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Peroral insulin delivery : new concepts and excipients

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Peroral Insulin Delivery: New Concepts and Excipients

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I dedicate this thesis to my family for their support and patients

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Objectives of the thesis:

In the past decades, the development of a suitable peroral peptide delivery system has been the focus of many pharmaceutical research groups. The recent advancements in biotechnology have led to synthesis, production and purification of a number of peptide and proteins such as insulin and octreotide. Application of these peptides in chronic diseases is limited due to their short half life in the blood circulation. Hence, numerous injections are required for these peptides to be therapeutically effective. The cost of these injections, their inherent challenges in production as well as patient compliance issues have led to major developments in the field of oral drug delivery systems. In comparison to the parenteral route, the oral drug administration is cheaper, more convenient for the patients and easier to manufacture. However, to develop a suitable peroral delivery formulation, a number of hurdles must be overcome. For a peptide drug of low bioavailability, the most important challenges in developing a successful delivery system are: a) to protect the peptide drug from the harsh environment of the stomach; b) to protect the

peptide drug against the enzymatic degradation in the small intestine; c) to prolong the transit time of the peptide drug in a specific area in the gastrointestinal tract; d) to overcome the poor permeability of the large, hydrophilic peptide drug across the small intestine. To overcome the above obstacles is a challenging task for designing and developing a suitable peroral peptide delivery system. Amongst the synthetic peptides, insulin has been studied the most due to an increase in the number of diabetic patients worldwide.

In this study, research is described on designing a novel peptide delivery system based on CO₂ gas empowered delivery (GEDD) system. In this system, hydrophilic drugs such as peptides and proteins are delivered to the intestinal tract where they are adhered to the mucus layer using the mucoadhesive polyethylene oxide (PEO) to prolong their residence time at the mucosal surface and trimethyl chitosan (TMC) as permeation enhancer will simultaneously trigger the opening of the tight junction and drug permeation by the paracellular pathway. In the GEDD system, the CO₂ gas acts as the driving force to push the delivery system to the absorbing membrane to form a protective layer around the delivery system to protect it

against the enzymatic degradation and also acts as a permeation enhancer that mechanically opens the tight junctions. The GEDD system was enterically coated with cellulose acetate phthalate (CAP) to protect the drug from the acidic pH of the stomach. In this study insulin was used as the model drug and an increase in insulin permeation using the GEDD system is due to the synergistic effects of both the CO₂ and TMC as mechanical and chemical enhancers, respectively. The GEDD system was tested in sheep's intestine using ex vivo studies and in rabbits for in vivo studies. The ex vivo and in vivo studies showed promising results for the future development of the system as peroral peptide delivery system. With respect to peptide drug delivery systems, the GEDD systems with its novel characteristics may have a promising future in oral peptide drug delivery.

PART I

General introduction and physiochemical aspects

Chapter 1

Peroral peptide delivery and absorption challenges using novel drug delivery systems: current status

1. Introduction

Rapid developments in biotechnology have posed new challenges for pharmaceutical scientists to develop peptide and protein drugs especially for the treatment of chronic diseases at sufficient amounts and at an affordable price. Peptides and proteins are highly potent and specific in their physiological activities. However, there are severe difficulties in administration of these active agents orally. Most peptide and protein drugs are unstable in the gastrointestinal (GI) tract and have poor oral absorption due to their size and hydrophilic properties. Such low absorption can be increased by the chemical modification of the polypeptide to render it more stable and probably by increasing its lipophilicity [1]. It is well documented that peptides and proteins are easily hydrolyzed and digested by acids and enzymes in the GI tract. Hence, to deliver peptide and proteins orally, it is necessary to protect these active agents from the harsh and unfavorable GI environment.

Oral peptide drug delivery is the most desirable and the preferred method of administering therapeutic agents. In addition, the oral medication is generally considered as the first choice for investigation

in the discovery and development of new pharmaceutical formulations due to convenience in administration, patient compliance and cost effective manufacturing process. However, not all new drugs and especially hydrophilic peptide and protein drugs can be administered orally, because of their sensitivity to gastric acid, their instability to gastrointestinal enzymes and their poor absorption. The overall process of oral delivery is frequently impaired by several physiological and pharmaceutical challenges that are associated with the inherent physicochemical nature of the drugs and/or the variability in GI condition such as pH, presence of food, transit times, as well as enzymatic activity in the GI tract.

In order to overcome the above obstacles, the ideal oral delivery system must release its contents in a pH-dependent fashion only at the optimal target region, remain in the optimal site long enough for the complete peptide and protein release to be absorbed across the intestinal epithelium, and have a reproducible therapeutic effect. Thus, site specific delivery is required to deploy the peptides and proteins intactly to specifically targeted parts of the body through a platform that can control their release by means of physiological and/or chemical triggers [2-4].

In recent years, a number of drug delivery companies specializing in protein delivery have evolved. Some of these companies are attempting to attain suitable formulations such as liposomes or microspheres to protect peptide and protein drugs from enzymatic degradation in the GI tract or use site-specific delivery to the colon to

by pass the harsh GI environment. Moreover, study of these problems and challenges is considered an important strategy for improving oral drug delivery that requires thorough understanding of the drug's physicochemical properties, GI physiology and biochemistry, polymer science, pharmacokinetics and pharmacodynamics.

2. Required parameters for effective oral protein delivery

In order to obtain sufficient peptide absorption in the gut, novel delivery systems must be developed that are able to overcome the barriers present in the oral route. The main barriers include: the acidic environment in the stomach, the digestive and proteolytic enzymes in the small intestine, the low permeability of the intestinal epithelium to large hydrophilic peptide molecules and finally the first pass metabolism of the peptides in the liver [5-8].

Protease inhibitors such as Aprotinin, Chymostatin, EDTA and Leupeptin can be used to locally deactivate the proteolytic enzymes of the GI tract. In order to be safe, however, these protease inhibitors have to be linked to high molecular weight hydrophilic matrices to avoid their absorption and possible cell toxicity [9-11].

Peptide and protein protection can also be achieved using chemical modification of the peptides at their $-NH_2$ or $-COOH$ terminus. Lipophilic derivatives of peptides such as monocaproyl and dicaproyl derivatives of insulin as well as glycosylated insulin can enhance the protein permeation across the intestinal membrane and hence protect the protein from deactivation [12].

Moreover, permeation enhancers have been used to reversibly open the tight junctions of the intestinal epithelium and allow the passive absorption of peptides and proteins by the paracellular pathway. Two main classes of materials including calcium chelators and surfactants are able to increase the permeability of tight junctions and thus improve the absorption of macromolecules and hydrophilic substances. While chelating agents may induce disruption of actin filaments by extracellular Ca^{+2} depletion, surfactants cause irreversible exfoliation of the intestinal epithelium. However, neither of these substances can be used as permeation enhancers for hydrophilic macromolecules such as peptides or proteins due to their interactions with phospholipid bilayer of the intestine and the resulting cell toxicity [13].

Mucoadhesive polymers such as chitosan and trimethyl chitosan (TMC) have mucoadhesive properties that enable them to attach to the intestinal membrane and interact with the actin filaments of the tight junction to reversibly open them and allow for the passage of hydrophilic peptides across the membrane. These mucoadhesive polymers are shown to be non toxic and due to their specific interaction with the actin filaments and lack of interference with the phospholipid bilayers of the enterocytes, their use is becoming very common for the induction of the paracellular transport of hydrophilic macromolecules within the opened water filled channels. Their possible use will be discussed in more detail later in this chapter.

Microparticulate and nanoparticulate drug delivery systems have attracted an immense attention as novel carriers for the delivery of lipophilic and hydrophilic substances as well as vaccines [14]. There is a strong belief that nanoparticles of appropriate size may pass the mucosal membranes intactly and deliver their drug load into the systemic circulation. In the case of hydrophilic drugs, nanoparticles should be able to protect such drugs from degradation in the intestinal fluids and improve their penetration and permeation across the intestinal mucosal epithelium [15-17]. Suitable nanoparticles have mucoadhesive properties which are due to their particle size and the particle's surface charge. However, more and more research shows that only a small fraction of nanoparticles is able to act as a carrier for hydrophilic drug molecules across the enterocytes and to deliver their drug load at the serosal site, which in most cases was not sufficient for a therapeutic effect. It was further shown that a substantial part of the nanoparticles may be internalized into the intestinal epithelial cells [18, 19]. Exception is the transport of antigen containing micro and nanoparticles across so-called M-cells with specified particle uptake mechanism capable to induce a sufficient high immunogenic response. More details about the use of micro and nanoparticulate systems will be given below. The following sections will highlight the possible use of mucoadhesive polymers with special emphasis on chitosan and its increasing family of derivatives as well as promising delivery systems for peroral peptide delivery.

3. Chitosan and its derivatives

Chitosan is a polysaccharide composed of two subunits, D-glucosamine and N-acetyl-D-glucosamine, linked together by $\beta(1-4)$ glycosidic bonds. Chitosan, a constituent of crustacean shells and being the second most abundant biopolymer after cellulose is derived from chitin by deacetylation. Chitosan attracts a lot of attention in the pharmaceutical research as a polymeric drug carrier. Chitin and chitosan are copolymers; however, chitin has a limited application because of its poor solubility and reactivity. Chitosan is a fully or partially deacetylated chitin derivative and is consequently soluble in acetic acid and other acidic solvents. This polymer has an apparent pK_a of about 6.5 and is soluble in acidic solutions with pH values lower than 6.5. In recent years, significant progress has been made in identifying substances, which may increase the absorption of drugs through the paracellular pathways at a wider pH range [20]. The intestinal epithelial cells express apical intercellular attachments, known as tight junctions, connecting the enterocytes with each other. They have some regulation mechanism for the paracellular absorption of hydrophilic compounds like glucose when present in higher amounts and allow the passage of macromolecules through their intercellular space after their opening is triggered externally by specified ionic interactions.

Chitosan with its mucoadhesive and non toxic properties can act as a significant absorption enhancer by opening the intercellular tight junctions of the epithelia and promoting the paracellular permeation

of hydrophilic macromolecules [21]. Nevertheless, chitosan has poor solubility at pH values above 6.5; therefore, water soluble chitosan derivatives, which are soluble in both acidic and basic physiological environments, are good candidates for improving the paracellular permeation of peptide and protein drugs in the whole GIT. However, just recently a chitosan product has been synthesized by controlled deacetylation process of chitin which results in a soluble chitosan at pH value 7.2. (Personal communication, Prof. Jörg Thöming, University of Bremen, Germany).

Different studies were carried out to synthesize and determine the antibacterial activities of quaternary ammonium salt of chitosan. These investigations showed that the antibacterial activities of quaternary ammonium salt of chitosan are much stronger than that of chitosan itself since the cationic charge of the ammonium salt is found to increase the interaction with the negative peptidoglycan residues of the bacterial cell surface and will make the bacterial membrane more permeable [22, 23].

Trimethyl chitosan (TMC) was initially synthesized and characterized by Sieval et al [24]. Further in-vitro and in-vivo studies of the intestinal absorption of octreotide by quaternized chitosan showed that TMC is able to increase the permeability of octreotide across the Caco-2 cell monolayer [25]. Moreover, Thanou et al. have studied the effect of TMC on the intestinal permeation of buserelin. This investigation has shown that TMC, as permeation enhancer, similar to chitosan, is able to open tight junction in a reversible way and

increase the permeation of buserelin across the intestinal epithelia both in vitro and in vivo [26]. Moreover, Jonker and coworkers have studied the intestinal paracellular permeation enhancement with TMC of various substitution degrees [27]. Their studies clearly demonstrated that their TMCs were able to enhance the intestinal permeation in a neutral pH environment. Furthermore, it was shown that the degree of quaternization of the derivative has a major impact on its permeation enhancing properties across the intestinal epithelia. Due to their unique properties, such as their permeation enhancing effect and enzyme inhibitory capabilities, chitosan and its derivatives also act as antimicrobial agents [23]. These investigations have shown that growth inhibition of chitosan against microorganisms, such as fungi and bacteria depends on the molecular weight of chitosan [22].

Avadi et al. have synthesized two new derivatives of chitosan, namely triethyl chitosan (TEC) and diethyl methyl chitosan (DEMC) using a factorial design approach to achieve the optimum experimental conditions [28, 29]. They have investigated the effect of TEC on permeation of brilliant blue across intestinal epithelia (ex vivo). Their investigation indicated a significant increase in absorption of sodium fluorescein and brilliant blue in the presence of TEC compared to chitosan. TEC bearing a positive charge is therefore also able to interact with the tight junctions of colon and increase the permeation of the model drug via the paracellular pathway [28]. Moreover, the enhancing effect of DEMC on insulin absorption from the ascending colon in rats was investigated in vivo [30]. These studies have shown

that there was a significant decrease in blood glucose levels after 60 min, when a mixture of insulin and polymer was injected into the ascending colon. They have claimed that chitosan derivatives may induce a redistribution of cytoskeleton of F-actin. Furthermore, it may be possible that the polymers interact with the proteins of the tight junction (e.g. occludin and claudin) leading to disruption of tight junction integrity and a higher permeation of the insulin across the intestinal membrane. Their investigation has shown that DEMC with cationic character is compatible with insulin and no precipitation or aggregation phenomena were observed during the preparation of insulin-DEMC mixture in phosphate buffer solution at pH 7.2 [30, 31].

Bayat et al. have synthesized and fully characterized dimethyl ethyl chitosan (DMEC), another new chitosan derivative with a substitution degree of $50\pm 5\%$ [32]. In their investigations, insulin nanoparticles were prepared using the polyelectrolyte complexation method between insulin and DMEC or chitosan for further ex vivo and in vivo studies in rats [33]. They have shown that insulin in the nanoparticulate form is more permeable across the rat's colon to decrease the blood glucose level mostly due to the endocytotic pathways whereby the insulin containing nanoparticles are transported across the colonic membrane resulting in an approximately 2- fold reduction in blood glucose level compared to the free insulin [34]. Trimethylated and triethylated 6-NH₂-6-deoxy chitosan with substitution degrees of $50\pm 5\%$ were synthesized and

characterized by our group [35]. These derivatives contain 2 alkyl groups at their C₂ and C₆ positions and are water soluble. The permeation enhancing effect as well as the cytotoxicity of the C₂-C₆ methylated 6-NH₂-6-deoxy chitosan was compared with those previously studied with TMC. It was shown that the new chitosan derivative, trimethylated 6-amino-6-deoxy chitosan, at 0.5% concentration, reduced cell numbers in water soluble tetrazolium (WST-1) cytotoxicity assay compared to the control, indicating a certain enhanced cytotoxicity as compared to TMC. Moreover, despite its higher zeta potential due to the presence of two positively charged methyl groups per monomer units, trimethylated 6-amino-6-deoxy chitosan did not significantly reduce the TEER in confluent monolayers as compared to the control. This could be explained by the higher susceptibility of Caco-2 cells in a 24 hrs culture to the cytotoxic polymer properties compared to fully differentiated epithelial cells forming tight monolayers. Moreover, the bulky size of the polymer with reduced flexibility of its backbone and the possibility of the two positive groups' repulsion may cause a less significant reduction in TEER (unpublished results).

The bioavailability, biodegradability and the extensive studies on chitosan and its numerous derivatives have made them, as multifunctional polymeric permeation enhancers, good candidate polymers for oral peptide/protein delivery.

4. Novel Drug Delivery systems

Today, the design and development of novel peroral delivery systems for peptides and proteins are the main goal of many pharmaceutical researchers. The low oral bioavailability of these peptide and proteins due to their low permeation across the intestinal epithelium, the harsh environment of the gastric pH, their rapid degradation by the proteolytic enzymes and their rapid clearance due to the first pass effect are the major drawbacks of developing a successful delivery system, however the delivery of the hydrophilic peptide drug is not difficult to achieve but to enable its absorption in the intestinal tract is the crucial part. Hence, the delivery system has not only to overcome the harsh pH of the stomach and the enzymatic degradation of the proteins in the GI tract, but to also increase the permeation of these molecules across the GI epithelium either by opening the tight junctions and increasing the paracellular transport or by increasing the endocytotic passage of the molecules through intracellular transport. In order to achieve this, the delivery system must be able to attach to a specific site in the GI tract long enough for the drug to permeate across the epithelium before the delivery system is being detached by the peristaltic movements of the gut [36].

A number of peroral delivery systems were designed using liposomes, beads, adhesive drug delivery systems, superporous hydrogels, chitosan and its derivatives as well as nanoparticles to protect the

drugs from the harsh environment of the GI tract and prolonging the drug's transit time at a specific site of the GI tract for an optimum drug bioavailability.

4.1. Liposomes

Long circulating macromolecular carriers such as liposomes can exploit the enhanced permeability and retention effect for the protein drugs. Liposomes are vesicles consisting of one to several, chemically active lipid bilayers. Drug molecules can be encapsulated and solubilized within these bilayers. Different types of phospholipids such as phosphatidyl choline or phosphatidyl inositol may be used in liposomal carriers. Liposomes are prepared by sonication, reverse phase evaporation or film formation [37, 38]. Among different types of liposomes, dehydrated-rehydrated vesicles are most commonly used in protein drug delivery due to the ease of preparation and low amount of stress applied to the proteins [39]. The liposomes can be easily decorated with targeting moieties, e.g., antibodies, hence delivering the protein drugs to their specific target site [40, 41]. The liposomal composition, encapsulation efficiency, the rate of drug release from lipid bilayers, size and the surface charge are all important factors in successful liposomal drug delivery [38]. Stefanov et al. have used liposomes prepared from phosphatidylcholine (PC) and cholesterol (CH) for oral insulin delivery. They have reported a significant reduction in blood glucose levels in diabetic rats. Further investigations with liposomes containing insulin in rats and dogs showed reduction in blood glucose levels [43, 44]. Although

liposomes with their organized structures have some advantages as drug delivery systems, the extensive leakage of water-soluble drugs entrapped in liposomes during the GIT passage, the low drug entrapment, the heterogeneity of the vesicle size, the poor reproducibility and instability of formulations are some of the disadvantages of using liposome as peptide/protein drug delivery system.

4.2. Microtablets

Microtablets with diameters of 0.5-3mm containing permeation enhancers and/or enzyme inhibitors were designed and investigated for the peroral delivery of protein and peptide drugs. The permeation enhancers must be released rapidly from the dosage form and prior to the release of the peptide over a wide area across the epithelium. In order for the peptide to pass through the epithelium, the site of opening of the paracellular pathway must coincide with the site where the peptide is released from the dosage form [45]. Hence, multiple unit dosage forms (MUDFs) were designed to control the release of the drug [46, 47]. The minitablets can be then filled in gelatin capsules and enteric coated to be protected from the acidic condition of the stomach. Microtablets are easy to manufacture, can be used in defined sizes and strengths and show low variability within a batch [48]. In a study by Van der Merve et al. minitablets containing TMC permeation enhancer and Desmopressin (1-(3-mercaptopropionic acid)-8-D-arginine Vasopressin monoacetate (DDAVP) were designed. The release of both DDAVP and TMC from different

formulations of multi tablets was investigated [45]. The results suggested that suitable DDAVP release was obtained only from formulations that were too big to be fit inside the largest available gelatin capsule rendering them unsuitable for in vivo usage.

4.3. Microspheres

Spherical microspheres, prepared by complexation between oppositely charged macromolecules such as chitosan and negatively charged molecules such as tripolyphosphate (TPP) or alginates have received a lot of attention as drug delivery vehicles for protein drug delivery purposes [49, 50]. These microspheres can protect the drugs from the hostile environment of the GI tract, improve drug absorption via the paracellular route and control the drug release at a specific site [51-53]. Lueßen et al. and Kotzé et al. have applied drug containing chitosan microspheres on Caco-2 cell monolayers and showed a strong increase in the transport of buserelin, insulin and vasopressin derivative [20, 54]. A number of investigations were done by Shu et al. and Mutara et al. for controlled release drug delivery [55, 56]. They showed that variables such as drug concentration, type and concentration of chitosan, the pH of TPP solution, volume of the internal and external phases, gelation time as well as drying conditions can all determine the fate of drug release from chitosan beads. Avadi et al. have used enteric coated capsules containing Brilliant Blue chitosan beads as model hydrophilic drug for colon drug delivery [57]. The γ -scintigraphy images have demonstrated that Eudragit S coated capsules containing Brilliant Blue loaded-chitosan

beads are suitable for colon drug delivery. It can be thus concluded that the non toxic chitosan microspheres and beads can increase the bioavailability of the peptide and protein drugs by protecting them from degradation, when they are able to mucoadhesively attach to a specific site on the intestinal tract and to increase drug permeation by opening the tight junctions via the paracellular pathway.

4.4. Mucoadhesive Drug Delivery Systems

Mucoadhesion is the attachment of any type of polymer to the mucus layer via strong interaction between the functional groups of the polymer and those of the mucosa lining of the tissue. The mucoadhesive bonding is attained mostly by physical, chemical and more importantly through H-bonding. Hence, the presence of hydroxyl, carboxyl and H-bond forming functional groups strongly contributes to the strength of mucoadhesion [58, 59]. The formation process of mucoadhesive bonds include 1) wetting and swelling of the polymers, 2) interpenetration of the mucoadhesion polymer chains and entanglement of the polymer and mucin chains, 3) interfacial interaction of functional groups, 4) formation of weak chemical bonds [60].

The use of mucoadhesive drug delivery systems results in a controlled drug release and attachment at a specific site of the body. Increasing the residence time of the drug delivery systems at the site of absorption in the body may result in prolonging their action. As the GI tract is covered by a mucus layer, the mucoadhesive drug delivery system must be able to attach to a specific site in order to be

beneficial. Acrylic acid based polymers have been used extensively for mucoadhesive applications. Their strong bond strength in contact with tissues allows localization of the drug at the site of absorption, increasing residence time at the absorbing tissue and increasing drug bioavailability. Their responsive behaviour to different pH values allows the drug to be released at the desired site of the GI tract [58, 61, 62].

In order to increase mucus interpenetration, adhesion promoters such as polyethylene glycol (PEG) may be employed, which are not mucoadhesive but contribute to the adhesion process. Moreover, these tethered promoters may be grafted onto polymeric surfaces such that at the one end they are covalently attached to the polymer surface and the other end is free. These grafted chains are able to diffuse into the mucus layer and enhance the mucoadhesiveness of the system [63, 64].

Peppas et al. have done extensive studies on the design and the effect of network morphology of polyethylene glycol (PEG) tethered copolymers as novel mucoadhesive drug delivery systems [60]. They have suggested that the performance of the copolymer is due to the synergistic effect of both polymers: the backbone polymer providing the hydrogen bonds between the hydrogel and mucus layer as well as the adhesive promoter that contributes to the mucoadhesion by increasing the chain interpenetration.

