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THE EFFECT OF MOLECULAR WEIGHT AND DIFFERENT DEGREE OF DEACETYLATION OF CHITOSAN ON THE IN VITRO AND IN VIVO RELEASE OF ORAL INSULIN PREPARATIONS

By

Qutuba G. Hessen

Petra University Library



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A thesis submitted in
Partial fulfillment of the
Requirements for the Degree of
Master of Science
In Pharmaceutical Sciences

at

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Amman-Jordan

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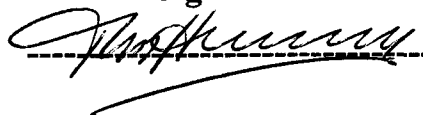
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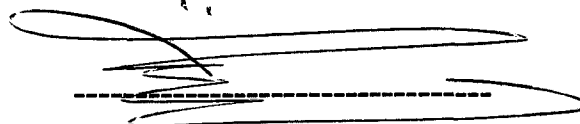
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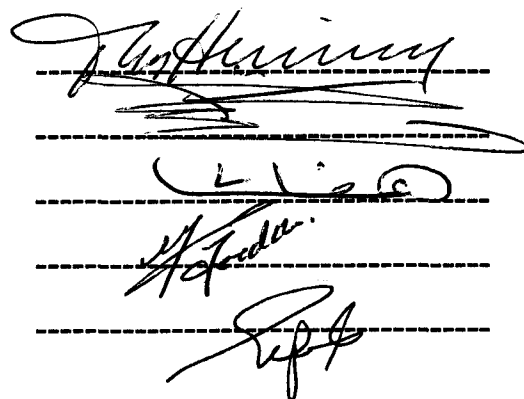
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Abstract

The Effect of Different Molecular Weight and Degree of Deacetylation of Chitosan On The In vitro and In vivo Release of Oral Insulin Preparation

By

Qutuba G. Hessen

There are many barriers to delivering insulin perorally. One of the major obstacles is the harsh environment of the GIT that cause destruction of insulin. The hydrophilicity and high molecular weight of insulin represented other obstacle for its permeability across the intestinal mucosa. There are many ongoing investigations to improve the oral bioavailability of peptide and protein formulations. Chitosan has been shown to be potential in delivering oral and other mucosally administered proteins due to its excellent mucoadhesive and permeation enhancing effects across the biological surfaces. Previous studies have demonstrated the possibility of formulating an oral insulin delivery system by combining the advantages of nanoencapsulation and the use of oily vehicle. These studies revealed a necessity to well understanding the effect of molecular weight and degree of deacetylation on the formulated system. Thus, different molecular weights (1.3, 6, 13, 18, 30 kDa) and degrees of deacetylation (100, 80, 75, 65, 55 DDA%) were prepared and characterized.

In vitro studies revealed that the low molecular weight chitosan, compared to high molecular weight chitosan, had pronounced increment in its solubility due to the shortage in length of chitosan chains. This shortening of chitosan chains had its impact towards reducing the size of the formulated nanoparticles. On the other hand, and in relation to changing DDA%, the executed in silico studies explained that the flexibility of chitosan chains was increased as the DDA% decreased. At this point of

flexibility, the chitosan chains had the ability to wrapping on the insulin molecule and applying its construction power to reduce the particle size of the PEC to the nano-scale

In vivo results, using STZ diabetic rat model, demonstrated that low molecular weights of chitosan significantly enhanced the hypoglycemic effect of oral insulin. In addition; each molecular weight of chitosan had its optimum DDA%. At this DDA%, it was thought that chitosan had the best conformation to form the smallest particle size when compared to other DDA% of the same molecular weight. As a result, the lowest used molecular weight (1.3 kDa / 80%DDA) revealed to possess the best insulin release compared to the other investigated higher molecular weights.

In conclusion, the current study exposed the importance of optimizing the molecular weight of chitosan in relation with its DDA% in oral insulin delivery formulation.

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Abbreviation

DDA%	Degree of Deacetylation
Cs	Chitosan
STZ	Streptozotocin
Labrasol®	PEG-8 caprylic/capric glycerides
Plurol®	polyglyceryl-6 dioleate
IR	Infrared Spectroscopy
DSC	Differential Scanning Calorimetry
NMR	Proton Nuclear Magnetic Resonance
M.W.	Molecular Weight
min.	Minute
r.p.m.	Rotating per minute
MP	megapixel
gm	Gram
mg	Milligram
nm	Nanometer
RSD	Relative standard deviation
S.C.	Subcutaneous injection
kg	Kilogram
μmole	Micromole
M	Molarity
SEM	Scanning Electron Microscope
km	Molar ratio

Chapter One

Introduction

1. Introduction

The emerging technique of combinatorial chemistry, along with a growing knowledge of biochemistry of human body, has lead to an ever-increasing number of therapeutic proteins in the treatment of diseases. However, these proteins often lack durability that more traditional small molecule pharmaceuticals possess. Where simple therapeutic agent, such as Aspirin, can be taken orally and reach the blood stream intact, the lager or more delicate protein must often be delivered directly into the bloodstream through injection. In the case of insulin, less than 0.1% of the orally dosed insulin reaches the bloodstream intact (Banting F. & Best H., 1998).

