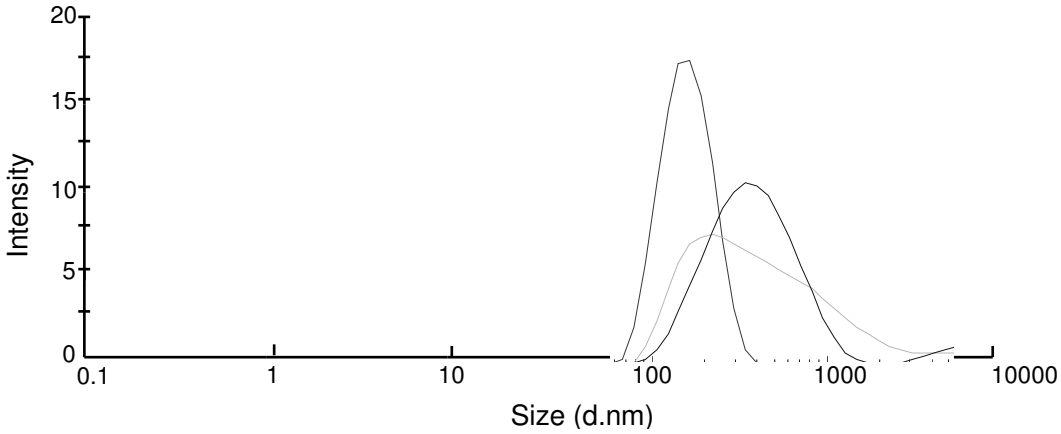


Fig. 3.7 Particle size vs. intensity for insulin stabilizer loaded PLGA nanoparticles prepared using 1% DMAB and 1% PVA employing 1% MH as stabilizer at 2% insulin loading. Colour codes; red: DMAB, green: PVA. All samples were analyzed in triplicates.

Table 3.3 Entrapment efficiencies of stabilizers at different concentrations in insulin nanoparticles calculated using back titration method.

Stabilizer	*Theoretical content (mg)*	*Actual content (mg)*	*Entrapment Efficiency (%)*
MH			
0.5%	0.5	0.175	35.0
1%	1	0.580	58.0
2%	2	1.610	80.5
ZC			
0.5%	0.5	0.115	23.0
1%	1	0.549	54.9
2%	2	1.410	70.5
MC			
0.5%	0.5	0.135	27.0
1%	1	0.245	24.5
2%	2	0.367	18.3

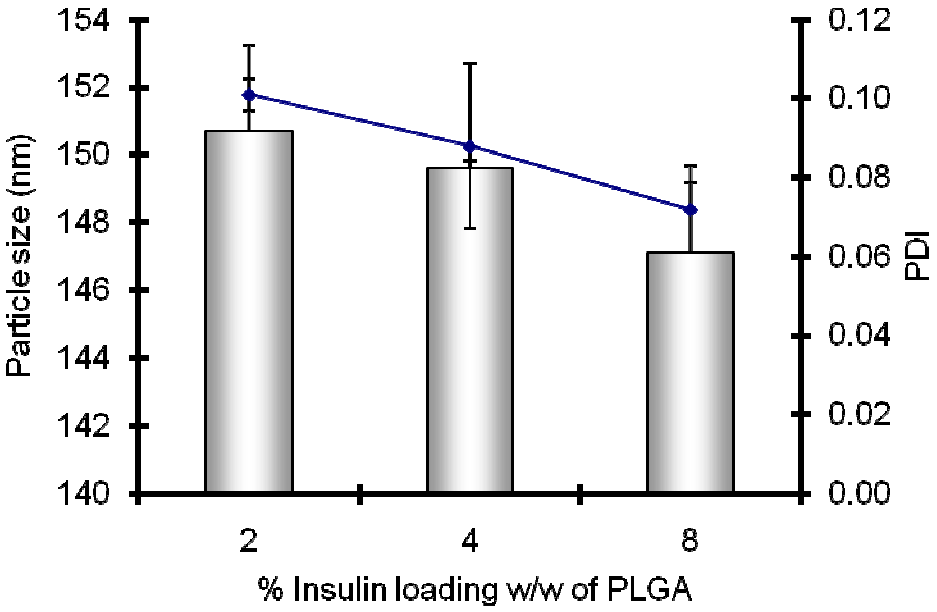


Fig. 3.8 The effect of initial insulin load on particle characteristics that are made of 1% DMAB as surfactant and 2% MH as stabilizer. Data represents mean with standard deviation at n=3.

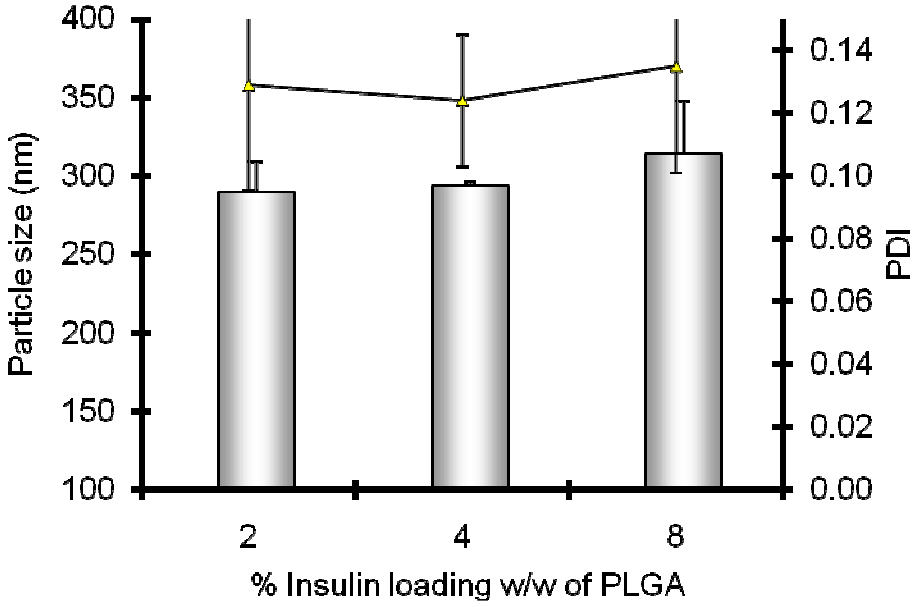


Fig. 3.9 The effect of initial insulin load on particle characteristics that are made of 1% PVA as surfactant and 2% MH as stabilizer. Data represents mean with standard deviation at n=3.

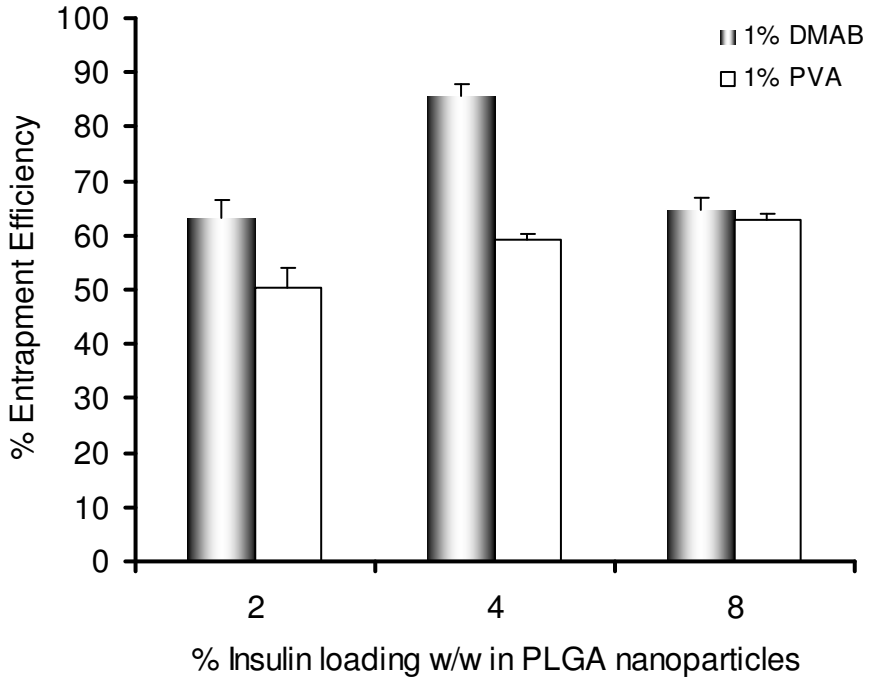


Fig. 3.10 Entrapment efficiency of insulin MH coencapsulated PLGA nanoparticles prepared using two different surfactants: DMAB and PVA at 1 % concentration using 1% MH as stabilizer. Data represents mean with standard deviation at n=3.

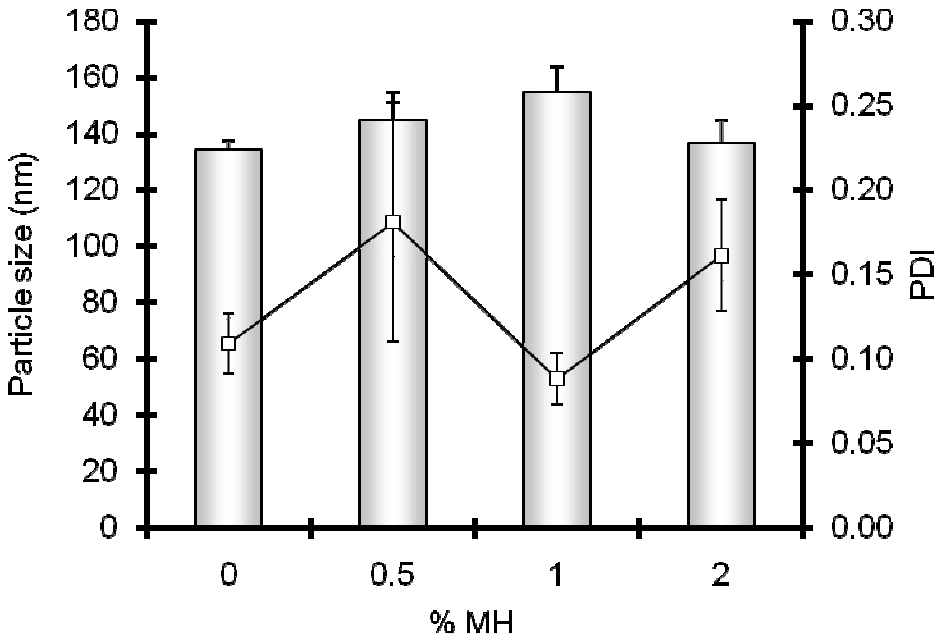


Fig. 3.11 Effect of MH concentration on size and PDI of insulin nanoparticles prepared using 1% DMAB. Data represents mean with standard deviation at n=3.

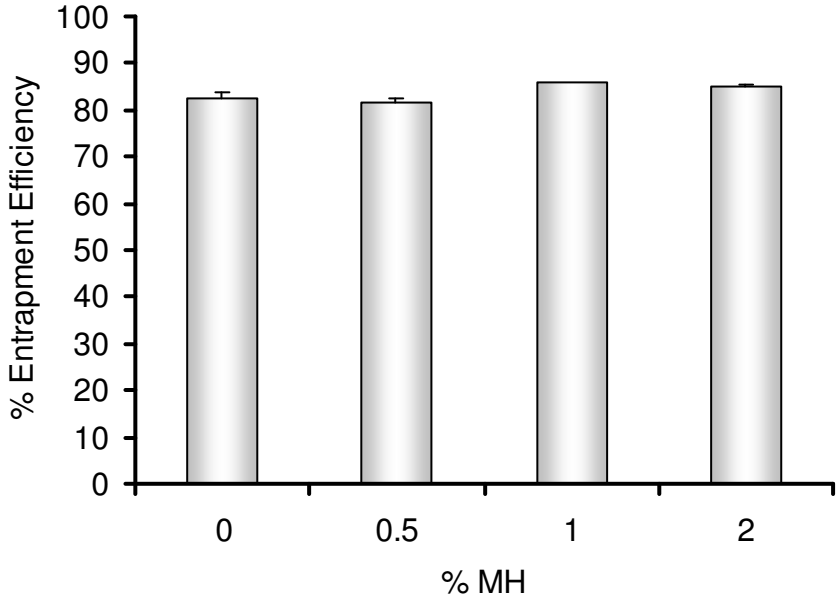


Fig. 3.12 Effect of MH concentration on entrapment efficiency of insulin in PLGA nanoparticles prepared using 1% DMAB. Data represents mean with standard deviation at n=3.

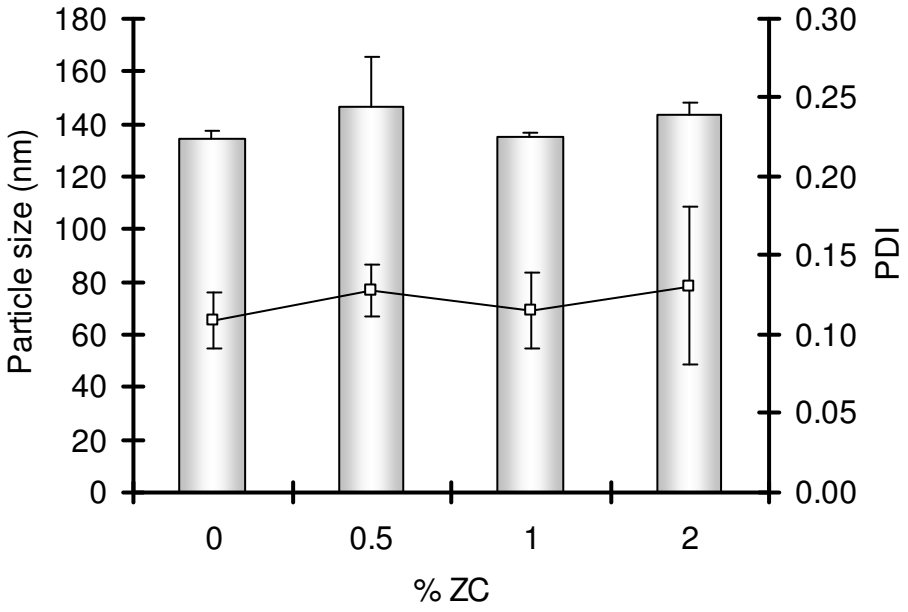


Fig. 3.13 Effect of ZC concentration on size and PDI of insulin nanoparticles prepared using 1% DMAB. Data represents mean with standard deviation at n=3.

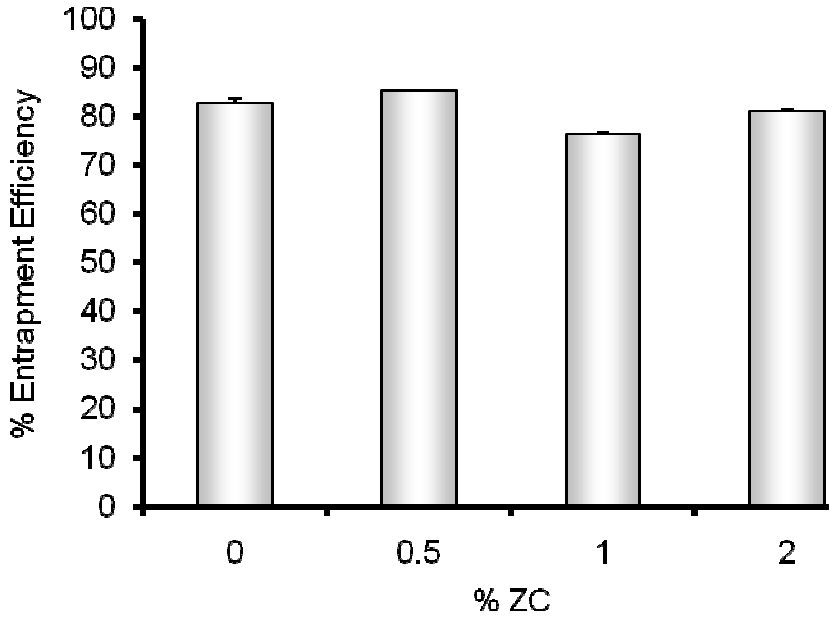


Fig. 3.14 Effect of ZC concentration on entrapment efficiency of insulin in PLGA nanoparticles prepared using 1% DMAB. Data represents mean with standard deviation at n=3.

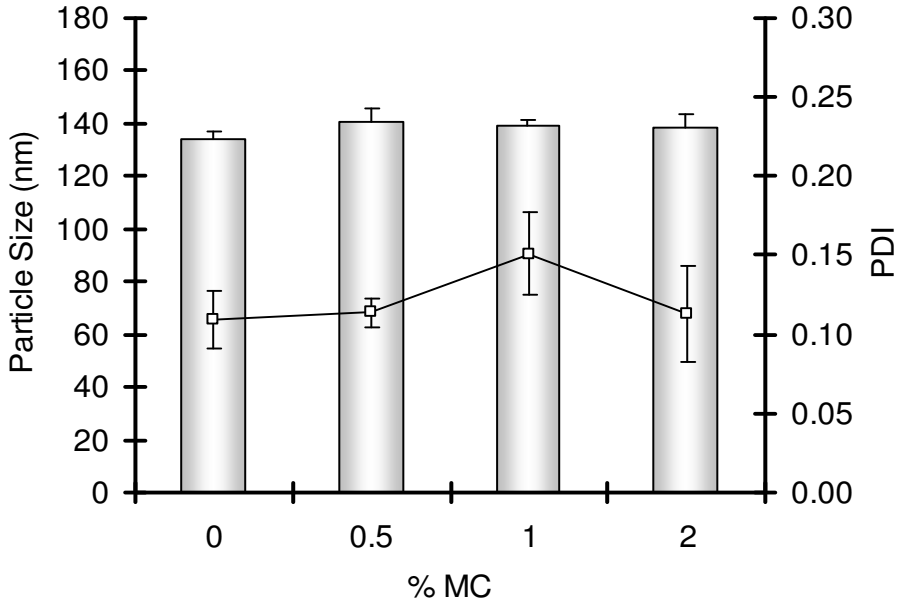


Fig. 3.15 Effect of MC concentration on size and PDI of insulin nanoparticles prepared using 1% DMAB. Data represents mean with standard deviation at n=3.

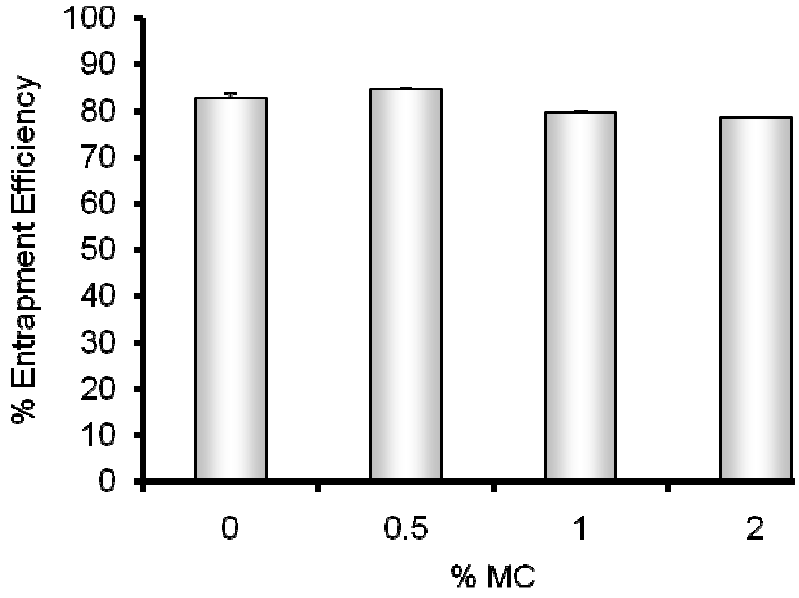


Fig. 3.16 Effect of MC concentration on entrapment efficiency of insulin in PLGA nanoparticles prepared using 1% DMAB. Data represents mean with standard deviation at n=3.