Furthermore, oral insulin delivery was investigated using hydrogels of poly(methacrylic acid-g-ethylene-glycol) P(MAA-g-EG) by Peppas et al. [65]. A hypoglycemic effect combined with insulin absorption was observed in rats. These results could be due to the ability of the complexes to entrap and protect the drugs in their network structure. At the pH present in the small intestine, these complexes dissociate and the network swells and releases the peptide [66-68]. Moreover, these hydrogels were shown to decrease the TEER (transepithelial electrical resistance) across the Caco-2 cell models with no sign of cytotoxicity [68]. The in vivo studies were done in male Sprague-Dawley rats using a closed loop absorption method. 25IU/kg of human recombinant insulin was incorporated into the polymer and infused in an isolated ileal segment. The control sample contained the polymer without insulin. At predetermined intervals blood samples were withdrawn from the jugular vein, serum was collected and the insulin levels were determined by enzyme immunoassay. The insulin bioavailability of the formulation was measured using subcutaneous injections. The bioavailability of insulin was shown to increase to 6.2% compared to the control [65]. The mucoadhesion of their delivery system is mediated by weak, non covalent bonds such as hydrogen bonds, van der Waal's forces and ionic interactions resulting in mucoadhesion that may not be strong enough to localize the hydrogels at the specific site for a sufficiently long time. This suggests that although p(MAA-g-EG) hydrogels are promising carriers for oral insulin delivery the bioavailability of the protein is still too low for the system to be commercialized.

Thiolated polymers are another promising class of mucoadhesive with their capability to form strong covalent bonds through the disulfide binding of the polymers with the mucus gel layer of the mucosa. Thiomers are mucoadhesive polymers with thiol bearing side chains [69]. The disulfide bonds are formed between both the cysteine side of the polymers and glycoproteins of the mucus layer as well as the thiomers themselves leading to a strong adherence to the mucus gel layer [70]. At physiological pH values, the oxidation of the thiol groups results in gelling properties due to the formation of inter- and intramolecular disulfide bonds. Studies on thiolated poly (acrylic acid) in comparison to the unmodified polymer have shown that the mucoadhesive properties of the polymer measured using tensile studies and by using rotating cylinder method were 20 fold increased. Furthermore, the residence time of the polymer in the small intestine was prolonged by up to 3-fold through immobilization via the thiol groups [71, 72]. Thiolated chitosan was shown to improve mucoadhesion by more than 100- fold [73, 74]. Recently, dosage forms based on thiomers using peptide microparticles were generated via solvent evaporation emulsification method. Because of the formation of the disulfide bonds within the particles they did not disintegrate under physiological conditions for 48hrs and the mucoadhesive properties of the microparticles were improved 3- fold due to immobilization of the thiol groups compared to the control group consisting of the peptide alone [75]. Kast et al. have used thiolated polycarbophil for oral delivery of low molecular weight heparin (LMWH) in rats. In their investigation, they have shown that

the absorption of LMWH was significantly increased using the thiolated polymer in comparison to the unmodified polymer. They have reported a $19.9 \pm 9.3\%$ bioavailability in rats compared to the intravenous application [76].

Marschütz et al. have investigated the oral insulin delivery using thiolated polycarbophil in diabetic mice. The results indicated that a significant decrease was observed in blood glucose level when insulin was administered using the thiolated polymer in comparison to the unmodified polymer [77]. Similar results were obtained when pegylated insulin was incorporated in thiolated polycarbophil and orally administered to mice. The results indicated that the decrease in blood glucose level was maintained up to 24 hrs and the oral pharmacological efficacy of this mucoadhesive system compared to the s.c. injection was 7% [78]. Accordingly, while thiomers seem to be suitable for oral peptide delivery, their low stability in aqueous form as well as their biosafety issues are amongst the disadvantages of these systems as oral delivery vehicles. Furthermore the rapid mucus turnover may be responsible for a reduced residence time at the site of absorption despite the formation of covalent –S-S- bonds.

4.5. Nanoparticles

Today, a vast number of investigations have been focused on nanoparticles and their role as drug delivery vehicles. Nanoparticles were first introduced in the mid seventies by Birrenbach and Speiser [79]. The preparation of nanoparticles was simple, the particles

formed were relatively stable and easily freeze-dried; hence, biodegradable polymers were found useful and further developed for drug delivery. Polymeric nanoparticles have the advantages of protecting the protein and peptide drugs from chemical and enzymatic degradation in the GIT, increasing their stability and absorption across the intestinal epithelium as well as controlling the drug release [80-83]. A number of techniques such as polymerization, nanoprecipitation, inverse microemulsion can be used to prepare polymeric nanoparticles; however, most of these methods involve the use of organic solvents, heat and vigorous agitation which may be harmful to the peptide and protein drugs [84, 85]. More recently the ionic gelation technique is used as the most favorable method for producing peptide and protein nanoparticles. The nanoparticles prepared by this method have a suitable size and surface charge, spherical morphology as well as a low polydispersity index indicative of a homogenous size distribution. The lack of using organic solvents, sonication or harsh conditions during preparation reduces the damage to the peptide and proteins and makes this method a favorable one for the preparation of protein loaded nanoparticles [86, 87]. Chitosan nanoparticles with excellent biodegradable and biocompatible characteristics have been used extensively as drug delivery vehicles [88]. However, due to poor solubility of chitosan at pH above 6.0, its quaternized derivatives such as trimethyl chitosan, triethyl chitosan, diethylmethyl chitosan and dimethylethyl chitosan, which are soluble at the intestinal pH, have been used to prepare nanoparticles loaded with insulin [24, 28, 29, 32]. Chitosan nanoparticles loaded with

insulin were prepared by mixing the positively charged polymer with the negatively charged insulin and nanoparticle formation occurred via electrostatic interaction. Studies have shown that insulin in nanoparticulate form was more likely to be delivered across the GI tract than in its free soluble form [33, 34, 89]. The particle size and surface charge are critical factors in nanoparticle absorption. Size is a determining factor for both uptake and biological fate of the particulate systems. Moreover, a size dependent phenomenon exists in the gastrointestinal absorption of the particles. Studies have shown that particles with a size of 100nm were taken up 6- times more than particles with 100 μ m by the absorptive cells [90-92]. Hydrophobic particles are absorbed more readily than hydrophilic ones. Thus increasing the hydrophobicity of particles may enhance their permeability through mucus but decreases the translocation through and across the absorptive cells [93]. In the GIT, the particles interact with the mucus before coming into contact with the absorptive cells. Positively charged particles are more prone to uptake as they can associate with the negatively charged functional groups in the mucus [94]. Accordingly, biodegradable, hydrophobic nanoparticles with sizes between 100-200 nm and positive surface charge may be good candidates for uptake by the epithelial cells. The disadvantage of the nanoparticles is their bigger size in comparison to the soluble protein molecules and the fact that a lot of particles may get trapped inside the cells within the cellular membrane such as the Golgi apparatus or the endoplasmic reticulum being unable to pass across the cells intactly. Accordingly, while the use of nanoparticles is highly

recommended for gene therapy their use for peptide drug delivery and absorption is debatable also with respect to the minimal drug load they can carry with them in comparison to bigger particles.

Even though all of the above mentioned delivery systems gave reasonable results when studied in-vitro and ex-vivo, they were only tested in-vivo using small animals such as mice and rats with intestinal diameters much less than that of humans. For a delivery system to be useful for commercialization, it must have reasonable bioavailabilities in bigger animals such as pigs or dogs with intestinal diameters closer to that of the humans. The small intestinal diameter of smaller animals allows the delivery system to easier come in direct contact with the intestinal wall where in bigger animals and humans reaching the absorbing surface still with full mucoadhesiveness is a big challenge. Moreover, the amount of mucus produced in bigger animals is higher than in the GIT of smaller animals. It was shown for nanoparticles developed by Peppas et al. that they lose their mucoadhesive properties in contact with soluble mucins present in the GIT of larger animals before reaching the absorbing surface and are no longer able to open the tight junctions and allow for the paracellular transport of the drugs [95]. Hence, to overcome the above obstacles, delivery systems using superporous hydrogels and gas empowered delivery systems were designed and examined in larger animals such as pigs, rabbits and humans.

4.6. Super porous hydrogels (SPH) based delivery systems

SPH and SPH composites were synthesized by Park et al and adopted by Dorkoosh et al. for intestinal drug delivery [96, 97]. These polymers are able to swell very rapidly up to 200-fold of the original volume upon sucking up the gut fluids and are then able to attach mechanically to the gut wall and bring the dosage form in close proximity of the site of absorption. Consequently, these polymers not only increase the residence time of the dosage form at a specific site in the gut; but also by absorbing the gut fluids they decrease the enzymatic activity. The SPH and SPHC delivery systems were prepared either by inserting the core inside the conveyor system (i.e. core inside, c.i.) or attaching to the surface (i.e. core outside, c.o.). The core consists of the peptide/protein drug, such as octreotide or insulin. These formulations were then placed in gelatin capsules and enterically coated with Eudragit S100. Hence, the delivery system could safely pass through the acidic environment of the stomach and thereafter dissolved in the *responsive* pH of the small intestine allowing for the polymer to swell and mechanically attached to the gut wall. The mechanical pressure on the intestinal cells opens the tight junctions allowing for the paracellular transport of the peptides across the intestinal membrane [98]. In vivo studies in pigs of a weight of about 25 kg have shown that using this delivery system, the bioavailability of octreotide was increased up to 16% in comparison with the peroral administration of octreotide without polymer [99]. However, the synthesis and fabrication of the delivery systems is based on SPHs or SPHCs technology, which is difficult and currently

not commercially feasible on mass production scale. Moreover, their big size (i.e. capsule size 000), is not easily swallowed and may cause variable residence times in the stomach of the patient.

4.7. Gas Empowered Drug Delivery system (GEDD)

The latest approach for a novel drug delivery device is using Gas Empowered Drug Delivery (GEDD) system to deliver hydrophilic drugs such as peptide and proteins to the intestinal tract and enhance their absorption across the intestinal wall by pushing the active compounds together with the mucoadhesive polymer polyethylene oxide (PEO) and TMC as penetration enhancer to the absorbing membrane of the gut tissue using CO₂ gas. The drug will be then adhered to the mucus layer together with the PEO to prolong the residence time at the mucosal surface and TMC as permeation enhancer will simultaneously trigger the opening of the tight junctions for enhancing drug permeation by the paracellular pathway. The mucoadhesive remnants of the delivery system slide down the mucus membrane and will be shed off at the latest in the large intestine where the dosage form is degraded and expelled. In the GEDD system CO₂ acts mainly as the driving force to push the delivery system to the absorbing membrane. Additionally, it can form a layer around the delivery system protecting it from enzymatic and proteolytic degradation. Furthermore, the CO₂ bubbles may act as a permeation enhancer that mechanically opens the tight junctions [100]. In order to protect the drug from the acidic pH of the stomach, the GEDD system was enterically coated with cellulose acetate

phthalate (CAP). An increase in insulin permeation using this delivery system may be due to the synergistic effect of both the CO₂ and TMC in the form of mechanical and chemical enhancement, respectively. The advantage of this delivery system over the superporous hydrogel is its ease of production in large scale.

5. Conclusion

Numerous investigations were done to develop suitable peroral delivery systems; nevertheless, to date no single delivery system has been designed to cover all the criteria required for peptide drug delivery. Developing a suitable peroral drug delivery system requires a multi task planning. The delivery system must first and foremost overcome the harsh acidic environment of the stomach. Secondly, it must protect the peptide and protein drugs from the enzymatic degradation in the intestine. Once in the intestinal tract, it must be able to adhere to a specific site long enough to be effective. As the delivery system attaches to the intestinal epithelium it must act as an enhancer to open the tight junctions and enhance the drug permeation across the intestinal wall via the paracellular pathway. Multifunctional polymers such as polyacrylates and chitosans with their multiple derivatives show promising properties as penetration enhancers for the paracellular absorption route of hydrophilic macromolecules by reversibly open the tight junctions; however, their high viscosity and slow dissolution rates make the development of suitable delivery systems using these polymers very difficult. The bioavailability of peptide and proteins crossing the intestinal wall

must be enough to have a therapeutic effect. Moreover, the delivery system must be easy to prepare and to scale up in order to be appropriate for commercialization. In short, the concept of a suitable peroral peptide delivery system is still far from reality and requires a lot of investigations to become reality.

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Chapter 2

Synthesis, characterization and antibacterial effects of trimethylated and triethylated 6-NH₂-6-Deoxy Chitosan

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Abstract

Chitosan, a biodegradable and biocompatible polymer, has attracted great attention in the pharmaceutical and biomedical fields specially due to its properties to reversibly open the tight junctions of the epithelial tissues to allow for paracellular transport of hydrophilic macromolecules. However, chitosan exhibits low solubility at pH values above 6 that may prevent its enhancing effects at the site of intestinal absorption. Hence, a number of alkylated chitosan salts have been synthesized and characterized. These derivatives have been shown to have good solubility at neutral pH and act as effective permeation enhancers. In this study two new derivatives of chitosan, C2-C6 trimethyl 6-amino-6-deoxy chitosan and C2-C6 triethyl 6-amino-6-deoxy chitosan were synthesized and characterized using ¹H-NMR and FTIR spectra. Moreover, the zeta potential and the antibacterial properties of these polymers were compared to chitosan, trimethyl chitosan (TMC) and triethyl chitosan (TEC). Our results suggest that both C2-C6 trimethyl and triethyl 6-amino-6-deoxy chitosan, as highly water soluble polymers, have higher positive surface charge than chitosan, TMC and TEC. Moreover, the new synthesized polymers show higher antibacterial activity against gram-positive *Staphylococcus aureus* bacteria. Consequently, these polymers with substitution degrees of 50-60% may be good candidates for the enhancement of peptides in mucosal drug delivery.

1. Introduction

Deacetylation of chitin, the second most abundant biopolymer isolated from insects, crustaceans, and fungi, leads to poly[b-(1-4)-D-glucosamine] and is called chitosan [1]. Chitosan has been used extensively in biomedical fields in the form of sutures, wound covering and as artificial skin. Chitosan, a natural polysaccharide (Figure 1(a)) having similar structure to glucosamine, is a non toxic, biocompatible, and biodegradable polymer. Both the amine (NH_2) and or hydroxyl (OH) groups of chitosan can interact with transition metal ions and organic compounds [2]. In the acidic environment below pH 6.5, chitosan acts as an unbranched cationic biopolymer and carries a positive charge. Many uses of chitosan are based on its positive charge which attracts negatively charged materials. Most living tissues (e.g., skin, bone, and hair), polysaccharides (e.g., alginate), polyanions, bacteria, and fungi, as well as enzymes and microbial cells are negatively charged. Hence, chitosan can be used to remove toxic and contaminating bioburden materials such as proteins and heavy metals for safety reasons [3]. It has been purposed that chitosan salts influence the function of the tight junctions in the intestine in such a way that paracellular transport of large hydrophilic compounds such as proteins and peptides becomes possible [4]. The increase in the transport of these compounds could be attributed to an interaction of the positively charged C-2 amine groups of chitosan with the negatively charged sites on all membranes and tight junctions [4]. Due to their unique properties such as their permeation enhancing effect, enzyme inhibitory capabilities, and antimicrobial

effects, chitosan and its derivatives are able to increase absorption especially of hydrophilic drugs, reduce enzymatic barriers, and act as antimicrobial agents [5]. However, chitosan has poor solubility at pH values above 6.5 that limits its enhancing effect at the site of action in the intestinal tissue. Moreover, at this pH, chitosan loses its positive charge density, aggregates, and precipitates from the solution [6]. To overcome the above obstacle, many chemical modifications were done on chitosan [7, 8]. Trimethylation of chitosan is a useful strategy to overcome the low solubility of chitosan in the intestinal milieu. Trimethyl chitosan (TMC) was synthesized by Sieval et al. and reported to be water soluble, non cytotoxic, and act as permeation enhancer. The TMC with the degree of quaternization of 60% was shown to be a potent enhancer of the paracellular transport of hydrophilic markers and peptide drugs in vitro in Caco-2 cell monolayer and also an effective intestinal absorption enhancer of peptides in vivo after internal administration in rats [9, 10]. Avadi et al. [11] synthesized and characterized triethyl chitosan (TEC) with different quaternization degrees and studied their pharmacological effects ex vivo. It was shown that unlike chitosan but like TMC, the solubility of TEC is not pH dependent and is soluble in water at room temperature. Furthermore, they showed that TEC is able to enhance the absorption of hydrophilic compounds more than chitosan.

A second derivative of chitosan, diethylmethyl chitosan (DEMC), was synthesized by Avadi et al. using a modified two-step procedure. The highly quaternized DEMC showed rapid and complete solubility in water at room temperature as well as higher antibacterial effect

compared to chitosan. Furthermore, ex vivo studies on DEMC showed enhanced absorption of hydrophilic model drugs through tight junctions [12]. Moreover, the enhancing effect of DEMC on insulin absorption in the ascending colon section in rats was investigated in vivo [13]. These studies have clearly demonstrated the DEMC enhancing effect in opening the tight junction of colonic epithelia in ex vivo and in vivo model studies [14]. Chitosan derivatives, with absorption enhancing properties, have the following advantages: (a) the intestinal absorption of these polymers is expected to be negligible due to their hydrophilic characteristics and (b) their paracellular permeation enhancing activity along the enterocytes is reversible [10]. Moreover, interesting modifications, for example, substitution of the amino groups in the C-6 position instead of the hydroxyl group, may result in two sites for quaternization and perhaps higher enhancing properties as well as more pronounced antibacterial effects. In particular, N-phthaloyl chitosan is regarded as one of the most useful precursors for such characterization due to its high solubility in polar aprotic solvents [15]. The aim this study was to synthesis 6-amino-6- deoxy chitosan and trimethylation and triethylation of the amino groups in C-2 and C-6 position for antimicrobial and drug delivery purposes.

2. Materials and Methods

Chitosan (98% deacetylated, low viscosity of 1% w/v solution) was purchased from Primex, Iceland. Phthalic anhydride, dimethyl formamide, tosyl chloride, dimethyl sulfoxide, N-methyl pyrrolidone, hydrazine monohydrate, methyl iodide, ethyl iodide, and sodium iodide were purchased from Sigma (Vienna, Austria). The *Staphylococcus aureus* ATCC 29737 was purchased from Persian Type Culture Collection (PTCC). The other materials used were of pharmaceutical and analytical grade and used as received.

2.1.Preparation of 6-Amino-6-Deoxy Chitosan

Initially, 8.3 g (5.6mM) phthalic anhydride was added to 60mL of dimethyl formamide (containing 5% w/v) and then 3.0 g chitosan was added to the solution and magnetically stirred for 8 h at 120°C. The phthaloyl chitosan precipitate was obtained by adding cold water, the precipitate was then washed with methanol for 1 h, filtered, and dried at 40°C. In the second step of the reaction, 4.0 g of phthaloyl chitosan was dissolved in 80mL pyridine and 26.0 g tosyl chloride was added to the solution. To obtain a viscous solution, the mixture was stirred for 17 h. In order to precipitate the obtained product, 1000mL of cold water were added and the precipitate was washed with 200mL ethanol and 200mL of diethyl ether, respectively, and dried at room temperature. In the third step, 6.0 g of product was added to 180mL DMSO and 30mL solution of ammonia (25% w/v) and stirred for 24 h at 80°C. After the mixture was cooled down, it was precipitated with acetone, washed with diethyl ether, filtered, and dried at room

temperature. In the last step, 350.0 mg of the previous product (stage 3) was added to 7.0mL of N-methyl pyrrolidone (NMP) and stirred at 100°C for half an hour magnetically under nitrogen condition. Consequently, 10.0mL hydrazine monohydrate was added to the mixture and stirred for 12 h at 100°C. Finally the mixture was cooled down and ethyl acetate was added stepwise to obtain precipitation. The obtained precipitate was washed with diethyl ether after filtration and dried at room temperature.

2.2.Trimethylation and Characterization of C2–C6 of the 6-Amino-6-Deoxy Chitosan

The preparation was one step synthesis according to Avadi et al. [11] with minor modifications. Briefly, the obtained 6-amino-6-deoxy chitosan obtained (400 mg) was dispersed in 15mL NMP and stirred magnetically for 4h at room temperature. Aqueous sodium hydroxide solution (2.5mL), sodium iodide (900 mg), and 7.0mL of methyl iodide were added. The mixture was heated to 60–65°C, for 6 h under magnetic stirring. The quaternized polymer was precipitated with acetone (300mL) and dried at room temperature. For exchanging I⁻ with Cl⁻, the polymer was dissolved in 10.0mL of 5% sodium chloride aqueous solution. The polymer was precipitated with acetone (100mL), filtered, and dried to obtain a water-soluble powder. The solubility of the polymer obtained was comparable to TMC and reported to be 1.0% w/v. The ¹H-NMR spectrum was obtained in D₂O using a 500MHz spectrometer (Bruker AC 500) and the degree of quaternization was calculated. The FTIR spectrophotometer (Perkin–

Elmer) was used to record the polymer spectrum. The samples were prepared as KBr pellets with 0.25mm thickness (1mg in 100 mg of KBr).

2.3.Triethylation and Characterization of C2–C6 of the 6-Amino-6-Deoxy Chitosan

The method of preparation was one step synthesis according to Avadi et al. [11]. Briefly, 200mg of the obtained 6-amino-6-deoxy chitosan were dispersed in 8.0mL of NMP and magnetically stirred at room temperature. After 4h, 1.2mL aqueous sodium hydroxide solution, 480 mg sodium iodide, and 3.0mL ethyl iodide were added. The mixture was heated at 60-65⁰C for 6h under gentle magnetic stirring. The obtained product was precipitated using acetone and separated by centrifugation. For exchanging I⁻ with Cl⁻, the polymer was dissolved in 5.0mL of 5% sodium chloride and stirred magnetically at room temperature for 30 min. The polymer was precipitated with acetone (100mL), filtered, and dried to obtain a white, water-soluble powder. The solubility of the polymer obtained was comparable to TEC and reported to be 1.0% w/v. The ¹H-NMR spectrum was obtained in D₂O using a 500MHz spectrometer (Bruker AC 500) and the degree of quaternization was calculated.

2.4.Measurement of the Zeta Potential and the Antibacterial Activity of the Trimethylated and Triethylated C2–C6 of the 6-Amino-6-Deoxy Chitosan

The zeta potential of the polymers was compared with chitosan using a zeta sizer (3000 HAS, Malvern Instruments Ltd, UK). The antibacterial effect study was done by comparing the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the new polymers to chitosan, TMC and TEC as described previously by Avadi et al. In order to obtain polymer concentrations of 1000 to 62.5 $\mu\text{g/mL}$, chitosan was dissolved in 0.25% acetic acid and the derivatives were dissolved in water. The organism used in this study was *S. aureus* ATCC 29737. The experiments were done in triplicate.

3. Results and Discussion

3.1.Characterization of C2–C6 Trimethylated and Triethylated 6-Amino-6-Deoxy Chitosan

Figure 1 represents the schematic picture of chitosan (A) 6-amino-6-deoxy chitosan (B) C2–C6 trimethylated 6-amino-6-deoxy chitosan (C), and C2–C6 triethylated 6-amino-6-deoxy chitosan (D). The FTIR spectra of chitosan (a) in Figure 2) has peaks at 897 cm^{-1} and several bands around $1050\text{--}1150\text{ cm}^{-1}$ indicating the stretching vibration of C–O related to primary and secondary hydroxyl group. Moreover, a strong peak at 1588 cm^{-1} is observed that indicates the bending vibration of NH_2 group. This peak is also present in (b) of Figure 2, 6-amino-6-deoxy chitosan, the precursor for the C2–C6 alkylated 6-

amino-6-deoxy chitosan. However, this peak is not observed in C2–C6 N-alkylated 6-amino-6-deoxy chitosan (c) in Figure 2) due to conversion of the NH_2 to 3 N-alkyl groups. The peaks presented at 1649 and 1320cm^{-1} are characteristic of N-acetylated chitin and reported to be the amide I and III bands, respectively.