1.1. Oral delivery of protein

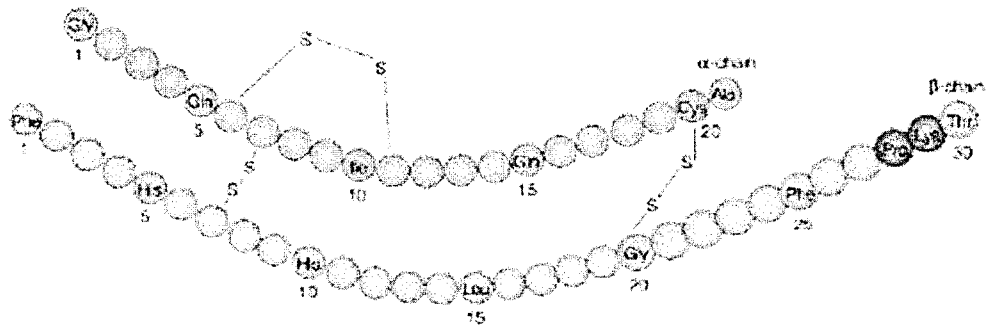
The oral delivery of therapeutic proteins has often been referred to as the 'Holy Grail' of drug delivery (Peppas N.A. *et al.*, 2004). Therapeutic proteins that are characterized by poor permeability across mucosal membranes commonly possess one or more of the following physicochemical characteristics: low octanol /water partitioning, presence of charged or hydrogen-bonding functional groups, and high polar surface area (Chornet E. & Dumitriu S., 1998a). However, therapeutic proteins are most frequently delivered by injection because of their large molecular weight (>3500 Da.), their hydrophilicity that prevent them to cross the lipophilic barrier of mucosal walls and the sensitive three-dimensional structure required for proteins to remain biologically active (Peppas N.A. *et*

al., 2004). Oral protein delivery is an ideal therapy because it would increase patient compliance and comfort over injection, better mimic physiologic delivery of proteins, provides a simple means of administration, reduce costs, and potentially improve the efficacy of a therapeutic treatment. Although oral protein delivery could be beneficial for many individuals, there are two key challenges to making it a successful therapy: (1) maintaining the functionality of the protein and (2) increasing the bioavailability of the drug.

Insulin, calcitonin, heparin, erythropoietin, interferon, and human growth hormone are just a few examples of therapeutic proteins actively being investigated as candidates for oral delivery. While all of those proteins are important for treating various diseases and illnesses, the focus of this research was using insulin as a model protein for oral delivery.

1.2. Insulin and insulin therapy

Insulin is a protein composed of two polypeptide chains (5800Da.) which are covalently bound by disulfide bonds between cysteine residues (Jintapattanakit A. *et al.*, 2007a) as show in (Figure 1.1) . Insulin is secreted by β -cells of pancreatic islets in response to high blood glucose levels. Insulin is a hormone, which is necessary for glucose uptake in skeletal muscle and adipose tissue, and it also stimulates the formation of glycogen from glucose in the liver. In addition, insulin inhibits gluconeogenesis, thus slowing the hepatic glucose uptake. In contrast to insulin, glucagon is a hormone secreted by the α -cells of the pancreatic islets in response to low blood sugar. Glucagon induces the liver to secrete glucose by breaking down glycogen, thus raising the blood sugar level (Ross S.A. *et al.*, 2004).



Insulin monomer



insulin dimer



insulin hexamer

Figure 1.1: Insulin molecular structure

1.3. Diabetes Mellitus

Diabetes Mellitus (DM) is a debilitating disease that is defined as the presence of high blood glucose levels due to either deficiencies in insulin production, insulin action, or both. There are approximately 150 million cases of diabetes mellitus in worldwide (Armour T.A. *et al.*, 2005). The increased glucose level can cause irreversible damage to the patient. Complications of the disease can include retinopathy, nephropathy, neuropathy, heart diseases and even death (Abolfotouh M.A., 1999).

More specifically, *Type 1 Diabetes Mellitus* (or insulin dependent diabetes mellitus, IDDM) is classified as an autoimmune destruction of the pancreatic β cells, or the insulin-producing cells of the body. The exact cause of the disease is unknown, but it thought to be induced by both genetic and environmental factors. Type 1 Diabetes is called Juvenile Diabetes as the onset of it begins in childhood (Hammami M.M., 1997a; Salsali A. & Nathan M., 2006a). *Type 2 Diabetes Mellitus* (or non-insulin dependent diabetes mellitus, NIDDM) is diagnosed when a patient begins to become resistant to insulin production and the patient may even eventually lose the ability to produce insulin. Age, obesity, family history of diabetes, race/ethnicity, and inactivity are all factors that make certain individuals more prone to developing type 2 diabetes (Yap W.S. *et al.*, 1998; Salsali A. & Nathan M., 2006b). *Gestational Diabetes (GDM)* is defined as any abnormality in glucose levels noted for the first time during pregnancy. During pregnancy, the placenta and placental hormones create an insulin resistance that is most pronounced in the last trimester. Risk assessment for diabetes is suggested starting at the first prenatal visit. High-risk individuals should be screened immediately.