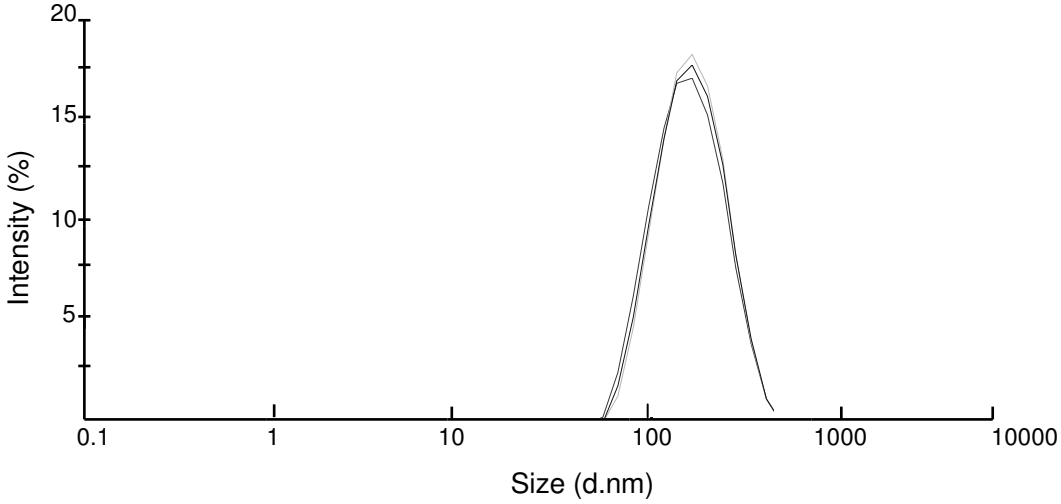


Fig. 3.17 Particle size distribution of insulin MH coencapsulated PLGA nanoparticles prepared using 1% DMAB at 2% insulin loading. MH concentration is 2%. Data shows the overlapping of triplicates.

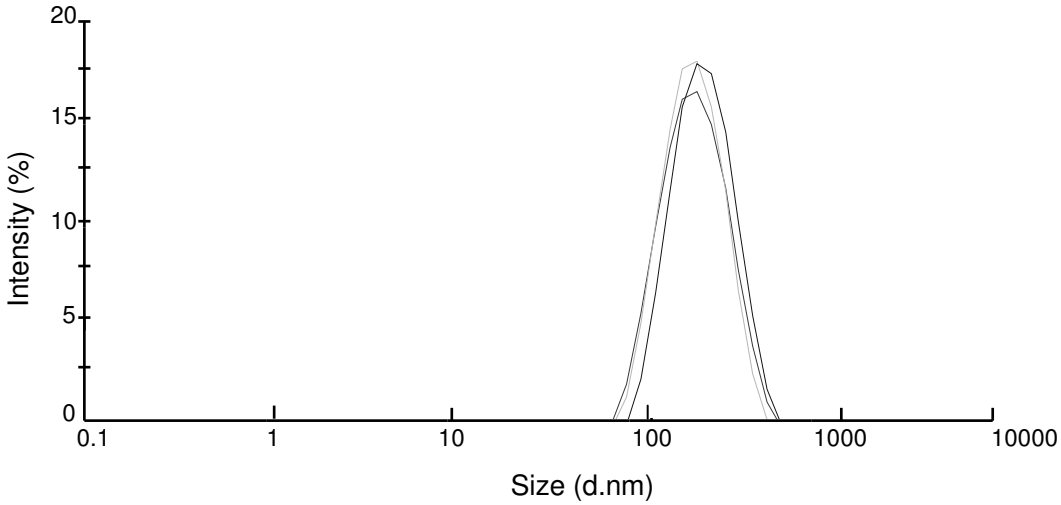


Fig. 3.18 Particle size distributions of insulin MH coencapsulated PLGA nanoparticles prepared using 1% DMAB at 4% insulin loading. MH concentration is 2%. Data shows the overlapping of triplicates.

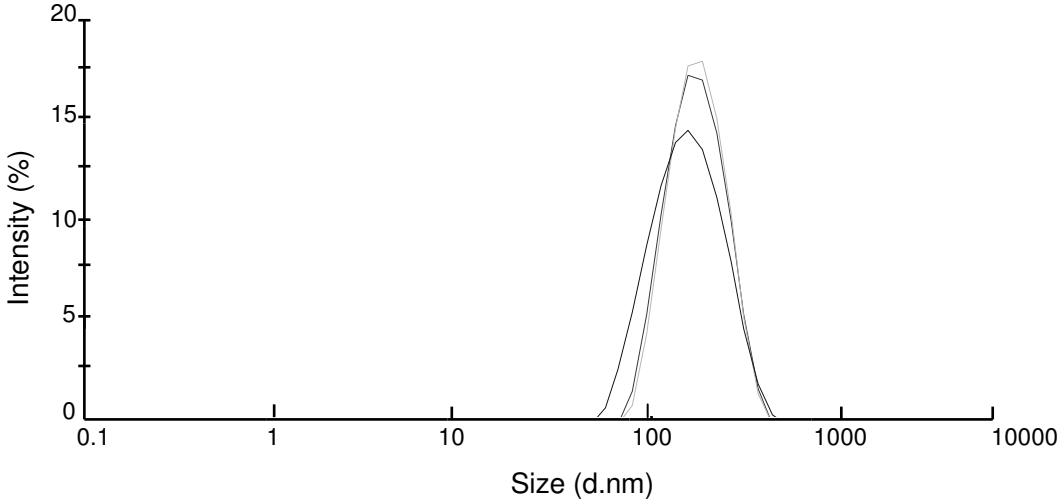


Fig. 3.19 Particle size distributions of insulin MH coencapsulated PLGA nanoparticles prepared using 1% DMAB at 8% insulin loading. MH concentration is 2%. Data shows the overlapping of triplicates.

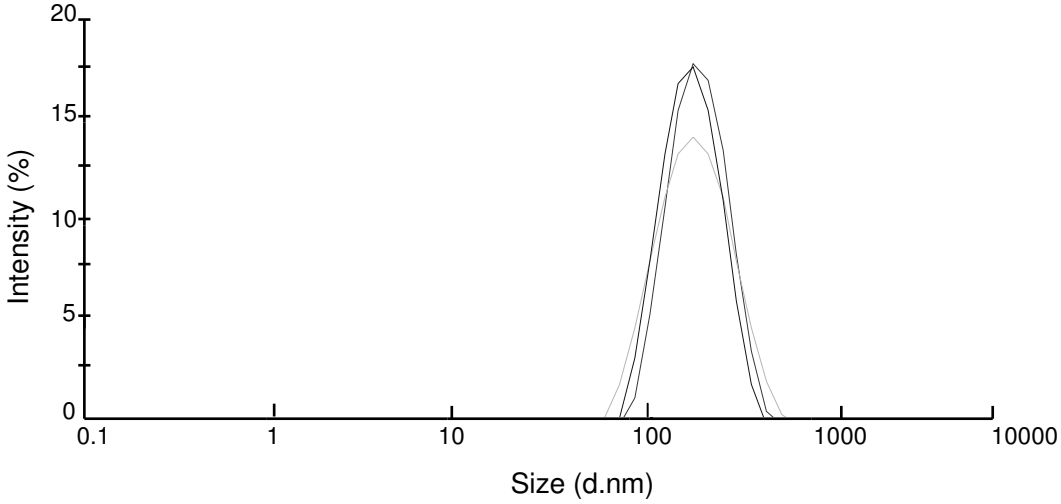


Fig. 3.20 Particle size distribution of insulin loaded PLGA nanoparticles prepared using 1% DMAB at 2% insulin loading without addition of stabilizer MH. Data shows overlapping of triplicates.

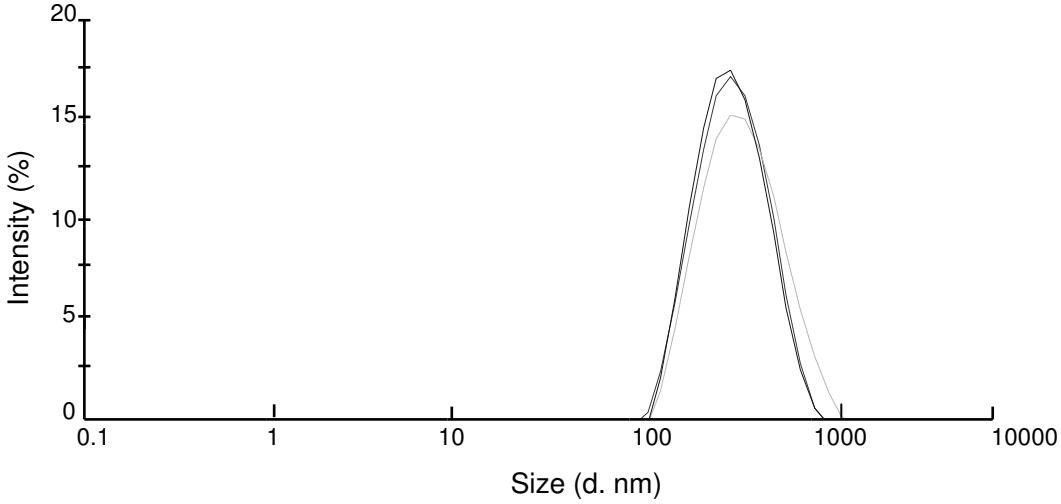


Fig. 3.21 Particle size distributions of insulin MH coencapsulated PLGA nanoparticles prepared using 1% PVA at 2% insulin loading. MH concentration is 2%. Data shows the overlapping of triplicates.

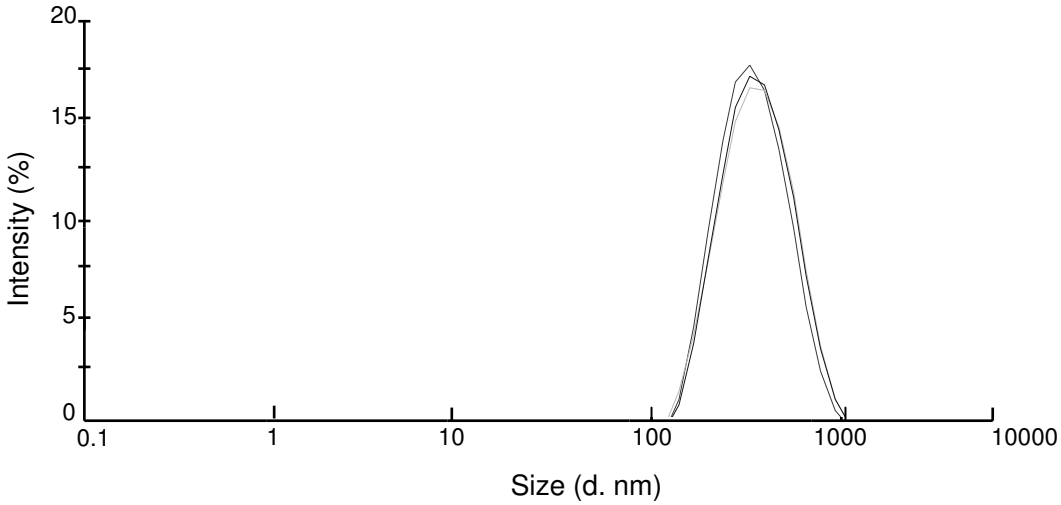


Fig. 3.22 Particle size distributions of insulin MH coencapsulated PLGA nanoparticles prepared using 1% PVA at 4% insulin loading. MH concentration is 2%. Data shows the overlapping of triplicates.

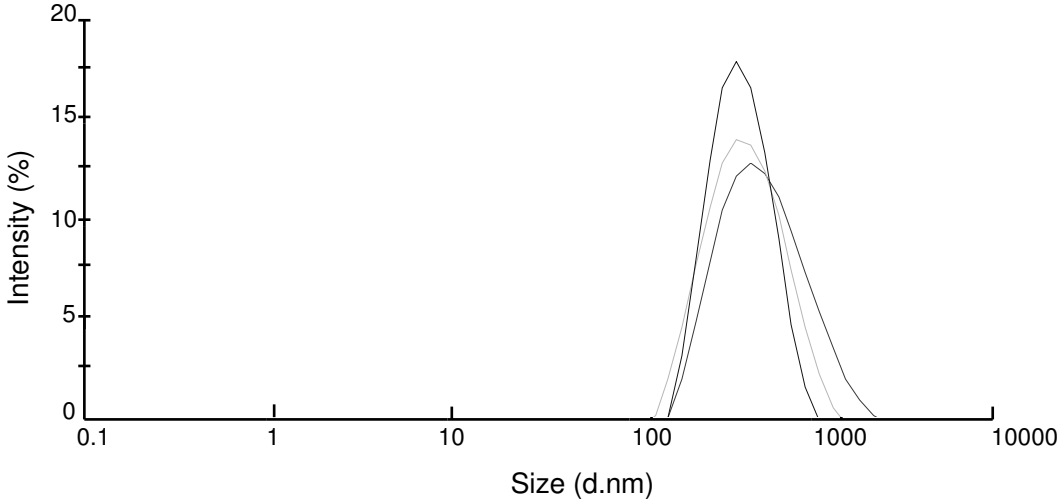


Fig. 3.23 Particle size distributions of insulin MH coencapsulated PLGA nanoparticles prepared using 1% PVA at 8% insulin loading. MH concentration is 2%. Data shows the overlapping of triplicates.

4.3 FTIR studies for evaluating insulin's stability in nanoparticles.

Figure 3.24 shows the typical FTIR profile of lyophilized bovine insulin. The spectrum showed major protein bands: amide A and amides I, II and III positioned at 3309.2, 3067.2, 1653.7 and 1240 cm^{-1} respectively. When second derivatization was carried out overlapping peaks were resolved showing α-helix content (1654 cm^{-1}) and also β-sheet and β-turn minor components (1684 and 1632 cm^{-1}), and these were in agreement with literature findings (Furato *et al*., 1998; Sarmento *et al*., 2007). When insulin was entrapped in PLGA nanoparticles there was only a slight change in the amide band positioning. However, the main peaks corresponding to β-sheet and α-helix remained unchanged as seen in second derivative spectra, which indicates that the protein secondary structure was not significantly altered. Since the concentration of insulin in the nanoparticles was low as compared to the native insulin used as the control, there was an overall decrease in the intensity of bands especially due to overlapping of broad bands of trehalose

and PLGA (Fig. 3.25). The IR spectrum of insulin in the nanoparticles was extracted by subtraction method and compared with spectrum of native insulin. The overlay shows the preservation of amide A and amide bands I, II and III (Fig. 3.26). To further confirm preservation of secondary structure of insulin in nanoparticles, second derivative IR analysis was carried out for enhanced resolution that allows identification of individual peaks in the IR spectrum of proteins. Hence the second derivative IR spectrum of native insulin was compared with second derivative IR spectra of insulin (Fig. 3.27) extracted from nanoparticles after subtraction procedure and the similarity of the spectra suggests that the secondary structure was preserved during and after entrapment into nanoparticles.

Ionic interactions that occur between opposite charges of the protein and the polymers could be responsible for small rearrangements of the protein structure and a simple subtraction of empty nanoparticles spectrum may not be complete because the protein also slightly affects the polymers through ionic interactions (Sarmento *et al*., 2007). In addition to this small change in secondary structure of insulin due to processing conditions like solubilisation in acid before adding to primary emulsion, slight exposure at oil-water interface and the stress due to two homogenization cycles cannot be overruled. The shape of the amide I band of globular proteins is characteristic of their secondary structure. The deconvolution of amide I band is based on the assumption, that a spectrum of single bands (each narrow band is characteristic for a secondary structure) is broadened in the liquid or solid state. Therefore the bands overlap and cannot be distinguished in the amide envelope. A curve fitting procedure is applied to estimate quantitatively the area of each component representing a type of secondary structure along with integration of area of respective peaks and

quantification of secondary structures in insulin can be carried out using this methodology (Susi and Byler, 1983; Byler and Susi, 1986, Surewicz and Mantsch, 1988; Dong *et al.,* 1990) as in insulin the absorption associated with the Amide I band that leads to stretching vibrations of the C=O bond of the amide and the absorption associated with the Amide II band which primary leads to bending vibrations of the N-H bond, play a major role in hydrogen bonding that stabilizes the secondary structure of insulin and any change in secondary structure (denaturation) causes shift in these band positions. No significant change in band positioning (especially amide I and II) was observed in insulin extracted from nanoparticles compared to native insulin which confirmed significant preservation of secondary structure of insulin.

4.4 Transmission electron microscopy

Morphological characterization of nanoparticles was acquired by TEM, to complement the dynamic light scattering results. TEM images of the formulations are presented in Figure 3.28 which confirmed that nanoparticles were well dispersed without any aggregation, spherical in shape, appreciably homogeneous and with submicron sizes. The diameters of the nanoparticles determined by TEM were slightly smaller than determined by dynamic light scattering and this can be attributed to the differences involved in sample preparation: dynamic light scattering being carried out in the liquid state where nanoparticles are relatively hydrated with TEM involving a drying step which results in nanoparticle shrinkage and smaller size compared with light scattering (Liu *et al*., 2007).

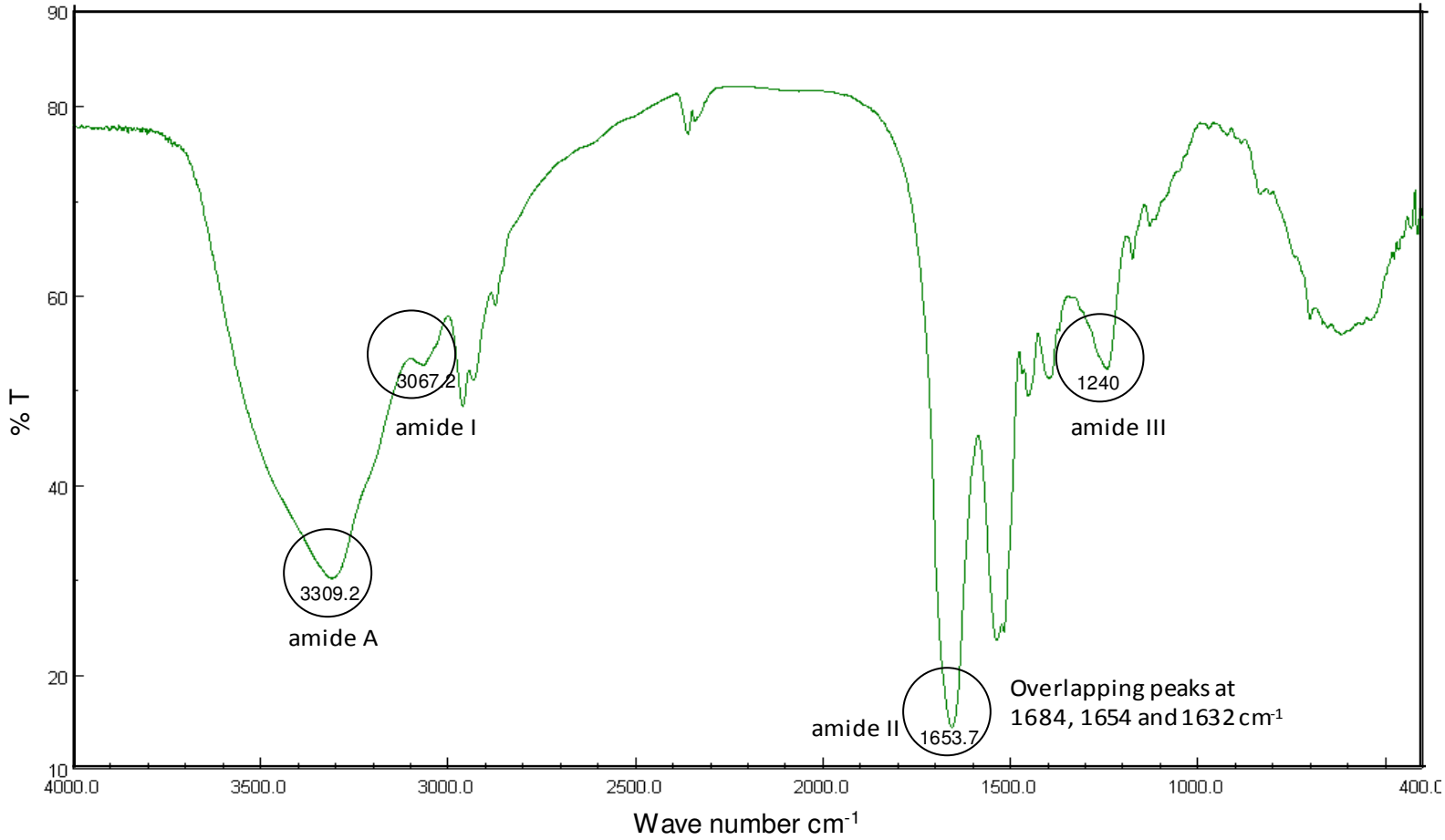


Fig. 3.24 Typical solid state IR spectrum of lyophilized bovine insulin in potassium bromide disc.