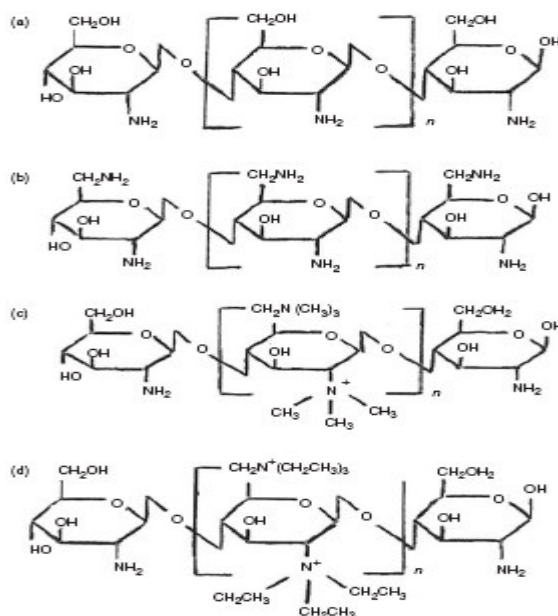


Figure 1. Chemical structures of chitosan (a), 6-NH₂-6-deoxy chitosan (b), C2–C6 methylated chitosan (c), and C2–C6 ethylated chitosan (d).

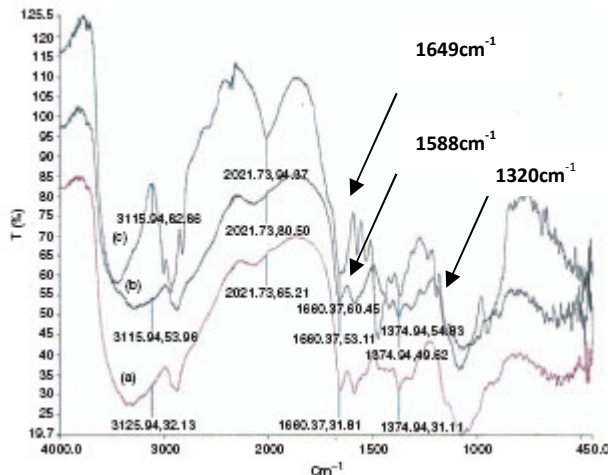


Figure 2. The FTIR spectra of chitosan (a), 6-amino-6 deoxy chitosan (b), and C2-C6 *N*-alkylated 6-amino-6-deoxy chitosan (c).

Figures 3 and 4 represent the ^1H -NMR spectra of low molecular weight chitosan and C2–C6 trimethylated 6-amino-6-deoxy chitosan in a 2% $\text{CF}_3\text{COOH}/\text{D}_2\text{O}$ solution and D_2O , respectively. According to Figure 3, the peak at 1.9 ppm was attributed to the methyl protons from the acetylated chitosan, the peak at 3.2 ppm was attributed to the proton ring connected to the C–OH group and finally the peak at 4.9 ppm was attributed to the proton on the anomeric carbon. According to Figure 4, the intense band at 4.6 ppm was due to D_2O (solvent). The peaks at 4.4–3.6 ppm were attributed to the glucosamine units and the double peak at 3.6 and 3.2 ppm was attributed to the hydrogen groups of the methyl units substituted at C2–C6; the sharp peak at 2.6 ppm was attributed to dimethylated chitosan and the integral of these signals were used to calculate the degree of quaternization. The signal

at 1.97 ppm was due to the methyl protons from the acetylated chitosan. The dual peak at 3.6–3.2 did not shift or change when the solution was acidified with CF_3COOD . This proved that the indicated band belonged to TMC and not to the dimethylated chitosan. The degree of quaternization was calculated to be 65% using the integral of chitosan backbone protons as well as CH_3 of the methyl group. Figure 5 represents the ^1H -NMR spectrum of C2–C6 triethylated 6-amino-6-deoxy chitosan. According to this figure, the intense band at 4.7 ppm was attributed to the D_2O used as the solvent. The peaks at 3.6–4.3 ppm were attributed to the glucosamine units. The double signal at 1.3 was attributed to the CH_3 groups of the ethyl substituents while the CH_2 groups of the quaternized site were superimposed by the 2-H and the 6-H protons of the polysaccharide backbone. The degree of quaternization was calculated to be 51% using the integral of chitosan backbone protons as well as CH_3 of the ethyl group.

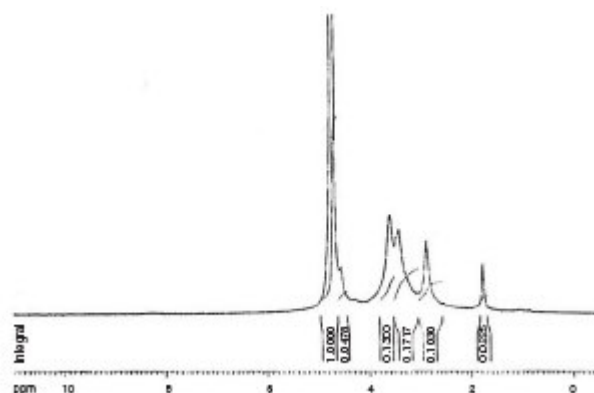


Figure 3. The ^1H -NMR spectrum of low molecular weight chitosan in 2% $\text{CF}_3\text{COOH}/\text{D}_2\text{O}$.

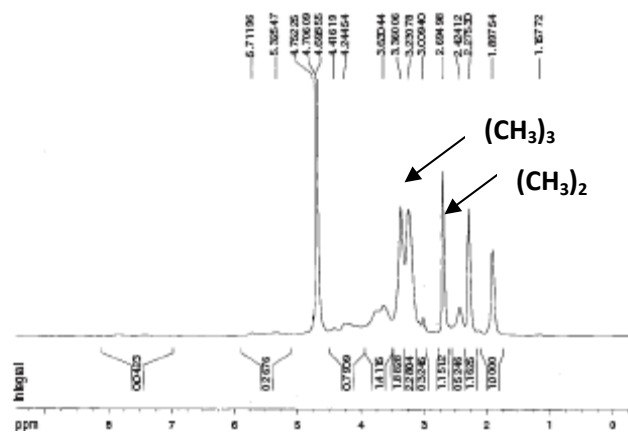


Figure 4. The ^1H -NMR spectrum of C2-C6 trimethylated 6-amino-6-deoxy chitosan in D_2O .

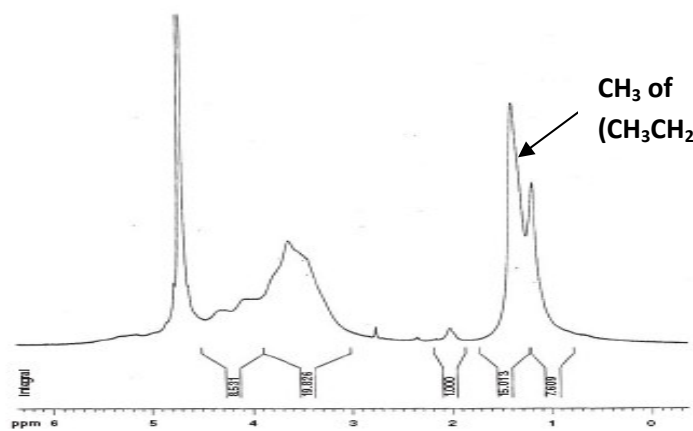


Figure 5. The ^1H -NMR spectrum of C2-C6 triethylated 6-amino-6-deoxy chitosan in D_2O .

3.2.Zeta Potential Analysis

In Table 1, the zeta potential of the obtained C2–C6 trimethylated 6-amino-6-deoxy chitosan and C2–C6 triethylated 6-amino-6-deoxy chitosan was compared to chitosan, TMC and TEC. Accordingly, the zeta potential of chitosan was shown to have the least positivity and C2–C6 trimethylated 6-amino-6-deoxy chitosan showed the highest positive zeta potential. As expected the zeta potential of TMC is higher than chitosan but lower than the C2–C6 trimethylated 6-amino-6-deoxy chitosan. This may be explained as chitosan by nature contains a less positive surface charge while TMC contains a higher positive surface charge. However, having two methylated groups substituted on the NH₂ positions of the C2 and C6 result in a higher zeta potential than either the chitosan or TMC. The zeta potential of TEC was less than its C2–C6 triethylated form and C2–C6 triethylated 6-amino-6-deoxy chitosan had a zeta potential lower than C2–C6 trimethylated 6-amino-6-deoxy chitosan but higher than TMC. The zeta potential positivity of the polymers had the following order: C2–C6 trimethylated 6-amino-6-deoxy chitosan> C2–C6 triethylated 6-amino-6-deoxy chitosan>TMC>TEC>chitosan. This may be useful for future in-vitro, ex vivo, and in vivo studies where the polymer's surface charge plays an important role in its penetration and permeation enhancing effects of the large, hydrophilic drugs, such as insulin. The higher positivity of the surface charge in a polymer may result in a higher ability to interact with the tight junctions and cellular membrane components to increase the

paracellular permeation of hydrophilic compounds. Although the size and the configuration of the polymers are also important factors in their interaction with the cellular components, methyl groups are usually very compact and show less steric hindrance in comparison with the ethyl substituted groups.

Table 1. The zeta potential of chitosan, TMC, TEC, and C2–C6 alkylated 6-amino-6-deoxy chitosan.

Polymer	Zeta potential (mV)
Chitosan	+24.2 ± 2.1
TMC	+43.2 ± 1.5
C2–C6 trimethylated 6-amino-6-deoxy chitosan	+57.7 ± 3.8
TEC	+39.1 ± 1.2
C2–C6 triethylated 6-amino-6-deoxy chitosan	+52.2 ± 1.4

3.3. Antibacterial Studies

The antibacterial effects of C2–C6 trimethylated and triethylated 6-amino-6-deoxy chitosan against Gram-positive bacteria, *S. aureus*, were compared to chitosan, TMC and TEC. According to Table 2, the C2–C6 trimethylated 6-amino-6-deoxy chitosan has the highest antibacterial effect represented by the lowest minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 62.5 and 25 µg/mL, respectively. C2–C6 triethylated 6-amino-6-deoxy chitosan and TMC showed the second highest inhibitory effect after the C2–C6 trimethylated form. The inhibitory effect of TEC was

lower than TMC and both C2–C6 trimethylated and triethylated 6-amino-6-deoxy chitosan and finally chitosan showed the least inhibition only at 1000 µg/mL. These results correlate very nicely with the results obtained from the zeta potentials. Accordingly, chitosan with the least positive surface charge showed the least bacterial inhibition; conversely, the C2–C6 trimethylated 6-amino-6-deoxy chitosan, with the highest zeta potential, showed the highest antibacterial activity. This new obtained derivative, with its high zeta potential, probably has the highest ability to bind to the negative peptidoglycans on the bacterial cell wall and may induce severe morphological alterations in Gram-positive bacteria. TEC showed less inhibition than TMC and this can be explained by the fact that the ethyl groups are larger in size than the methyl substituted groups and hence the binding of TEC to the bacterial cell wall is sterically hindered. The fact that C2–C6 triethylated 6-amino-6-deoxy chitosan had higher inhibition than TEC could be due to the presence of two positive sites of the polymer being able to bind to the bacterial cell and increase their inhibition. The results from MIC indicate that Gram-positive bacteria exhibited discontinuity and rupture of the cell wall, which may be explained by the enhanced autolysis activity in the presence of the polymer. The more positive the surface charge, the better the ability of the polymer to interact with the bacterial cell wall and to result in autolysis. Since the MIC and MBC are close in value, one can state that chitosan and its derivatives are bactericidal and not bacteriostatic under these experimental conditions.

Table 2. The antibacterial effect of chitosan, TMC, TEC, and C2–C6 alkylated 6-amino-6-deoxy chitosan.

Polymer	MIC (μg/mL)	MBC(μg/mL)
Chitosan	1000	1000
TMC	125	250
C2–C6 trimethylated 6-amino-6-deoxy chitosan	62.5	250
TEC	250	500
C2–C6 triethylated 6-amino-6-deoxy chitosan	125	250

4. Conclusion

In this study, C2–C6 trimethylated 6-amino-6-deoxy chitosan and C2–C6 triethylated 6-amino-6-deoxy chitosan were synthesized for the first time to our knowledge and further characterized using ¹H-NMR and FTIR spectra. The obtained polymers had a degree of quaternization of approximately 60 and 50%, respectively and were rapidly and completely soluble in water at room temperature (1.0% w/v). Moreover, the zeta potential and the antibacterial activity of these polymers were compared to chitosan, TMC and TEC. The new polymers had higher zeta potential than either chitosan, TMC or TEC. Moreover, their antibacterial activities revealed higher inhibition against Gram-positive bacteria, *S. aureus*. In conclusion, these polymers may be good candidates for drug permeation and penetration enhancers in the intestinal mucosal layer. However, in vitro, ex vivo, and in vivo studies are required to further characterize these polymers in detail.

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Chapter 3

Synthesis of N, N-dimethyl N-ethyl Chitosan as a Carrier for Oral Delivery of Peptide Drugs

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ABSTRACT

N, N-dimethyl N-ethyl chitosan (DMEC), a quaternized derivative of chitosan was synthesized based on a modified two-step method via a 2^2 factorial design to optimize the preparative conditions. The degree of deacetylation of the starting chitosan was determined by FTIR and NMR methods and was 95%. In the first step of the synthesis, mono-ethyl chitosan was prepared by introducing an ethyl group onto the amine group of chitosan via a Schiff base and in the next step methyl iodide was added to produce DMEC which was water soluble in a pH range of 4–8. The DMEC polymers with different degrees of quaternization were obtained and fully characterized using FTIR and ^1H -NMR spectroscopic methods. Based on ^1H -NMR calculations, the degree of quaternization was 52% by optimizing the two-step process.

1. Introduction

Recent studies have shown that mucoadhesive polymers, like chitosan, are useful carriers for the oral delivery of peptide drugs [1]. These agents are able to open the intercellular tight junctions between the enterocytes and to increase the paracellular permeation of hydrophilic macromolecules such as peptides and proteins. Chitosan is a mucoadhesive polymer (Figure 1(a)) obtained by deacetylation of chitin, an abundant polysaccharide isolated from insects, crustaceans, and fungi [2]. Chitosan has favourable characteristics, such as biocompatibility, biodegradability, low toxicity, and mucoadhesive properties. Because of its superior properties, chitosan is widely used as a pharmaceutical excipient [3]. The physical properties of chitosan

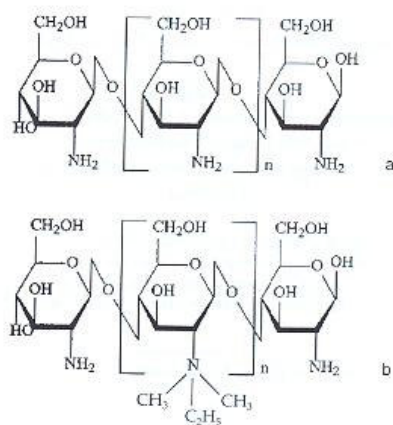


Figure 1. Structure of (a) chitosan and (b) DMEC.

depend upon the degree of N-acetylation and the distribution of the N-acetyl groups [4]. Using new biotechnology, peptide analogues that are resistant to enzymatic degradation were prepared. Despite the improved stability of these compounds against enzyme degradation, the hydrophilicity and molecular size of these analogs remain a serious problem with respect to absorption through the intestinal epithelium. The main barrier to the passage of macromolecules, such as proteins and peptides, is the lipophilic nature of the intestinal membrane. Calcium chelators and surfactants with extracellular Ca^{+2} depletion, disruption of actin filaments, and exfoliation of the intestinal epithelium are known to increase the intestinal permeability. However, the toxicity and nonspecific mechanisms of these agents limit their application as safe absorption enhancers for hydrophilic drugs [5].

Several studies have shown that chitosan is able to enhance the permeation of macromolecules across the intestinal barrier by reversibly opening the tight junctions and allowing a better absorption of orally administered drugs [6, 7, 8, 10]. However, its limited solubility, which is restricted to dilute acidic aqueous media, allows its application in the nose but not in the intestinal tract where the pH values are higher than 6.5. Chitosan, being a cationic polysaccharide, contains free amino groups and is insoluble in water at neutral pH. In acidic pH, the amino groups undergo protonation, thus making it ionic and soluble in an aqueous media that has a pH value lower than 6.5 [9]. Since chitosan is only soluble in acidic environments, it is

incapable of enhancing the absorption in the small intestine, the main absorption area in the gastrointestinal tract [11]. The preparation of chitosan derivatives possessing permanent positive charges was envisaged to improve its permeation enhancer activity.

The introduction of permanent positive charges into the chitosan chains can be accomplished by preparing quaternary ammonium chitosan salts by the quaternization of the amino groups of the parent polymers. This may be accomplished by reductive alkylation or direct extensive N-alkylation of chitosan [10]. Quaternized derivatives of chitosan have been evaluated to overcome the limited solubility of chitosan and its reduced effectiveness as absorption enhancer at neutral pH or weakly alkaline values such as those found in the intestinal tract [12]. Several studies have shown that the degree of quaternization of these polymers influences their drug absorption enhancing properties [13]. Hamman et al. showed that trimethyl chitosan as a quaternized derivative of chitosan with a degree of quaternization above 22% was able to enhance absorption in a neutral condition (pH 7.4). However, the maximum absorption enhancing activity was reached with a degree of quaternization of 48%. The degree of quaternization of 50–60% for quaternized derivatives of chitosan has been shown to be optimum for the permeation enhancing effect [14]. Consequently, the aim of the present work is to introduce DMEC a new quaternized derivative of chitosan (Figure 1(b)) as a carrier for oral delivery of peptide drugs. A factorial design approach was followed to achieve the optimum experimental conditions for the synthesis of DMEC.

2. Materials and Methods

Chitosan (98.1% deacetylated, viscosity of 1% w/v solution, 22mPas) was obtained from Primex, Iceland. Methyl iodide, sodium borohydride and acetaldehyde were obtained from Sigma (Germany). Sodium hydroxide, N- methylpyrrolidone (NMP), sodium iodide and other materials were purchased from Merck (Germany) as pharmaceutical and analytical grades and were used as received.

2.1.Determination of the Degree of Deacetylation of Chitosan

Infrared and NMR spectroscopic techniques were used for the determining the degree of deacetylation (DD) according to previously reported methods [15, 16]. It has been shown by IR spectroscopy using the fully N-acetylate chitosan (chitin) that the ratio between hydroxyl absorbance relative to carbonyl absorbance was 1.33. On assumption of that value, this ratio is zero for fully deacetylated chitosan, and that there is a direct linear relationship between the N-acetyl group content and the absorbance of the amide I band, the percentage of the acetylated amine group was determined by:

$$\text{N-acetyl \%} = (A_{1655}/A_{3450}) \times 100/1.33 \quad (1)$$

Using NMR spectroscopy, the degree of acetylation was determined by comparing the area under the curve of the acetyl group at 1.9ppm with the sum of the area under the curve of the other protons [16]. The value obtained by FTIR (i.e. DD 0.95) was confirmed by NMR

(DD 0.94). Therefore, the DD value of 0.95 was assumed to be the correct value and not the one as given by the supplier.

2.2.Molecular Weight Determination

One method of determining the molecular weight of a polymer is the Mark–Houwink equation where η is the intrinsic viscosity, α and k are constants of the equation and M_w is the molecular weight of chitosan.

$$[\eta]=k \times M_w^{\alpha} \quad (2)$$

In order to determine the average M_w of chitosan, five different concentrations of chitosan solution in acetic acid–sodium acetate buffer were prepared. The relative viscosity was obtained with a capillary viscometer at $30 \pm 0.05^\circ\text{C}$. The intrinsic viscosity ($\eta=1.050\text{cm}^3/\text{g}$) was determined and the M_w (10.3×10^5) of the chitosan was calculated based on the Mark–Houwink equation [17] where, $k=1.64 \times 10^{-30} \text{DD}^{14}$ and $\alpha= -1.02 \times 10^{-2} \text{DD} + 1.82$, respectively.

2.3.Preparation of N, N-dimethyl N-ethyl Chitosan

DMEC was synthesized by a two-step method reported by Kim et al. [18]. In summary, 700 mg of chitosan were dissolved in 1% w/v acetic acid (original pH 3.7 ± 0.5). Then specific amounts of acetaldehyde were added to the chitosan solution and after 1.5h of stirring, the pH of solution was adjusted to 4.5 by adding 1M NaOH solution. Then 2.0mL of a 10% sodium borohydride solution were added and magnetically stirred for 2h. The first step was to obtain mono-ethyl chitosan precipitate by adding 1 M NaOH solution and adjusting the pH of the solution to 10.

The precipitate was washed with distilled water and then Soxhlet extracted with ethyl alcohol and ether (1:1 v/v) for 3 days to give ethyl chitosan in high yield (degree of substitution 90%). In the second step, 200mg of ethyl chitosan were dispersed in 10mL of NMP for 5h. Then NaOH solution (1.0 or 2.0mL of a 30% w/v solution) methyl iodide (3.0 or 5.0mL) and sodium iodide (600mg) were added to the dispersion. The reaction was carried out with stirring for 5 h at 60°C. Finally, acetone was added to precipitate the quaternized chitosan derivative which was collected. To exchange I⁻ against Cl⁻, the polymer was dissolved in 4 mL of 10% aqueous sodium chloride solution and then filtered. The polymer was precipitated with acetone, centrifuged and dried to obtain a white water soluble powder. The ¹H-NMR spectrum was obtained in D₂O using a 400MHz spectrometer (Varian Unity Plus) and the degree of quaternization was calculated. In step 2 the synthesis was optimized based on a 2² factorial design. The independent variables were the amount of NaOH (30% solution) and methyl iodide and the degree of quaternization is the response factor as the dependent variables.

3. Results and Discussion

A two-step synthesis of trimethyl chitosan was reported by Kim et al. [18]. We followed a similar approach with some modifications to synthesize DMEC. In the first step, mono-ethyl chitosan was prepared by introducing an ethyl group into the amine group of chitosan via Schiff's base followed by reducing the C=N bond and in the next step methyl iodide was added to produce N, N-dimethyl N-ethyl chitosan.