Screening may be deferred in lower risk women until the 24th to 28th week of gestation (Jeha G.S. *et al.*, 2005). *Type 3 Diabetes Mellitus* refers to multiple other specific causes of elevated blood glucose: non-pancreatic diseases, drug therapy, etc (Hammami M.M., 1997b).

1.4. Alternative routes of insulin administration

The 1922 discovery by Banting and Best that insulin could be used to treat diabetic patients is often recognized as “one of the greatest achievements of modern medicine” (Ashcroft F.M. & Ashcroft S.J.H., 1992). Since then, overwhelming amounts of research have contributed to understanding diabetes as a disease and the role that insulin plays in that disease, but little has changed about the way insulin is administered. It still remains today that the primary route of administration for insulin is by injection, just as it was almost 85 years ago. A few variations of injection technique have been developed, most notably the insulin pump and insulin pens (Hanaire-Broutin H. *et al.*, 1992; Ziv E. & Hoffman A., 1997; Baillot-Rudoni S. *et al.*, 2006). Insulin pumps are mechanical pumps attached to catheters. The flow of insulin into the body can be directly controlled by the pump speed and the concentration of the insulin solution. It has shown ability to accurately deliver insulin in its active form. The problem with this system is the size of the pump. Also, the catheter needs to be continually cleaned and sterilized when the pump is disconnected to avoid infection. The insulin pen is a small pen-shaped device with small needle that can administer exact amount of insulin to the body. While it is easier to use than the standard needles, it is still considered as an invasive method for insulin delivery to the body.

Another common route of insulin delivery system being developed is through transdermal systems (Lee S. *et al.*, 2004; Park E.J. *et al.*, 2007). The use of ultrasound enhancement technique has led to the development of this system. Transdermal transport is usually limited to the small weight compounds, but low-frequency ultrasound shows a several orders of magnitude increase in insulin transport across the skin. Researchers continue to improve this process, but it is currently not efficient enough for clinical use.

A third investigated process is the transmucosal delivery of insulin into the blood stream. There are a number of mucosal surfaces possible for transport: nasal (Yu S. *et al.*, 2004; Krauland A.H. *et al.*, 2006), buccal (Luo Y. *et al.*, 2005; Starokadoms'kyi P.L., 2006), pulmonary (Heinemann L. *et al.*, 2001; Pfutzner A. & Forst T., 2005b), oral (Jintapattanakit A. *et al.*, 2007b; Elsayed A. *et al.*, 2009c), vaginal (Richardson J.L. *et al.*, 1992; Degim Z. *et al.*, 2005) and rectal (Bakth S. *et al.*, 1980; Hauss D.J. & Ando H.Y., 1988). The nasal, pulmonary and oral routes are leading candidates for transmucosal delivery.

Numerous researchers have developed delivery for insulin via the nasal route. The high surface area of the nasal cavity cause a wide range in the amount of insulin reaches the systemic circulation. Researchers were able to monitor a response in glucose level of the body after dosage, but the insulin delivered was too low. Further research into enhancement of insulin transport via the nasal passage has shown an increase in blood insulin when chemical enhancers were used over more traditional nasal administration (Pillion D.J. *et al.*, 2002).

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Pulmonary insulin delivery requires the addition of absorption enhancers to achieve good insulin delivery (Hussain A. *et al.*, 2003;Pfutzner A. & Forst T., 2005a). But recently, some studies have shown the possibility of an immunological response to the added enhancer used in pulmonary delivery system (Rave K. *et al.*, 2007;Hsu E. & Feghali-Bostwick C.A., 2008).

Oral delivery is perhaps the most attractive delivery route alternatively to parenteral delivery due to general acceptance of orally administered drugs. Oral administration of insulin may be beneficial to the patients as it can mimic the physiological fate of insulin and might provide a better glucose homeostasis. Insulin absorption through the GIT can be affected by its particle size, stability, release profile and residence time at the site of absorption.

There are many obstacles that faced the insulin delivery systems when passed along the gastrointestinal tract (Figure 1.2). In the GIT, the acidity of the stomach and the proteolytic enzymes represented destructive barrier and cause denaturation of the insulin like the other proteins and polypeptides. The other obstacles to the insulin absorption are its hydrophilicity and high molecular weight, which hindered its permeability across epithelial cells through lipid-bilayer cell membranes to reach the blood stream. In other words, insulin has low permeability through the intestinal mucosa.