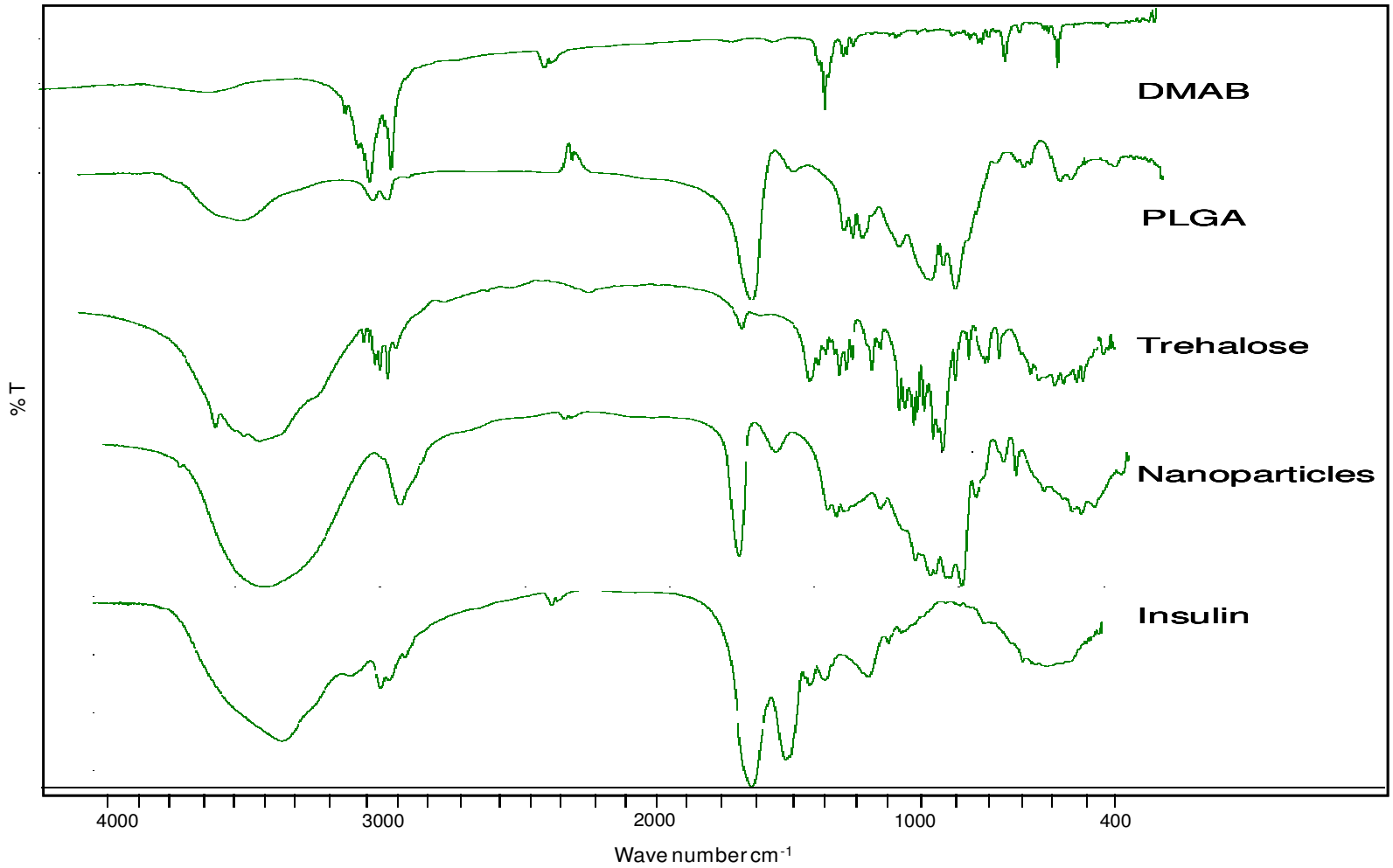


Fig. 3.25 Simple overlay of IR spectra

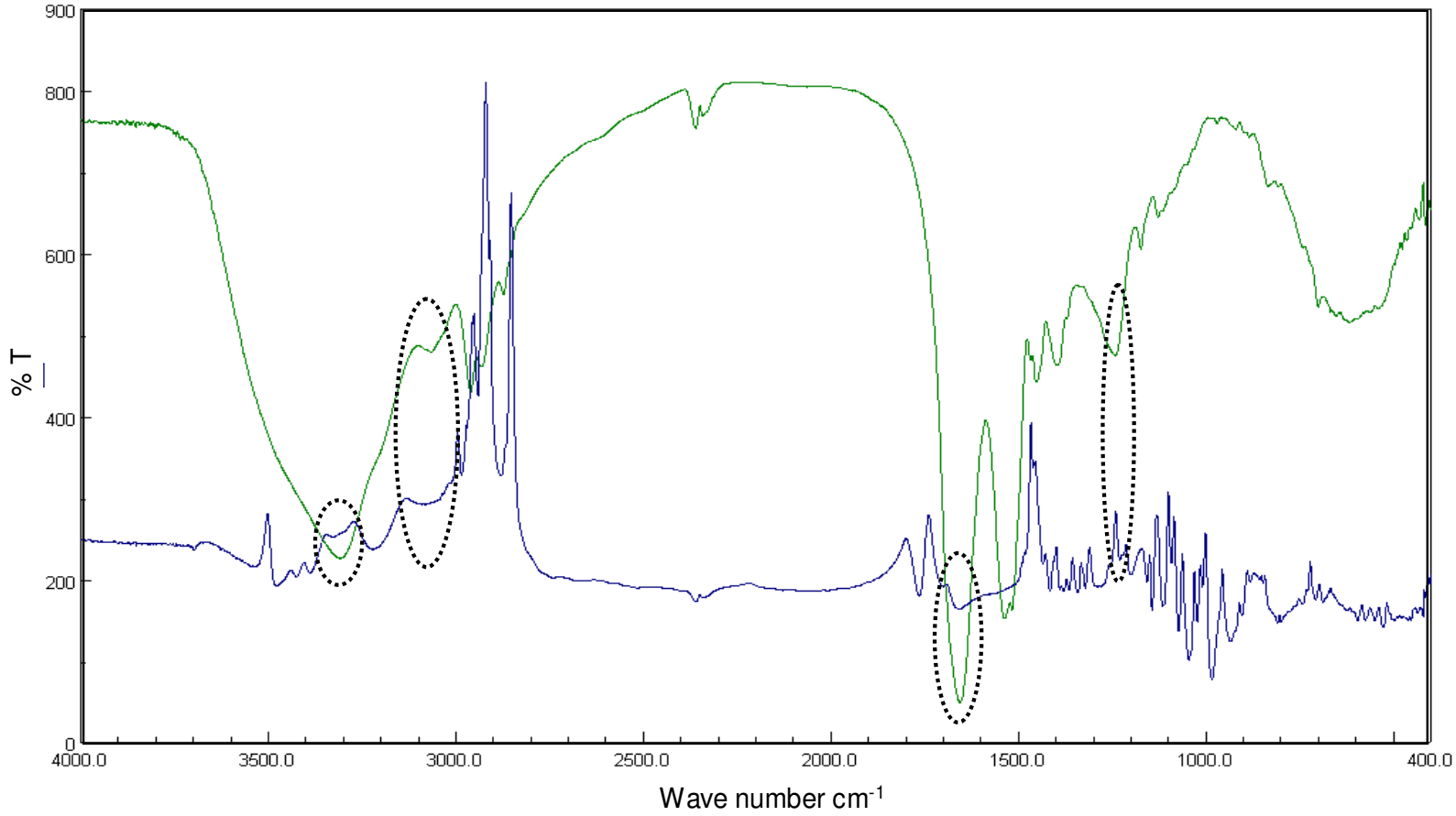


Fig. 3.26 Overlay of spectra. The green is native insulin and the blue shows the spectrum of insulin extracted from spectrum of nanoparticles by subtraction method. The encircled regions show amide A and amide bands I, II and III respectively starting from the left.

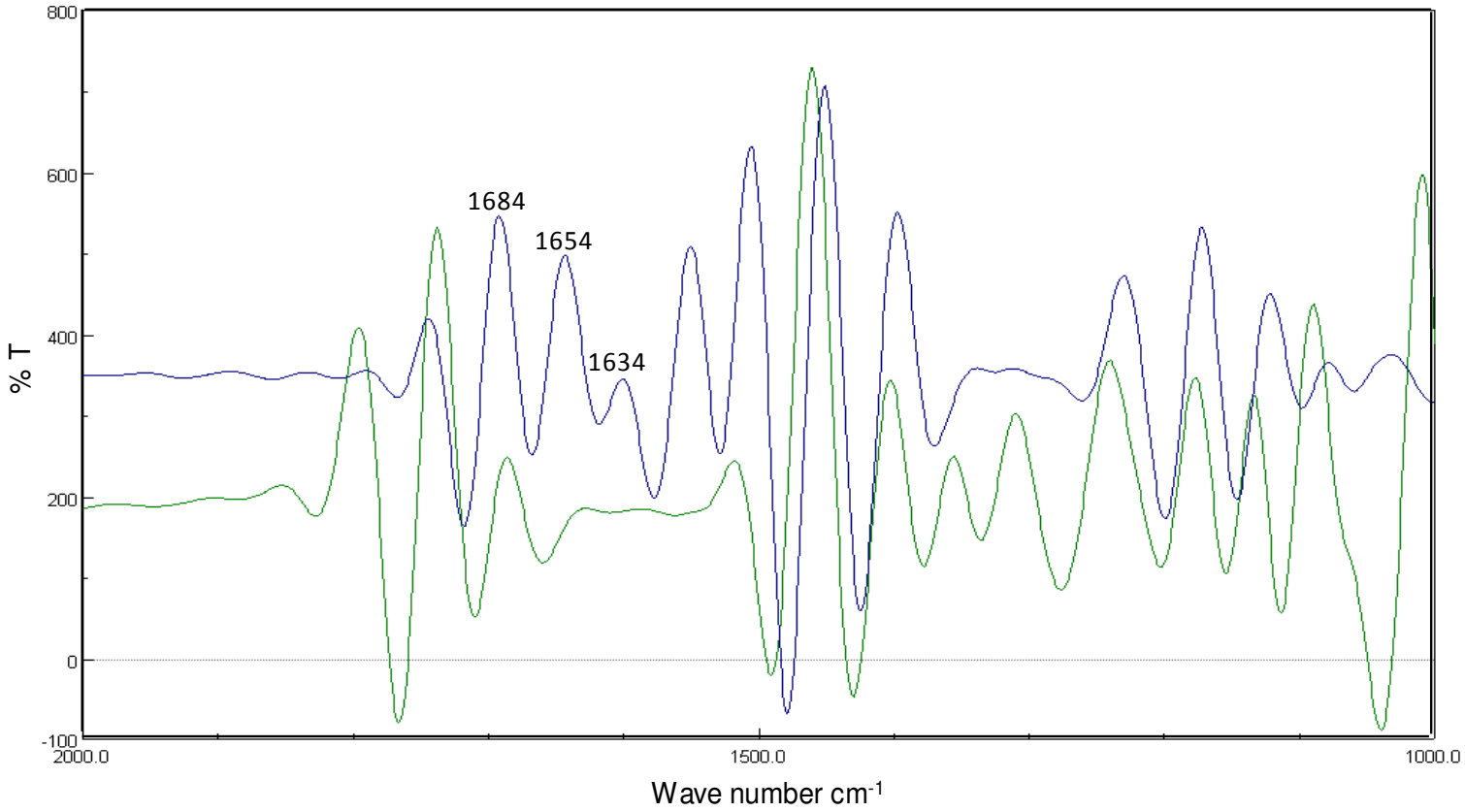


Fig. 3.27 Second derivative IR spectrum of native insulin (in green) and the second derivative IR spectra of insulin extracted from spectrum of nanoparticles by subtraction method (in blue), demonstrating a considerable degree of overlap between the two suggesting preservation of insulin structure in nanoparticles.

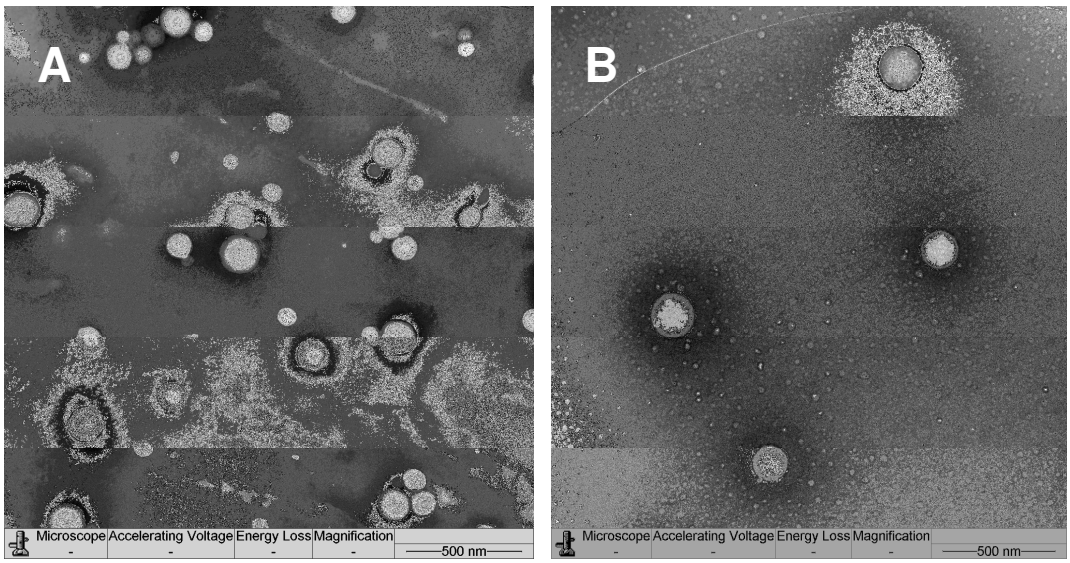


Fig. 3.28 TEM micrograph of (A) blank nanoparticles prepared using 2% MH and 1% DMAB (B) insulin nanoparticles at 4% insulin loading prepared using 2% MH and 1% DMAB. Magnification of 10000X (Scale=500 nm).

4.5 In vitro stability/release studies

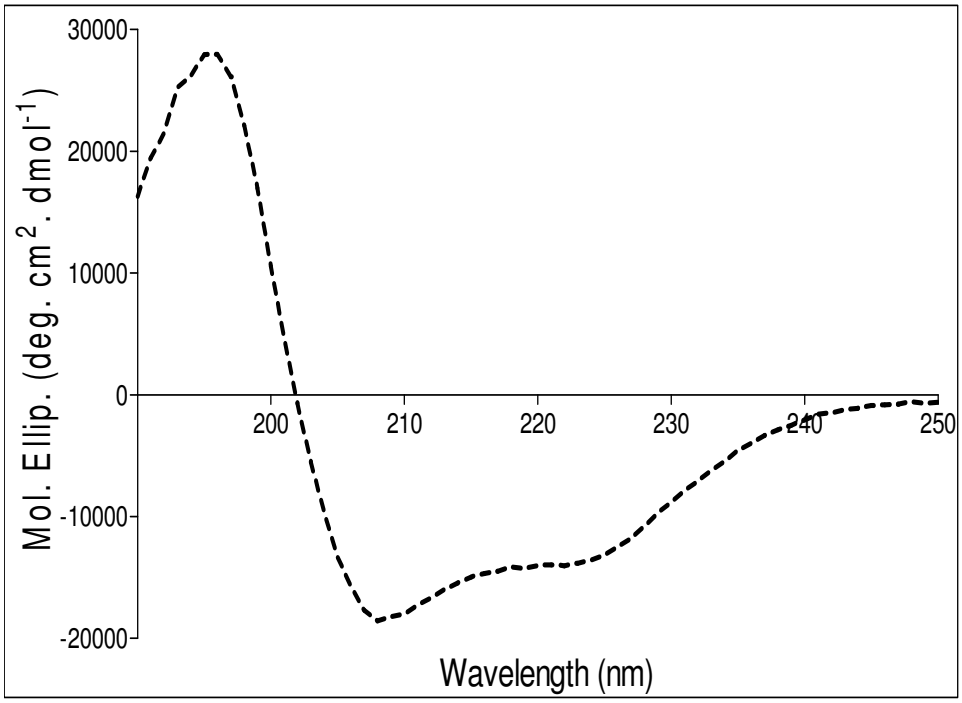
Complete degradation of native insulin was observed when subjected in SGF for 2 h and even in SIF, more than 90% of insulin was degraded. In modified SGF and SIF (without pepsin and pancreatin respectively) native insulin suffered a considerable degradation of about 35% and 45% respectively when incubated as a suspension and this was confirmed by the CD where a compared to native insulin solution, complete loss of insulin's extremas (196, 209 and 222 nm) was observed (Fig.3.29). On the other hand when nanoparticles were incubated in modified SGF for 2 h followed by incubation in modified SIF for 6 h, the cumulative release of insulin in simulated fluids was 3% from nanoparticles. Both types of nanoparticles (with and without 2% MH) showed almost preservation of 97% (Fig. 3.30) of entrapped insulin.

In insulin monomer, the A chain represents two sections of α-helix whereas B chain contains a larger section of α-helix, together these helices represent the active region of the molecules and loss of CD signal at 209 and 222 nm is

strongly corroborated with disappearance of α-helical configuration and loss of signal at 196 nm with β-sheet confirmation thus leading to inactivation/denaturation of insulin. Complete degradation of native insulin was observed in SGF and this was due to the presence of enzyme pepsin along with 0.1 N hydrochloric acid which together contributed to significant changes in the secondary structure of insulin. Pepsin digests up to 20% of ingested amide bonds of insulin by cleaving preferentially after the N-terminal of aromatic amino acids such as phenylalanine, tryptophan, and tyrosine and it exhibits preferential cleavage for hydrophobic, preferably aromatic, residues in P1 and P1' positions. Pepsin cleaves $Phe^{1}Val$, $Gln^{4}His$, $Glu^{13}Ala$, $Ala^{14}Leu$, $Leu^{15}Tyr$, $Tyr^{16}Leu$, $Gly^{23}Phe$, $Phe^{24}Phe$ and $Phe^{25}Tyr$ bonds in the B chain of insulin (Foltman, 1981). Even in SIF, native insulin suffered more than 90% degradation due to presence of pancreatin. The chief proteolytic enzyme present in pancreatin is trypisn which is an endopeptidases, i.e. the cleavage occurs within the polypeptide chain rather than at the terminal amino acids located at the ends of polypeptides. Trypisn redominantly cleaves insulin at the carboxyl side of the amino acids lysine and arginine (Keil-Dlouha, 1971).

Even in non enzymatic physiological fluids, native insulin suffered appreciable degradation due to one or more possible reasons; the strong acidic pH of modified SGF and slightly basic pH of SIF causing significant deamidation (Brange *et al*., 1992; Fisher and Porter, 1981; Darrington and Anderson, 1994), the temperature of incubation (37°C), presence of phosphate ions and due to strong adsorption of insulin on the glass walls of incubation tubes as hydrophobic domains in insulin hold strong affinity for hydrophobic surfaces and often in contact together leads to insulin degradation (Sluzky *et al*., 1991). Insulin was incubated as a suspension with

simulated media to obtain its stability profile in simulated gastric and intestinal fluids. The primary reason for not incubating insulin as a solution with simulated media was due to the fact that insulin is soluble in 0.1 N hydrochloric acid and basic solutions and at the same time theses strong acidic and basic environments causes insulin degradation via deamidation, formation of covalent dimers and aggregation (Brange *et al*., 1992; Fisher and Porter, 1981; Darrington and Anderson, 1994). In addition to this, solubilising insulin in any aqueous environment slights unfolds the molecule and makes it more reactive and prone to degradation especially at lower pH due to increase in its molecular mobility (Strickley and Anderson, 1997). Therefore using control as insulin solution would have lead to higher degradation in non enzymatic and enzymatic simulated fluids, making it difficult to conclude the contribution in denaturation from simulated media itself. The observations suggest the protective effect of polymer preventing insulin denaturation. This further suggests the suitability of the polymer nanoparticles for oral delivery of insulin where insulin degradation in the gastric pH is one of the major concerns. Moreover, the slow degradation rate of the polymer prevented insulin release from the matrix indicating the potential of polymeric nanoparticles in sustaining the release of insulin in physiological conditions.