The second step of the synthesis was based on a nucleophilic substitution of chitosan amine protons with methyl groups of methyl iodide in the presence of sodium iodide and sodium hydroxide in a water/NMP medium. The counterion I⁻ was exchanged with Cl⁻ by dissolving the quaternized polymer in a 10% NaCl solution to obtain DMEC chloride. Studies have shown that an inorganic base, such as an aqueous NaOH solution, was better than organic bases (amines); the NaOH has a larger pK_a than chitosan for neutralizing the hydroiodic acid produced during the reaction [19]. Sodium hydroxide is a strong base, able to immediately neutralize the acid liberated and avoids the protonation of the unreacted NH₂ groups. High concentrations of NaOH solution are able to produce higher substituted polymers; however, it also results in O-alkylation, which decreases the solubility of the reaction product. If O-alkylation did occur, then the ¹H-NMR spectrum of the derivative would have had two sharp peaks at 3.5 and 3.45 ppm for 3- O-methyl and 6-O-methyl, respectively [20]. Since 95% DD chitosan was used in our experiments, higher NaOH concentration (2.0mL of 30% solution) resulted in greater quaternization (Table 1) without undesired effects on other sites of the chitosan molecule. The optimal reaction temperature for the second step was about 60°C. Higher temperatures caused O-alkylation and degradation of the chitosan. Methyl iodide and sodium hydroxide were reported to be the most effective reaction variables in the synthesis of quaternized chitosan derivatives [21–23]. Therefore, we varied the concentration in two levels based on a concise experimental design (Table 1) and precisely conducted four

reaction runs in triplicate; the degree of quaternization was selected as a dependent variable (Table 1). Therefore, we obtained DMEC polymers with different degrees of quaternization that were controlled by the basic conditions for the reaction. The optimum amounts of the reactants were found to be 5.0mL of methyl iodide and 2.0 mL of 30.0% aqueous NaOH (formulation S4 in Table 1) which gave a degree of 52.2% quaternization. Since sodium iodide was reported to be necessary to adjust the overall concentration of reactants in the reaction medium [24], no noticeable change was observed by changing the amount in the range from 480 to 600mg.

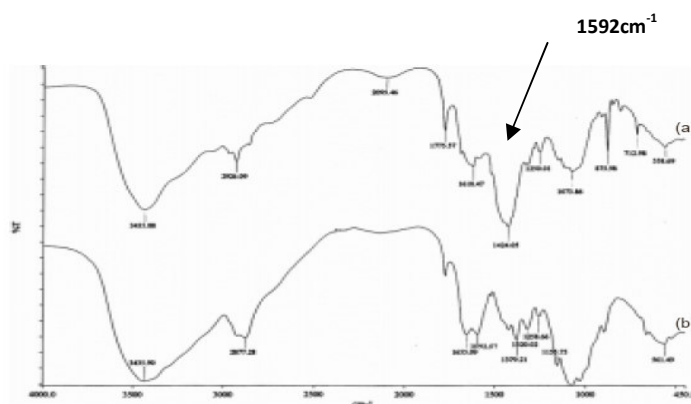


Figure 2. FTIR spectra of (a) DMEC and (b) chitosan.

Table 1. Reaction parameters for degree of quaternization.

Formulation	Reaction conditions (NaOH ml/Mel ^a ml)	Degree of quaternization ^b
S1	1.0/3.0	32.3 ± 2.75
S2	1.0/5.0	35.5 ± 3.94
S3	2.0/3.0	41.7 ± 3.71
S4	2.0/5.0	52.2 ± 3.10

^aMethyl iodide.

^bDegree of quaternization based on H-NMR integral calculation.

3.1.Characterization of DMEC

The FTIR spectrum of chitosan (Figure 2(b)) shows C—O peaks that were assigned to the saccharide structure at 898 and 1,155cm⁻¹ and an amino characteristic peak at ~1,592 cm⁻¹ (bending of NH). The absorption bands at 1,655 and 1,320cm⁻¹ are characteristic of N-acetylated chitin and reported to be the amide I and III bands, respectively [25]. The peak at 1,592cm⁻¹ disappeared in Figure 2(a) after conversion of NH₂ to N-alkyl. The ¹H-NMR spectrum of DMEC chloride and its extended spectrum are shown in Figures 3 and 4, respectively. The signal at 1.3ppm was attributed to CH₃ groups of the ethyl substituent, while H2–H6 protons of the polysaccharide backbone superimposed the —CH₂— groups of the ethyl group between 3.15–4.2ppm. The intense band at 4.70ppm was related to HDO. In this region, as observed more clearly from an extended spectrum, two different anomeric protons (H1) appeared at 5.07 and 5.35ppm. These were attributed to mono N-acetyl glucoseamine units.

The integral of CH₃—of the ethyl group versus the other protons was used to calculate the degree of quaternization [26].

In a follow-up experiment, it was found that the peak at 2.97ppm assigned to the methyl group of the quaternized amino group did not shift or change when the solution was acidified with a droplet of CF₃COOD. This indicated that only negligible amounts of mono and/or diethylated chitosan were obtained under the preparative conditions used. For ethyl chitosan (Figure 5), a triplet at 0.99ppm was assigned to the **CH₃—CH₂—** group in the D₂O/CF₃COOH. The area under this peak and the sum of the integrals of other peaks at 2.7–3.8 and the anomeric hydrogens at 4.5 and 4.6ppm were used to calculate the yield of the alkylation of the chitosan —NH₂ groups.

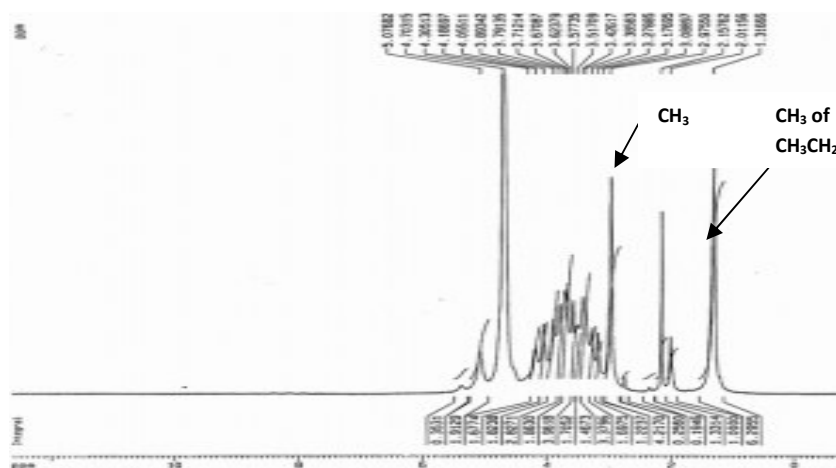
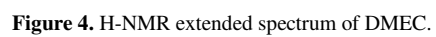


Figure 3. H-NMR spectrum of DMEC



4. Conclusion

In this study, N, N-dimethyl N-ethyl chitosans (DMECs) with different degrees of quaternization were synthesized. A modified two-step procedure and a factorial design approach were employed to prepare N, N-dimethyl N-ethyl chitosan with suitable degrees of quaternization. Under optimized conditions (methyl iodide, 5mL; NaOH 30%, 2mL; 60°C, 5h), 52% quaternization was obtained. Since several studies have shown that quaternized derivatives of chitosan with quaternization degrees of 50–60% are able to enhance the absorption of hydrophilic compounds, therefore, it seems that this new polymer could be a suitable absorption enhancing agent for oral delivery of peptide drugs. However, more studies, such as CaCO-2 cell permeation and *ex vivo* studies, are necessary to fully evaluate the potential of DMEC as a delivery platform for oral peptide delivery and absorption.

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PART II

In-vitro studies

Chapter 4.

Preparation, characterization and antibacterial activities of chitosan, *N*-trimethyl chitosan (TMC) and *N*-diethylmethyl chitosan (DEMC) nanoparticles loaded with insulin using both the ionotropic gelation and polyelectrolyte complexation methods.

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Abstract

TMC and DEMC, quaternized derivatives of chitosan, have been shown to have penetration enhancement properties and able to open the tight junctions of the intestinal epithelia at neutral and alkaline pH environments. The use of the nanoparticulate systems has the advantage of protecting the peptidic drugs from the harsh environment of the gastrointestinal tract. Hence, the aim of this study was to synthesize and characterize TMC and DEMC, both with quaternization degrees of $50\pm 5\%$, which were then used to prepare insulin nanoparticles with two different methods: ionotropic gelation and the polyelectrolyte complexation (PEC) techniques. The obtained nanoparticles were then characterized for size, zeta potential, insulin loading and release as well as antibacterial activities. The results showed that nanoparticles prepared by the PEC method had higher insulin loading efficiency and zeta potential than those made by the ionotropic gelation method and may subsequently be used for further in vitro, ex vivo and in vivo studies. Moreover, the antibacterial studies suggest that the polymers in free form have higher antibacterial activity against Gram-positive bacteria than in the nanoparticulate form.

1. Introduction

Oral drug delivery is the most favourite route for drug administration. In the past decade, biodegradable polymers such as chitosan and its quaternized derivatives have been studied extensively for their role as multifunctional permeation enhancers to enhance the permeation of hydrophilic macromolecules in peroral drug delivery. Chitosan, a natural, biocompatible and biodegradable polymer that has been used in biomedical fields in the form of sutures, wound covering, and as artificial skin (Dodane and Vilivalam, 1998; Domb and Bentolila, 1998; Varum et al., 1991; Rinaudo and Domard, 1989). Chitosan has mucoadhesive properties which are mediated by the spreading ability of chitosan over the mucus layer and additionally through its positive ionic interactions with the negative charges of the mucus or of the cell surface membranes (Lehr et al., 1992, 1993). Furthermore, it has been shown that chitosan and its derivatives can act as antibacterial agents against both Gram-negative and Gram-positive bacteria (Henriksen et al., 1996; Jung et al., 1999). Chitosan is a polycation with an apparent pK_a 6.5; hence, in neutral and basic environments prevailing in the intestine, the chitosan molecules lose their charge and precipitation will occur. However, studies have convincingly shown that quaternized derivatives of chitosan, synthesized by introducing alkyl groups to the $-NH_2$ -group of the chitosan structure, are drastically more soluble in the neutral and alkaline environments of the intestine and hence more useful for drug delivery of peptides and proteins (Avadi et al., 2004; Thanou et al., 1999). Moreover, the soluble chitosan salts may act as permeation enhancers to increase the

transmucosal absorption of peptide 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 permeation of hydrophilic compounds (Park et al., 2002).

Today, biotechnological processes enable us to produce in large amounts endogenous peptides and proteins that are required for the treatment of chronic diseases. Although the peroral route for drug delivery is the most convenient and desirable one for patients, the harsh environment of the gastrointestinal tract, the high molecular weight of the peptides as well as their hydrophilicity which prevents them to cross the lipophilic barrier of the mucosal walls, are major problems in developing an effective delivery system. Special strategies are hence required to overcome the above obstacles. It is the current opinion that nanosized polymeric particles, designed as delivery platform for those hydrophilic peptides, have the highest chance to succeed in overcoming these hurdles. Moreover, nanoparticles are capable of protecting drugs from degradation, improving permeation and penetration of the drugs across the mucosal surface as well as controlling the release of the encapsulated or adsorbed drugs (Schipper et al., 1997; Florence et al., 1995; Takeuchi et al., 2001). The uptake of chitosan nanoparticles seems to be related to their size and the superficial charge; hence, the higher the superficial charge, the stronger the affinity of the nanoparticles for the negatively charged cell membrane (Janes et al., 2001; Sakuma et

al., 1997). Various techniques are available for producing nanoparticles including solvent evaporation, interfacial polymerization and emulsion polymerization (Sakuma et al., 1999; Julienne et al., 1992; Ibrahim et al., 1992); however, most of these techniques involve the use of organic solvents, heat and vigorous agitation that may be harmful to peptides (Leroux et al., 1995). In 1997, the first chitosan nanoparticles were prepared by Alonso et al. using ionotropic gelation of chitosan (polycation) with tripolyphosphate TPP (polyanion). Ever since, almost all chitosan nanoparticles were prepared accordingly. However, recently polyelectrolyte complexation (PEC) resulting from self-assembly of proteins with natural and synthetic polymers has drawn increasing attention (Calvo et al., 1997). PEC is formed when oppositely charged polyelectrolytes are mixed and interact via electrostatic interactions. Both processes are easily applied; they have the advantage of not using sonication or organic solvents, both harmful for the proteins and peptides. The PEC formation results in an optically homogeneous and stable nano-dispersion (Mao et al., 2001). At a pH below 6.5, chitosan becomes positively charged due to protonation of the amino groups; on the other hand, most proteins become negatively charged at pH above 6.5. Hence, the electrostatic interactions between both entities at a suitable pH value can be used as driving force for PEC formation. The aim of the present work was to develop nanoparticulate systems based on chitosan, diethylmethyl chitosan (DEMC) and trimethyl chitosan (TMC) using both the polyelectrolyte complexation and the ion gelation methods and to load the nanoparticles with insulin. The

nanoparticles were characterized in terms of particle size, zeta potential, insulin loading and release as well as stability for further in vitro, ex vivo and in vivo investigations. Moreover, the antibacterial effects of these polymers were compared both in free polymer solution and in the nanoparticulate form.

2. Materials and methods

2.1. Materials

ChitoClear® chitosan (viscosity 1% (w/v) solution, 22mPa.s) was purchased from Primex, Iceland. Human insulin was a generous gift from Exir Pharmaceutical Company (Lorestan, Iran). Ethyliodide, methyl iodide, and sodium borohydride were obtained from Sigma (Vienna, Austria). Sodium hydroxide, *N* methyl pyrrolidone (NMP) and sodium iodide were purchased from Merck (Darmstadt, Germany). *Staphylococcus aureus* ATCC 29737 was obtained from Persian Type Culture Collection (PTCC, Iran). The antibiotic broth # 5273 was purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and were used as received.

2.2. Synthesis and characterization of N-trimethyl chitosan

TMC with degree of substitution of $55\pm 5\%$ was synthesized as previously described using minor adjustments (Sieval et al., 1998). Briefly, low molecular weight chitosan was dispersed in a basic solution of methylpyrrolidone and sodium hydroxide, methyl iodide and sodium iodide were, subsequently, added at 60°C for 6h. The polymer was then precipitated with acetone and separated by

centrifugation. For exchanging I⁻ with Cl⁻, the polymer was dissolved in aqueous NaCl (5.0%, w/w) solution and subsequently, precipitated with acetone and dried to obtain a water-soluble white powder with quantitative yield of 98% and substitution degree of 50±5%. The ¹H NMR spectrum was obtained in D₂O using a 500MHz spectrum (Varian unity plus) and the degree of quaternization was calculated.

2.3. Preparation and characterization of N-diethylmethylchitosan

DEMC was prepared by a two-step method reported previously by Avadi et al. In summary, low molecular weight chitosan was dissolved in 1% acetic acid solution and formaldehyde solution. After stirring for 120 min, the pH of solution was adjusted to 4.5 by adding 1M NaOH solution. Methyl chitosan precipitate was obtained by adding 1M NaOH solution and adjusting the pH of solution to 7.0. The precipitate was washed with distilled water and Soxhlet-extracted with ethyl alcohol and diethyl ether (1:1 v/v) for 72h. The ¹H NMR spectrum of methyl chitosan was obtained in CF₃COOD and D₂O as solvent. In the second step, methyl chitosan was dispersed in *N*-methyl pyrrolidone and subsequently, sodium hydroxide, ethyl iodide and sodium iodide were added. The reaction was carried out under gentle stirring for 5h at 60°C. Finally acetone was added and the precipitate of the chitosan derivative was collected. For exchanging I⁻ with Cl⁻, the polymer was dissolved in NaCl solution (5.0%, w/w) and subsequently precipitated with acetone, centrifuged and dried to obtain a white, water-soluble powder. The ¹H NMR spectrum was

obtained in D₂O using a 500MHz spectrometer (Varian unity plus) and the degree of quaternization was calculated to be 55±5%.

2.4. Preparation and characterization of nanoparticles with chitosan, TMC, and DEMC using polyelectrolyte complexation and ionotropic gelation method

The polyelectrolyte complexation method was used to make nanoparticles from chitosan and its two derivatives. To obtain such a complex, the pH of chitosan and its derivatives were adjusted to 5.0 so that over 90% of the amine groups were protonated and the insulin pH was adjusted to 8.0. Chitosan was dissolved in 0.25% acetic acid and the derivatives were dissolved in water. The pH was, subsequently, adjusted to 5.0 using 1N NaOH. The insulin powder was dissolved in 0.01N HCl to obtain a solution of 1.0 mg/mL and the pH was adjusted to 8.0 using 1N NaOH. The insulin was added to the polymer solution at a 1:1 ratio in a beaker under gentle magnetic stirring at room temperature. Once an opalescent suspension was formed, the samples were centrifuged at 14,000 rpm for 30 min at 4°C and the pellet was freeze-dried overnight to obtain a white powder and further examined as nanoparticles (Shirui et al., 2005). Chitosan-insulin nanoparticles were also prepared by an ionotropic gelation method (Julienne et al., 1992). Briefly, insulin (1.0 mg/mL) was pre-mixed with TPP (1.0 mg/mL) at a 1:4 ratio. A solution of chitosan (1.0 mg/mL) was prepared in 0.25% acetic acid. The pre-mixed solution of TPP-Insulin was added to chitosan solution drop-wise at a ratio of 1:3 and under gentle magnetic stirring at room temperature.

Chitosan derivatives, TMC and DEMC, were dissolved in water to obtain a solution of 1.0 mg/mL and the pH was adjusted to 5.2 using 0.01N NaOH. Insulin (1.0 mg/mL) was pre-mixed with TPP (1.0 mg/mL) at a 1:4 ratio. The pre-mixed solution of TPP-Insulin was added to derivative solution drop-wise at a ratio of 1:4 and under gentle magnetic stirring at room temperature. Once a stable colloidal suspension was obtained, the samples were centrifuged at 14,000 rpm for 30 min at 4 °C and the pellet was freeze-dried overnight to obtain a white powder. Particle size and the zeta potential of the nanoparticles were measured using a Malvern Zetasizer (3000HS, UK) of the freshly prepared suspension of polymer-insulin nanoparticles. The morphology of the nanoparticles was observed by TEM-100CXII.

2.5. Insulin loading of the nanoparticles

The freeze-dried samples were used to measure the loading efficiency of insulin in the nanoparticles. The amount of insulin associated with the PEC was calculated by the difference between the amount of insulin added to the suspension and the amount found in the freeze-dried samples in the case of the direct method. For the indirect method, the calculation was done by measuring the difference between the total amount of insulin added to the solution and the amount of insulin in the free form in the supernatant. The quantity of insulin was measured using a HPLC-UV apparatus (Waters 600 Controller, USA) at 210 nm. Isocratic elution was performed using 28% acetonitrile and 72% buffer containing 0.1M KH_2PO_4 and 1%

triethylamine adjusted to pH 3.0 with phosphoric acid (Dorkoosh et al., 2002). The column used was C8 (Capital HPLC, UK) 250mm×4.6mm equipped with a C8 (Capital HPLC) 10mm×4.6mm precolumn. Flow rate and injection volume were 1.5 mL/min and 50µL, respectively. The detection limit was 0.10µg/mL. The amount of insulin was quantified by peak integration and insulin association efficiency was calculated as follows:

a) Direct method

$$\text{Loading efficiency} = \frac{\text{Amount of insulin detected} \times 100}{\text{Total amount of insulin}}$$

b) Indirect method:

$$\text{Association efficiency} = \frac{\text{Total amount of insulin} - \text{Free insulin in supernatant} \times 100}{\text{Total amount of insulin}}$$

All experiments were done in triplicate.

2.6. Insulin release studies

A comparative insulin release study was done in three different pH values of 1.5, 3.0 and 6.8 with 0.01N HCl, PBS (pH 3.0 and 6.8), respectively. A known amount of freeze-dried nanoparticles was transferred to a 25mL tube that was designed to fit into the Erweka apparatus (DT6, Heusenstamm, Germany) and 10mL of the

respective dissolution buffer was added to the tube. The temperature and rotation were adjusted to 37°C and 50 rpm, respectively. At predetermined time of 5, 15, 30, 45, 60, 90, 120, and 240 min 0.5mL of sample was removed and replaced with fresh pre-warmed buffer. The samples were further analyzed using HPLC.

2.7. Stability test

In order to determine the stability of the nanoparticles made by the PEC and ionotropic gelation methods, the freeze-dried samples, kept at -4.0 °C, were analyzed monthly for 6 months and the amount of insulin was measured using HPLC. Briefly, 1.0 mg of freeze-dried samples was analyzed for the insulin content and the results were compared with the initial loading obtained on the first day of the experiment.

2.8. The antibacterial effect of chitosan and its derivatives as free polymer and in nanoparticulate form

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined as described previously (Avadi et al., 2004). Chitosan was dissolved in 0.25% acetic acid and the chitosan derivatives were dissolved in water. The concentrations of the polymers used in this study were 1000–62.5 µg/mL. The micro-organism was *S. aureus* ATCC 29737. The experiments were performed in triplicates.

To compare the inhibitory properties of the polymers with their nanoparticle forms, the PEC method was used and the nanoparticles

were applied as suspensions form. As antibacterial standard tetracycline hydrochloride was used. Four concentrations (1000, 500, 250 and 125 μ g/mL) of TMC and the standard tetracycline were prepared in water. Since TMC had shown the highest antibacterial effect among the polymers, it was used for the comparison studies with the tetracycline. The Kirby-Bauer diffusion method was used to study the inhibitory zones at different antimicrobial concentrations. Accordingly, a standard concentration of staphylococcus bacteria (1.0 μ g/mL) was uniformly spread on the surface of an agar plate (antibiotic broth #1). Consequently, 4 discs each impregnated with a predetermined concentration of the polymers were placed on the surface of the medium. During incubation, polymers diffuse outward from the disc at a rate inversely proportional to their size, forming a concentration gradient around each disc. A clear zone of inhibition around an antimicrobial disc reflects the degree of susceptibility of the micro-organism to the polymer.

3. Results and discussion

3.1. Characterization of trimethyl chitosan chloride and diethylmethyl chitosan chloride

The ^1H NMR spectra of chitosan, TMC and DEMC are presented in Fig. 1a, b and c, respectively. In Fig. 1b, the signal at 1.9 ppm is attributed to the acetyl group of the chitin; the peak at 3.6 represents the $\text{N}(\text{CH}_3)_3$ group together with a smaller peak at 3.4 ppm assigned to the $\text{N}(\text{CH}_3)_2$ group. According to the peak assignment and intensity the degree of quaternization was calculated to be $50\pm 5\%$. In Fig. 1c,

the signal at 1.3 ppm is attributed to the CH₃ group of the diethyl substituted N-atom; while H₂–H₆ protons of the polysaccharide backbone superimpose the CH₂ groups. The intense band at 4.8 ppm is related to the solvent HDO. The integral of the CH₃ of the ethyl groups versus the other protons was used to calculate the degree of quaternization to be 50±5%. The zeta potential of the polymers was measured using a Malvern Zeta Sizer and the results are presented in Table 1. As shown in this table, chitosan has the least positive zeta potential and TMC has the highest positive zeta potential among the derivatives. DEMC has a zeta potential in between. The positivity of the chitosan in zeta potential relates to the portion of the chitosan that has been protonated and dissolved in water. In the alkylated salts of chitosan, TMC and DEMC, the -NH₂-group was converted to a quaternary ammonium group and that describes the higher positivity of the derivatives in comparison to the chitosan itself. The higher positivity of TMC compared to DEMC can be explained by the fact that methyl groups have a smaller volume than the ethyl groups which makes the positive charge of the TMC more accessible.