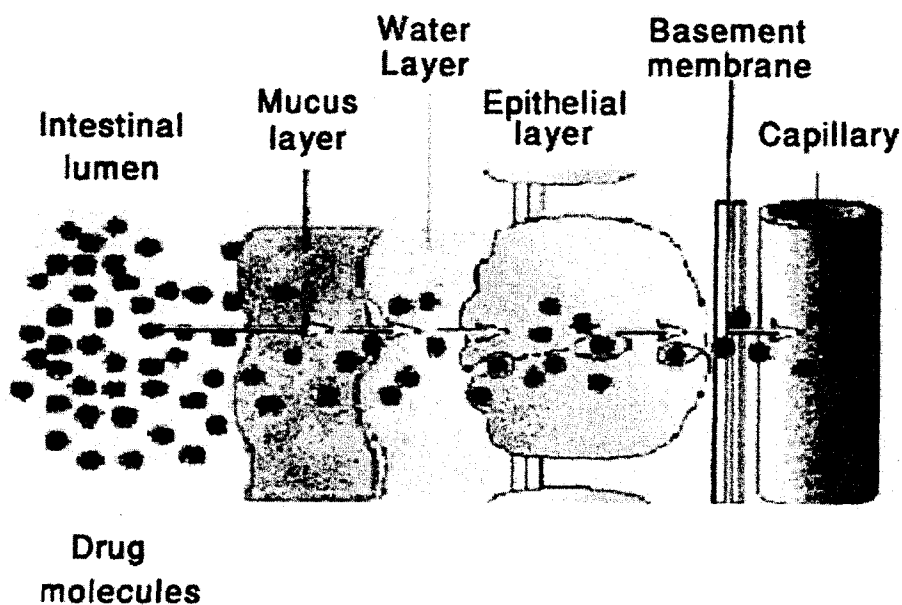


Figure 1.2: Gastrointestinal Barriers for oral delivery of proteins

1.5. Strategies for oral delivery of insulin

Various approaches have been proposed to overcome enzymatic and absorption barriers and to attain better oral bioavailability, including the use of surfactants (Toorisaka E. *et al.*, 2003), permeability enhancers (Tirumalasetty P.P. & Eley J.G., 2005), protease inhibitor (Del Curto M.D. *et al.*, 2009), enteric coatings (Zhao W.G. *et al.*, 2007), carrier systems (Chalasani K.B. *et al.*, 2007) and chemical modifications of insulin (Hashizume M. *et al.*, 1992). Of these systems, microsphere encapsulation was the most oral drug delivery vehicle that has the potential to surpass both the enzymatic and physical barrier of GI tract.

The first method of oral insulin delivery was with the addition of proteolytic inhibitor such as diisopropylfluorophosphate (Danforth E. *et al.*, 1959) or naturally occurring aprotinin (Owens D.R. *et al.*, 1988). While some decrease in blood glucose level was obtained, these reductions were far short of the decrease required.

Researches on encapsulating insulin into liposomes (Wu Z.H. *et al.*, 2005) showed large decrease in the blood glucose level, but the results were inconsistent. Enteric coating techniques with acrylic polymers were used to create a controlled release from oral insulin system (Foss A.C. *et al.*, 2004).

A wide range of biodegradable and conventional polymers has been investigated as possible oral insulin delivery systems (Guo B.L. & Gao Q.Y., 2007a; Nakamura K. *et al.*, 2008; Badwan A. *et al.*, 2009). These systems must protect the insulin as it passes through the stomach, and were design to release their insulin and successfully transport it across the gastrointestinal lining. Most systems developed to this point either protect the insulin while in transit or aided in the transport of insulin across the cell layer of the upper part of small intestine, none have accomplished both.

It was demonstrated that lectin modified solid lipid nanoparticles containing insulin orally administered to rats resulted in relative bioavailabilities of between 4.99% and 7.11% (Zhang N. *et al.*, 2006). Another group successfully maintained plasma glucose level at pre-diabetic levels for 11 hours after oral administration of chitosan and insulin nanoparticles to diabetic rats (Ma Z. *et al.*, 2005). In addition, microparticles composed of poly(methacrylic acid) and poly(ethylene glycol)(PEG) and containing insulin that

were orally administrated to type 1 diabetic rats resulted in suppressed post-prandial blood glucose levels (Morishita M. *et al.*, 2006).

1.6. Nanotechnology and Nanoparticle

Nanotechnology is a multidisciplinary field, which covers a vast and diverse array of devices derived from engineering, biology, physics and chemistry (Mauro Ferrari, 2005).

Our work is related in general to nanotechnology, involved the fabrication of nanoparticles of insulin by utilizing one of biodegradable polymer. Nanoparticles are generally defined as particles between 10 nm and 1000 nm. In the prior art, it is known that orally administered nanoparticles can be absorbed, albeit in small quantities, not only the way of membranous epithelial cells (M-cell) of Peyer's patches in the gut-associated lymphoid tissue, but also by the much more numerous gut enterocytes (Florence A.T. *et al.*, 1995).

Nanoparticles drug delivery utilizing degradable polymers provides a more efficient and less risky solution to many drug delivery challenges. The advantages of using these systems for drug delivery are many, wherein the most important are that these systems are absorbable, degradable and, in addition, might provide protection against the environment of gastrointestinal tract. Thus, these systems might be useful for oral delivery of active compounds sensitive to acid and enzymes in the human gut, specifically protein and peptide drugs, such as insulin. One example for a biodegradable polymer is called chitosan.

1.7. Chitosan

Chitosan is a linear co polymer consisting of β (1-4)-linked 2-amino-2-deoxy -D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) units (Figure 1.3). Chitosan is the second most abundant natural polymer after cellulose; obtained by alkaline N-deacetylation of chitin (Figure 1.3), which is the primary structural component of the outer skeletons of many marine creatures such as crustaceans, crab, shrimp shells and many other species such as insects and fungi (Tadashi Uragami & Seiichi Tokura, 2006).