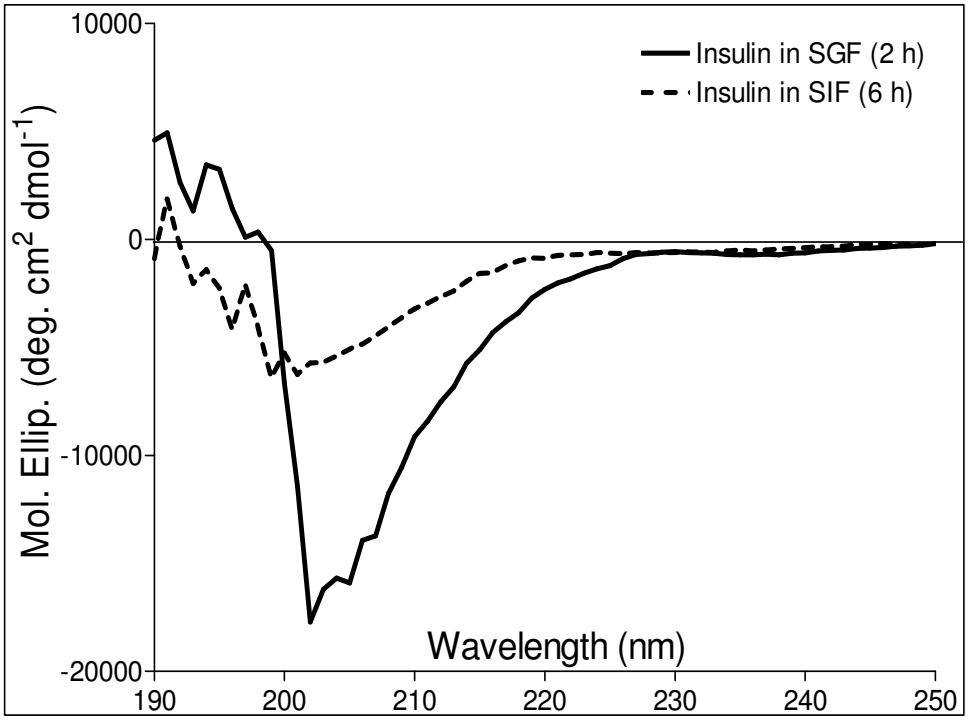


Fig. 3.29 Far UV (190-250 nm) CD spectrum of insulin solution (top) and CD spectrum of insulin after incubation in SGF for 2h and in SIF for 6 h separately at 37°C (bottom).

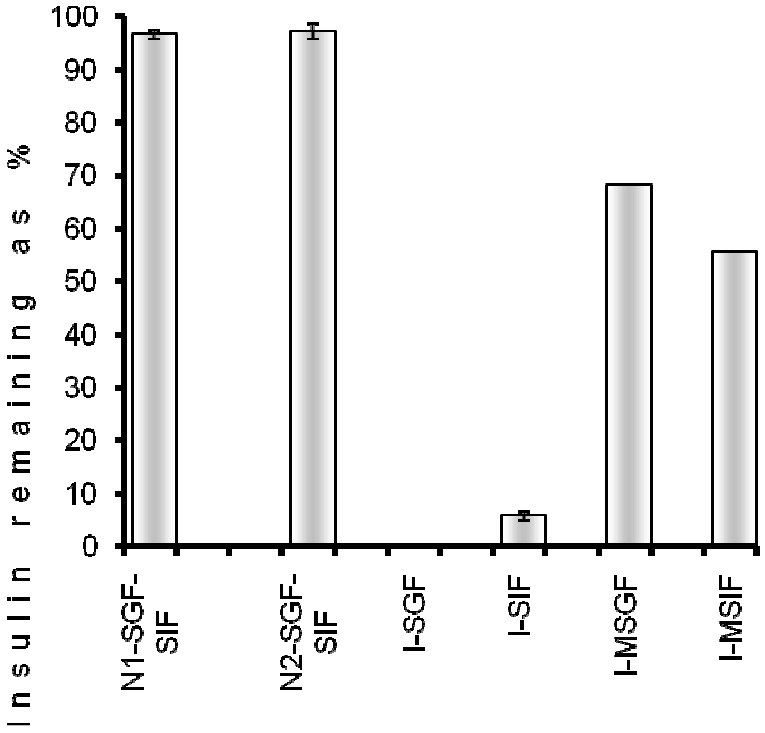


Fig. 3.30 The protective effect of nanoparticles on entrapped insulin in simulated physiological fluids. N1-SGF-SIF=insulin remaining in nanoparticles (nanoparticles equivalent to 2 mg insulin) prepared using 1% DMAB without MH, after 2 h incubation in SGF followed by 6 h incubation in SIF at 37°C; N2-SGF-SIF=insulin remaining in nanoparticles (nanoparticles equivalent to 2 mg insulin) prepared using 1% DMAB and 2% MH after 2 h incubation in SGF followed by 6 h incubation in SIF at 37°C; I-SGF=insulin remaining in suspension (amount equivalent to 2 mg insulin) after 2 h incubation in SGF at 37°C; I-SIF=insulin remaining in suspension (amount equivalent to 2 mg insulin) after 6 h incubation in SIF at 37°C; I-MSGF=insulin remaining in suspension (amount equivalent to 2 mg insulin) after 2 h incubation in modified SGF (without pepsin) at 37°C; I-MSIF=insulin remaining in suspension (amount equivalent to 2 mg insulin) after 6 h incubation in modified SIF (without pancreatin) at 37°C. Data represents mean with standard deviation at n=3.

5. Conclusions

1. The developed spectrofluorimetric method was qualitative, quantitative, rapid, sensitive and selective for insulin estimation *in vitro* and gave a good correlation coefficient (R^2=0.9968) with linearity in the range 1-10 μg/ml compared to previously reported method where detection range from 10-200 μg/ml was reported. Moreover, the developed method also indicated as the compatibility monitoring method as illustrated in results of chapter 2.
2. Double emulsion was the method of choice for preparing insulin stabilizer coencapsulated PLGA nanoparticles as compared to other methods for nanoparticle preparation, w/o/w double emulsion method least exposes insulin to the organic phase and affords good stability to insulin.
3. Among various surfactants screened for preparing insulin stabilizer coencapsulated nanoparticles, 1% DMAB was the surfactant of choice as it was compatible with insulin at this concentration (refer chapter 2), lead to smaller particles (average size $\underset{\sim}{}$ 150 nm) and lower PDI values (0.06-0.1) with appreciable entrappment efficiency of 84%.
4. With 1% DMAB as surfactant, an increase in insulin loading from 2-8% resulted in decrease in particle size and PDI of nanoparticles but no linear trend was observed in entrapment efficiency of insulin in nanoparticles and this was attributed to the size reduction equilibrium phenomenon. With 1% PVA as surfactant, increase in insulin loading from 2-8% resulted in an increase in particle size and entrapment efficiency but no specific trend was observed in PDI of nanoparticles and this was caused due to increase in insulin adsorption on to

nanoparticles which is a usual trend observed when loading of peptides and proteins is increased in PLGA nanoparticles.

5. Co-encapsulation of different concentrations (0.5-2%) of stabilizers: MH, ZC and MC had no effect on particle size, PDI and entrapment efficiency of insulin in nanoparticles. The co-encapsulation of stabilizers was evident from their quantification in the PLGA nanoparticles and moreover, the two homogenization steps assured their entrappment in nano meter size within PLGA nanoparticles. With increase in stabilizer loading, an increase in stabilizer entrapment was observed for MH and ZC due to increase in adsorption of stabilizers whereas a reverse trend was seen for MC due to more portioning in the aqueous phase.
6. FTIR and second derivative FTIR analysis revealed preservation of secondary structure of insulin during and after nanoparticle preparation. The overlap obtained both in first derivative spectra and in second derivative spectra revelead that molecular structure of insulin was not modified and this assured its pharmacological activity inside PLGA nanoparticles.
7. TEM analysis of nanoparticles indicated that the particles were spherical in morphology, homogeneous without aggregation and were in submicron range and the results were in agreement with results of dynamic light scattering studies.
8. *In vitro* stability/release studies suggested the protective effect of polymer preventing insulin denaturation and suggested that due to slow release of insuli from PLGA nanoparticles in simulated gastric and intestinal fluids for 2 and 6 h respectively, 97% of insulin was preserved from enzymatic and acidic degradation.

9. Based on the results of this chapter, nanoparticles prepared with 1% DMAB as surfactant, at 4% insulin loading and with 2% MH and ZC as stabilizers were selected for subsequent studies. The reason for selecting this composition was due to the fact that nanoparticles with 1% DMAB, 4% insulin laoding and 2% stabilizer gave optimum size with high insulin entrappment efficiencies. In addition to this, the protective action of stabilizers against PLGA acidic microclimate induced insulin damage was concentration dependent and since stabilizer loading had no profound effect on nanoparticle characteristics with MH and ZC showing increase in entrappment with stabilizer loading along with compatibility with insulin at their higher concentration investigated in the study, these excipients were selected in 2% w/v concentration to formulate particles further for *in vivo* studies.

CHAPTER 4

Chapter 4: *In vivo* evaluation of oral insulin nanoparticles

Aim: To study the *in vivo* profile and efficacy of oral insulin nanoparticles

1. Introduction

Animal models have been used extensively in field of diabetic research from discovery to development of anti-diabetic drugs. Earlier studies used pancreatectomized dogs to confirm the pivotal role of pancreas in glucose regulation and homeostasis leading to the discovery of insulin (Rees and Alcolado, 2005) but this method was not reliable as incomplete removal of pancreas caused hypertrophy of remaining tissue which produced sufficient amounts of insulin for fulfilling natural metabolic needs. Today, most of the experiments are carried out in rodents though some studies are still carried out in higher species. Type 1 diabetes in humans is characterized by the specific depletion of β-cell mass in the pancreas which is primarily associated with auto-immune response (Dahlquist, 1998). This necessitates the need for a specific strategy to generate experimental diabetes in laboratory animals which can mimic the diabetic pathology in humans. Many methodologies have been used for generating Type 1 diabetes and its clinical symptoms in rodents and chief among them is the use of chemical toxins like streptozotocin (STZ) and alloxan.

STZ is a nitrosourea derivative isolated from strains of soil bacterium called *Streptomyces achromogenes*. It is a powerful alkylating agent (anti-cancer compound) with broad spectrum antibiotic and anti-neoplastic activity (Bono, 1976). STZ interferes with normal glucose homeostasis by specifically destroying β-cell mass in pancreas thereby depleting insulin leading to generation of Type 1 diabetes. Several mechanisms have been reported for diabetogenic activity of STZ and chief among them include blockage of

Glucose transport (Wang and Gleichmann, 1998), dysregulation of glucokinase function (Zahner and Malaisse, 1990) and induction of multiple DNA strand breaks (Bolzan and Bianchi, 2002). A single large dose of STZ produces diabetes in rodents, probably as a result of direct toxic effects which are mediated via free radical damage (Kanitkar *et al*., 2007). Among other agents, STZ is the most widely used diabetogenic compound because it induces insulinopenic diabetes (Price *et al*., 1996) in which the rodent's immune system plays the major role in generation of pathology, as in case of Type 1 diabetes in humans where the primary manifestation of disease is due to autoimmune response (Notkins and Lernmark, 2001). The STZ model of diabetes is extensively used for studying the immunological pathways that lead to β-cell death and insulitis (Mensah-Brown *et al*., 2002). Apart from spontaneous generation of diabetic condition which parallels diabetes in humans, STZ also offer other advantages over other diabetogenic models; this model is independent of the immune status of animal and works even in the absence of functional B and T cells (Reddy *et al*., 1995) and unlike other diabetes models, diabetes can be reliably transferred to syngenic recipients by the transfer of splenocytes (Arata *et al*., 1994).

STZ in long term leads to development of insulin dependent diabetic complications (Brownlee, 2001) which are characterized by changes in levels of certain pro-inflammatory and inflammatory biomarkers which in long term mediate the development of macrovascular and microvascular diabetic complications and development of abnormal lipid profile. Therefore, detailed *in vivo* investigations were performed in healthy animals for blood and tissue levels, followed by the ability of oral insulin nanoparticles in managing the hyperglycaemia and complications thereof in STZ diabetic rat model.

2. Materials

Bovine serum insulin (nominal strength 27 units/mg), STZ and DMAB were purchased from Sigma (St. Louis MO, US). PLGA 50:50 (Molecular weight 35-45 K Da) was purchased from Boehringer Ingelheim (Germany). Ethyl acetate was purchased from Fisher Scientific Ltd. (UK). MH and ZC were purchased from Fischer Scientific (UK). Accu-Check® Aviva Nano Glucometer was purchased from Roche Diagnostics (Germany). Bovine serum insulin specific ELISA kits were purchased from Mercodia (UK). Rat Tumour necrosis factor α (TNF-α) kits were purchased from Assay Designs (US). Cholesterol, high density lipoprotein (HDL) and Triglyceride (TG) quantitation kits were purchased from Source Biosciences Autogen (UK). Rat Interleukin 6 (IL-6) assay kits were purchased from R & D Systems (Europe). Rat C-reactive protein (CRP) assay kits were purchased from BD Biosciences Pharmigen (US). Distilled water was prepared in house and all the reagents used were of analytical grade.

3. Methods

3.1 Pharmacokinetics of oral insulin nanoparticles in healthy rats

Pharmacokinetic studies were carried out in healthy male Sprague Dawley (SD) rats weighing between 250-300 g with n=3 animals per time point within a group. Based on literature reports (Damge *et al.,* 2008), dose of insulin was kept at 20 IU/kg for all the groups including the subcutaneous route of administration so as to compare the pharmacokinetic profile of insulin from two different formulations utilizing two different routes of administration. The selected dose was safe as indicated by earlier studies reported in literature which used insulin at a dose of 15 IU/kg per day for 92 days in BB Wistar rats (Gotfredsen *et al*., 1985) whereas Rastogi and co-workers reported

a dose of 30 IU/kg subcutaneously in diabetic rats without producing insulin over dosage (Rastogi *et al*., 2009), though some researchers believe that this dose is controversial and may produce insulin over dosage in rats and hence doses much lesser than 20 IU/kg are also reported in literature (El Sayed *et al*., 2009) . All animal experiments were performed according to project licence duly approved by the Home Office (UK). Animals were divided into 5 groups. Group I received plain insulin solution orally in phosphate buffer saline pH 7.4. Group II and III received oral insulin nanoparticles prepared using 2% MH and 2% ZC respectively. Group IV was administered plain insulin solution intravenously in phosphate buffer saline pH 7.4 and group V received plain insulin solution subcutaneously in phosphate buffer saline pH 7.4. For each formulation, animals were further classified (A, B and C) in sets of 3 for generating pharmacokinetic profile over 3 days (Table 4.1).

Table 4.1 Study design for pharmacokinetic analysis of formulations.

Groups	*Formulations at a dose of 20 IU/kg*	*Blood sampling time points from 0 h with n=3 animals for A, B and C individually*			*Rats*
		A (0-24 h)	B (at 48 h)	C (at 72 h)	
I	Solution, oral	✓	✓	✓	9
II	NP1, oral	✓	✓	✓	9
III	NP2, oral	✓	✓	✓	9
IV	Solution, i.v.	✓	×	×	3
V	Solution, s.c.	✓	✓	✓	9

A (0-24 h): sampling time points were 0.5, 1, 6, 12 and 24 h, sacrifice at 24 h

Blood sampling in A group was carried out at 0.5, 1, 6, 12 and 24 h from 0 h, following sacrifice at 24 h. Sampling in B group was carried out at 48th h from 0 h, following sacrifice at 48 h and likewise for C group, blood was sampled at 72 h from 0 h followed by sacrifice at 72 h. For group IV, blood samples were withdrawn till 24 h only and after which those animals were sacrificed.

All blood samples were collected using tail prick technique. Blood samples were centrifuged at 10000 *g* for 5 min and plasma was separated and stored at -20°C until further analysis. Different tissues were collected after sacrificing the animals at said time points for studying the tissue distribution profile of insulin from different formulation groups. All tissues were cleaned, removing any blood and connective tissue and were then weighed and to which phosphate buffer saline was added (volume used equals 4 times the weight of the tissue). Each tissue was then homogenised for 2 min at 25000 rpm followed by centrifugation at 14000 *g* for 30 min. The pellet obtained was rejected and supernatant was stored at -20°C until further analysis. Insulin was quantified in blood samples and in tissue homogenates using bovine serum insulin specific ELISA kits.

3.2 Pharmacodynamics of oral insulin nanoparticles in diabetic rats

Pharmacodynamic studies were carried out in diabetic male SD rats weighing between 250-300 g with n=6 animals per group. All animal experiments were performed according to project licence duly approved by the Home Office (UK). Animals were made diabetic by a single intraperitoneal injection of STZ (in 10 mM ice cold citrate buffer adjusted to pH 4.5) at a dose of 55 mg/kg. All rats were allowed to stabilize for two days and housed 12 h dark-light cycle with access to food and water *ad libitum*. Only the rats with plasma glucose levels (PGLs) $\geq$ 2.5 mg/ml were considered diabetic and used in the study.

Animals were divided into 5 groups; Group I received oral insulin nanoparticles at a dose of 20 IU/kg, group II was administered oral insulin nanoparticles at a dose of 60 IU/kg and group III was given oral insulin nanoparticles at a dose of 120 IU/kg. Considering no appreciable differences

in particle characteristics prepared using MH or ZC, ZC was selected as the stabilizer for nanoparticles used in the *in vivo* studies. Group IV received insulin nanoparticles subcutaneously at a dose of 20 IU/kg. Group V received plain insulin solution (in phosphate buffer saline pH 7.4) subcutaneously at 20 IU/kg. Blood (50 µl) was withdrawn from tail vein at 2, 6, 12, 24, 48 and on 72 h from all the groups. PGLs were estimated using the Accu-Check Aviva Nano® Glucometer. Blood samples were centrifuged at 10000 rpm for 5 min and plasma was separated and stored at -20°C until further analysis. Insulin was quantified in blood samples using ELISA kits.

3.3 Evaluation of oral insulin nanoparticles in diabetic complications

STZ causes development of diabetic complications in the long run because it deprives the body from endogenous insulin by destroying β-cells in the pancreas. Diabetic complication studies were carried out in diabetic male SD rats weighing between 250-300 g with n=6 animals per group. All animal experiments were performed according to project licence duly approved by the Home Office (UK). Animals were made diabetic by a single intraperitoneal injection of STZ (in 10 mM ice cold citrate buffer adjusted to pH 4.5) at a dose of 55 mg/kg.

All rats were allowed to stabilize for 15 days and housed 12 h dark-light cycle with access to food and water *ad libitum*. Only the rats with PGLs $\geq$ 2.5 mg/ml were considered diabetic and were used in the study. Animals were divided into 5 groups; treatment started from the sixteenth day of diabetes induction and was as follows: group I comprised of normal untreated animals, group II served as untreated positive control, group III received insulin nanoparticles orally at a dose of 120 IU/kg for 15 days daily, group IV received plain insulin solution (in phosphate buffer saline) subcutaneously at

a dose of 20 IU/kg for 15 days daily and group V received blank PLGA nanoparticles orally for 15 days daily. All the treatment groups had once a day administration of respective formulation. After 31 days from the diabetes induction, animals were sacrificed and blood was collected. Blood samples were centrifuged at 10000 rpm for 5 min and plasma was separated and stored at -20°C until further analysis. Evaluation of treatment efficacy was carried out by assaying the serum levels of pro-inflammatory and inflammatory markers in the blood samples which comprised of TNF-α, IL-6, CRP, cholesterol, TG and HDL, the levels of which were estimated using their respective assay kits.

Statistical analysis

Statistical analysis of the pharmacokinetic and pharmacodynamic data was performed via unpaired T test using SigmaStat 3.0 software (Jindal Scientific); a p value < 0.05 was considered significant. For comparing the means between the groups in diabetic complication study, one way analysis of variance (one way ANOVA) was performed followed by Tukey's post hoc modification for evaluating significance difference between the groups; a p value < 0.05 was considered significant.