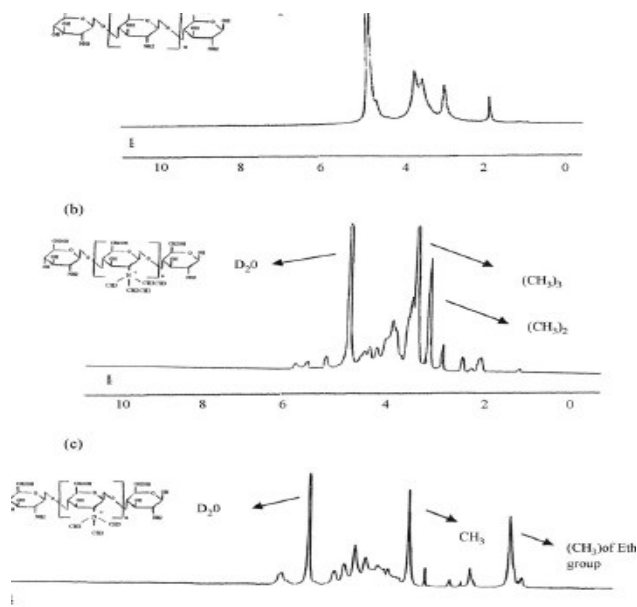


Fig. 1. The ^1H NMR spectrum of chitosan (a), TMC (b) and DEMC (c).

Table 1

The zeta potential of chitosan, TMC and DEMC in free form and the nanoparticle forms

Polymer	Zeta potential (mV)
Chitosan	+24.2
Chitosan np	+15.9
TMC	+43.2
TMC np	+22.0
DEMC	+40.0
DEMC np	+19.0

3.2. Nanoparticle characterization

3.2.1. Insulin loading

Table 2 presents the diameters (nm), zeta potential and the insulin loading of the nanoparticles prepared by both the ionotropic gelation

and the PEC methods. The size of the nanoparticles made by either method is comparable and is around 200 ± 20 nm. Also the size of the insulin nanoparticles prepared with chitosan was not different with the ones made by TMC or DEMC. Thus, one can state that there was no distinct effect on the size of the nanoparticles dependent on the preparation method. The insulin loading of the nanoparticles made by PEC was shown to be higher than the nanoparticles prepared by the ionotropic gelation method. In the PEC method, the electrostatic interactions between the negatively charged insulin and the positively charged polymer are used as the driving force for the formation of the nanoparticles. Consequently, in this method, the insulin is directly interacting with the positive polymer and this can assign to the higher insulin loading in the obtained nanoparticles. In the ionotropic gelation method, the main interaction is between the negative phosphate groups of the tripolyphosphate (TPP) and the positive amino groups of the polymers. As the TPP (negative charge) interacts with chitosan (positive charge), a matrix system is obtained. The hydrophilic insulin is entrapped in this network and tends to diffuse to the external medium. This can explain the lower insulin loading efficiency of the nanoparticles using the ionotropic gelation method. Furthermore, using the PEC method, the insulin loading efficiency of the nanoparticles prepared with the chitosan was higher than in the ones prepared by the chitosan derivatives, TMC and DEMC. One can argue that in the PEC method when the pH of the polymers is adjusted to 5.2 the NH_2 -groups of the chitosan are mostly protonated and are better accessible to interact

with the insulin. With the quaternized derivatives, the N⁺ atoms are surrounded by methyl and ethyl groups and are hence less available to interact with insulin. This may account for the higher insulin loading of chitosan in comparison to the derivatives. According to Table 2, the zeta potential of the insulin nanoparticles made by the PEC method is higher in comparison to the nanoparticles prepared by the ionotropic gelation method. The zeta potential is a good indicator of the available surface charges on the nanoparticle. The positivity of the nanoparticles is important for their interaction with the cellular membrane components and the tight junctions in triggering the paracellular permeation of the hydrophilic compounds. In the nanoparticles made by the PEC method, the ratio of negative charge (insulin) and the positive charge of the chitosan and its derivatives remains in the favour of the positive charge of the polymers because only a fraction is neutralized by binding insulin. However, in the ionotropic gelation method, the ratio of TPP, both the negatively charged TPP and the insulin bind with the positive charged polymers resulting in a lower positivity of the nanoparticles.

Table 2

The results of insulin loading, size of nanoparticles, polydispersity and zeta potential of the nanoparticles prepared by both ionotropic gelation and PEC methods using chitosan, TMC and DEMC

Polymer	Insulin loading (%)	Size (nm)	Poly dispersity	Zeta potential (mV)
Chitosan (ionotropic)	50 $\pm$ 5	250 $\pm$ 20	0.46	+15.9
Chitosan (PEC)	90 $\pm$ 5	220 $\pm$ 20	0.25	+23.1
TMC (ionotropic)	55 $\pm$ 5	215 $\pm$ 20	0.1	+22.0
TMC (PEC)	70 $\pm$ 5	195 $\pm$ 20	0.32	+29.0
DEMC (ionotropic)	50 $\pm$ 5	203 $\pm$ 20	0.23	+19.0
DEMC (PEC)	75 $\pm$ 5	220 $\pm$ 20	0.45	+26.0

3.2.2. Morphology of the nanoparticles

According to the TEM picture shown in Fig. 2a and b, the morphology and the size of the nanoparticles are comparable in both ionotropic gelation and PEC methods. Morphologically, the nanoparticles look round to oval in shape and have a relatively smooth surface.

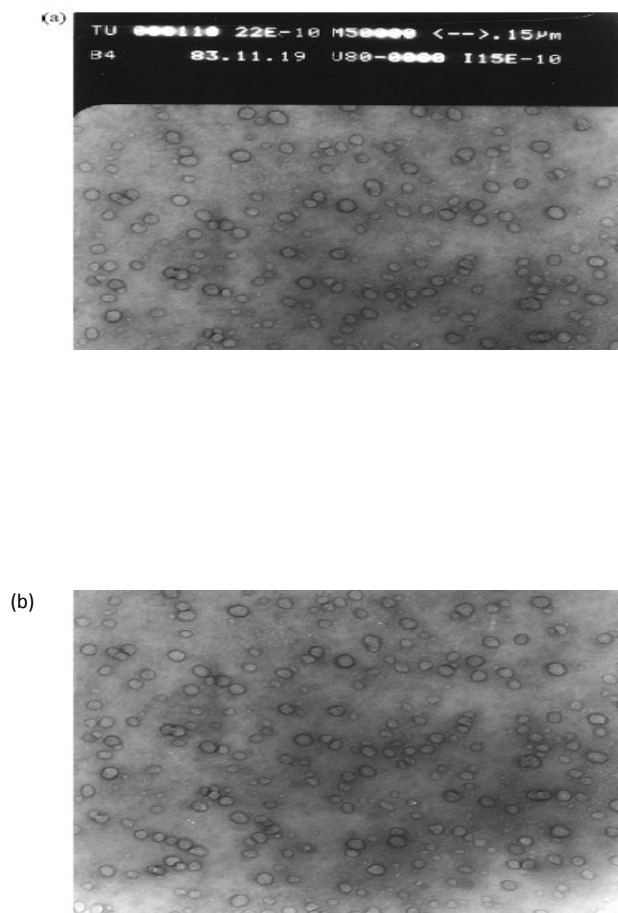


Fig. 2. The TEM picture of the insulin loaded nanoparticles made by the ionotropic gelation (a) and the PEC method (b).

3.2.3. The stability of nanoparticles

The stability of nanoparticles prepared by both ionotropic gelation and the PEC methods was followed up to 6 months and the results indicate that the insulin was stable in both methods for this period of

time and a $10\pm 4\%$ decrease in loading was observed (data not presented).

3.3. Insulin release studies

Fig. 3 shows a comparison between the insulin released from the nanoparticles made by the PEC method at three different pH media: 0.01N HCl, phosphate buffer pH 3.0 and phosphate buffer pH 6.8. Each study was done three times. In 0.01N HCl, a burst release of insulin in the first minutes of the study (about 70%) was observed and after 30 min almost 100% of the insulin was released from the nanoparticles. When phosphate buffer pH 3.0 was used, the burst release was less, approximately 20% and a maximum of 50% insulin release was observed after 45 min. Finally with phosphate buffer 6.8, a burst release of about 15% was observed and the maximum insulin release was 40% after 45 min.

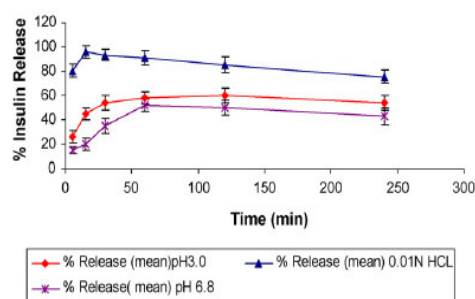


Fig. 3. The release profile from insulin nanoparticles using PEC method at 3 different pH mediums (0.01N HCL, PBS pH 3.0 and PBS pH 6.8).

Finally with phosphate buffer 6.8, a burst release of about 15% was observed and the maximum insulin release was 40% after 45 min.

This observation can be best described by the fact that both insulin and the polymer are soluble in the acidic pH (0.01N HCl) and hence a better insulin release was observed. Using phosphate buffer pH 3.0, although both insulin and polymer are soluble, their solubility is lower than in the HCl medium and consequently a lower insulin release is observed from the nanoparticles. Finally, the lowest insulin release in phosphate buffer pH 6.8 can be described by the poor solubility of both insulin and the polymer at this pH. The release profiles of insulin from nanoparticles prepared by the ionotropic gelation method at pH media 3.0 and 6.8 were similar to those of the PEC method (data not presented). Figs. 4 and 5 show the insulin release from nanoparticles made from chitosan, TMC and DEMC by both the ionotropic gelation method and the PEC method, respectively. According to these figures the insulin release profiles from the chitosan, TMC and DEMC nanoparticles prepared by the ionotropic gelation and the PEC methods were very similar in 0.01N HCl. Fig. 6 compares the insulin release from the nanoparticles made by both ionotropic gelation and the PEC methods in 0.01N HCl medium. According to Fig. 6, a burst release is observed early during the release study from the nanoparticles made according to the PEC method. The release of insulin was slower from nanoparticles made according to the ionotropic gelation method.

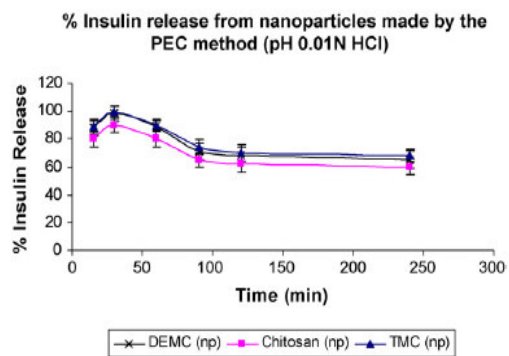


Figure 4. Comparison of insulin release from chitosan, TMC and DEMC nanoparticles prepared by the PEC method in 0.01NHCl

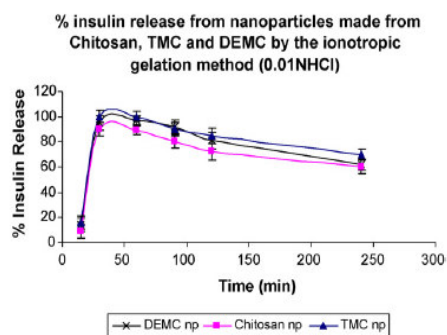


Figure 5. Comparison of insulin release from chitosan, TMC and DEMC nanoparticles prepared by the Ionotropic gelation method in 0.01NHCl

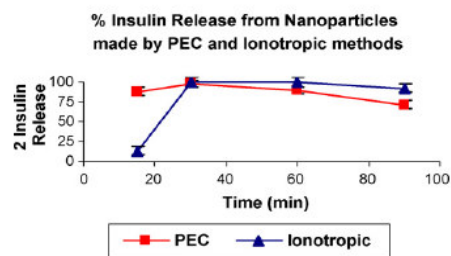


Fig. 6. Comparison of insulin release from chitosan nanoparticles prepared by both the ionotropic gelation and the PEC methods in 0.01N HCl.

In both methods, the maximum insulin release was 100% and comparable. The release studies were done with nanoparticles made from chitosan, TMC and DEMC the results were comparable in all three polymers. The difference in the insulin release profiles between the PEC and the ionotropic gelation method is that the nanoparticles prepared by the ionic gelation method due to the additional cross linking of the polymer with TPP possess a denser matrix structure and hence a lesser availability of the incorporated insulin at the beginning of the release experiments.

3.4. Comparison of the antibacterial effect of chitosan, TMC and DEMC in free form and as nanoparticles

Table 3 presents the antibacterial effect of chitosan and the two chitosan derivatives, TMC and DEMC, in free form and as nanoparticle form prepared by the ionotropic gelation method. According to the results, TMC shows the highest inhibition and chitosan the least inhibition against *S. aureus*. Since the chitosan derivatives as quaternary ammonium compounds, have a stronger

positive charge than chitosan, their increased antibacterial effects can be attributed to the formation of polyelectrolyte complexes between the polymers and the negative peptidoglycans of the bacterial cell wall. This interaction may in turn disrupt the cell wall and result in the inhibition of the bacterial growth. As for the derivatives, with N-substitution degrees of approximately 50%, the charge densities are very close to each other; thus, their different inhibitory effects may be explained by the size of the substituted group. In another word, as the trimethyl group is smaller than the ethyl group, it may react better with the bacterial cell wall in comparison to the more voluminous diethylmethyl (DEM) group. Also, less steric hindrance may be expected when TMC with three methyl groups interacts with the bacterial cell wall in comparison to the (DEM) group containing 2 ethyl groups and a methyl group. The antibacterial effect of the polymers was compared to their nanoparticle forms as well and the results are presented in Table 3. The results show that the nanoparticles have less inhibitory effect than the polymers in free form. One can assume that since the nanoparticles are prepared by electrostatic interactions, the amount of positive charge available for interacting with the bacterial cell wall is reduced. This can be explained by a lower zeta potential of the nanoparticles in comparison to the free polymer form.

Table 3

The antibacterial effect of chitosan, TMC and DEMC in free form and as nanoparticles forms against Gram-positive *Staphylococcus aureus*

Polymer	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)
Chitosan	1000	1000
Chitosan np	2000	2000
TMC	250	250
TMC np	500	500
DEMC	500	500
DEMC np	1000	1000

In all three cases, the antibacterial effect of the nanoparticles was about one dilution lower than their respective free polymer forms. Fig. 7 presents the difference between TMC (1000 $\mu\text{g/mL}$) and tetracycline hydrochloride (1000 $\mu\text{g/mL}$) in the inhibitory effect against *S. aureus*. Evidently, the inhibition zones produced by tetracycline are much bigger than the ones from TMC which indicates higher inhibition potency by tetracycline. Using the diffusion disc method, no zones were seen at lower concentrations of TMC. This can be best described by the interaction and probably entanglement of the polymer chains with the agar which further reduce the ability of the polymer to diffuse through the agar. As expected chitosan and DEMC have lower inhibitory effects than TMC compared to tetracycline.

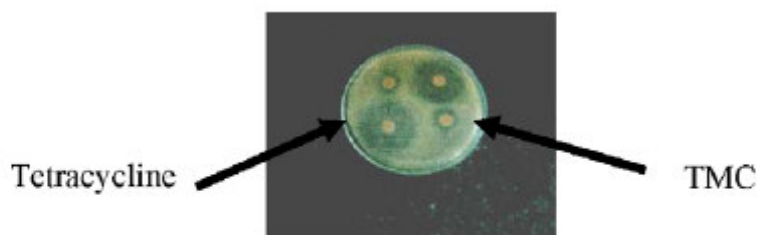


Fig. 7. The comparison of TMC and tetracycline antibacterial effect against *Staphylococcus aureus* using the disc method.

4. Conclusions

In summary, the results suggest that while both the ionotropic gelation and the PEC methods were easy to be used for the production of insulin loaded nanoparticles, the PEC method seems to be more suitable for future studies. The obtained nanoparticles by the PEC method had higher insulin loading efficiency and zeta potential both required for an effective permeation enhancement across the intestinal epithelium. Moreover, the antibacterial studies suggest that the nanoparticles have less inhibition effect on Gram-positive bacteria than the polymers in free soluble form since nanoparticles have less positive charge available to bind to the negative bacterial cell wall. However, among the polymers used, TMC, with the highest zeta potential and smallest alkyl groups showed the highest antibacterial inhibition against *S. aureus*.

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PART III

Caco-2 cell studies

Chapter 5

Permeation enhancer effect of chitosan and chitosan derivatives: comparison of formulations as soluble polymers and nanoparticulate systems on insulin absorption in Caco-2 cells.

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Eur. J. Pharm. Biopharm 70:270-278 (2008)

Abstract

In this study four quaternized derivatives of chitosan trimethyl chitosan (TMC), diethylmethyl chitosan (DEMC), dimethylethyl chitosan (DMEC) and triethyl chitosan (TEC) with degree of substitution of approximately $50\pm 5\%$ were synthesized and their effect on the permeability of insulin across intestinal Caco-2 monolayers was studied and compared with chitosan both in free-soluble form and in nanoparticulate systems. Transepithelial electrical resistance (TEER) studies revealed that all four chitosan derivatives in free soluble forms were able to decrease the TEER value in the following order TMC>DMEC>DEMC=TEC>chitosan, indicating their abilities to open the tight junctions. Recovery studies on the TEER showed that the effect of the polymers on Caco-2 cell monolayer is reversible and proves the viability of cells after incubation with all polymers. A similar rank order was also observed when measuring the zeta-potentials of the various polymers in solution form. Transport studies of insulin together with soluble polymer across Caco-2 cell layers showed the following ranking: TMC>DMEC>DEMC>TEC> chitosan which is in agreement with the strength of the cationic charge of the polymer. In comparison to the free- soluble polymers, the nanoparticles prepared by ionic gelation of the chitosan and its quaternized derivatives had much lower effect on decreasing the TEER by opening of the tight junctions. This can be explained by the reduced available amount of

positive charge at the surface of the nanoparticles. In accordance with these results, the insulin loaded nanoparticles showed much less permeation across the Caco-2 cell monolayer in comparison to the free-soluble polymers. Mass balance transport studies revealed that a substantial amount of the nanoparticles has been entrapped into the Caco-2 monolayer or attached to the cell surface. It can thus be stated that while free-soluble polymers can reversibly open the tight junctions and increase the permeation of insulin, the nanoparticles had basically only a low effect on opening of the tight junction and the paracellular transport of insulin across the Caco-2 cell monolayer. These data convincingly show that nanoparticles consisting of chitosan and its quaternary ammonium derivatives loaded with insulin are less effective in facilitating paracellular transport across Caco-2 cell monolayers than the corresponding free polymers.

1. Introduction

The low oral bioavailability of the peptide and protein drugs is mainly due to their poor absorption through the gastrointestinal epithelium as well as the rapid hydrolytic and enzymatic degradation in contact with the gastrointestinal fluids [1, 2]. In order to overcome the above obstacles, different delivery platforms have been developed for hydrophilic drugs. An effective strategy to improve peptide permeation is the incorporation of suitable permeation enhancers in the delivery system. Modern permeation enhancers are multifunctional polymers with mucoadhesive properties, the ability to locally inhibit intestinal enzymes, reversibly open the tight junctions, show no toxicity and they are ideally not absorbed. Hence they do not show perturbances of the membranes of the epithelial cells and are therefore specific for inducing only the paracellular hydrophilic drug transport. The intestinal epithelium regulates the passage of natural compounds and acts as a barrier for paracellular passive transport of large hydrophilic molecules. This absorption barrier is composed of a single layer of columnar epithelial cells joined at the apical surface by a tight junctional complex. The junctional complex forms a continuous seal, which segregates the apical from and the basolateral compartment and conveys size and charge selectivity due to the presence of negative charge in its structure [3, 4]. Nanoparticulate drug delivery systems have attracted an immense attention as novel carriers for the delivery of lipophilic and hydrophilic substances as well as vaccines [5]. There is a strong belief that nanoparticles of

appropriate size can pass the mucosal membranes intactly and deliver their drug load into the systemic circulation. In the case of hydrophilic drugs, nanoparticles should be able to protect such drugs from degradation in the intestinal fluids and improve their penetration and permeation across the intestinal mucosal epithelium [6-8]. Suitable nanoparticles have mucoadhesive properties which are due to their particle size and the particle's superficial charge [9]. It has been shown that nanoparticles may be internalized into the intestinal epithelial cells [10].

Chitosan, a natural polyaminosaccharide obtained by *N*-deacetylation of chitin is a non-toxic, biocompatible and biodegradable polymer that has good mucoadhesive properties in acidic environments [11-13]. Studies have shown that chitosan can promote the nasal absorption of insulin in rats and sheep and further enhance the paracellular transport of peptides in vitro and in vivo by opening the tight junctions [14-17]. However, chitosan is a polycation with an apparent pKa of 6.5 and it loses its charge and precipitates in neutral and basic environments as prevailing in the intestine. Studies have shown that only protonated soluble chitosan in its uncoiled configuration can trigger the opening of the tight junctions and facilitate the transport of hydrophilic compounds [18, 19]. Hence, chitosan can be used as an enhancer only in the proximal part of the intestine where the pH is close to its pKa value. Subsequently, quaternized derivatives of chitosan, synthesized by introducing various alkyl groups to the NH₂-group of the chitosan molecule

structure, were studied extensively. These derivatives were characterized to be drastically more soluble in neutral and alkaline environments of the intestine and hence more useful for drug delivery and absorption across the intestinal epithelium of the jejunum and ileum than the mother compound chitosan [19, 20]. The permeation enhancing properties of these chitosan derivatives have been attributed to their ion pair interactions with the tight junctions and cellular membrane components to increase the paracellular permeation of hydrophilic compounds [20]. Trimethyl chitosan (TMC), Dimethylethyl chitosan (DMEC), Diethylmethyl chitosan (DEMC) and Triethyl chitosan (TEC) were synthesized by partial quaternization of chitosan as described by Sieval et al., Bayat et al. and Avadi et al., respectively [21-24]. The polymer charge density, determined by the substitution degree is a key factor in obtaining both the mucoadhesion and penetration enhancement towards the intestinal epithelium [25, 26]. Chitosan derivatives were synthesized from low molecular weight chitosan and all had the same degree of quaternization of approximately $50 \pm 5\%$ which has been shown for TMC to have the highest penetration rate across the intestinal epithelium in vitro [25, 27]. The molecular weight of the synthesized derivatives did not change significantly.

Accordingly, the aims of this study were to develop and compare nanoparticulate systems based on chitosan, TMC, and the newly described DMEC, DEMC and TEC loaded with insulin generated by the polyelectrolyte complexation (PEC) method described previously

[28] with a weight ratio of polymer to insulin of 50% (w/w) which has been described by the same authors as optimal and to measure their intestinal transmucosal insulin transport across Caco-2 cell layers. The flux data were compared afterwards with the insulin fluxes across Caco-2 cell layers obtained after application of the various aqueous insulin/polymer solutions. The obtained results convincingly show that nanoparticles consisting of chitosan and its quaternary ammonium derivatives loaded with insulin are much less effective in facilitating paracellular transport across Caco-2 cell monolayers than the corresponding free polymers.

2. Materials and Methods

2.1. Materials

ChitoClear[®] chitosan (viscosity 1% w/v solution, 22 mPa.s, 98% deacetylated) was purchased from Primex, Iceland. Human insulin was a generous gift from Exir Pharmaceutical Company (Lorestan, Iran). The TMC, DMEC, DEMC and TEC were synthesized in our laboratory as described previously [21-24].