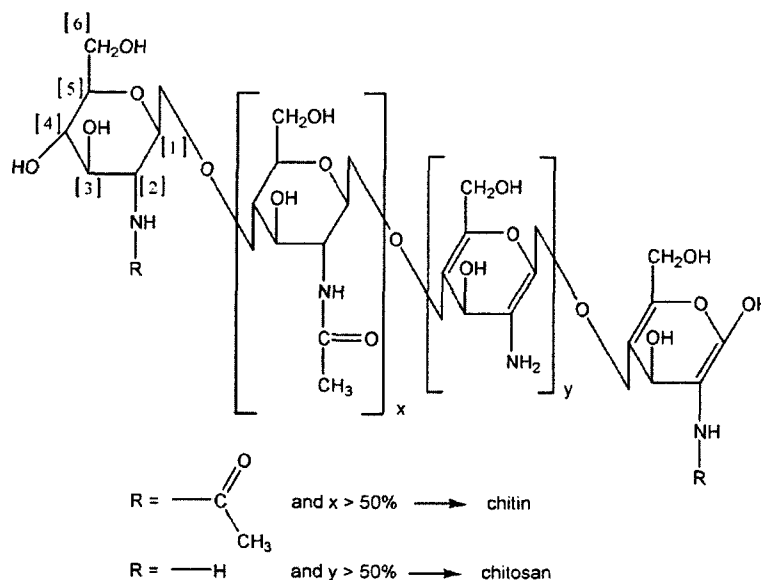


Figure 1.3: Skeleton structure of glucosamine unit

Chitosan is degraded by lysozyme present in the various mammalian tissues that lead to the production of N-acetyl-D-glucosamine and D-glucosamine, which also play an important physiological role in the in vivo biochemical processes. The physiochemical properties of chitosan polymer can manipulate through two parameters: the molecular weight (M.W.) (which is the sum of the weights of the atoms of which it is made) and the degree of deacetylation (DDA%)(which is defined in term of the percentage of primary amino groups in the polymer backbone). The DDA% of typical commercial chitosan is usually between 70%-95% and the M.W. between 10-1000 kDa. Furthermore, The properties and biological role of chitosan is dependent on its DDA% and M.W. These two parameters influence the conformation that adopt be the polymer in the solution. Low molecular weight chitosans were more stiff and extended than that of higher molecular weight one. Many studies discussed the influence of degree of deacetylation of the polymer chains on the conformation in the solution; some researchers indicated that a high expansion and stiffness with decreasing DDA% while other reported that no effect was express by different DDA% on chitosan arrangement (Gao Qun & Wan Ajun, 2006).

Chitosan is a hydrophilic polycation polysaccharide with a pKa of 6.5; thus, the polymer will be protonated in acidic environments and lose its charge and precipitate in neutral and basic medias. Since only the non-acetylated amino groups bind to protons, the charge density of polyelectrolyte depends on the ratio of the two monomers in the chains. When the acetylamine group is replace by an amine group, the linear biopolymer's solubility in aqueous solutions can increase. Chitosan is much easier to process than chitin, but the stability of chitosan materials is generally lower, owing to their more hydrophilic

character and, especially, pH sensitivity (Harish Prashanth K.V. & Tharanathan R.N., 2007).

In fact, the solubility is a very difficult parameter to control: It is related to the molecular weight, DAA%, the ionic concentration, the pH, the nature of the acid used for protonation, and the distribution of acetyl groups along the chain, as well as the conditions of isolation and drying of the polysaccharide. The distribution of acetyl groups along the chain (random or blockwise) may influence the solubility of the polymer and also the inter-chain interactions due to H-bonds and the hydrophobic character of the acetyl group (Marguerite Rinaudo, 2006b).

Chitosan have some cytotoxic properties, and the extent of this toxicity is dependant upon their molecular weight, degree of deacetylation and salt form (Carreno-Gomez B. & Duncan R., 2000;Kean T. & Thanou M., 2009). The toxicity of chitosan increase with increasing in M.W. and DDA%, and chitosan hydrochloride is the most toxic form. Chitosan with much lower molecular weight showed substantially decreased toxicity in addition to the good solubility in physiological pH environment (Azarmi S. *et al.*, 2008).

The advantage of chitosan over other polysaccharides (cellulose, starch, galactomannans, etc.) is that its chemical structure allows specific modifications without too many difficulties at the C-2 position. Specific groups can be introduced to design polymers for selected applications (Marguerite Rinaudo, 2006b). The net cationicity as well as the presence of reactive functional groups (1 amino and 2 hydroxyl groups per glucosamine unit) in the molecule makes chitosan a sought-after biomolecule. Low solubility of chitosan in both water and organic solvents resulted in many studies aimed at making