4. Results and discussion

4.1 Pharmacokinetics of oral insulin nanoparticles in healthy rats

Figure 4.1 shows plasma insulin concentration (μIU/ml) versus time (h) profile for orally administered nanoparticles and plain insulin solution in healthy SD rats at a dose of 20 IU/kg. Orally administered insulin nanoparticles demonstrated a higher C_{max} (maximum concentration attained in plasma) ~ 4.79 $\pm$ 0.99 μIU/ml and a longer T_{max} (time to attain maximum

plasma concentration) 24 h compared with orally given plain insulin solution (C_{max}~ 1.84 ± 0.11 μIU/ml and T_{max}~1 h) and sustained the release of insulin over 3 days whereas plain insulin was not detected after 24 h. Both oral nanoparticle formulations (NP1 and NP2 displayed similar pharmacokinetic profile. Subcutaneous group (Fig. 4.2) displayed a C_{max}~ 322.47 ± 70.19 μIU/ml, T_{max}~ 1 h and an area under the curve (AUC) ~ 17411.43 ± 1389.70 μIUh/ml) whereas group administered with intravenous insulin solution showed highest C_{max} of 458.92 ± 10.57 μIU/ml with lowest T_{max} of 0.25 h. The main pharmacokinetic parameters obtained from all the formulations have been summarized in Table 4.2. AUC for nanoparticles showed almost a 6 fold increment from AUC generated after administering plain insulin solution indicating a significant enhancement of insulin's pharmacological bioavailability (compared with orally administered insulin) when given orally as nanoparticulate formulation. AUCs were generated using standard trapezoidal rule applicable for pharmacokinetics of oral formulations (Bourget and Delouis, 1993). Tissue distribution analysis revealed significant levels of insulin in various tissues over 72 h in nanoparticulate group (Fig. 4.3 for NP1 and Fig. 4.4 for NP2 formulations) whereas group administered with plain insulin solution orally had very low levels of insulin which were not detected after 24 h (Fig. 4.5). In the intravenous group, higher levels of insulin were observed till 24 h after which insulin was not detected whereas in subcutaneous group, insulin was detected till 72 h (Fig. 4.6) with levels much higher than nanoparticles group. The intravenous group demonstrated highest levels of insulin in various tissues that was detected till 24 h (Fig. 4.7). The enhanced absorption of insulin administered as nanoparticles compared to plain insulin solution at same dose can be possibly attributed to one or more of the following reasons along with unique uptake mechanism of

nanoparticle uptake by M cells of Peyer's patches; (a) huge specific surface area of nanoparticles and as a result better uptake (Bhardwaj *et al*., 2005); (b) altered permeability of the intestinal mucosa because of DMAB (Peetla and Labhasetwar, 2009) which promotes improved permeation of insulin nanoparticles across the gastric barrier; (c) reduction in the interfacial tension between the formulation and lipophillic mucosal layers and (d) due to enhanced stability of insulin inside nanoparticles against gastric proteases and acidic microenvironment. The oral administration of plain insulin resulted in detectable levels in the blood suggesting a possible role of specific insulin receptors in intestinal enterocytes and rapid internalization by the epithelial cells to the interstitial space from which it reached the blood circulation (Bendayan *et al*., 1994; Ziv *et al*., 2000).

This study was an attempt to demonstrate the potential of nanoparticles in delivering insulin orally when compared to plain insulin solution administered orally at the same dose though a more rational approach should have involved the comparison of oral insulin nanoparticles with the best oral nanoparticulate formulation of insulin or with the clinically accepted route of insulin administration i.e. the subcutaneous route. Moreover, nanoparticles were supposed to modify its pharmacokinetic profile because of the unique properties of these carriers. Insulin as plain solution is not absorbed from the gut due to its high molecular weight and hydrophilicity and moreover it undergoes degradation by acidic pH and enzymes when given orally. Administered as a subcutaneous injection, insulin undergoes rapid metabolism and exerts a half life around 5-6 min and is mainly degraded in liver after exerting its pharmacological action but administered orally as nanoparticles, insulin is protected from harsh gut pH and enzymes by phenomenon of entrapment into nanoparticles and because

of unique uptake mechanism of nanoparticles, it is absorbed systemically to an appreciable extent and these circulating carriers in blood because of slow degrdadation profile release insulin at a very low pace thereby modifying its apparent resident time *in vivo*. Insulin levels observed after oral administration of nanoparticles were actually lower than endogenous insulin levels observed after feeding. One of the things to be noted here is that the insulin quantified in rat blood was the external bovine insulin only as the ELISA used in the present study was specific for bovine insulin determination and would not detect the inherent rat insulin. Moreover, the plasma insulin concentrations of diabetic rats are around 4.796 μIU/ml (Ratnam *et al.*, 2008) and the formulations at a dose of 20IU/kg were able to deliver around 5 μIU/ml. Clinically speaking, the formulations were not efficacious in providing insulin levels required to normalize the enhanced glucose levels and to achieve a higher glucose reduction which would normalize hyperglycaemia, a higher dose of insulin in nanoparticles is required. Group administered with plain insulin solution showed very low plasma levels of insulin which were not detected after 24 h. Tissue distribution analysis revealed lower insulin levels in different organs after 24 h. In contrast to other tissues evaluated for tissue distribution of plain insulin solution, higher insulin levels were observed in the intestinal tissue at the end of 24 h and this was in agreement with the fact that the second major challenge to oral insulin delivery is the physical barrier of intestinal epithelium where mucous/glycocalyx layer reduces access to the surface of epithelial cells causing severe retardation in diffusion rate of large molecules greater than approximately 5000 Da and due to this large molecules like insulin are not effectively degraded by brush-border enzymes and this layer keeps the majority of them in the lumen (Sood and Panchagnula, 2001). In

addition to this, mucous/glycocalyx layer is impermeable to protein transport in general and the reason for this is its inherent defensive nature to exclude other macromolecular species such as bacteria and viruses which may enter the GI tract (Aoki *et al.,* 2005). Altered tissue distribution profile of insulin from nanoparticles resulted in increased retention of insulin in tissues compared to other formulation groups (Fig. 4.3-4.7). Tissue distribution analysis was carried out after sacrificing the animals after which the primary organs were surgically removed, washed with excess of buffer or distilled water, pat dried and weighed and finally homogenized with known volume of buffer to solubilize the drug from the tissues (Li *et al*., 2001; Manjunath and Venkateswarlu, 2005; Esmaeili *et al.,* 2008; Bhardwaj *et al.,* 2009). The prepared tissue homogenates were centrifuged and their supernatants were analyzed for estimating the drug content in the tissue. A more rational approach for tissue distribution analysis would be to perform a cardiac perfusion in anaesthetized animals using saline before surgically removing the tissues as this eliminates the possibility of co-estimating the drug levels in blood entrapped in tissues as simply washing the organs with buffer or water would wash away the surface bound blood with internal entrapped blood still existing in the tissues. After their uptake nanoparticles circulate in the blood and many processes aid their extravasation in tissues. In some specialized tissue like liver, the endothelium is fenestrated, thus allowing material up to 100 nm to pass from the endothelium to the underlying parenchymal cells. In other tissues like spleen, the endothelium is discontinuous, having larger fenestrations, and also lacks basement membrane, allowing exit of very large particles. In contrast, in the brain the endothelial junctions are particularly tight and effective, reducing still further the ability of materials to pass through (the blood brain barrier). In

addition to the paracellular route of exit from the circulation, some nanoparticles can also pass through the endothelial cells in a process known as transcytosis, a process involving caveolae. This allows the passage of macromolecules through the endothelium, and is thought to be size dependent, so larger macromolecules pass through less easily than smaller molecules.

Insulin is an important endogenous hormone and apart from its primary regulatory activity of lowering blood suagr in event of hyperglycaemia, the molecule is involved in several other biological processes both at cellular and at molecular level. Altered tissue distribution of insulin from nanoparticles is correlated with attainment of insulin levels in different tissues of the body, where insulin action is involved in various other normal functions of the associated organ and this fact bears physiological significance as insulin is required by almost all the different tissues in the body for carrying out some unique pharmacological activities. Insulin is required in spleen for lipogenesis (Hulstijn *et al*., 1997), it modulates the pancreas with respect to amylase, lipase and trypsin content in pancreatic juice (Takayama *et al*., 2003), prevents cardiomyopathy in cardiac tissues (Bell, 2003). In liver, insulin stimulates glycogenesis by promoting the enzyme glycogen synthase which is responsible for conversion of glucose to glycogen (Bernaert *et al*., 1977). In brain, insulin increases glucose uptake in cortical areas, promotes memory and learning, prevents dementia, and is required for normal synaptic functioning and brain plasticity (Biessels *et al*., 2004). In lungs, insulin regulates energy homeostasis and inflammation by modulating levels of PPAR-α and PPAR- δ (Shalev *et al*., 1996) whereas in kidneys insulin is required physiologically for the proliferation of renal cells and stimulates the

production of other important growth factors such as insulin-like growth factor-1 and transforming growth factor β (Kempson, 1996).

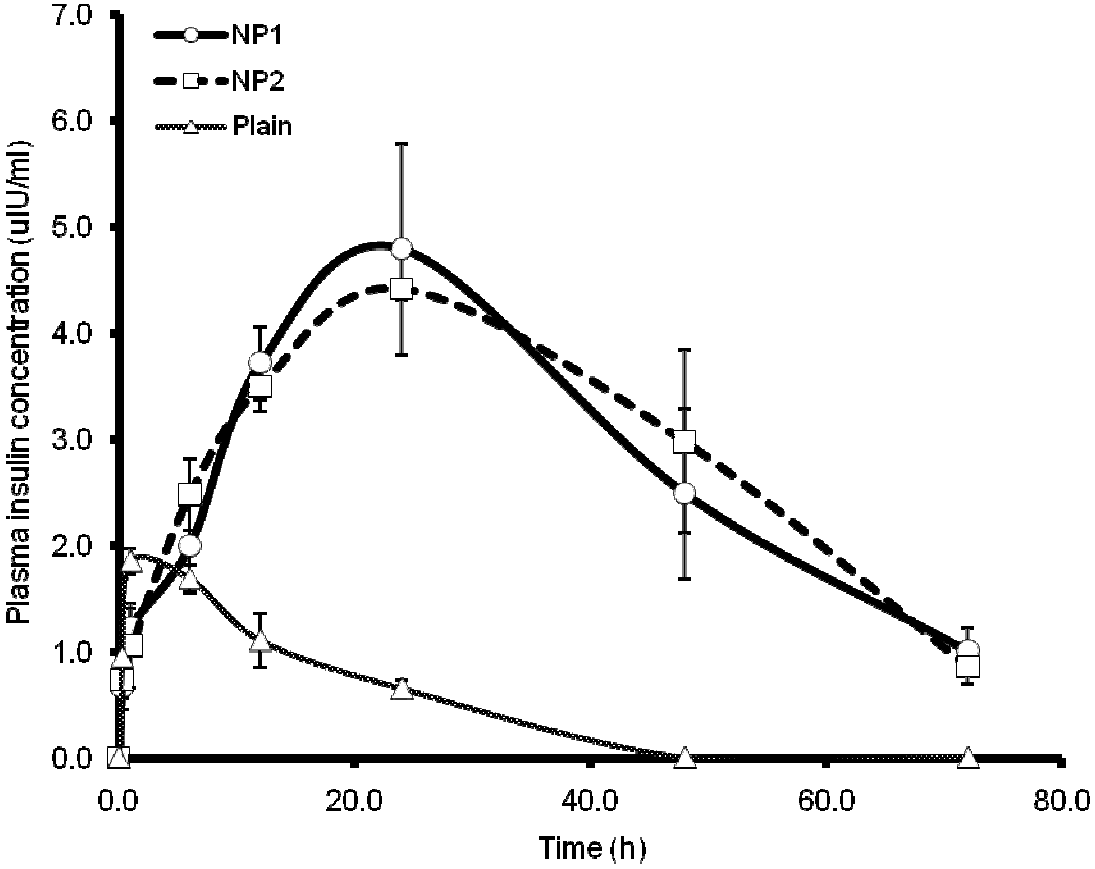


Fig. 4.1 Plasma insulin concentration (μIU/ml) vs. time (h) for insulin formulations in healthy SD rats at a dose of 20 IU/kg. NP1: insulin nanoparticles prepared using 1% DMAB and 2% MH, administered orally; NP2: insulin nanoparticles prepared using 1% DMAB and and 2% ZC, administered orally; plain: insulin solution in phosphate buffer saline pH 7.4, given orally. Data represents Mean with standard deviation at n=3.

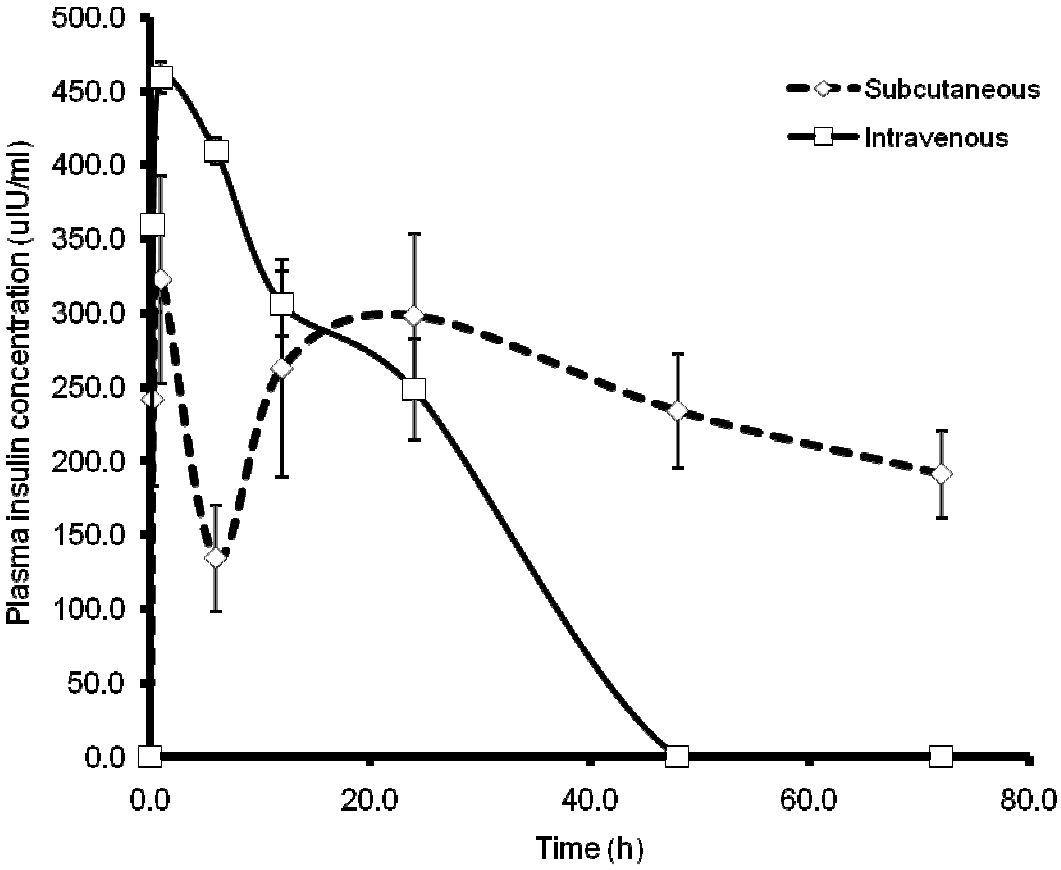


Fig. 4.2 Plasma insulin concentration (μIU/ml) vs. time (h) for insulin formulations in healthy SD rats at a dose of 20 IU/kg. Subcutaneous: plain insulin solution in phosphate buffer saline pH 7.4, given subcutaneously; Intravenous: plain insulin solution in phosphate buffer saline pH 7.4, administered intravenously via tail vein. Data represents Mean with standard deviation at n=3.

Table 4.2 Pharmacokinetic parameters of insulin formulations in healthy SD rats at a dose of 20 IU/kg.

Groups	***Route***	***C_{max} (μIU/ml)***	***T_{max} (h)***	***AUC $_{0\text{-}t}$(μIU h)/ml***
NP1	oral	4.79 ± 0.99	24.0	206.32 ± 44.49
NP2	oral	4.40 ± 0.09	24.0	209.52 ± 28.58
Subcutaneous	s.c.	322.477 ± 70.19	1.0	17411.43 ± 1389.70
Intravenous	i.v.	458.925 ± 10.57	0.25	11036.36 ± 566.69
Plain*	oral	1.848 ± 0.11	1.0	36.69 ± 2.51

Data presented as Mean ± standard deviation (n=3).

C_{max}: maximum plasma concentration, T_{max}: time taken to attain C_{max}, AUC: Area under the plasma concentration–time curve; AUCs (0-t) were calculated using the standard trapezoidal rule.

s.c.=subcutaneous

i.v.=intravenous

*$p<0.05$ vs. plain insulin solution

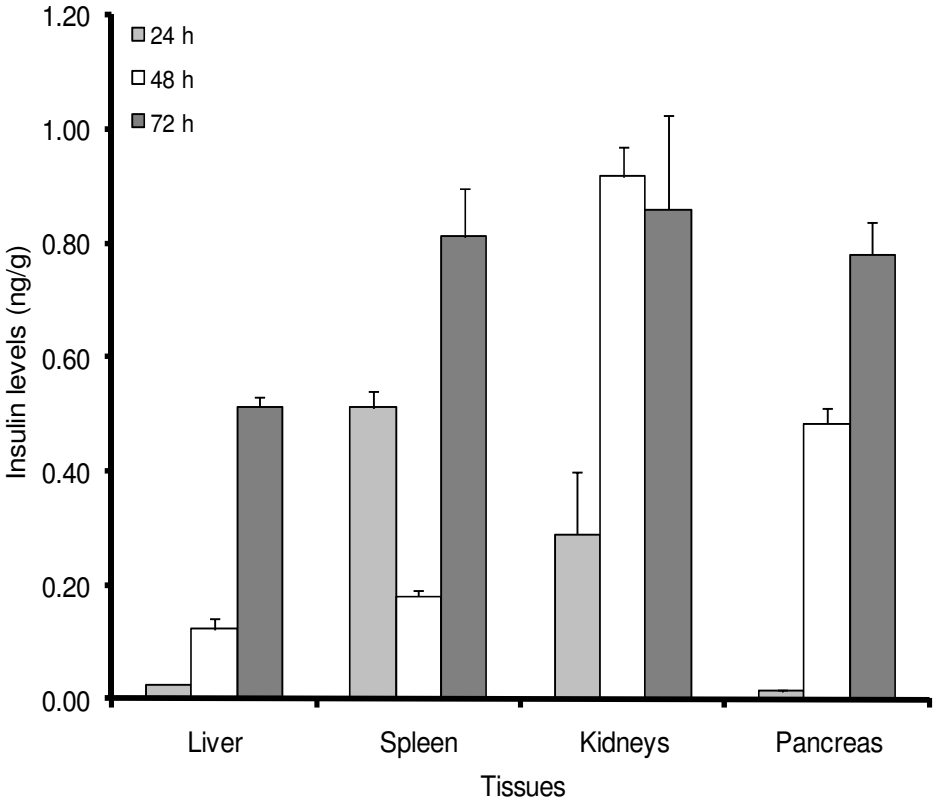


Fig. 4.3 Tissue distribution profile of insulin from orally administered nanoparticles (NP1) over 72 h in healthy SD rats. The nanoparticles were prepared using 1% DMAB as surfactant and 2% MH as stabilizer. Insulin dose was 20 IU/kg. Data represents Mean with standard deviation at n=3.