2.2. Determination of the molecular weight of chitosan and its derivatives

The biopolymer analysis was performed with a triple detection size-exclusion-chromatography (SEC³) on a TDA max SEC system (Viscotek, USA). The detection was operated by a differential refractometer at $\lambda=660\text{nm}$ and a right angle light scattering detector (RALS) with a 3mW He/Ne laser at $\lambda=670\text{nm}$. A dn/dc value of

0.163 was used. Intrinsic viscosities were measured on a 4 capillary, differential Wheatstone bridge viscometer. A degassed 0.3M AcOH/0.3M AcONa buffer (pH=4.5) with 1% ethylene glycol was used as eluent. To ensure a low light scattering noise level the eluent was filtrated through a 0.2 μ m filter. Chitosan samples dissolved in the eluent at a concentration of 0.3-1mg/ml were filtrated through a 0.45 μ m filter prior to analysis to remove aggregates. Injection volume varied from 10 to 100 μ l and the flow rate was maintained at 0.7 ml/min.

2.3. Preparation and characterization of insulin loaded nanoparticles

The insulin loaded nanoparticles were prepared from chitosan, TMC, DMEC, DEMC and TEC using polyelectrolyte complexation as described earlier [28]. The complex formation was a result of the positively charged polymer and the negatively charged insulin. To obtain such a complex, a solution of chitosan and each quaternized derivatives was prepared in 0.25% acetic acid and water, respectively and the pH was adjusted to 5.0 so that more than 90% of the amine groups were protonated. The insulin powder was dissolved in 0.01N HCl to obtain a solution and the pH was adjusted to 8.0 using 1N NaOH. The insulin solution was subsequently added to the polymer solution at a 1:1 ratio (w/w) in a beaker under gentle magnetic stirring at room temperature. The concentration of the polymer and insulin was adjusted to obtain a final insulin concentration of 2.0 mg/mL. An opalescent, colloidal suspension was immediately formed to indicate

the formation of nanoparticles. The morphology of the nanoparticles was observed by TEM -100CXII (JEOL, Tokyo, Japan). Insulin loading was calculated by measuring the difference between the total amount of insulin added to the solution and the amount of insulin in the free form in the supernatant. The quantity of insulin was measured using a HPLC-UV apparatus (Waters 600 Controller, Milford, Massachusetts, USA) at 210nm. Isocratic elution was performed using 28% acetonitrile and 72% buffer containing 0.1M KH₂PO₄ and 1% triethylamine adjusted to pH 3.0 with phosphoric acid [29]. The column used was C8 (Capital HPLC, Edinburg, Scotland) 250 × 4.6mm equipped with a C8 (Capital HPLC, Edinburg, Scotland) 10 × 4.6mm precolumn. Flow rate and injection volume were 1.5mL/min and 50 µl, respectively. The detection limit was 0.10µg/mL. The amount of insulin was quantified by peak integration and insulin association efficiency was calculated as follows:

$$\text{Association efficiency} = \frac{\text{Total amount of insulin} - \text{Free insulin in supernatant}}{\text{Total amount of insulin}} \times 100$$

The characteristics of the obtained nanoparticles are presented in Table 1.

Table 1

The mean molecular weight (M_w) and intrinsic viscosities ($[\eta]$) of chitosan and its alkylated derivatives

Polymer	M_w (kg/mol)	M_n (kg/mol)	M_w/M_n	η (dl/g)	Rh (nm)	M-Ha	Zeta-potential
TMC	105	40	2.76	1.15	11.3	0.73	43.2 $\pm$ 1.1
DEMC	123	47	2.61	1.58	13.4	0.72	40.0 $\pm$ 1.2
TEC	135	59	2.28	2.37	15.9	0.65	39.9 $\pm$ 2.1
DMEC	121	49	2.83	3.03	23.9	0.69	41.1 $\pm$ 1.1
Chitosan	126	36	3.47	2.82	16.0	0.72	24.2 $\pm$ 0.9

 M_w , molecular weight, $[\eta]$, intrinsic viscosity, number average M_n , Rh, hydrodynamic radius (nm), MH- a , Mark-Houwink constant a .

2.4. Zeta potential measurement

Particle size and the zeta potential of the nanoparticles were measured using a Malvern Zeta sizer (3000 HS, Malvern, Worsc, UK) with a folded capillary cell (DTS, 1060). The zeta potential of the free soluble polymers was measured by dissolving chitosan in 0.25% acetic acid, while the derivatives were dissolved in deionized water. The obtained solutions (2.0mg/mL) were then diluted at a 1:10 ratio using deionized water that was passed through a 0.22 μ filter and ultrasound for 6-10 minutes. The measurements were done on the polymer solutions after 20 minutes to account for possible polymer aggregation. It should be noted that zeta potential measurements are not dependent on the size of the particles but the surface charge of the particles. The nanoparticle suspensions were diluted in pre-filtered deionized water as described above to obtain a dilution of 0.01 mg/ml as recommended by the instrument's instruction to avoid multiple scattering.

2.5. Caco-2 cell cultures

Caco-2 cells, with a passage number 36-42, were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were cultured on polycarbonate membrane filters (pore size 0.4 μm , area 4.7 cm^2) in Corning Transwell 6-well plates (Corning Incorporated, Europe) at a seeding density of 10^4 cells/ cm^2 . The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma, pH 7.4), supplemented with 1% v/v non-essential amino acids, 10% v/v heat denatured fetal calf serum (FCS), 160 U/mL benzylpenicillin and 100 U/ml streptomycin sulfate (Sigma Chemical, St. Louis, MO, USA). The culture medium was added to both the apical and the basolateral compartments and was changed every second day for 21 days. The cells were maintained at 37°C, in an atmosphere of 95% air and 5% CO_2 at 90% relative humidity. One hour before the experiments the medium was changed to the transport medium: Hank's balanced salt solution (HBSS) buffered with 30mM *n*-(2-hydroxyethyl) piperazine-*n*-(2-ethanesulfonic acid) (HEPES) at pH 5.5 and the cells were allowed to equilibrate for one hour.

2.6. Measurement of the insulin release from the nanoparticles

The in vitro release profiles of insulin from all nanoparticles have been described in previous publications [30] and will be only shortly mentioned. The release curves of insulin obtained with all 5 polymers are the same and can be superimposed. In 0.01N HCl as dissolution medium, approximately 90% of the insulin has been released within

30min. At pH 6.8 (PBS buffer), about 50% of insulin is released within 30 min.

2.7. Measurements of the transepithelial electrical resistance (TEER)

The transepithelial electrical resistance (TEER) was measured using a Milicell[®] ERS meter (Millipore Corp. Bedford, MA, USA) connected to a pair of chopstick electrodes to ensure the integrity of the monolayers formed on the filters. TEER measurements were also performed during the experiments to check the effect of soluble polymers and the nanoparticles on opening the tight junctions at predetermined time intervals of 0, 1, 2, 4, 8 and 24 hours. The experiments were done in triplicate.

2.8. Permeability studies

For the transport studies insulin was used in free form, in the presence of free soluble polymer and in the form of nanoparticle suspensions prepared from chitosan, Trimethyl chitosan (TMC), Dimethylethyl chitosan (DMEC), Diethylmethyl chitosan (DEMC) and Triethyl chitosan (TEC). Consequently, 2.0 mL of 2.0 mg/mL final concentration of insulin and 0.25mg/mL polymer were added to the apical side of the cell culture dish. Samples of 200 µl were withdrawn from the basolateral part at predetermined time of 15, 30, 45, 60, 120 and 240 minutes and replaced with equal volumes of fresh HBSS-HEPES. The samples were analyzed for the insulin content using the HPLC method as described previously [29]. After completion of the transport studies, the samples were carefully removed from the apical

part and the cell monolayers were rinsed with HBSS-HEPES and this medium was then replaced with culture medium. The monolayers were allowed to incubate for 24 hours at 37⁰C in regular cell culture conditions. The TEER was monitored during this period up to 24 hrs. Results were corrected for dilution and expressed as cumulative transport with time. All the experiments were done in triplicate.

Apparent permeability coefficients (P_{app}) were calculated according to the following equation:

$$P_{app} = (dQ/dt) \cdot (1/A \cdot C_0 \cdot 60)$$

where P_{app} is the apparent permeability in cm/s, dQ/dt is the permeability rate, A is the diffusion area of the monolayer (cm²), and C_0 is the initial concentration of the insulin. Statistical differences were calculated using one way ANOVA at a significant level of $P < 0.05$.

2.9. Mass Balance Studies

After the completion of the transport study, samples were removed from both the basolateral and the apical compartments. The samples were analyzed for insulin contents using HPLC as described previously [29]. The measured insulin on the apical part was compared to the initial insulin on the apical part as well as the amount permeated from the basolateral compartment to decide upon the fate of the insulin.

2.10. Trypan blue exclusion

After the completion of the insulin transport experiments, both the apical and the basolateral compartments of the monolayer were rinsed gently with sterile 0.01 M phosphate buffered saline (PBS) pH 7.4. A solution of 0.1 % trypan blue in PBS buffer was added to the apical part and the monolayers were incubated for 30min at 37⁰C. Subsequently, the solutions were removed from both sides and the monolayers were rinsed gently with PBS to remove excess dye. The filters were cut carefully from the inserts and transferred on a coverslip. The monolayers were then checked by a light microscopy for the exclusion of trypan blue. If the intact monolayers showed no inclusion of dye they were considered to be viable, if the cells, however, showed dye inclusion they were considered to be damaged.

3. Results and Discussion

3.1. The polymer characterization

Table 1 presents the result of zeta potential, the molecular weight and intrinsic viscosities of the polymers. Accordingly, there was a significant difference between the zeta potential of chitosan and the derivatives ($p < 0.05$). The zeta potential of the chitosan derivatives, however, was very close to one another. This may be explained as chitosan by nature contains a less positive surface charge while the derivatives contain a higher positive surface charge. The higher the positivity of the surface charge in a polymer may result in a higher ability to interact with the tight junctions and cellular membrane

components to increase the paracellular permeation of hydrophilic compounds. Although the size and the configuration of the polymers are also important factors in their interaction with the cellular components, methyl groups are usually very compact and show less steric hindrance in comparison with the ethyl substituted groups.

Moreover, the Mw and the intrinsic viscosities of chitosan and chitosan derivatives are presented in Table 1. The results suggested that the molecular weight of the synthesized derivatives did not change significantly. The derivatives showed a lower intrinsic viscosity in comparison with the initial chitosan. This can be due to the fact that the derivatives have a more dense structure and become heavier through the synthetic reactions.

3.2. Morphology of the nanoparticles

According to the TEM picture shown in Figure 1, morphologically the nanoparticles look round to oval in shape and have a relatively smooth surface.

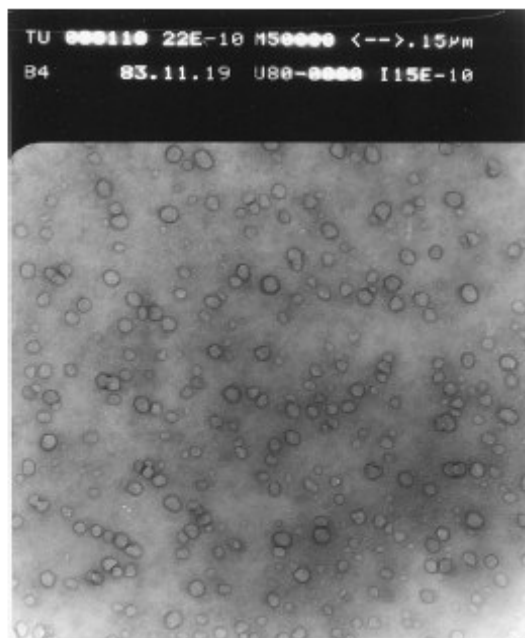


Fig. 1. The TEM picture of the insulin-loaded nanoparticles made by the PEC method.

3.3. Effect of polymers in free form and in nanoparticle suspension on TEER of Caco-2 cell monolayer

The effects of soluble polymers and nanoparticles in opening the intestinal tight junctions were studied by measuring the TEER values across the Caco-2 cell. The results are presented as the percentage of the initial values at $t=0$ min and are shown in Figure 2. According to this figure, the effect of the free-soluble polymers in opening the tight junction was immediate. After the apical addition of the free soluble polymers to the monolayers together with the insulin solution, a significant TEER decrease was observed within the first hour which remained stable during the course of the experiment. Moreover, among the polymers used chitosan had the least effect on opening the

tight junctions (about 75% of the initial value) and TMC had the strongest effect (about 45% of the initial value). The effect of the other 3 polymers namely, DMEC, DEMC and TEC was comparable and was about 60% of the initial value. These results were significant in comparison to the free insulin used as the negative control ($p < 0.05$) that had no effect on the reduction of the TEER. Hence, the chitosan derivatives with a substitution degree of about $50 \pm 5\%$ were able to significantly decrease the TEER at pH values present in the small intestine. This ability of chitosan derivatives to decrease the TEER can be explained by the possible interactions of their positive surface charge with the anionic components of the glycoproteins on the surface of the epithelial cells or the fixed negative charges of the interior of the tight junctions [31]. It is known that the surface charge of the molecules is independent of their size. Hence it is possible to measure the surface charge of the polymer solutions which are not really solutions but macromolecular dispersions. The surface charge density of the polymers has a direct effect on their ability to interact with the components of the tight junction, i.e. to open the paracellular pathway and increase the permeability of hydrophilic peptide drugs. The results obtained from the zeta potential of the polymers, Table 1, correlate very well with the ability of chitosan and each derivative to reduce the TEER and open the tight junction. The TEER measurement after the apical application of the nanoparticle suspensions on top of the monolayers indicate that the TEER was reduced much less than in the presence of free-soluble polymer (Figure 3). Accordingly, the polymers in the nanoparticulate forms

had much less effect on opening of the tight junction and hence a much lower reduction was observed in the TEER measurement during the course of the experiment. This is also explained by the surface charge density of the nanoparticles. According to Table 2, the zeta potential of chitosan and its quaternized derivatives in the nanoparticle form is less than in their free soluble form. As the nanoparticles were prepared by the polyelectrolyte complexation, i.e. the positive charge of the polymers interacted with the negatively charged insulin to result in the nanoparticle suspension. Subsequently, the amount of available positive charge on the surface of the nanoparticles was less in comparison to that of the free-soluble polymers. Moreover, it can be stated that nanoparticles – if at all - are mainly transported via an intracellular pathway, i.e. endocytosis or transcytosis across the Caco-2 cells and not along the paracellular pathway. This is in accordance with the study of Lim et al. [10]. These authors also have shown that chitosan nanoparticles can reduce the TEER, however to a stronger extent than what was found in this study. However, this can be due to difference in size and zeta potential of the obtained nanoparticles in their study. Their conclusion stating that basically no insulin was transported across the cellular monolayer using nanoparticles correlates quite well with our findings.

Table 2

The results of insulin loading, size of nanoparticles (np), polydispersity and zeta potential of the nanoparticles prepared by PEC method using chitosan, TMC, DMEC, DEMC and TEC ($n = 3$)

Nanoparticles	% Insulin loading	Size (nm)	Poly dispersity	Zeta potential (mV)
Chitosan (np)	90 ± 5	220 ± 20	0.25	$+15.9 \pm 0.5$
TMC (np)	70 ± 5	195 ± 20	0.32	$+22.0 \pm 1.2$
DMEC (np)	75 ± 5	205 ± 15	0.40	$+21.2 \pm 1.2$
DEMC (np)	75 ± 5	220 ± 20	0.45	$+19.0 \pm 0.9$
TEC (np)	69 ± 5	210 ± 10	0.20	$+21.0 \pm 1.0$

nm, nanometer, mV, millivolt.

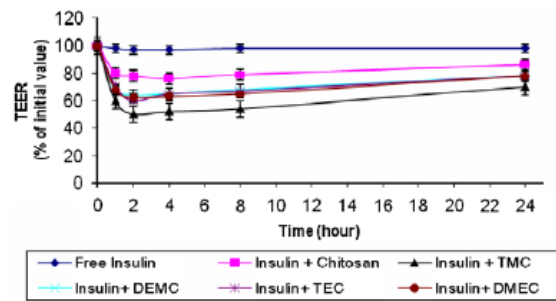


Fig. 2. Effects of free-soluble polymers on TEER of Caco-2 cell monolayer. After 240 min, the monolayers were rinsed with HBSS-HEPES and culture medium was applied on the monolayers. Data are expressed as mean $\pm$ SD of 3 experiments the difference ($p < 0.05$) is considered significant.

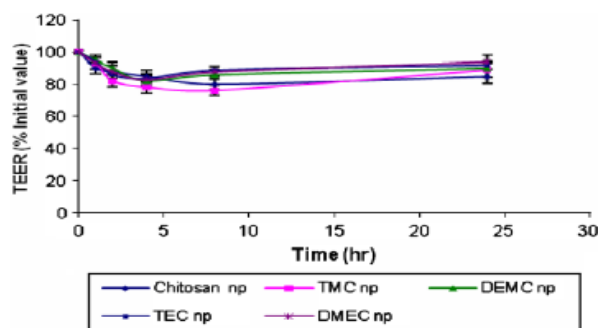


Fig. 3. Effects of nanoparticles on TEER of Caco-2 cell monolayer. After 240 minutes, the monolayers were rinsed with HBSS-HEPES and culture medium was applied on the monolayers. Data are expressed as means $\pm$ SD of three experiments the difference ($p < 0.05$) is considered significant.

3.2. Insulin transport studies

Human insulin was used as a relevant model drug to determine the increase of paracellular permeability within Caco-2 cell monolayers using the polymers in free-soluble and in nanoparticulate forms. As shown in Figure 4, the chitosan and to a greater extent its derivatives caused a significant increase in the transport of insulin across the monolayer. According to Figure 4, TMC showed the highest increase on the permeability of insulin and chitosan had the least influence on the transport of insulin across the Caco-2 cell monolayer. The influence of DMEC, DEMC and TEC in inducing permeation of insulin across the monolayer was within a narrow range. The following ranking: TMC>DMEC>DEMC=TEC>chitosan could be obtained. These results correlate well with the results obtained from the TEER measurements. TMC with the highest reduction of the TEER values was also most effective in inducing the permeation of insulin across the monolayer. Conversely, chitosan with the weakest

reduction on the TEER across the monolayer caused the least permeation of insulin across Caco-2 cells. Moreover, the other three derivatives with the same effect on the TEER had very similar influence on the permeation of insulin.

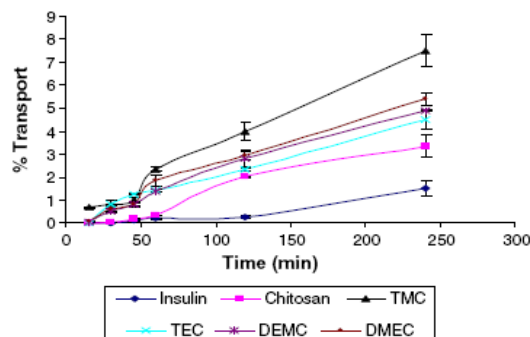


Fig. 4. Cumulative transport of insulin across Caco-2 cell monolayers using free-soluble polymers in HBSS-HEPES medium. Data are expressed as means $\pm$ SD of three experiments the difference $p < 0.05$ is considered significant.

In Figure 5, the effect of nanoparticles on the induction of paracellular transport of insulin across the Caco-2 cell monolayer is presented. According to this Figure, the effect of nanoparticles in facilitating insulin transport across the monolayer was much lower than that of the soluble polymers. The TMC nanoparticle has a slightly higher ability to transport insulin than chitosan and the other derivatives. This, however, is not significant and may be explained by the increase in the cationic charge of TMC- nanoparticles' zeta potential. The decrease in permeation with nanoparticles in comparison to the free soluble polymers can be explained by the much lower TEER reduction caused by the nanoparticles.

Additionally, the enhancing ability of polymers in the nanoparticulate and soluble forms can be explained by the strong difference in the surface charge density of free polymers compared to the nanoparticles. Release studies of insulin from the nanoparticles in 0.01N HCL showed a quick insulin release up to 90% of the incorporated dose within 30 min and to about 50% in PBS buffer pH 6.8 (data not shown). As a result of these release profiles the insulin transport across the Caco-2 cell layers is controlled by the transport across the opened tight-junctions and not by the insulin release from the nanoparticles.

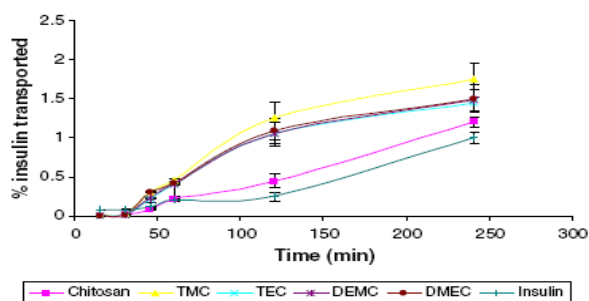


Fig. 5. Cumulative transport of insulin across Caco-2 cell monolayers using nanoparticles in HBSS-HEPES medium. Data are expressed as means $\pm$ SD of three experiments the difference $p < 0.05$ is considered significant.

The calculated apparent permeability values of the free-soluble polymers and nanoparticles prepared with different polymers are presented in Table 3. According to this table, insulin and TMC

showed the lowest and highest apparent permeability values, respectively. The values for DMEC, DEMC and TEC, although significantly increased in comparison to chitosan, are lower than that of TMC. Chitosan also showed an increase in permeation enhancement of insulin, however, its apparent permeability value is the lowest of all polymers investigated. Moreover, the apparent permeability coefficients of the nanoparticles prepared with different polymers indicate that, even though the nanoparticles are effective in permeating insulin across the Caco-2 cell monolayer, their enhancing effect is less pronounced than the free-soluble polymer.

Table 3
Apparent permeability (P_{app}) for Insulin transport across Caco-2 cell monolayers using free-soluble polymer and nanoparticulate suspensions ($n =$ three experiments) the difference $p < 0.05$ is considered significant

Polymer	Average $P_{app} \times 10^{-8}$ cm/s
Insulin	0.3 ± 0.05
Chitosan (np)	0.2 ± 0.19
TMC (np)	0.45 ± 0.12
DMEC (np)	0.39 ± 1.1
DEMC (np)	0.35 ± 0.21
TEC (np)	0.40 ± 0.25
Chitosan	0.8 ± 0.4
TMC	1.84 ± 1.1
DMEC	1.33 ± 0.9
DEMC	1.11 ± 0.82
TEC	1.2 ± 0.55

3.3. Mass Balance Studies

Mass balance transport studies measuring the apical and basolateral insulin concentrations after application of the insulin nanoparticles revealed that a substantial amount of the nanoparticles must have been entrapped into the Caco-2 cell monolayers and/or they are

attached to the surface of the cells and the insulin has not been transported across the cell monolayer. In the case of the free-soluble polymer, the amount of insulin present on the apical part was higher than the amount found with the nanoparticles; as a result of this, the insulin transported across the Caco-2 cell monolayer was also found to be higher than in the nanoparticulate form. This can indicate that the transport of insulin using free soluble polymer is mainly via opening the tight junctions and via the paracellular pathway, while the insulin nanoparticles are mainly transported by the intracellular pathway. Previous studies have shown that once the nanoparticles are inside the cells they may get entrapped within the cellular components such as the Golgi apparatus and endoplasmic reticulum [31]. Also our in-vitro release studies indicate that only 50% of the insulin is released from the nanoparticles at pH 5.0 within the first hour [data not presented]. This may also account for the lower permeation of insulin nanoparticles across the Caco-2 cell monolayer in comparison to the free-soluble polymer. The apical and the basolateral concentrations and the differences between both values regarding the total amount of added insulin are summarized in Table 4. This difference is less pronounced in the amount of insulin on the apical and the basolateral compartment with the free soluble polymer indicating that the permeation of insulin in the presence of free soluble polymer is predominantly transported via opening the tight junction and not through the intracellular pathway. Degradation of insulin cannot be fully ruled out as it can count for some of the insulin missing. However, as the missing amount with free insulin is only

0.5%, for the free-soluble polymers between 2.9-12% and for nanoparticles it is about 19-27%, it can be assumed that with nanoparticles a substantial amount of insulin (intactly or degraded) is entrapped within the Caco-2 cells.