water-soluble derivatives of chitosan using chemical modification techniques. For example, sulfonation (Bannikova G.E. *et al.*, 2002), quaternarization (Polnok A. *et al.*, 2004), carboxymethylation (Wongpanit P. *et al.*, 2005), succinate derivative (Aiedeh K. & Taha M.O., 2001), phthalate derivative (Aiedeh K.M. *et al.*, 2006), and N- and O-hydroxyalkylation (Donges R. *et al.*, 2000; Richardson S. & Gorton L., 2003). In addition, a variety of graft copolymerization of chitosan with lactic acid (Yao F.L. *et al.*, 2003), mono-iodoacetic acid (Aiedeh K.M. *et al.*, 2007), poly acrylic acid (Cho S.M. & Choi H.K., 2005), vinyl pyrrolidone (Mangiapia G. *et al.*, 2007), Polyethylene glycol (PEG) (Zhang X. *et al.*, 2008), 3-o-dodecyl-D-glucose (Ngimhuang J. *et al.*, 2004), and N-isopropylacrylamide (Guo B.L. & Gao Q.Y., 2007b) were presented and evaluated as practical biomedical materials. Furthermore, the chitosan conjugated with many drugs and components to enhance their bioavailability such as proteins (Elsayed A. *et al.*, 2009b) and anticancer drugs (Dufes C. *et al.*, 2004)

1.8. Properties of chitosan

Both chitin and chitosan, in native as well as modified forms, are used in a wide range of possible applications including the production of value added food products, preservation of foods from microbial deterioration, formation of biodegradable films, recovery of waste material from food processing discards, purification of water, clarification and deacidification of fruit juices (Utai Klinkesorn & Yaowapa Namatsila, 2009). The biological properties of chitosan include non-toxic, weight loss product, hemostaticity, antitumor, antimicrobial activity, antiviral activity (Caiqin Qin *et al.*, 2006; Rekha M.R. & Chandra P., 2009). The US Food and Drug Administration (FDA) has approved chitosan

for fruit juice clarification, protein recovery from food process waste, edible coatings, and as an additive for animal feed. Industrially, chitosan has been used as a vehicle for directly compressed tablets, as a binder, as a disintegrant, for the production of controlled release solid forms or for improvement of drug dissolution, encapsulation and controlled release of drugs, and stabilization of emulsions (Andre Sao Pedro *et al.*, 2009).

From biopharmaceutical point of view, chitosan has special features that made it receiving a great deal of interest for medical and pharmaceutical applications especially as oral delivery system. These features can be summarized as follow:

A) Biocompatibility and Biodegradability

Chitosan has been widely used in food industry and is approved as a food additive. Chitosan has also been marketed throughout the world as a safe component in non-medical products. It has also been studied in the biomedical field and has been found to be highly biocompatible (Muzzarelli R. *et al.*, 1988). Chitosan is considered biodegradable, can be degraded into N-acetyl glucosamine by general lysozyme in the body, which is subsequently excreted as carbon dioxide via the glycoprotein synthetic pathway (Muzzarelli R.A., 1997). From a regulatory point of view, authorities have also approved chitosan, and a monograph relating to chitosan hydrochloride was included in the fourth edition of the European Pharmacopoeia (2002).

B) Mucoadhesive Properties

Recently, chitosan microspheres has been considered as a promising candidate in orally or other mucosally administrations for improving the transport of bio-macromolecules

such as peptides, proteins, oligonucleotides and plasmids across biological surfaces. This is mainly due to the mucoadhesive property of chitosan that improve the drug adsorption of paracellular route, which mediated by the spreading ability of chitosan over the mucus layer (Sadeghi A.M. *et al.*, 2008b).

Mucoadhesive polymers are synthetic or natural macromolecules capable of attaching to mucosal surfaces (Figure 1.4). Mucoadhesive polymers may fulfill the desirable features of a prolonged residence time at the site of drug absorption owing to increased contact with the absorbing mucosa, resulting in a steep concentration gradient to favor drug absorption, and localization in specified regions to improve the bioavailability of drugs (Filipovic-Grcic J. *et al.*, 2001). The basic components of mucus are mucin glycoprotein, which form an unstirred gel layer over the epithelial cells of the mucosa. For optimum mucoadhesion, there has to be an intimate contact between the adhesive and the substrate and interpenetration of the polymer chains with the mucin glycoprotein network. Chitosan interacts with mucin by multiple modes, such as by molecular attractive forces formed by electrostatic interaction between positively charged chitosan and negatively charged mucosal surfaces (Andre Sao Pedro *et al.*, 2009).