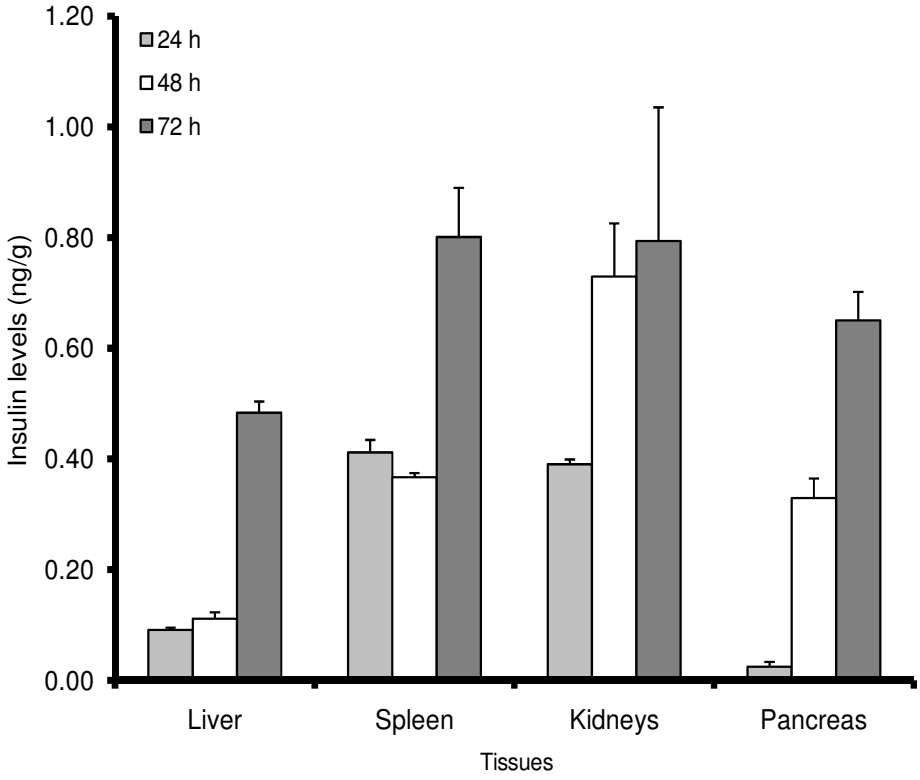


Fig. 4.4 Tissue distribution profile of insulin from orally administered nanoparticles (NP2) over 72 h in healthy SD rats. The nanoparticles were prepared using 1% DMAB as surfactant and 2% ZC as stabilizer. Insulin dose was 20 IU/kg. Data represents Mean with standard deviation at n=3.

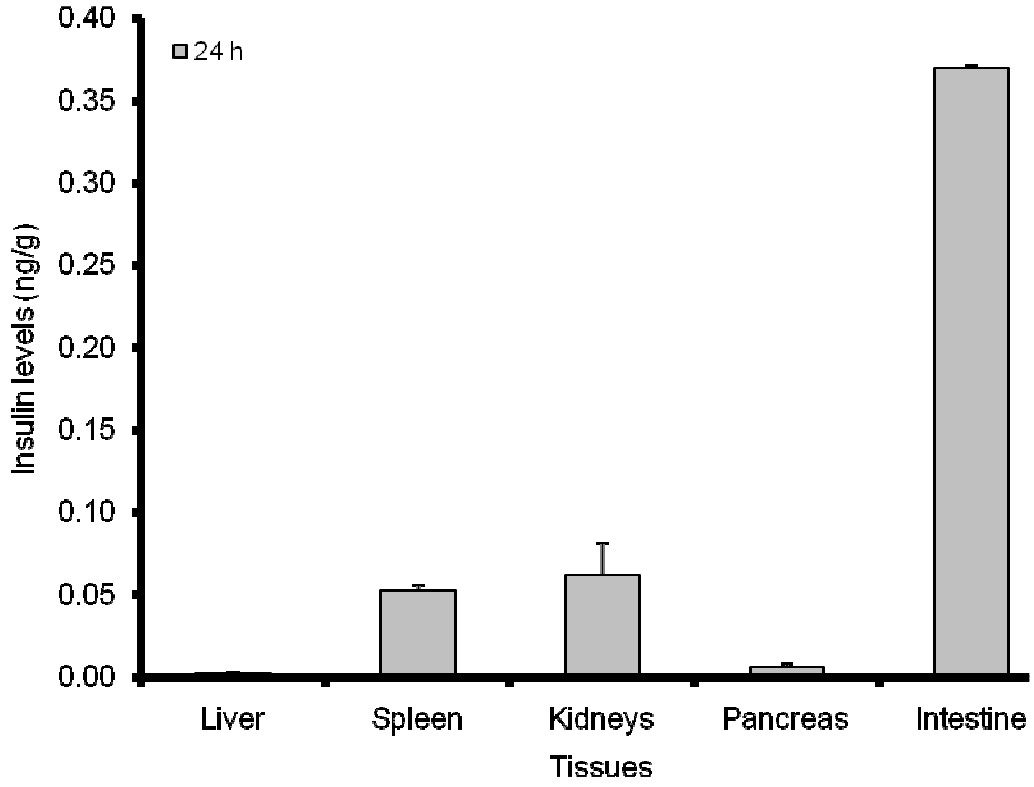


Fig. 4.5 Tissue distribution profile of insulin from orally administered plain insulin solution (in phosphate buffer saline pH 7.4) at 24 h in healthy SD rats after which insulin was not detected. Insulin dose was 20 IU/kg. Data represents Mean with standard deviation at n=3.

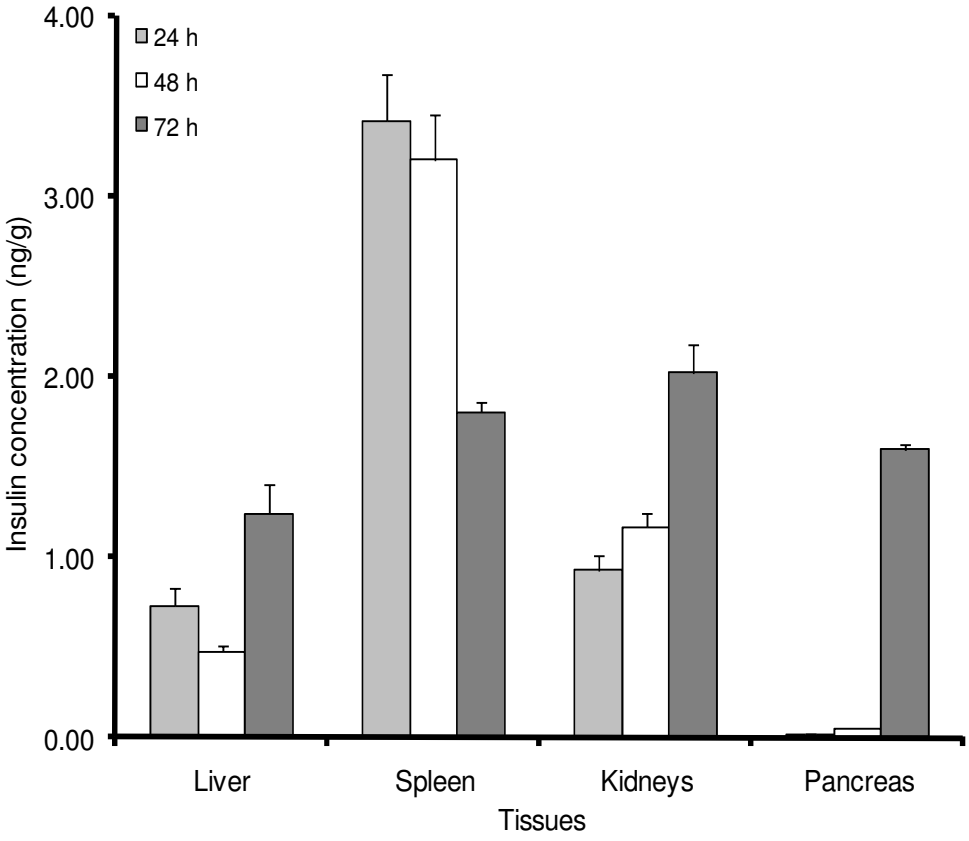


Fig. 4.6 Tissue distribution profile of insulin from subcutaneously administered insulin solution (in phosphate buffer saline pH 7.4) over 72 h in healthy SD rats. Insulin dose was 20 IU/kg. Data represents Mean with standard deviation at n=3.

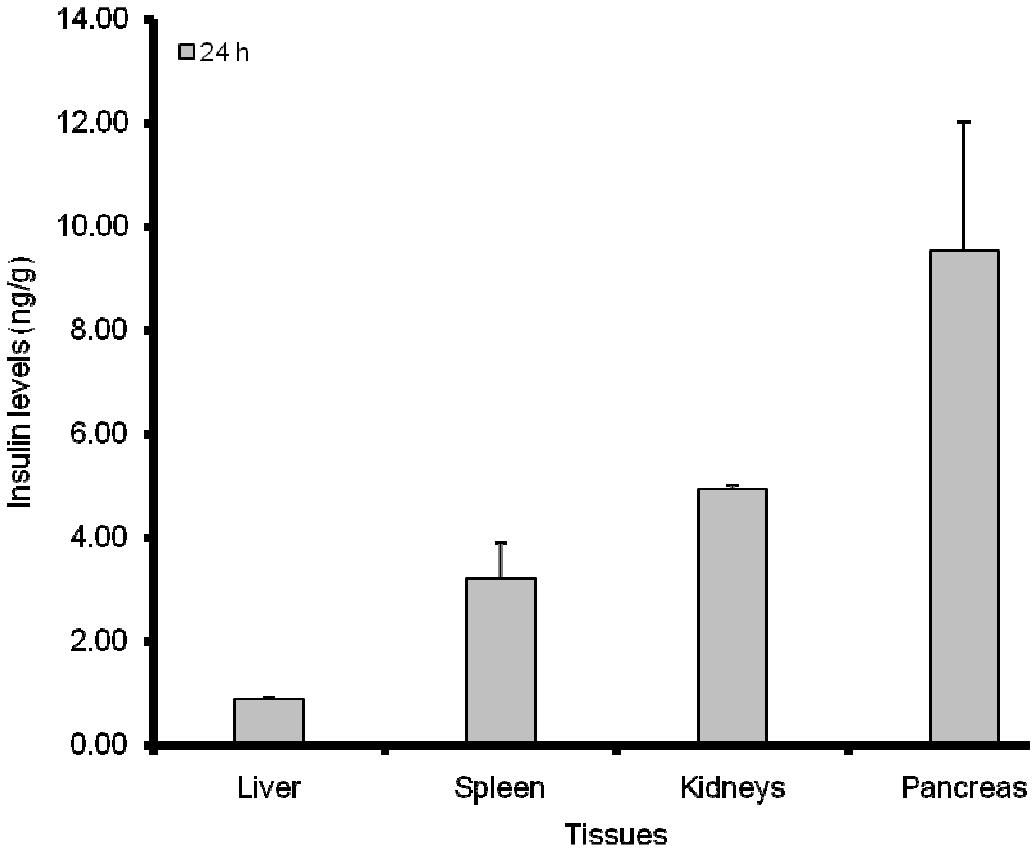


Fig. 4.7 Tissue distribution profile of insulin from intravenously administered (through tail vein) insulin solution in phosphate buffer saline pH 7.4 over 24 h in healthy SD rats. Insulin dose was 20 IU/kg. Data represents Mean with standard deviation at n=3.

4.2 Pharmacodynamics of oral insulin nanoparticles in diabetic rats

PGL of formulation treatment groups are shown in Fig. 4.8 which showed a decrease and then increase in PGL upon subcutaneous administration of insulin nanoparticles, oral administration of insulin nanoparticles and subcutaneous administration of plain insulin solution, all at a dose of 20 IU/kg in diabetic rats. In control group, there was no change observed in the PGL till 72 h although there were regular insignificant fluctuations observed whereas in group treated subcutaneously with insulin nanoparticles at a dose of 20 IU/kg, PGL was decreased to 3.24 mg/ml by 6 h (corresponding to 33.4% reduction in PGL) and it returned to basal levels by 72 h, also altered tissue distribution of insulin was observed at the end of 72 h (Fig. 4.9). On the other hand, orally administered insulin nanoparticles at a dose of 20 IU/kg showed PGLs of 3.75 mg/ml at 24 h (corresponding to 21.11% reduction in PGL compared with PGL values before the treatment at 0 time point) and it returned to the basal levels by 72 h. Group administered subcutaneously with plain insulin solution at 20 IU/kg showed the maximum reduction in PGL with values as low as 1.1 mg/ml by 2 h (corresponding to 76.59% reduction in PGL compared with PGL values before the treatment at 0 time point) and the basal levels were returned by 48 h. All the glucose reduction values in treatment groups were biologically and statistically significant. Insulin nanoparticles (both subcutaneously and orally administered) displayed a sustained hypoglycaemic activity illustrating the pharmacodynamic effect of the formulation.

The study demonstrated the potential of oral insulin loaded PLGA nanoparticles in reducing hyperglycaemia in STZ induced diabetic rats. It is evident from the profile that nanoparticles were absorbed from the intestine soon after their oral administration as their glucose lowering potential

started after 2 h though values were still in the diabetic range. Nanoparticles released insulin slowly in the blood depending upon rate of PLGA degradation *in vivo* with maximum release around 24 h where maximal blood glucose reduction of 21.1% was obtained corresponding to 3.75 mg/ml glucose concentration compared to the glucose concentration before the nanoparticle treatment at time 0 h. The glucose reduction obtained was statistically significant but enhanced glucose levels were not normalized with physiological levels and this may be attributed to combination of different reasons which include; lack of efficacy of nanoparticles in delivering insulin orally at given dose which might be further attributed to variable absorption profiles of nanoparticles as their uptake is strongly size dependent, the slow degradation rate of polymer causing insufficient insulin release and degradation of insulin bound on nanoparticle surface, together causing lowering of plasma glucose to a small extent without normalizing the levels. In addition to this lack of normalization of glucose levels can also be remotely associated with the experimental design as throughout the study animals were not fasted and this might have acted as a continuous glucose challenge which might have possibly caused insufficient lowering of glucose levels as for fasted rats a significant fall in glycaemia on oral nanoparticle administration has been observed by other investigators (Damge *et al*., 2007). Study based on oral efficacy of insulin nanoparticles in lowering enhanced glucose levels in diabetic rats which are fasted overnight would reveal a better picture.

For diabetic treatment normalization of enhanced glucose levels is the therapeutic goal of the therapy and in such a scenario where glucose levels are reduced and not normalized, possibly a higher dose of insulin in nanoparticles or higher number of nanoparticles or a high frequency of

administration of same dose would be required to achieve glucose level normalization. Compared to oral group, group administered subcutaneously with insulin nanoparticles showed a relatively fast peak action at 6 h with glucose reduction higher than orally administered group (33.34% vs. 21.11%) but here again glucose levels were not normalized and the animals had hyperglycaemia. It is postulated that when nanoparticles are injected subcutaneously, the fat layer in the subcutaneous tissue impairs their instant absorption and they act as depot formulations via this route. Moreover, it is possible that insulin is released from nanoparticles in the subcutaneous tissue and gets absorbed in the underlying blood capillaries and hence gets rapidly metabolized. From all the nanoparticulate groups at doses of 20, 60 and 120 IU/kg, the biological activity lasted for over 72 h post oral administrations. The possible implications of this phenomenon could be primarily lack of efficacy of oral insulin nanoparticles in providing rapid insulin release and that to normalize the enhanced glucose levels as at 20 and 60 IU/kg doses, glucose levels were not normalized and animals were still diabetic unlike at 120 IU/kg where enhanced glucose levels were normalized with physiological levels. This study was based on a single dose administration and it provided merely one to two point assessment of the problem. More extensive studies are required to titrate the required dose of insulin in nanoparticles and then translating it into its pharmacological action as there are no doubts that insulin therapy provides benefits for patients suffering from diabetes but it comes on its own costs which include; selection of right dose and timings, adjusting dosage and timing to fit food intake timing, amounts, and types, adjusting dosage and timing to fit exercise undertaken, adjusting dosage, type, and timing to fit other conditions, for instance the increased stress of illness (Becker, 1998). The

pattern of insulin release in the body more or less operates on feed back mechanism where high plasma glucose concentrations trigger insulin release which subsequently not only lower the glucose levels but normalizes them and lower glucose concentration trigger off the release. For nanoparticles to be therapeutically effective in type 1 diabetes, they should be able to release insulin within 30 min to 1 hr after food intake and with relatively short timing for anti-hyperglycaemic activity mimicking the physiological insulin spikes in blood.

Moreover, when dose of orally administered insulin nanoparticles was increased, subsequent reductions in PGLs were observed (Fig. 4.10). Thus orally administered insulin nanoparticles displayed dose dependency and a maximum reduction in PGL (corresponding to 74% compared with PGLs at 0 h) was observed with 120 IU/kg dose of oral insulin nanoparticles without any change in the T_{max} and similar trend was observed in corresponding tissue distribution profiles at the end of 72 h (Fig. 4.11). All the orally administered nanoparticles formulations at different dose showed same T_{max} of 24 h with only change in the C_{max}, which more or less increased linearly with increase in dose of insulin in orally administered nanoparticles (Fig. 4.12). No significant change was observed in the pharmacokinetics of oral insulin nanoparticles in diabetic animals compared with healthy animals at same dose of 20 IU/kg.

Orally administered nanoparticles displayed dose dependent lowering of glucose in STZ induced diabetic rats. An interesting observation was that there was no change in their pharmacokinetic parameters like T_{max} and elimination profile, the only change that was observed was in C_{max} and tissue distribution profile which more or less increased linearly with increase in insulin dose in nanoparticles. A possible reason for this phenomenon could

be that higher dose of nanoparticles resulted in higher number of nanoparticles reaching systemic circulation. A similar dose response and pharmacokinetic relationship was reported by other investigators where RNA was directly delivered in the systemic circulation using nanoparticles and on increasing the dose of nanoparticles, a similar trend in pharmacodynamic and pharmacokinetic profile was observed (Davis *et al*., 2010). Moreover, dose dependent response from oral insulin nanoparticles can be correlated with the nanoparticle properties of protecting insulin from harsh gut environment and thereby facilitating its uptake.

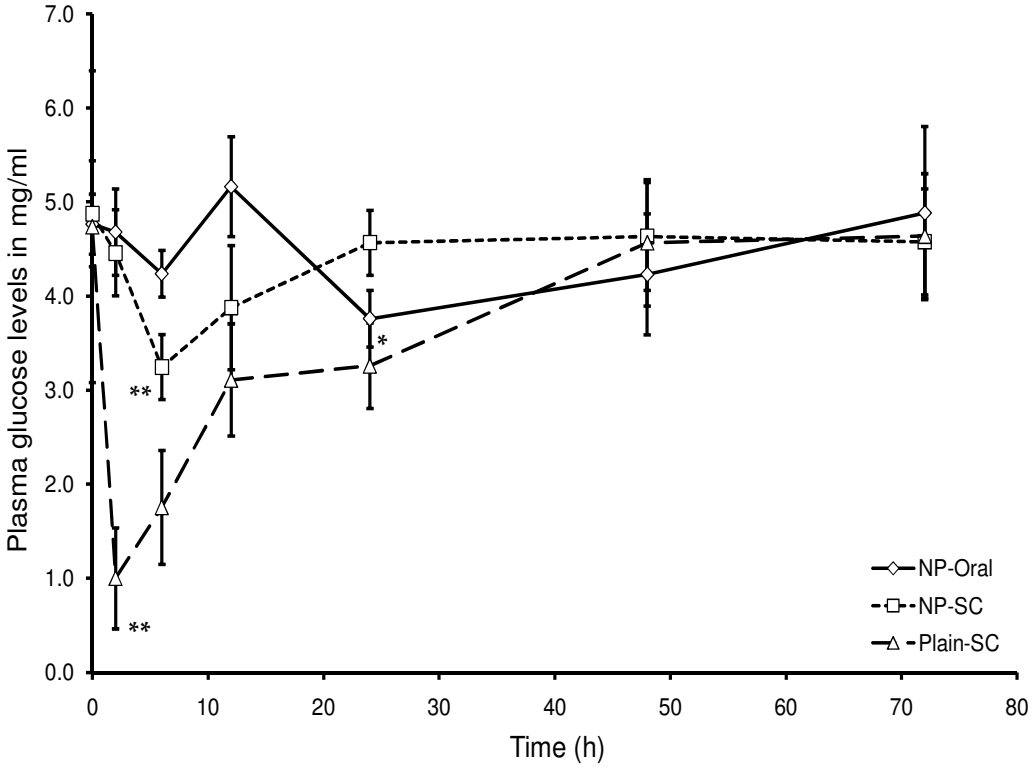


Fig. 4.8 Plasma glucose levels from single dose of insulin nanoparticles administered via oral (NP-Oral), subcutaneous route (NP-SC) and from plain insulin solution (in phosphate buffer saline pH 7.4) administered via subcutaneous route (Plain-SC) at a dose of 20 IU/kg in STZ induced diabetic rats. Insulin nanoparticles were prepared using 1% DMAB and 2% ZC. Data represents Mean with standard deviation at n=6. *p <0.05 and **p<0.01.