Table 4
Insulin concentration in the apical and basolateral compartments (incubation time 240 min, $n = 3$)

Polymer	μg Insulin apical part (t_0)	μg Insulin basolateral part	μg Insulin apical part ($t = 240$ min)	μg Insulin apical + basolateral	% Insulin missing
Insulin	2000	30 ± 0.3	1960 ± 20	1990 ± 20.3	0.5
Chitosan	2000	67 ± 0.5	1874 ± 53	1941 ± 53.5	2.9
TMC	2000	150 ± 0.7	1690 ± 62	1840 ± 62.7	8.0
DMEC	2000	108 ± 0.4	1788 ± 75	1896 ± 75.4	5.2
DEMC	2000	90 ± 0.3	1657 ± 76	1747 ± 76.3	12
TEC	2000	98 ± 0.4	1758 ± 74	1856 ± 74.4	7.2
Chitosan (np)	2000	24 ± 0.1	1591 ± 66	1615 ± 66.1	19
TMC (np)	2000	35 ± 0.1	1380 ± 46	1415 ± 46.1	29
DMEC (np)	2000	30 ± 0.2	1456 ± 55	1476 ± 55.2	26
DEMC (np)	2000	29 ± 0.1	1490 ± 65	1519 ± 65.1	24
TEC (np)	2000	29 ± 0.2	1440 ± 74	1469 ± 57.2	27

3.4. Cytotoxicity Studies

To determine the integrity of the cytoplasmic membrane after application of the polymers, trypan blue staining was used. Staining the Caco-2 cells with 0.1% trypan blue, revealed a less than 1% intracellular dye uptake by the cells [data not presented]. This demonstrates that the Caco-2 cells were still viable after the completion of the transport experiments in the presence of polymers. Hence, it can be stated that the polymers were not cytotoxic at the concentration used in this experiment.

4. Conclusion

In this study four chitosan derivatives TMC, DMEC, DEMC and TEC were synthesized and tested for their effect on the intestinal permeation of insulin in comparison to chitosan itself. Moreover,

these derivatives were used to make nanoparticle systems by the polyelectrolyte complexation method. The transport efficiency of insulin in free form, in the presence of free- soluble polymers, across the Caco-2 cell monolayer was shown to be higher than its loaded form in nanoparticles prepared with the same chitosan derivatives. Our study clearly shows that the chitosan derivatives in free soluble form have higher positive surface charge and can be consequently considered as good permeation enhancers for hydrophilic drugs through the paracellular route in suitable drug delivery systems. The chitosan derivatives in nanoparticle form have less positive surface charge and their interactions with tight junction is limited and hence the drug transport across the monolayer is more likely through the transcellular pathway rather than by tight junction opening. However, further studies are required to prove the mechanism by which the nanoparticles are able to transport peptide drugs across the intestinal membrane. Mass balance studies of the insulin from the apical to the basolateral side of the Caco-2 cells have shown that a significant amount of the nanoparticles must be entrapped within the Caco-2 cell monolayers. In conclusion, it can be stated that for oral drug delivery of hydrophilic compounds like insulin and other peptides targeted to the intestinal tract, the chitosan derivatives in the free soluble forms are more suitable as drug enhancers than in their nanoparticle forms loaded with insulin.

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PART IV

In vivo evaluation of the novel
drug delivery system

Development of a Gas Empowered Drug Delivery System for Peptide Delivery in the Small Intestine

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Abstract

The aim of this investigation was to design a novel Gas Empowered Drug Delivery (GEDD) system for CO₂ forced transport of peptide drugs together with mucoadhesive polymers to the surface of the small intestine. The GEDD effect of the core tablet was achieved using CO₂ gas to push insulin together with the mucoadhesive excipients poly(ethyleneoxide) (PEO) and the permeation enhancer trimethyl chitosan (TMC) to the surface of the small intestine. The in-vitro insulin release showed that almost 100% of the insulin was released from enterically coated tablets within 30 minutes at pH 6.8. The designed GEDD system was shown to increase the insulin transport by approximately 7 times in comparison with the free insulin across sheep's intestine ex-vivo. Three different peroral formulations were administered to male rabbits: F1 containing no TMC or PEO, F2 containing PEO but no TMC and F3 containing both PEO and TMC. The administrations of insulin using the formulation F1 resulted in a low F_R value of $0.2\% \pm 0.1\%$, while the formulations F2 and F3 resulted in a much higher F_R values of $0.6 \pm 0.2\%$ and $1.1\% \pm 0.4\%$, respectively. Hence, the insulin permeation achieved by the GEDD system is primarily due to the enhancing effect of TMC and the mucoadhesive properties of PEO both of which synergistically increase the bioavailability of insulin.

1. Introduction

In the past decade, a large number of endogenous peptides and protein drugs are produced in biotechnological companies for the treatment of chronic diseases. The majority of these drugs are administered by parenteral injection which is inconvenient, time and money consuming as well as dangerous. Oral drug delivery is the most convenient way for self administration of drugs, allowing a wide range of dosage adjustments [1]. Hence, a lot of research is focused on design and development of novel oral drug delivery systems for both hydrophilic, large macromolecules such as peptide and protein drugs and also small hydrophilic drug molecules with poor bioavailability.

Effective oral delivery of therapeutic peptides and proteins to the small intestine poses a great challenge in drug delivery system design. The harsh environment of the stomach, the presence of proteolytic enzymes in the small intestine, the hydrophilicity of the peptide molecules and its large molecular size as well as the poor membrane permeability have led to an intense investigation for site-specific drug delivery systems [2-4]. In order to overcome the above obstacles, the ideal oral delivery system must release its contents pH dependently only at the optimal target region, remain in the optimal site long enough for the complete peptide and protein release to be absorbed across the intestinal epithelium, and have a reproducible therapeutic effect. Thus, site specific delivery is required to deploy the peptides and proteins intactly to specifically targeted parts of the body through a platform that can control their release by means of physiological or chemical triggers [5-7].

A number of sophisticated oral novel delivery systems have been developed for the delivery of peptides and proteins [8]. Most of these delivery systems,

however, are only designed to release the hydrophilic macromolecule at the desired site of action but do not take into account appropriate mechanisms to also facilitate absorption of the drug molecule which in most cases is then enzymatically degraded in the small intestine [9].

In order to actively improve drug absorption a lot of focus was given to the mucoadhesive polymers for oral delivery of peptide drugs. Mucoadhesive polymers are hydrophilic macromolecules with numerous hydrogen bond forming groups such as carboxyl, hydroxyl, amide and amine groups that need to be hydrated for swelling and to act as a mucoadhesive polymers on the mucus linings of the gut. Suitable mucoadhesive polymers like chitosans and their quaternary derivatives form intermolecular complexes with the glycoproteins of the mucus linings to form a stable adhesive layer with the mucus. This adhesion can further assist in the absorption of peptide drugs across the mucosal membrane of the gut by reversibly opening of the tight-junctions [10-12].

Chitosan, a biocompatible and biodegradable polymer has been used as an oral drug delivery vehicle. However, chitosan can be used as an enhancer only in the proximal part of the intestine where the pH is close to its pKa value of 5.5. Quaternized derivatives of chitosan were synthesized and shown in contrast to chitosan to be drastically more soluble in neutral and alkaline environments of the intestine and more useful for drug delivery and absorption across the intestinal epithelium of the jejunum and ileum [13, 14]. The permeation enhancing properties of these chitosan derivatives have been attributed to their ion pair interactions with the tight- junctions and cellular membrane components to increase the paracellular permeation of hydrophilic compounds [15]. The polymer charge density, determined by the substitution degree is a key factor in obtaining both the mucoadhesion

and penetration enhancement towards the intestinal epithelium [16, 17]. Among chitosan derivatives, TMC was shown to have the most permeation enhancing effect. Hence, TMC was synthesized from low molecular weight chitosan with a degree of quaternization of approximately 50 $\pm$ 5% which has been shown to have the highest penetration enhancing effect across the intestinal epithelium in vitro and in vivo [18].

However, the use of mucoadhesive polymers used as delivery platforms in the micro- and nanosize poses the problem that after the release of these mucoadhesive units they firstly have to swell before they can fully exert their mucoadhesive properties. Already during this swelling process the mucoadhesive sites can be de-activated by soluble mucins amply available in the intestinal fluids rendering in systems which are unable both to adhere to the mucous linings of the gut wall and to trigger the opening of the tight junctions. This holds especially for big gut lumina as those of the pig and humans [19, 20]. In order to overcome this drawback, Dorkoosh et al. have developed a time-and site- controlled delivery system that kept the drug mechanically attached to the intestinal site of absorption. They have used superporous hydrogel (SPH) and SPH composite (SPHc) polymers that were able to swell very quickly due to their highly porous structure and incorporated in these conveyor systems suitable delivery systems for the drug octreotide to get a desirable time-controlled release profile, required for absorption of peptides and proteins. They succeeded in getting high absolute bioavailabilities ($F= 8 - 16\%$) depending on the type of delivery system in an in vivo study in pigs [21-24]. However these systems were not suitable for industrial mass production.

In this study, an easier to produce system was developed namely a CO₂ empowered drug delivery (GEDD) system has been designed to quickly

release insulin and the mucoadhesive polymers (polyethylene oxide) (PEO) and trimethyl chitosan (TMC) and to push them by the CO₂ bubbles to the mucous linings without losing their mucoadhesiveness and potency to open the tight junctions. The delivery system is enterically coated to release its contents in the proximal part of the small intestine where the CO₂ gas directs the mucoadhesive PEO polymer to attach to the mucosa of the small intestine. Once attached, the permeation enhancer, TMC, will also bind to the mucosal surface and trigger the opening of the tight junctions of the epithelial cells of the small intestine and open the paracellular pathway to facilitate the transport of the insulin across the mucosal membrane.

Robinson et al. have done studies using CO₂ as absorption enhancing agent in the ex vivo intestine of rabbit and mouse and showed that the direct bubbling of CO₂ can enhance drug permeability by reversibly altering the paracellular pathway [25]. However in the newly designed GEDD systems described here, CO₂ is primarily not used as a penetration enhancer but as pushing agent of the drug containing excipients to the absorbing surface.

Hence, the aim of this study was to investigate 1) the mucoadhesive and permeation enhancing effect of the novel delivery system 2) the in-vitro insulin release from the delivery system in the two different milieus of stomach and intestine 3) to evaluate the effectiveness of the GEDD system as permeation inducer of insulin in the sheep's intestine in ex vivo studies and 4) to determine the bioavailability of peroral insulin using GEDD delivery system in an in vivo study in rabbits.

2. Materials and Methods

2.1. Materials

Chito Clear[®] chitosan (viscosity 1% w/v solution, 22 mPa.s) was purchased from Primex, Iceland. Human insulin was a generous gift from Exir Pharmaceutical Company (Lorestan, Iran). The Polyethylene oxide (Mw 900,000) was a gift from (Dow Chemicals, U.K.). TMC with the degree of substitution of $50 \pm 5\%$ was synthesized in our laboratory as described previously [26]. Cellulose acetate phthalate (CAP) was purchased from Trans Medica (Germany), Ac-Di-Sol was purchased from SMF (Netherlands). All the other materials were of pharmaceutical and analytical grades and used as received.

2.2. Formulation and design of the GEDD system

The delivery system was prepared based on a 2^3 factorial design. The variables were the percent polyethylene oxide (PEO) as mucoadhesive polymer, percent of citric acid and Na-bicarbonate (for CO₂ gas production), and Ac-Di-Sol as super-disintegrant. The response was the CO₂ gas production, tablet disintegration time as well as a mucoadhesive mass response. The variables were set as low and high values of 50% and 70%, 2.5% and 5.0% and 5.0% and 10% of the total weight for the amount of acid-base, Ac-Di-Sol and the PEO, respectively. As it was impossible to quantify the response of the CO₂ gas produced, designated (+) was used to evaluate the formulations 1-8. Accordingly one plus (+) corresponds to a low response and two (++) corresponds to a high response. The disintegration response (amount of Ac-Di-Sol) was evaluated by the disintegration time of the tablets in PBS of pH 6.8 at 37⁰C. Finally, the mucoadhesive response was measured by the amount of work (N) required to detach the dosage form from the sheep's intestinal mucosa in vitro as

described in section 2.5. According to the results obtained from the factorial design experiment, the following formulation was used for further studies: (46.7% citric acid, 23.3% Na-bicarbonate, 5% Ac-Di-Sol, 5% Avicel PH102, 10% PEO, 3.0% lactose 80, 1.5 % human insulin, 0.5% TMC, and 5.0% Na-benzoate as lubricant).

In a lab-scale formulation, absolute alcohol was used for a wet granulation with citric acid, Na-bicarbonate and lactose. TMC, PEO, Avicel, insulin and the Na-benzoate were then added step-wise to the obtained granules and mixed well using manual mixing. The granules were then pressed to tablets using a single, lab scale press machine with a round, concave 7mm punch at a $150 \text{ mg} \pm 7.5\%$ weight and hardness of 4-6 Kpa. Content uniformity of the tablets was determined according to the standard method of USP 30 where 10 tablets were used to assay the amount of insulin. The RSD of the tablets weights was below 6%. The percent of insulin was calculated from the following equation: $R_u/R_s \times 100 = \% \text{ Insulin in each tablet}$.

R_u and R_s are the respective areas under the curve for the test and standard samples (data not presented).

2.3. Preparation of enteric coated tablets

Once the tablets were pressed, they were enterically coated with a 3.0 mg sub- coat of a 3% solution of polyvinylpyrrolidone (PVP) K30 in absolute isopropanol and subsequently by 11mg of a 6% CAP solution containing diethyl phthalate as plasticizer per single tablet. The PVP layer was a protective layer to prevent the direct binding of the CAP to the tablets to avoid interactions of the acidic CAP with Na-hydrogen carbonate, and to smoothen the tablet surface at the edges to achieve a homogeneous enteric coating. The pan coating method was used to coat the tablets with an

Erweka pan apparatus (Heusenstamm, Germany) with 30 rpm and spraying 1.0mL coating solution per minute for 15-20 minutes. A suitable hot air drier was used to heat and dry the coating. In order to examine the effectiveness of the enteric coat layer, dissolution tests were performed using the standard USP 30 paddle apparatus. The rotation speed was set at 75 rpm and the dissolution medium was 500 mL 0.1N HCl for 2 hours, followed by phosphate buffer saline solution (PBS) of pH 6.8 at 37⁰C for 60 minutes. Six coated tablets were used and the disintegration behavior was investigated in both the acidic and alkaline medium.

2.4. Insulin release studies from the GEDD system

The insulin release from GEDD system was measured from both the uncoated as well as the enterically coated tablets. The coated tablets were placed in 25 mL tubes that were designed to fit into the Erweka apparatus (DT6, Heusenstamm, Germany). Initially 10 mL of 0.1N HCl was added to the tube and after 2 hrs, a sample was removed and analyzed. Subsequently, the tablets were transferred to tubes containing 10 mL of PBS buffer of pH 6.8. At predetermined time intervals of 5, 15, 30, 45 and 60 min, 0.5 mL of sample was removed and replaced with fresh pre-warmed buffer. The samples were further analyzed for insulin using HPLC as described previously [26]. The temperature and rotation were adjusted to 37 ⁰C and 75 rpm, respectively. The release from the uncoated tablets was only measured in the PBS buffer of 6.8. The release studies were repeated at least three times for each sample and the sink condition was considered in all cases.

2.5. Mucoadhesive properties of PEO after its release from the GEDD system

The mucoadhesive properties of the PEO used in the system were evaluated in a piece of sheep's intestine (jejunum). The experiment was performed according to the method described by Smart et al. [12] with some modifications. Briefly, a small section of fresh sheep's intestine was removed, quickly frozen and was cut into pieces of 3cm lengths, opened longitudinally to expose the mucous surface and gently washed with PBS buffer pH 6.0. A preliminary histological study indicated the absence of any major damage to the tissue caused by the freeze-thawing process. A layer of mucus was shown to be present on the inner surface of the intestine. The sections of intestine were mounted on a platform and secured using a plastic cap, exposing an 11mm diameter of the test surface. The exposed surface was equilibrated in PBS buffer of pH 6.8 for 1 min at 37 °C. The cap was elongated at one side to allow sufficient distance for the tablet to be detached. Using a cyanoacrylate adhesive, the GEDD tablets were individually attached to a 1.5g weight and placed in contact with the adhesive surface for 2 minutes. Subsequently, a brass ring was placed over the 1.5g weight. The ring was connected to the force and position sensor of the rheometer via a pulley system. After 2 min the contact platform was lowered at a rate of 2mm/min and the maximum detachment force was calculated.

2.6. The CO₂ induced insulin penetration ex vivo

The procedure was done according to Eichmann and Robinson [25] with minor modification. Briefly, male albino rabbits weighting between 2-3 Kg were fasted overnight but had access to water ad libitum. The animals were sacrificed by 10 mL i.v. injection of 5% sodium pentobarbital solution in the marginal vein. The abdominal wall was exposed and 8-10 cm of the ileum

was excised and placed on ice cold Krebs buffer solution. The intestine was washed with the Krebs buffer to remove the feces and food debris. The perfusion was set as described by Robinson et al. For the mucosal to serosal transport study, 5.0 mL of the insulin solution (2.0 mg/mL final concentration) was added to the donor chamber. Solutions were kept at 37°C by a circulating water bath. The CO₂ gas was bubbled at 100 mL/min directly onto the epithelial surface. At predetermined intervals of 0, 15, 30, 45 and 60 minutes, 1.0 mL of sample was removed from the receiver chamber and replaced with fresh medium. The insulin transport was measured as described previously [26]. The permeability coefficient (P) was calculated using the following formula:

$$P = \frac{V}{A \times C_0} \left(\frac{dM}{dt} \right)$$

Where V= volume inside the intestine, A= tissue surface area, C₀= Initial donor concentration, dM/dt = the amount of transported drug versus time. The experiment was repeated 3-5 times.

2.7. The evaluation of the permeation enhancing effect of chitosan and TMC

The procedure for evaluating the permeation enhancing effect was performed as described by Dorkoosh et al. and applied with some modifications [27]. On the day of the experiment, pieces of intestine were removed from the jejunum of freshly slaughtered sheep and washed with PBS (0.9% NaCl) to remove food residues and feces. The intestine was cut into 15 cm pieces and subsequently transferred to ice cold Krebs-Ringer bicarbonate buffer solution. One side of the intestine was tightly tied with a silk thread and the following solutions were added to different pieces of the

intestine: a) free insulin solution, b) free insulin and chitosan solution, c) free insulin and TMC solution. The insulin and chitosan were dissolved in 0.01% HCl and 0.25% acetic acid, respectively and then mixed together. The pH was slowly raised to 6.0 using 1N NaOH to prevent the precipitation of chitosan or insulin. The TMC was dissolved in water and the pH of the insulin polymer was adjusted to 6.8. Chitosan and TMC were used at a concentration of 0.5% w/v. The amount of insulin and polymer used were the same in the free form and in the GEDD tablet. The filled intestine was hooked onto a stainless steel support to keep it stretched and immersed in a container filled with 50 mL of Krebs buffer medium saturated with 95% O₂ and 5% CO₂. The container was placed in a water bath of 37°C with gentle movements. At predetermined times of 0, 15, 30, 60, 90 and 120 minutes 5mL samples were removed from the serosal part and replaced with fresh medium. The samples were analyzed by HPLC method as described previously [26] and the amount of insulin transported across the intestinal lumen was calculated

2.8. The efficiency of the GEDD system for insulin permeation using sheep's intestine (ex vivo studies)

The procedure used was as described in section 2.5 and the following formulas were added to the isolated sheep intestine a) free insulin solution (f1), b) free insulin and TMC solution (f2) , c) GEDD tablet containing Insulin without TMC (f3) d), GEDD tablet containing both insulin and TMC (f4). Consequently, 8.0 mL of the Krebs buffer pH 6.8 was added to the intestine and the other end of the intestine was also tied carefully. The filled intestine was hooked onto a stainless steel support to keep it stretched and immersed in a container filled with 50 mL of Krebs buffer pH 6.8 medium saturated with 95% O₂ and 5% CO₂. The container was placed in a water bath of 37°C with gentle movements. At predetermined times of 0, 15, 30,

60, 90 and 120 minutes 5mL samples were removed from the serosal part and replaced with fresh medium. The samples were analyzed by HPLC method as described previously [26] and the amount of insulin transported across the intestinal lumen was calculated. The apparent permeability coefficient (Papp) of insulin transported was calculated according to the approximated surface area in the intestinal lumen.

2.9. The in vivo studies using enteric coated GEDD tablets

The investigation was performed in healthy, male rabbits of 2-3 kg body weight. A week prior to the start of the experiment the rabbits were kept in the animal unit for environmental adaptation. The animals were fasted the night before each experiment with access to water ad libitum. The enteric coated GEDD tablets were administered using a custom made applicator via the oral route and placed in the stomach of the animals. Subcutaneous injection of insulin was done in the lateral skin of the hind limb.

The following formulations were administered to the rabbits as a cross-over setup (Table 1). For subcutaneous injection, the desired amount of insulin was initially dissolved in 0.1M HCl and added to 0.9% NaCl. The pH was then adjusted to 7.2 using 0.1M NaOH. The formulations were administered to animals at 48hrs intervals to ensure complete insulin washout. Blood samples were collected at the predetermined intervals of -10, 15, 30, 60, 90, 120, 240 and 300 minutes. The samples were kept on ice and centrifuged at 2000 rpm for 10 min at 4°C. The separated serum was kept at -20°C for further analysis.

Table 1. Administration of different insulin formulations

Type of Administration	Insulin Dose	Amount of Administration
Subcutaneous	0.2 IU/Kg	0.5 mL containing 0.016 IU insulin
F1 Tablet	20 IU/Kg	Dosage form containing 1.6 IU insulin
F2 Tablet	20 IU/Kg	Dosage form containing 1.6 IU insulin
F3 Tablet	20 IU/Kg	Dosage form containing 1.6 IU insulin
F4 Tablet	-	Dosage form without Insulin
Oral Solution	20 IU/Kg	Solution containing 1.6 IU insulin

(F1 contains: neither TMC nor PEO, F2 contains: PEO but no TMC, F3 contains: both TMC and PEO, F4 contains: all the ingredients of the tablet without PEO, TMC or insulin)

2.10. Measurement of insulin levels in blood plasma

The insulin concentration in serum was measured by a radioimmunoassay kit (Diaplus, USA). The analysis was done according to the manufacturer's protocol.