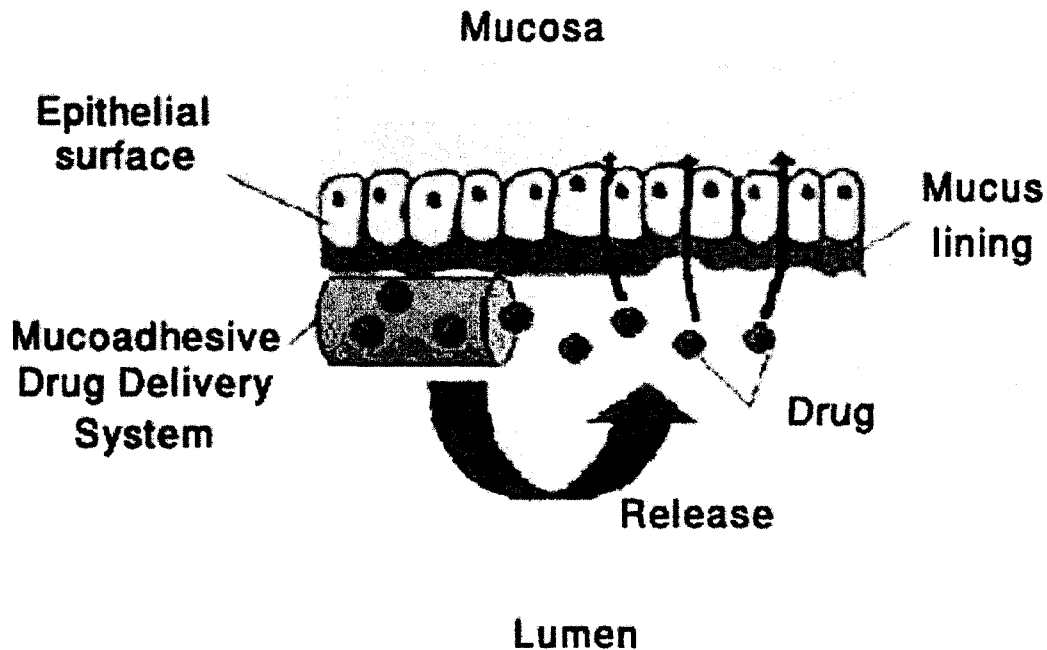


Figure 1.4: Mucoadhesive mechanism of chitosan

The adhesion of microspheres at the site of drug absorption offers various advantages for an improved uptake of therapeutic peptides. Firstly, in the case of orally or mucosally delivery of some delicate drug, the lumenally secreted proteases may degraded them on the way between the dosage form and the absorption membrane. Such degradable metabolism is strongly reduced by mucoadhesive formulations, because it provides an intimate contact with the intestinal mucosa. Secondly, the adhesion of microspheres containing drug on mucosal membrane provides a high concentration gradient of the drug towards the absorption membrane. This provides high driving force for the passive paracellular uptake. Thirdly, a prolonged residence time of microspheres on mucosal tissues such as the buccal, colonic or nasal mucosa leads to an extended time period of drug absorption and subsequently to an improved bioavailability (Wang L.Y. *et al.*, 2006).

C) Permeability Enhancer

Moreover, chitosan acts as a permeability enhancer to increase the transmucosal absorption of the drugs that normally do not pass the tight junctional barrier. The enhancing properties of chitosan and its derivatives have been attributed to their interactions with the tight junctions and cellular membrane components to reversibly open the tight junctions and hence to increase the paracellular permeability of hydrophilic compounds (Sadeghi A.M. *et al.*, 2008a).

As already mentioned, the mechanism of chitosan absorption enhancement has been suggested to be a combination of mucoadhesion and an effect on the gating properties of tight junctions from epithelium cells (Figure 1.5). Dodane *et al* (1999) studied the effects of chitosan on Caco-2 cells and observed, by confocal microscopy. They confirmed that chitosan induced redistribution of actin. Because actin has been shown to be important in regulating paracellular flow across cultured intestinal epithelia, the above effects of chitosan on epithelial barrier function might be due to a partial alteration of the cytoskeleton. The increased paracellular permeability was not accompanied by apparent changes in the junctional morphology. All these effects were reversible, indicating that chitosan had a transient effect on the cellular barrier. All these data suggest that chitosan could be used as a permeability enhancer without causing membrane perturbations and cause damage and irritate the intestinal mucosal membrane. It was concluded also that chitosan causes relatively mild and reversible effects on epithelial morphology, which makes it an advantageous absorption enhancing compound for mucosal delivery of drugs (Dodane V. *et al.*, 1999).

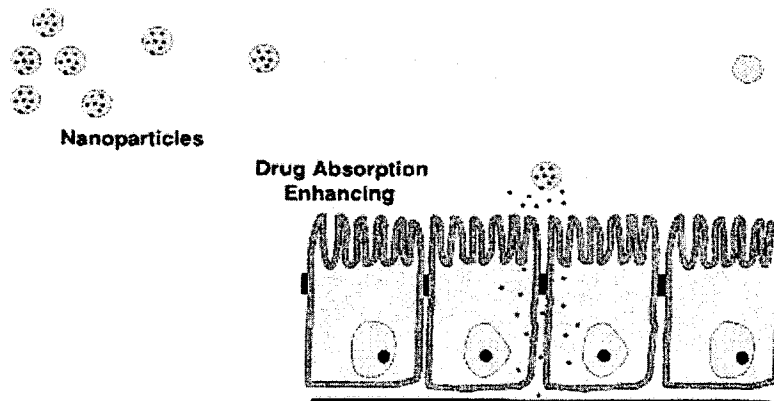


Figure 1.5: Permeability enhancing properties of chitosan

However, the chitosans used in these previous studies were macromolecular compounds and they generally had poor solubility in water at physiological pH, which brought much difficulty to use it in clinical application. To overcome these drawbacks of chitosan, we recently focused on chitosan oligomers, a new type of chitosan molecules, which have remarkably water-soluble characteristics due to their low molecular weights. Therefore, these chitosan oligomers can be expected to become useful absorption enhancer rather than conventional chitosans (Elsayed A. *et al.*, 2009d).