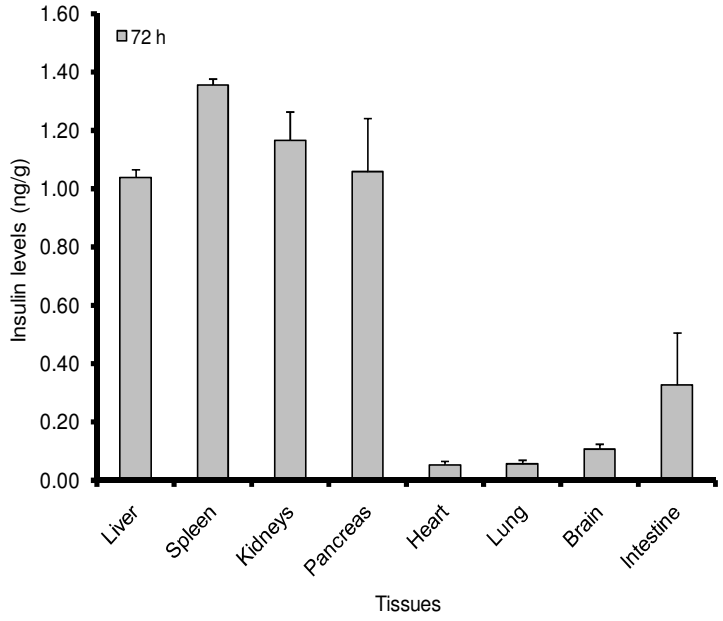


Fig. 4.9 Tissue distribution profile of insulin from single dose of subcutaneously administered insulin nanoparticles after 72 h in STZ induced diabetic rats. The nanoparticles were prepared using 1% DMAB as surfactant and 2% ZC as stabilizer. Insulin dose was 20 IU/kg. Data represents Mean with standard deviation at n=6.

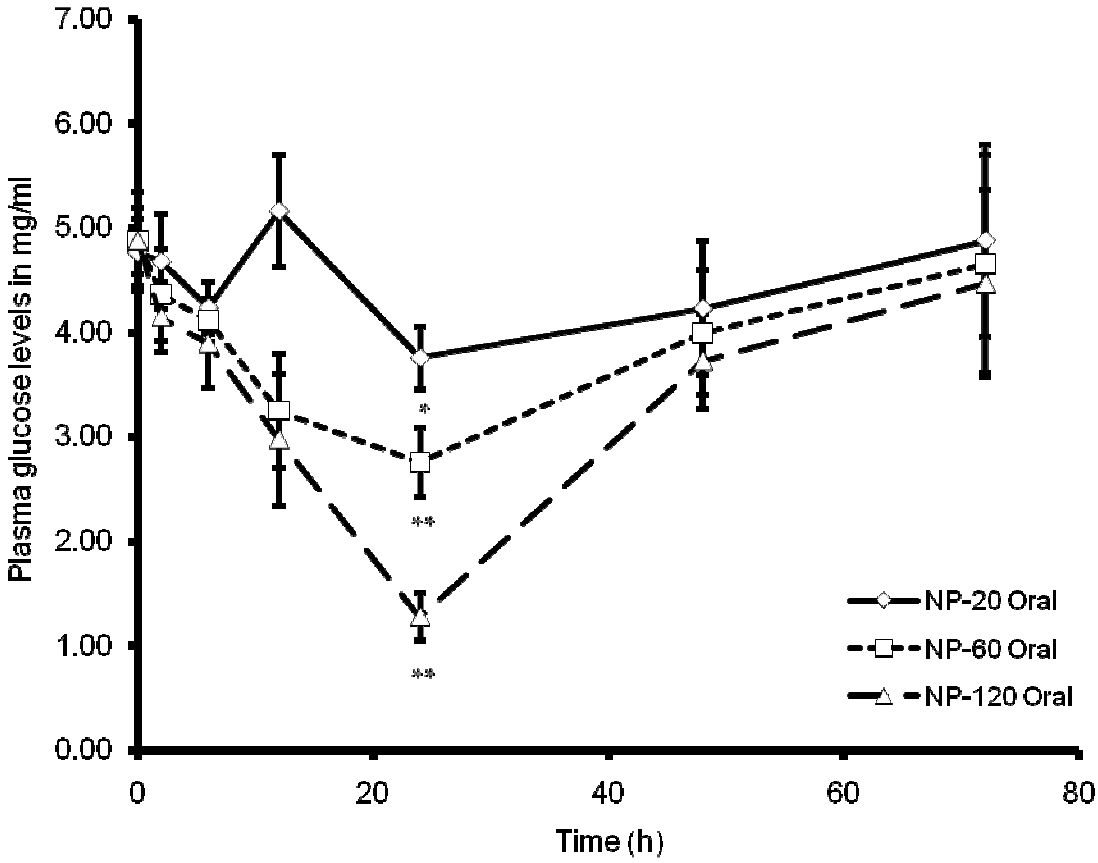


Fig. 4.10 Dose dependent glucose reduction from single dose of insulin nanoparticles administered orally in STZ induced diabetic rats. NP-20 Oral: insulin dose 20 IU/kg; NP-60 Oral: insulin dose 60 IU/kg; NP-120 Oral: insulin dose 120 IU/kg. All formulations were prepared using 1 % DMAB and 2 % ZC. Data represents Mean with standard deviation at n=6. *$p<0.05$ and **$p<0.01$.

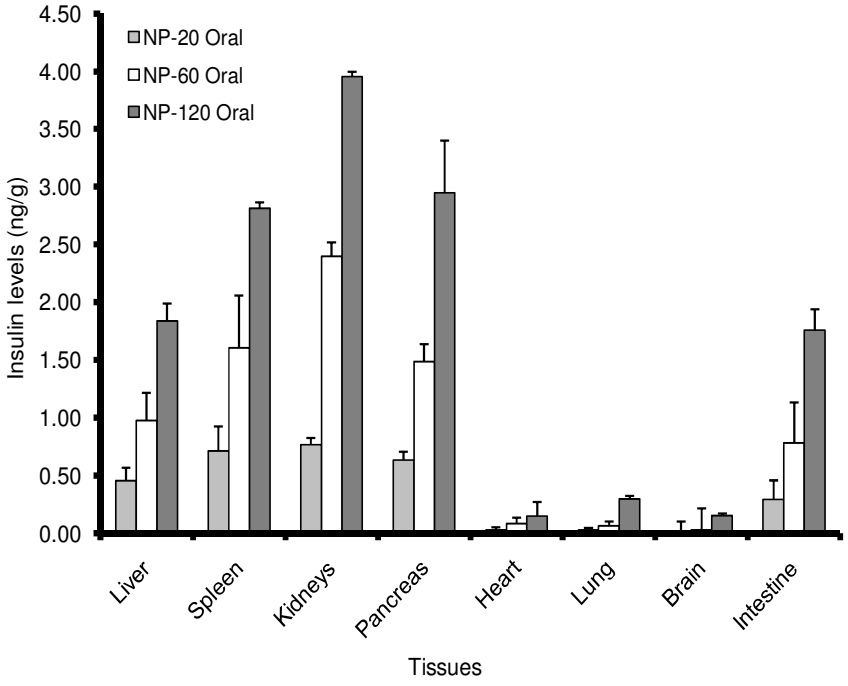


Fig. 4.11 Dose dependent tissue distribution from single dose of insulin nanoparticles administered orally in STZ induced diabetic rats after 72 h. NP-20 Oral: insulin dose 20 IU/kg; NP-60 Oral: insulin dose 60 IU/kg; NP-120 Oral: insulin dose 120 IU/kg. Nanoparticles were prepared using 1% DMAB and 2% ZC. Data represents Mean with standard deviation at n=6.

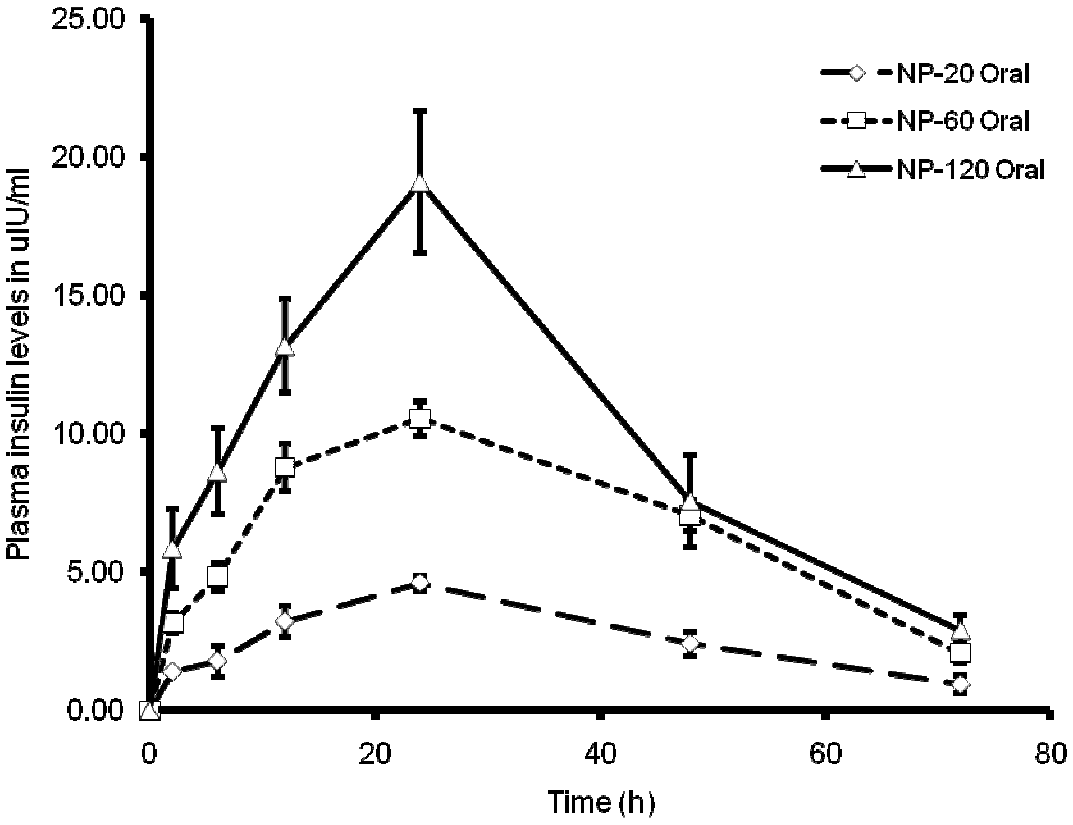


Fig. 4.12 Dose dependent pharmacokinetics from single dose of insulin nanoparticles administered orally to STZ induced diabetic rats. NP-20 Oral: insulin dose 20 IU/kg; NP-60 Oral: insulin dose 60 IU/kg; NP-120 Oral: insulin dose 120 IU/kg. Nanoparticles were prepared using 1% DMAB and 2% ZC. Data represents Mean with standard deviation at n=6.

4.3 Evaluation of oral insulin nanoparticles in diabetic complications

Diabetic complications are characterized by appearance of pro-inflammatory and inflammatory pathological biomarkers which initiate development of advanced pathologies pertaining to microvascular (retinopathy, neuropathy and nephropathy) and macrovascular complications like coronary artery disease, peripheral arterial disease and stroke (Fowler, 2008; Moore *et al*., 2009). Elevation in serum levels of IL-6, C-reactive protein and TNF-α is strongly correlated with generation of acute diabetic complications. These inflammatory biomarkers are elevated due to free radical generation inside the cells and role of insulin in reducing their levels is based upon the anti-inflammatory action of insulin (Hansen *et al*., 2003). Insulin exerts an inhibitory effect on the IL-6 signalling pathway by altering the post-translational modifications of the signal transducer and activator of transcription 3 (STAT3). Insulin reduces the tyrosine phosphorylation and increases the serine phosphorylation of STAT3, thereby reducing its nuclear localization and transcriptional activity (Andersson *et al*., 2007).

Results of our study showed that there was no change in serum IL-6 levels after treatment with subcutaneous insulin solution (Fig. 4.13) and oral insulin nanoparticles. The untreated diabetic control group exhibited serum IL-6 levels of 297.40 $\pm$ 34.01 pg/ml whereas group administered subcutaneously with plain insulin solution, IL-6 levels of 257.84$\pm$47.87 pg/ml were observed whereas group administered with oral insulin nanoparticles displayed IL-6 levels of 252.04 $\pm$ 31.05 pg/ml. No changes were observed in serum IL-6 levels with insulin treatment both as subcutaneous injection and oral nanoparticles. The results were statistically insignificant in all treatment groups and no change in serum IL-6 levels was observed with blank nanoparticles. C-reactive protein is known to play a crucial role in development of

cardiovascular diabetic complications which are mitigated through progression of atherosclerosis (Takebayashi *et al*., 2004). In addition to serving as a marker, CRP contributes to progression of atherosclerosis by activation of complement (Bhakdi *et al*., 1999) enhancement of monocyte chemotactic protein-1 (MCP-1) production (Pasceri *et al*., 2000), participation in low-density lipoprotein (LDL) uptake by endothelial macrophages (Zwaka *et al*., 2001) and other mechanisms. Several recent reports have indicated that insulin inhibits CRP which is produced by the liver in response to cytokines, such as TNF-α and IL-6. Effective insulin therapy for diabetic patients would inhibit atherosclerosis not only by suppressing glycation, but also more directly by decreasing pro-inflammatory cytokines and CRP. Based on this we evaluated the efficacy of the formulations in reducing the elevated serum CRP levels and the results showed the orally administered insulin nanoparticles and subcutaneously administered plain insulin solution were not able to bring down the elevated levels of serum CRP (75.43 $\pm$ 4.04 ng/ml for oral insulin nanoparticles and 77.08 $\pm$ 4.76 ng/ml for subcutaneous insulin treatment group). The values obtained were not statistically significant and no reduction in serum CRP levels was observed in group treated with blank nanoparticles (Fig. 4.14). Among other inflammatory markers known for their role in development of diabetic complications, TNF-α has been shown to anatgonize insulin signalling at the molecular level: by transiently inhibiting insulin induced ERK1/2 activation and by blocking insulin induced phosphorylation, TNF-α serves as one of the major anti insulin protein inside the cell (Goetze *et al*., 2000) leading to development of diabetic complications, mainly of cardiovascular nature. Earlier studies have shown that intensive insulin therapy with tight glucose control causes reduced

morbidity and mortality in inflammation-related critical illness like TNF-α induced myocardial infarction (Li *et al*., 2008).

The results of our study indicated that insulin treatment failed in facilitating the reduction of elevated serum TNF-α level in various treatment groups. Compared with untreated diabetic rats that displayed TNF-α level of 699.87 $\pm$ 37.43 pg/ml, treatment with subcutaneous insulin and oral insulin nanoparticles displayed serum TNF-α levels of 553.62 $\pm$ 45.56 pg/ml and 569.04$\pm$33.14 pg/ml respectively and the results obtained were not statistically significant (Fig. 4.15). Blank nanoparticles showed 672.79 $\pm$ 38.42 pg/ml. There is a considerable evidence of abnormal lipid profile in patients suffering from diabetes especially hyper-triglyceridemia and hypercholesterolemia (Weidman *et al*., 1982). Many underlying mechanisms have been suggested implicating the role of insulin deficiency as one of the major causes of disturbed lipid profile mainly decreased activity of lipoprotein lipases, increased lipolysis and increased triglyceride synthesis by liver. Studies done in past have shown that insulin treatment not only alters hyperglycaemic condition but also modifies the serum lipid status reverting it to normal physiological values (Das *et al*., 1993). The results showed no beneficial effect of insulin in ameliorating the hyperlipidemia in diabetic rats. Treatment with insulin nanoparticles failed to decrease hypercholesterolemia compared to the diabetic control (212.12 $\pm$ 33.02 mg/dl for untreated diabetic group compared with 175.73 $\pm$ 36.40 mg/dl for group treated with oral insulin nanoparticles) again here the values were statistically insignificant. Even no effect was observed in group treated subcutaneously with insulin solution (117.42 $\pm$ 40.56 mg/dl) and no effect was observed in group treated with blank nanoparticles (Fig. 4.16). Similarly, treatment with insulin nanoparticles orally and insulin solution

subcutaneously failed in reducing elevated triglyceride levels in diabetic rats (159.51 ± 13.64 mg/dl in subcutaneous insulin treatment group, 193.78 ± 27.62 mg/dl in group treated with oral insulin nanoparticles compared with untreated diabetic group that displayed serum TG levels of 255.32 ± 15.79 mg/dl) (Fig. 4.17). The results obtained were statistically insignificant.

No beneficial effect was observed in serum HDL levels of diabetic rats treated with subcutaneous insulin solution and oral insulin nanoparticles. HDL which are decreased in diabetic state, were not normalized by insulin treatment, both with subcutaneous solution and oral nanoparticulate formulation (37.29 ± 2.66 mg/dl in subcutaneous insulin treatment group, 25.13 ± 2.13 mg/dl in oral insulin nanoparticle treatment group compared with untreated diabetic animals that displayed serum HDL of 19.36 ± 3.08 mg/dl) (Fig. 4.18). Here again, no effect was observed with blank nanoparticles and the observed values failed to produce statistical significance.

Overall results pertaining to efficacy of oral insulin nanoparticles in lowering markers of diabetes associated complications, showed the failure of oral insulin nanoparticle formulation as well as of subcutaneous insulin formulation in modulating levels of TNF-α, CRP, IL-6, total cholesterol, triglycerides and HDL. No changes were observed in individual parameters after treatment with insulin (nanoparticles and subcutaneous injections) and results were statistically insignificant. Possible reasons could include; lack of efficacy of insulin nanoparticles in delivering the required amount of insulin *in vivo*, need for higher dose of insulin in oral nanoparticles and subcutaneous insulin solution, need for a better dosage regimen with enhanced frequency of drug administration and need for a better model of diabetic complications.

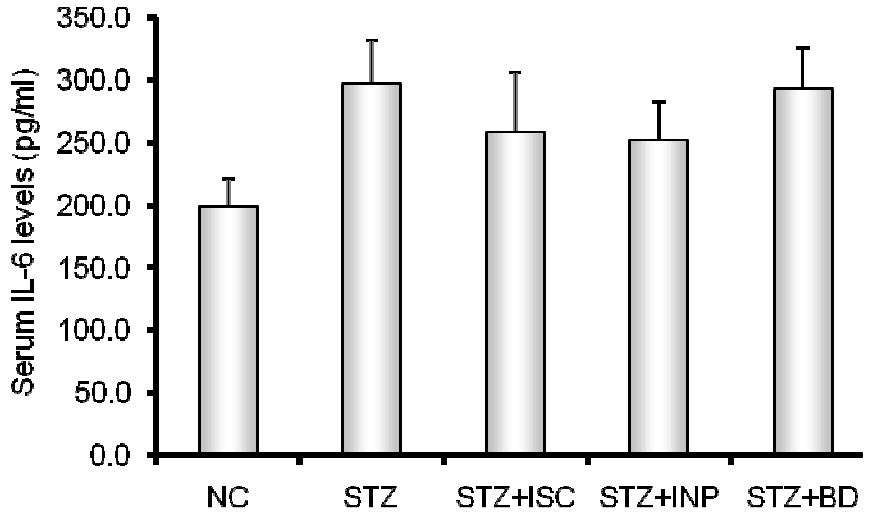


Fig. 4.13 Serum IL-6 levels (pg/ml) in STZ induced diabetic rats. NC: Negative control; STZ: Positive control; STZ+ISC: subcutaneous insulin at a dose of 20 IU/kg daily; STZ+INP: insulin nanoparticles (1% DMAB, 2% ZC) orally at a dose of 120 IU/kg daily; STZ+BD: Blank DMAB nanoparticles daily; Mean with SEM at n=6.

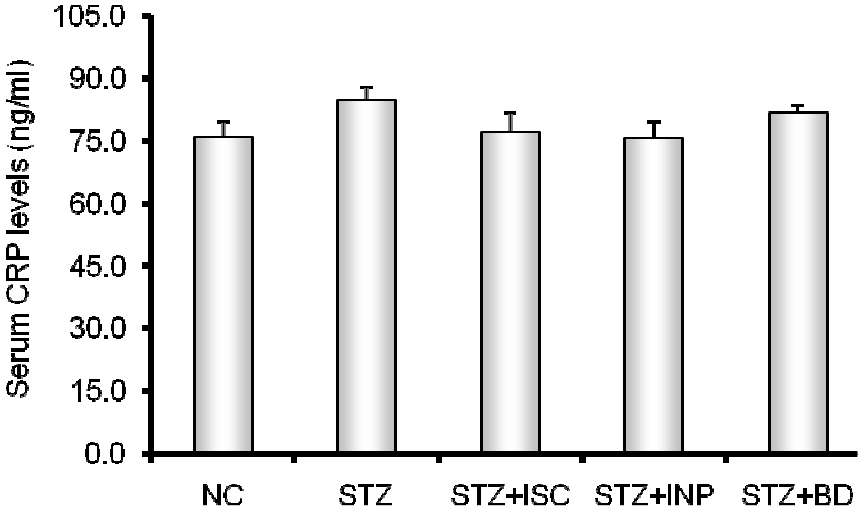


Fig. 4.14 Serum CRP levels (ng/ml) in STZ induced diabetic rats. NC: Negative control; STZ: Positive control; STZ+ISC: subcutaneous insulin at a dose of 20 IU/kg daily; STZ+INP: insulin nanoparticles (1% DMAB, 2% ZC) orally at a dose of 120 IU/kg daily; STZ+BD: Blank DMAB nanoparticles daily; Mean with SEM at n=6.

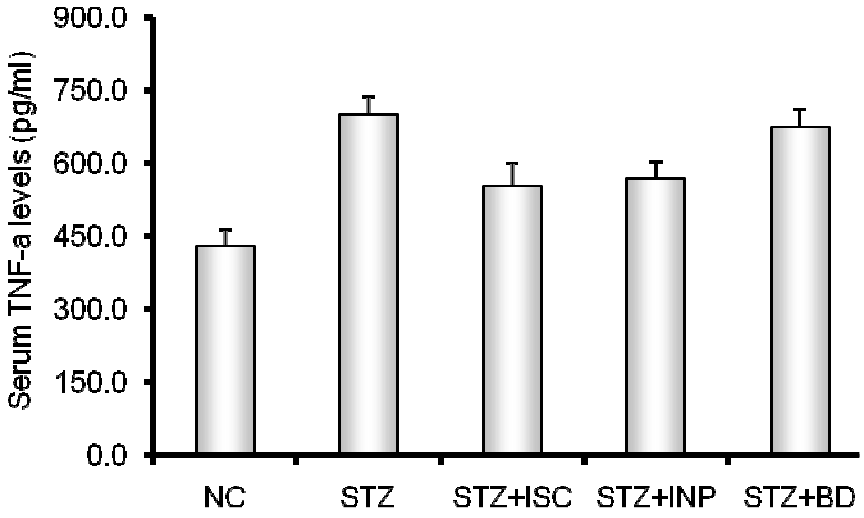


Fig. 4.15 Serum TNF-α levels (pg/ml) in STZ induced diabetic rats. NC: Negative control; STZ: Positive control; STZ+ISC: subcutaneous insulin at a dose of 20 IU/kg daily; STZ+INP: insulin nanoparticles (1% DMAB, 2% ZC) orally at a dose of 120 IU/kg daily; STZ+BD: Blank DMAB nanoparticles daily; Mean with SEM at n=6.

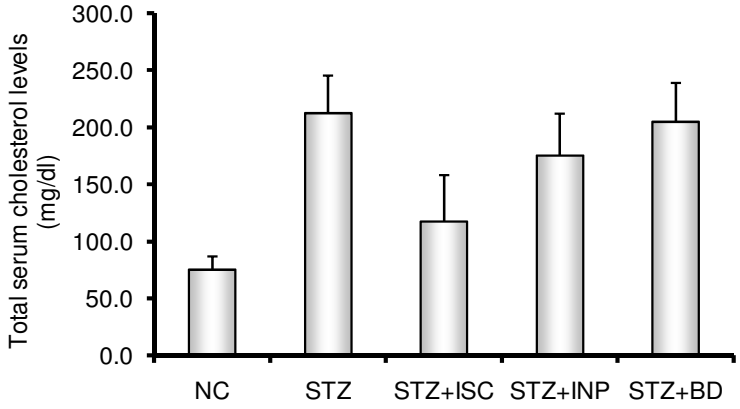


Fig. 4.16 Serum cholesterol levels (mg/dl) in STZ induced diabetic rats. NC: Negative control; STZ: Positive control; STZ+ISC: subcutaneous insulin at a dose of 20 IU/kg daily; STZ+INP: insulin nanoparticles (1% DMAB, 2% ZC) orally at a dose of 120 IU/kg daily; STZ+BD: Blank DMAB nanoparticles daily; Mean with SEM at n=6.

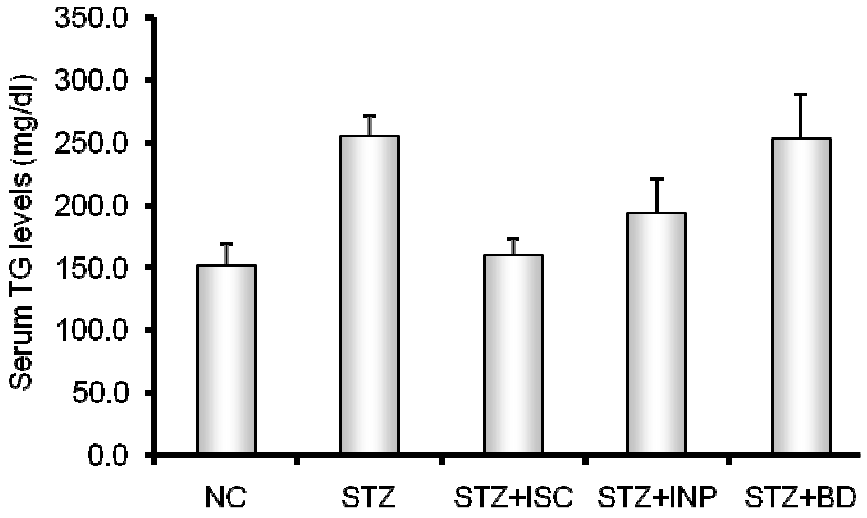


Fig. 4.17 Serum TG levels (mg/dl) in STZ induced diabetic rats. NC: Negative control; STZ: Positive control; STZ+ISC: subcutaneous insulin at a dose of 20 IU/kg daily; STZ+INP: insulin nanoparticles (1% DMAB, 2% ZC) orally at a dose of 120 IU/kg daily; STZ+BD: Blank DMAB nanoparticles daily; Mean with SEM at n=6.

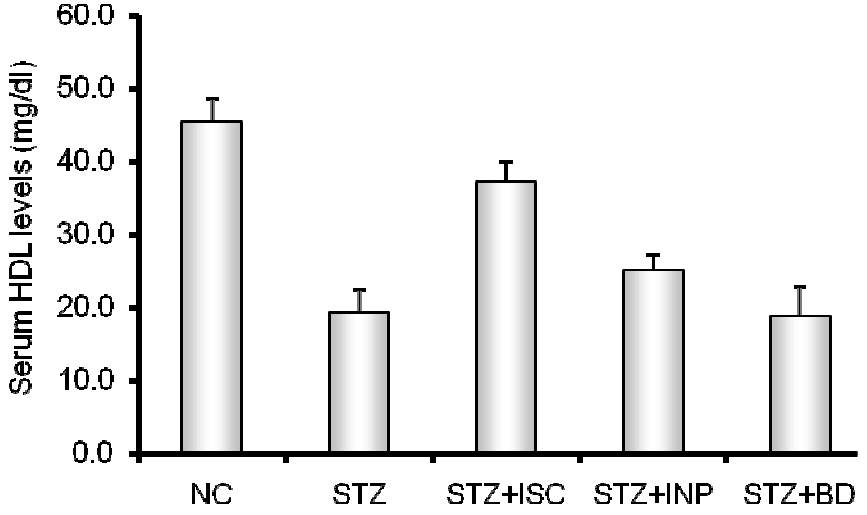


Fig. 4.18 Serum HDL levels (mg/dl) in STZ induced diabetic rats. NC: Negative control; STZ: Positive control; STZ+ISC: subcutaneous insulin at a dose of 20 IU/kg daily; STZ+INP: insulin nanoparticles (1% DMAB, 2% ZC) orally at a dose of 120 IU/kg daily; STZ+BD: Blank DMAB nanoparticles daily; Mean with SEM at n=6.

5. Conclusions

1. Pharmacokinetics of oral insulin nanoparticles in healthy SD rats revealed the ability of these polymeric carriers to enhance oral bioavailability of insulin 6 folds over simple insulin solution administered orally as demonstrated by the C_{max} values. Significant levels of insulin were detected till 72 h from oral nanoparticles whereas insulin from plain insulin solution was not detected after 24 h. Tissue distribution analysis revealed an altered pattern of insulin distribution in various tissues with oral nanoparticles compared to plain insulin solution at similar dose of 20 IU/kg over 3 days. The altered pattern of insulin residence with nanoparticles was attributed to their unique uptake mechanism, their filtration and compartmentalization in different organs due to their size and surface charge.
2. Pharmacodynamic assessment of oral nanoparticles showed modest anti-hyperglycaemic activity of formulations in STZ induced diabetic rats where glucose lowering was achieved without normalization of hyperglycaemic levels at a dose of 20 and 60 IU/kg, orally. Glucose normalization was only observed at a very high dose of 120 IU/kg. The study revealed lack of efficacy of formulation at low doses and this was attributed to the insufficient insulin release from slowly degrading PLGA nanoparticles, degradation of surface bound insulin and remotely due to constant glucose challenge due to continuous feeding. Dose dependent reduction in PGLs of STZ induced diabetic rats was observed with oral nanoparticles without any change in T_{max} though C_{max} increased almost linearly with increase in dose.

3. It was observed that pharmacokinetics of insulin from oral nanoparticles was independent of pathological state of the animals and similar blood levels of insulin were observed in healthy rats and diabetic rats at the same dose.
4. Both subcutaneous insulin and oral nanoparticles treatments were not successful in lowering the pathological markers of diabetic complication. Results demonstrated that though there were fluctuations in levels of pathological markers with insulin treatment (both subcutaneous injections and oral nanoparticles) but overall results were statistically compromised leading to failure of therapy.

General conclusions

General conclusions

The major objective of this dissertation to deliver insulin orally with the aid of polymeric nanoparticles was successfully achieved. The compatibility studies lead to selection of appropriate surfactants and stabilizers for formulation development. The prepared co-encapsulated nanoparticles were in 150 nm size ranges, homogeneous and spherical as revealed by TEM and displayed around 85% insulin entrapment. The type and concentration of stabilizer used for enhancing insulin's stability inside nanoparticles had no effect on particle characteristics. Prospect for further improving shelf life of nanoparticles using freeze drying was possible. Secondary structure of insulin was significantly preserved in nanoparticles as demonstrated by FTIR analysis. *In vitro* release/stability studies indicated the ability of nanoparticles in protecting encapsulated insulin from degradation in simulated gastric and intestinal fluids. Pharmacokinetic evaluation of nanoparticles in healthy rats revealed a 6 times enhancement of oral bioavailability, sustained release and modified tissue distribution profile over plain insulin solution at same dose. It was observed that pharmacokinetics of insulin from oral nanoparticles was independent of pathological state of the animals and similar blood levels of insulin were observed in healthy rats and in STZ induced diabetic rats at the same dose. The pharmacodynamic evaluation of nanoparticles in STZ induced diabetic rats revealed anti-hyperglycaemic activity of nanoparticles in a dose dependent manner, though elevated glucose levels were normalized with nanoparticles only at a dose of 120 IU/kg and not with 20 and 60 IU/kg dose. The nanoparticles failed to produce a change in levels of markers of diabetic complications in the same model. Therefore, the developed nanoparticles could be efficient for oral insulin delivery providing modest control over blood glucose levels. Moreover, oral delivery of insulin

would certainly provide better patient compliance and adherence to the therapy over injectable formulations making painless delivery of insulin possible.

Based on the results of the current study, the glucose reduction obtained with nanoparticles at a dose of 20 IU/kg was very modest. Even though there was a reduction in elevated plasma glucose values from 4.8 mg/ml to 3.75 mg/ml, glucose levels were not normalized with nanoparticles and the animals were still in clinically diabetic state. Moreover on escalating dose of nanoparticles from 20 to 60 IU/kg, more pronounced reduction in serum glucose levels was observed but here again the levels were not normalized with non diabetic values (around 1 mg/ml). The only normalization of elevated glucose levels was realized when nanoparticles were administered at a dose of 120 IU/kg. Increasing dose of insulin in nanoparticles lead to increase in plasma concentrations of insulin but no change was observed in T_{max} of the formulations and usually for insulin formulations a relatively short T_{max} is desired. Considering the above facts where glucose levels were normalized at a very high dose of 120 IU/kg, translation of nanoparticles into a marketed formulation is difficult at this stage and this is not only attributed to the high dose of insulin required and high amount of polymer dumping inside the body but also to the pharmacokinetic profile exhibited by nanoparticles as in ideal case glucose reduction from insulin regimens is expected to start soon after administration and desired time for action is usually not more than 30 min to 2 h. In this scenario, nanoparticles would afford glucose reduction 24 h post administration which would not be clinically desirable. Moreover, high insulin dose for glucose normalization as seen with 120 IU/kg formulation further questions economic feasibility of the formulation.

Clinical significance: The results of *in vivo* studies indicate in first place that oral delivery of insulin is possible adopting nanoparticulate approach. Secondly, the obtained pharmacokinetics of oral insulin nanoparticles in rats clearly demonstrated that with T_{max} around 24 h and C_{max} around 5 μIU/ml, nanoparticles afforded sustained release of insulin over 3 days. This regimen can still be adopted for preventive therapy as seen in case of Type 2 diabetes where an early insulin prescription results in significant reduction of morbidity and mortality associated with diabetes but for treating Type 1 diabetes, where a higher insulin load from nanoparticles with shorter time of action is desired, this dosage regimen is not suitable. Moreover, the doses used for pharmacodynamic study clearly showed that glucose normalization which is the clinical target of insulin therapy in Type 1 diabetes, was achieved only at a high insulin dose of 120 IU/kg whereas lower doses though caused reduction in elevated glucose levels, failed to normalize it completely. In the present study, oral nanoparticles were compared with plain insulin solution given orally at the same dose and the results indicated a 6 fold improvement in the AUC from nanoparticles. This exercise was undertaken to evaluate the potential of nanoparticles against plain insulin solution in enhancing the pharmacological bioavailability of insulin. A more rational approach would be to compare oral insulin nanoparticles with clinically used subcutaneous insulin injection at the same dose or with best nanoparticulate formulation via oral route, in reducing elevated glucose levels in a model of diabetes so as to gain better understanding of the efficacy of formulations under investigation.

Scientific significance: The present work focussed on development of PLGA based insulin nanoparticles for oral delivery. PLGA being the most biocompatible and biodegradable polymer was selected as carrier matrix for

nanoparticles. The issue associated with insulin's stability (secondary structure alteration due to change in microclimate pH caused by ageing or degrading PLGA matrix) was taken into consideration and additional stabilizers were screened and co-encapsulated with insulin to minimise this effect. Effect of stabilizer co-encapsulation on particle characteristics was studied along with *in vitro* stability studies and FTIR to confirm the stability of insulin in nanoparticles. Moreover, effect of stabilizer type on pharmacokinetics was also studied. We for the first time used DMAB as the surfactant for preparing insulin nanoparticles based on previous work from our lab where DMAB was extensively used as the surfactant in particle preparation process based on its safety and ability to produce smaller particle sizes with low polydispersity values. For developing PLGA based insulin nanoparticles and evaluating their potential in healthy and diabetic rats, a methodical plan was adopted. Before formulation development and optimization, extensive preformulation studies were carried out to assure the compatibility of insulin with formulation excipients as insulin being a reactive peptide is prone to denaturation by surfactants and stabilizers. Commonly used surfactants for preparing nanoparticles and stabilizers for minimizing insulin denaturation by PLGA matrix, were screened for compatibility with insulin adopting Circular dichroism, Fluorescence spectroscopy and Ultraviolet spectroscopy. This exercise led us to select most compatible excipients and even delineated their most effective concentration used in particle preparation process.

Future work

Future work

The future work should be aimed at the following.

1. Alter the hydrophobicity/hydrophilicity and molecular weight of the polymer to achieve better release profiles (Al-Tahami and Singh, 2007) thereby strict control over blood glucose and subsequently evaluate in type II diabetes model. The results of the present study indicated a slow release of insulin from PLGA nanoparticles. Moreover, the release was sustained over 3 days and the maximum amount of insulin delivered from the nanoparticles *in vivo* was lower than basal insulin levels found in species of rats investigated for evaluation of pharmacokinetic and pharmacodynamic performance of the system. Moreover, nanoparticles were not able to normalize enhanced glucose levels at a dose of 20 and 60 IU/kg, orally. Hence, alteration in polymer molecular weight and hydrophobicity/hydrophilicity should be carried to achieve faster (lower T_{max}) and higher release (higher C_{max}) of insulin from nanoparticles in order to enhance the efficacy of formulations and to facilitate rapid reduction in elevated glucose levels and control over generation of diabetic complications. Furthermore, early initiation of insulin therapy is viewed very beneficial in patients suffering from Type 2 diabetes. Latest clinical trials have revealed that that early initiation of therapy not only prevents short-term complications, but also reduces long-term morbidity and mortality and potentially alter the natural history of the disease (Glaser and Cerasi, 1999).
2. A systematic regulatory toxicology study. The current study focussed on development of PLGA based nanoparticles for oral delivery of

insulin. The *in vivo* behaviour of nanoparticles was analyzed via their pharmacokinetics and pharmacodynamic profile but a systematic regulatory toxicology study was not carried out. PLGA has been approved by US FDA for its use in medicinal products intended for human use. Apart from its safety for biomedical applications, a regulatory toxicology study is required to define its toxicity status when used in nanoparticles. This is because of quantum size effects and large surface area of nanoparticles as these carriers have unique properties compared with their larger counterparts. The smaller a particle is, the greater it's surface area to volume ratio and the higher its chemical reactivity and biological activity. The greater chemical reactivity of nanomaterials results in increased production of reactive oxygen species (ROS), including free radicals and this is one of the primary mechanisms of nanoparticle toxicity which has potential to induce oxidative stress, inflammation, and consequent damage to proteins, membranes and DNA. The extremely small size of nanomaterials also means that they much more readily gain entry into the human body than larger sized particles (Oberdorster *et al*., 2005). How these nanoparticles behave inside the body is still a major question that needs to be resolved. The behaviour of nanoparticles is a function of their size, shape and surface reactivity with the surrounding tissue. In addition to questions about what happens if non-degradable or slowly degradable nanoparticles accumulate in bodily organs, another concern is their potential interaction or interference with biological processes inside the body. Hence a regulatory toxicology study involving PLGA nanoparticles forms one of the most important future studies to be envisaged.

3. Test the potential of insulin nanoparticles for treating insulin deficiency mediated Alzheimer's disease. The tissue distribution profile of oral insulin nanoparticles revealed varied levels of insulin in different tissues of the body. In addition to this, insulin from nanoparticles was detected in almost all primary organs including the brain over 3 days. This gives an idea, that nanoparticles were able to cross the blood brain barrier due to their unique size and surface properties. Latest research has reported that damage to neurons exposed to amyloid beta-derived diffusible ligands (ADDL) was blocked by insulin (DeFeliche *et al.,* 2009). ADDLs are known to attack memory-forming synapses causing them to lose their capacity to respond to incoming information, resulting in memory loss and Alzheimer's disease. In such a scenario, insulin delivery to brain would be of clinical importance in treating the ADDL mediated memory loss and Alzheimer's disease. Since nanoparticles were able to traverse across brain as indicated by the insulin levels in the brain tissue, brain delivery of insulin via oral insulin nanoparticles can be experimented as a viable option.

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