1.9. Low Molecular Weight Chitosan (LMWC)

Unfortunately, in most published literatures concerning chitosan characterization, commercial samples were used to study their physical properties and surface activity and their influence by molecular weight, ionic strength, counter ion effect and deacetylation (Gao Qun & Wan Ajun, 2006). Indeed one has to be careful from concluding results because of the different impurities that are naturally constructed with chitosan in the main

skeleton of the marine creatures such as fluorescent marine materials, different cations, and other proteinaceous materials. Consequently, it was essential to clean commercial chitosan from the metal ions and the fluorescence materials. One of the traditional procedures that were applied to purify chitosan sample was by dissolving it in different types of acids that is followed by pH adjustment steps to precipitate the chitosan then filter it on porous membranes (with different pore diameters down to 0.45 μm). The polymer is then washed with water and dried (Marguerite Rinaudo, 2006b).

Low molecular weight chitosan (LMWC) can be prepared from high molecular weight chitosan (HMWC) by depolymerization using acidic depolymerization, oxidative, enzymatic, and ultrasonic degradation (Wu T. *et al.*, 2008). A major disadvantage of the ultrasonication method is that the rate of degradation was irregular during the time course of ultrasound treatment (Kasaai M.R. *et al.*, 2008). In the enzymatic degradation process more than 30 types of enzymes could be used for the degradation but it is difficult to be utilized for large-scale industrial processes (Ren D. *et al.*, 2005). Oxidative degradation is considered as a non specific method of low yield of the LMWC and there could be a presence of byproducts corresponds to the oxidation of chitosan (Zhao X. *et al.*, 2009).

Industrially, acidic degradation using hydrochloric acid (HCl) was preferred (Varum K.M. *et al.*, 2001). This method is usually simple, easy, reproducible and non random (Einbu A. *et al.*, 2007). Most of the previously reported procedures require the use of concentrated hydrochloric acid that produces low yield of LMWC and high yield of monomers (Knill C.J. *et al.*, 2009a).

1.10. Degree of Deacetylation (DDA%)

Chitosan versatility depends mainly on the high reactivity of its amino groups. Since, the degree of deacetylation depends mainly on the method of purification and reaction conditions (Baxter A. *et al.*, 1992; Borchard G., 2001). Therefore, it is essential to characterize chitosan by accurate determination of its degree of deacetylation prior to its utilization at the development stage of drug delivery systems (Borchard G., 2001).

The process of deacetylation involves the removal of acetyl groups from the molecular chain of chitin, leaving behind a complete amino group (-NH₂). While the acetylation reaction involves addition of acetyl groups to the chains of the polymer by reacting chitosan solution with acetic anhydride (Ac₂O) in specific molar ratio or for defined time intervals depend on the degree of deacetylation which is sought-after (Qi Zhao *et al.*, 2006).

1.11. Methods of preparation of chitosan nanoparticles

Different methods have been used to prepare chitosan nanoparticles. These include emulsion cross-linking, coacervation/precipitation, spray drying, emulsion coalescence method, reverse micellar method, and ionic gelation (Agnihotri S.A. *et al.*, 2004).

Chitosan, as a polyelectrolyte, is able to form electrostatic complexes under acidic conditions. Two different types of complexes are considered here: electrostatic complexes with an oppositely charged surfactant (SPEC) and polyelectrolyte complexes (PEC) (Marguerite Rinaudo, 2006b).

Proteins interact strongly with both synthetic and natural polyelectrolyte. These interactions may result in amorphous precipitates, complex coacervate, gels, fibers or the formation of soluble complexes (Chornet E. & Dumitriu S., 1998b). In order to form a PEC, both polymers have to be ionized and bear opposite charges. This means that the reaction can only occur at pH values in the vicinity of the pKa interval of the two polymers. The preparation of a PEC requires besides chitosan, only a protein. Interaction of chitosan and polyanions leads to spontaneous formation of nanoparticles in an aqueous environment without the need of heating or the use of organic solvents. No auxiliary molecules such as catalysts or initiators are needed and the reaction is generally performed in an aqueous solution, which represents the main advantage over covalently cross-linked networks and thus favors biocompatibility and avoids purification before administration.

The most important factors that have to be controlled during preparation of PEC are the pH of the solution, temperature, ionic strength and order of mixing (Majeti N.V. & Ravi K., 2000). In addition, there are secondary factors, related to the components that have to be considered, such as flexibility of polymers, M.W. and DDA% of chitosan (Berger J. *et al.*, 2004). In fact, protein-polyelectrolyte complexes are not new, and have been used extensively in biology over many years for protein purification, immobilization and stabilization of enzymes (Morawetz H. & Sage H., 1955). However, only recently, has it been used as a drug carrier, especially for DNA condensation and complexation (Lavertu M. *et al.*, 2006). The work of Sadeghi *et al.* (2008) showed that trimethyl chitosan and diethylmethyl chitosan nanoparticles prepared by the PEC method had higher insulin loading efficiency and zeta potential than those made by ionotropic gelation method.