2.11. Pharmacokinetic data analysis

Pharmacokinetic parameters, including the area under the curve (AUC) of the plasma concentration curve and mean insulin concentration after each administration (C_{ins} : average absorbed insulin at each time point), were obtained directly from the plasma insulin concentrations. The AUCs for each administration were calculated by the linear trapezoidal rule [28]. The

relative bioavailabilities of insulin after each administration were calculated according to the following formula

$$F_R = \frac{AUC_{\text{table t}} \times D_{\text{s.c.}}}{AUC_{\text{s.c.}} \times D_{\text{tablet}}} \times 100\%$$

Where F_R is the relative bioavailability and D is the administered dose.

2.12. Statistical analysis

The data are expressed as means $\pm$ S.D. Non-linear regression was used for ex vivo and in vivo studies. $P < 0.05$ was considered significant.

3. Result and Discussion

3.1. Formulation and design of the GEDD system using factorial design

In Table 2 the results of the factorial design experiments obtained from formulations 1-8 are presented. They were designed as follows: F1 (low acid-base, Ac-Di-Sol, and PEO content), F2 (high acid-base, low Ac-Di-Sol, and low PEO content), F3 (low acid-base, high Ac-Di-Sol, and low PEO content), F4 (low acid-base, low Ac-Di-Sol, and high PEO content), F5 (high acid-base, high Ac-Di-Sol, and low PEO content), F6 (high acid-base, low Ac-Di-Sol, and high PEO content), F7 (low acid-base, high Ac-Di-Sol, and high PEO content), F8 (high acid-base, high Ac-Di-Sol, and high PEO content). The CO_2 production could only be described as slow (+) and satisfactory (++) and the amount of gas produced is also dependent on the amount of gas forming agents in the tablet. As the gas is primarily intended to push the additional excipients of the GEDD system to the absorbing

surface of the gut tissue, the resulting mucoadhesiveness of PEO and TMC is a more important indicator of the efficiency of the GEDD system than its gas formation as such figure 1 gives a schematic representation of the function of the GEDD system.

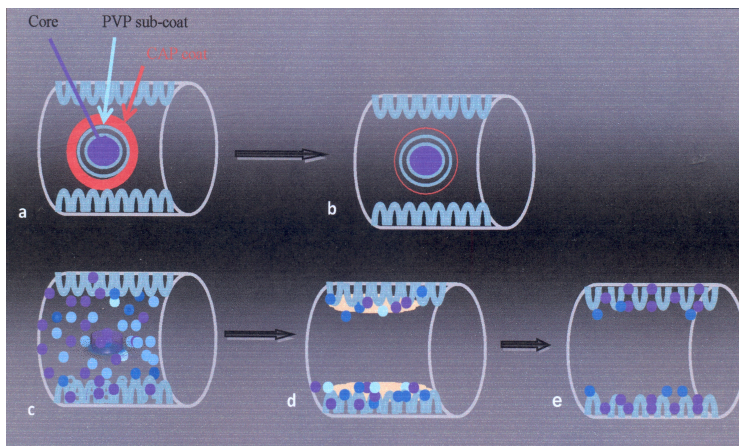


Figure 1. The schematic presentation of a GEDD system.

a) Enteric coated and PVP subcoated GEDD system in the stomach pH 1.2. b) The GEDD system reaching the small intestine at a pH of 6.8. The enteric coating starts to dissolve quickly at this pH. c) about 5 min later, the PVP subcoat is dissolving and the CO₂ gas formation starts to push the insulin drug and the excipients PEO and TMC to the absorbing surface of the intestinal wall (insulin, PEO and TMC) d) After about 10-15 min the tablet has completely dissolved and on the gut surface the mucoadhesive excipients together with insulin are attached. e) TMC induces the opening of the tight junctions and insulin is able to permeate into the underlying tissue by the paracellular route.

Table 2: Results of the factorial design experiment.

Formulation	Response		
	CO ₂ Production (+/++)	Disintegration Time (min)	Mucoadhesiveness F(mN)
F1 (50% Acid-base, 2.5% Ac-Di-Sol, 5.0% PEO)	+	2.5'	2055±244
F2 (70% Acid-base, 2.5% Ac-Di-Sol, 5.0% PEO)	++	1.8'	2014±345
F3 (50% Acid-base, 5.0% Ac-Di-Sol, 5.0% PEO)	+	1.3'	2024.±367
F4 (50% Acid-base, 2.5% Ac-Di-Sol, 10% PEO)	+	2.5'	2433±334
F5 (70% Acid-base, 5.0% Ac-Di-Sol, 5.0% PEO)	++	1.0'	1998±132
F6 (50% Acid-base, 5.0% Ac-Di-Sol, 10% PEO)	++	1.6'	2465±102
F7 (70% Acid-base, 2.5% Ac-Di-Sol, 10% PEO)	+	1.8'	2345±301
F8 (70% Acid-base, 5.0% Ac-Di-Sol, 10% PEO)	++	1.2'	2633±52.85

According to the results, the best gas production, tablet disintegration and mucoadhesion was obtained when the citric acid/sodium bicarbonate (CO₂ producing agents), Ac-Di-Sol (disintegrating agent) and PEO (mucoadhesive agent) contents were 70%, 5.0% and 10%, respectively. The disintegration time is slightly lower in the F8 formulation in comparison to the F5 formulation; however, the latter formula has a more suitable mucoadhesion than F5. Hence F8 formulation was chosen for further investigation as it had enough CO₂ power and Ac-Di-Sol for disintegration

while the mucoadhesive property of the tablet was strong enough for attaching the system to the intestinal lumen.

3.2. In vitro insulin release from the GEDD system:

The results of insulin release from the uncoated tablets in PBS buffer pH 6.8 were as follows: First a burst release was observed in the first 5 minutes where about 85% of the insulin was released into the buffer solution. After 10 minutes all insulin has been released in the medium (data not presented). This test was done to detect and eventually eliminate a possible interaction of the PEO polymer with insulin. The results clearly indicate that no such interaction has taken place.

Insulin release from the enterically coated tablets was also investigated both in the acidic medium of stomach at 0.1N HCl followed by pH 6.8 buffer representing the small intestine. In the acidic medium, only about 3% insulin was released after 2 hours which suggests that the enteric coat was sufficiently protecting the tablets at low pH values (Fig 2). Figure 2 also presents the percentage of insulin released in PBS at pH 6.8. Accordingly, only a small amount of insulin was released in the first 10 minutes and after 15 minutes a burst release of approximately 80% of insulin was observed. This corresponds with the time required for the enteric coat to be dissolved at pH 6.8. In 20 minutes almost 100% of the insulin was released from the GEDD system.

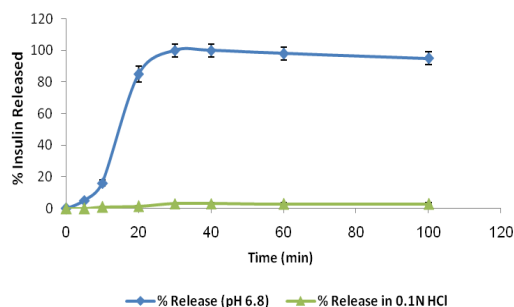


Figure 2. Percent of insulin released from the enterically coated GEDD tablet 2hrs in 0.1NHCl followed by PBS buffer pH 6.8 (n = 3).

3.3. Mucoadhesion of PEO after its release from the GEDD system to the sheep's intestine

The movement in the intestine is determined by 2 major forces: a) the horizontal force and b) the vertical force. The horizontal force is in the direction of the intestine to the rectum and pushes the food and the intestinal contents forward along the intestine. The vertical force is the contraction movements of the intestine [27]. For the drug delivery to be effectively attached to the intestine it preferentially should overcome the horizontal movements. Hence in this study a horizontal force was applied to evaluate the ability of PEO after its release from the GEDD tablets to attach to the intestinal wall. The results show that for the optimal formulation (formulation #8 in Table 1) a force of 2633 ± 53 mN was needed to detach the tablets remnants from the mucous membrane which is sufficiently high to easily allow the delivery systems to stay long enough to the gut wall for the insulin release.

3.4. The CO₂ enhancing properties in rabbit's intestine

Figure 3 presents the enhancing effect of the CO₂ on the insulin transport across the rabbit's intestinal tissue. The apparent permeability values of insulin in the absence and presence of CO₂ were $2.5 \pm 1.2 \times 10^{-7}$ (cm/sec) and $7.5 \pm 1.6 \times 10^{-7}$ (cm/sec), respectively ($n=3$, $p<0.05$). Previous studies by Eichmann and Robinson [25] using hydrophilic drugs have shown similar results. As was indicated by their studies the increased drug transport is most probably due to the mechanical effect of CO₂ on opening of the tight junctions and increasing the paracellular pathway. Moreover, these authors have shown that CO₂ has the ability to decrease the transepithelial resistance (TEER) across the duodenal tissue with no apparent toxic effect indicated by the results of the MTT test. However it should be kept in mind that the CO₂ effect in increasing the paracellular transport of insulin across the intestine is a desirable side effect for our system. Primarily the CO₂ production is aimed to push the mucoadhesive tablet excipients to the absorbing surface of the gut wall.

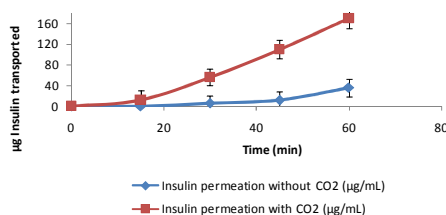


Figure 3. Insulin permeation across rabbit's intestine in the presence and absence of CO₂ ($n = 3$).

3.5. The effectiveness of the GEDD system for inducing insulin permeation across the sheep's intestine (ex vivo studies)

The result of the ex vivo studies are presented in Figures 4 and 5. In this study, the effect of chitosan and TMC as permeation enhancers of insulin across the sheep's intestine was investigated. According to these results, TMC with a higher positive surface charge shows a higher enhancing activity in comparison with chitosan. Without an enhancer only 1% of insulin was transported across the intestinal epithelium. With chitosan this amount was increased to about 4%, however, with TMC an increase of 7% in the insulin transport was observed. These data corresponds with our previous studies in Caco-2 cells [29] where it was shown that amongst different chitosan derivatives used, TMC showed the highest permeation enhancing effect. Moreover, the effect of the GEDD system in the transport of insulin in sheep's intestine was investigated. Figure 5 presents the results obtained from the ex vivo tests done in sheep's intestine using free insulin as the negative control (f1), free insulin in the presence of the TMC in the form of the solution (f2), the GEDD system with insulin in the absence and presence of the TMC (f3 and f4, respectively). According to figure 5 and in agreement with the ex vivo study, only about 1% of the free insulin was transported across the intestinal epithelium. The insulin containing GEDD tablet showed an increased permeation of about 3 times higher in comparison with the free insulin solution. This increase in transport can also be attributed to the enhancing effect of the CO₂ in mechanically opening the tight junction and increasing the paracellular transport of the insulin across the sheep's intestinal epithelium. The transport of insulin in the presence of the TMC in solution form resulted in a higher permeation in comparison to free insulin or the delivery system without the TMC. This indicates that the enhancing effect of TMC is more pronounced than that of the CO₂ gas. The

best enhancing effect was observed with the insulin and TMC containing GEDD tablets. Insulin transport was significantly higher ($p<0.05$) than the other forms and was found to be about 7%. This increase in insulin permeation may be due to the synergistic effect of both the CO_2 and TMC in the form of mechanical and chemical enhancement, respectively. The apparent permeability for insulin using the GEDD tablet in the presence of TMC had the highest value of $2.7\pm0.18\times10^{-6}$ (cm/sec) which is significantly higher than the P_{app} of free insulin $2.1\pm0.03 \times 10^{-7}$ (cm/sec). The GEDD tablet containing only insulin had a P_{app} of $4.4\pm0.83 \times 10^{-7}$ (cm/sec) and free insulin with soluble TMC had a P_{app} $4.1\pm0.65 \times 10^{-7}$ (cm/sec), respectively ($n=3$, $p<0.05$).

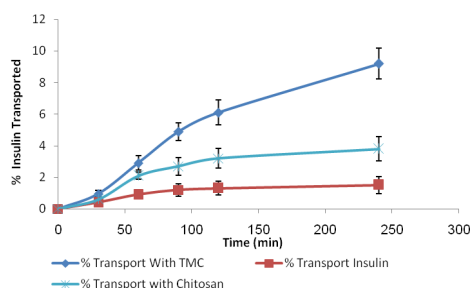


Figure 4. The effect of chitosan and TMC on insulin transport across the sheep's intestine ex vivo ($n= 3$).

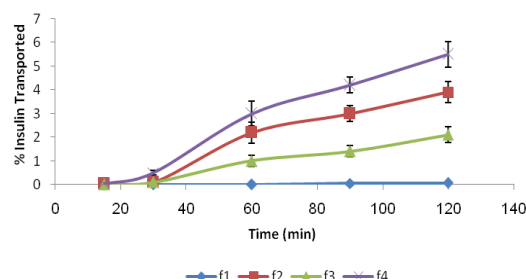


Figure 5. Effect of the GEDD system on the transport of the insulin across the sheep's intestine (n=3).

3.6. The in vivo studies

Table 1 contains all relevant data about the administration of the different insulin formulations used for the in vivo study. The mean plasma insulin concentration versus time profiles obtained after s.c and peroral administration of the different oral dosage forms are presented in Figure 6. S.c. injection showed a rapid increase in insulin concentration up to $6.34 \pm 1.7 \mu\text{IU}$ within 30 minutes post administration followed by a steady state and finally a drop in insulin concentration. The reason for a rapid insulin increase in plasma could be due to the presence of numerous endothelial capillaries present at site of administration in the rabbit. The insulin molecules can quickly diffuse through the endothelial layer and result in an increase plasma concentration. In formulation F1, without TMC or PEO, as expected no significant increase in insulin plasma concentration was detected. This could be due to the presence of the proteolytic enzymes in the intestine that can inactivate the protein as soon as it is released in the intestine. Moreover, the large molecular size of the insulin and its hydrophilic properties are factors that limit the passage of insulin through the intestinal epithelium resulting in low plasma concentration. The low

amount of insulin detected in plasma could be due to the effect of CO₂ gas produced that may act as a mechanical enhancer to open the tight junctions. In formulation 2, containing PEO and no enhancer, a significant increase in insulin concentration is observed in plasma 30 minutes post administration as depicted in Figure 6. Accordingly, it can be concluded that the transit time of the dosage form in the stomach of the fasting rabbits was very brief and once in the intestine, the enteric coat was quickly dissolved. As was presented in Figure 2, the insulin was released from the enteric coated tablets in approximately 20 minutes at pH 6.8 which correlates very well with the in vivo results. Once the enteric coat has dissolved in the intestine, the PEO will interact with the mucus layer and prolong the insulin presence at the site of the absorption. The presence of PEO can only increase the residence time of the insulin at the site of the absorption and the large, hydrophilic insulin molecule still has to overcome epithelial barrier for transport across the membrane. Hence only a small increase in the amount of insulin plasma concentration is observed and a steady state is reached very quickly followed by a drop in the concentration of insulin. In formulation 3, (the complete GEDD tablet formulation) containing both the TMC and PEO, the initial part of the graph followed the same trend as in F2 due to the presence of the PEO. However, a lag time of approximately 60 minutes is needed to for the released TMC to completely dissolve and open the tight junctions for the paracellular transport of the insulin. Also a good correlation is observed between these results and the ex vivo studies, depicted in Figure 4, where it was shown that after 90 min the amount of insulin transported across the membrane was increased and reached its maximum at 240 min. Accordingly, one can conclude that the presence of both TMC and PEO is necessary for insulin transport across the intestinal membrane: PEO with its mucoadhesive characteristic can prolong the

insulin transit time at the site of absorption and TMC as permeation enhancer can act on opening the tight junctions and increasing the paracellular transport of the insulin along the enterocytes. The administration of oral insulin as negative control resulted in no significant insulin plasma concentration (data not shown). This is mainly due to degradation and inactivation of insulin in the stomach and intestine. The oral administration of placebo tablets to rabbits indicates no significant increase in insulin concentration in plasma concentration which indicates that the stress level had no effect on the results obtained in this study (data not presented). Mean insulin concentrations in plasma C_{ins} after F2 and F3 administration were $2.3\mu\text{U/mL}$ and $4.8\mu\text{U/mL}$, respectively which are significantly higher than C_{ins} after F1 administration ($1.3\mu\text{U/mL}$). These results indicate that F2 and to a higher degree F3 formulations are able to increase the intestinal absorption of insulin. The relative bioavailabilities of insulin in rabbits were calculated to be $0.2\%\pm 0.1$, $0.6\pm 0.2\%$ and 1.1 ± 0.4 using the F1, F2 and F3 formulations, respectively. These results indicate that F2 and F3 tablet (containing only PEO and PEO + TMC, respectively) show a 3 fold and 5.5 fold enhancements in comparison to the F1 tablet containing no PEO or TMC. The bioavailability of insulin in vivo is more pronounced when the formulations were compared to the oral insulin (data not presented). Moreover, these values are expected to be higher with diabetic induced rabbits. Hence it is recommended to either use diabetic induced animals or a non-endogenous peptide for a more precise evaluation of the potency of the delivery system.

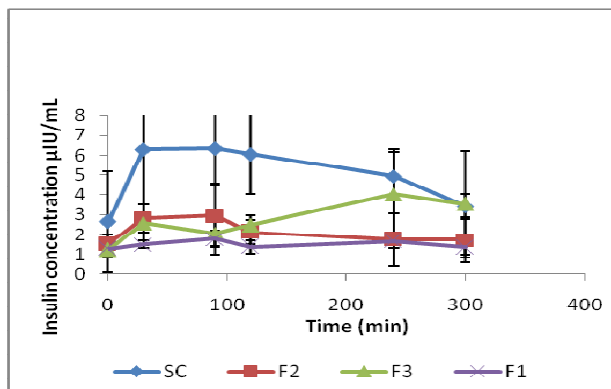


Figure 6. In vivo studies of insulin absorption in rabbits using different GEDD formulations (n= 6).

Conclusions

In this study a novel Gas Empowered Drug Delivery (GEDD) system was designed for oral insulin delivery to the small intestine. The designed delivery system has shown to be able to push by the CO₂ formation of the mucoadhesive excipients PEO and TMC of the enteric coated tablet to the absorbing gut membrane and to have consecutively both mucoadhesion and permeation enhancing effects. While the permeation enhancing effect of the system is thought to be due to both mechanical enhancement resulting from the CO₂ gas and the chemical enhancement of the TMC, the mucoadhesion is a result of the PEO polymer used in the formulation. This bioactive polymer is used to deliver peptide drugs to the mucous membrane by attaching to the gut wall. The GEDD system developed so far was shown to

enable an insulin transport of about 7.0% of the applied dose ex-vivo. The bioavailabilities of insulin in vivo in rabbits were shown to be 0.2% $\pm$ 0.1, 0.6 $\pm$ 0.2% and 1.1 $\pm$ 0.4% using the formulation containing a) no PEO or TMC (F1), b) formulation containing PEO alone (F2), and c) formulation containing both PEO and TMC (F3), respectively. Further animal studies using either big animals such as pigs of about 40kg body weight or humans are required for full assessment of the effect of the GEDD system in-vivo. Additionally the GEDD system developed so-far was made in lab-scale format. Industrial scale production (which is in progress) with optimized enteric coating and with other hydrophilic drugs like bisphosphonates or low molecular weight heparin (LMWH) will further improve and increase the versatility of the system.

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Summary

Oral drug delivery is the most convenient and preferred route for drug administration. Novel developments in biotechnology and advances in molecular biology have led to the introduction of a number of natural and synthetic peptides into the pharmaceutical markets specifically intended for the treatment of chronic diseases.

Amongst these peptides, insulin has attracted the most attention due to an increasing number of diabetic patients worldwide. Insulin, a large hydrophilic macromolecule as hexamer, has been used as the model drug in many of the recently published peroral drug delivery systems. However many of these delivery system are only designed for peptide release. But peptide release is not the primary challenge: it is to achieve its reproducible absorption into the systemic circulation. Hence the following points are the mandatory key elements a successful delivery system must fulfill:

- 1) Drug survival in the acidic medium of the stomach and preventing the proteolytic enzymes attack in the intestine
- 2) Inhibition of the brush border proteases
- 3) Attachment to the intestinal wall and increase the residence time of the delivery system
- 4) Opening the tight junctions and allowing paracellular transport at the correct time.

Several delivery systems have been designed to meet as much as possible the above given parameters. In this thesis, a novel Gas Empowered Drug Delivery (GEDD) system was developed using polyethylene oxide (PEO) as mucoadhesive agent and trimethyl chitosan (TMC) as polymeric permeation enhancer with the ability to specifically open the tight-junctions between the enterocytes. The designed system introduces novel approaches for intestinal absorption of insulin used as the model drug. The main advantages of this system are i) its ability to protect the hydrophilic drug from the acidic environment of the stomach and the proteolytic enzymes of the intestine by using enteric coatings for protection in the stomach, and the CO₂ gas formation for protection of the drug in the intestinal environment, ii) the immediate release of the peptide at the specific targeting site using the produced CO₂ gas to propel the drug and the mucoadhesive polymers PEO and TMC to the absorbing surface, iii) prolonging the transit time of the dosage form in the intestine by their firm attachment to the luminal wall, iv) opening the tight junctions using trimethyl chitosan (TMC) as permeation enhancer and v) increasing the peptide drug transport along the paracellular pathway. Furthermore this system can be easily mass produced.

In order to find the optimal polymeric permeation enhancer based on chitosan, a systematic approach has been undertaken in synthesizing a series of chitosan derivatives to evaluate the optimal chemical structure of a polymeric multifunctional permeation enhancer for the paracellular absorption of the peptide drug. Additionally a study has

been performed to investigate the use of nanoparticulate insulin complexes with these quaternary chitosan derivatives in comparison with the free, non- bound chitosan derivatives to optimize insulin delivery and absorption across the intestinal tissue. The results of these investigations are described in the following chapters.

Part I. General introduction and physicochemical aspects

chapter 1 reviews the principles of peroral peptide drug delivery and summarizes the most important oral delivery systems developed in recent years including: gel and liquid state liposomes, microparticles, nanoparticles, superporous hydrogels, and others including the use of mucoadhesive nanosized delivery systems. Moreover, this chapter describes the advantages and disadvantages of the above developed delivery systems and results in the description of a more suitable, easier to produce gas empowered drug delivery (GEDD) system.

The synthesis and characterization of two new chitosan derivatives namely C₂, C₆ 6-amino, 6-deoxy methyl and ethyl chitosan derivatives are described in chapter 2 of this book. The synthesized polymers were characterized using ¹H-NMR and FTIR techniques. The substitution degree of each polymer was calculated to be 50±5%. The zeta potential and the antibacterial activities of these polymers were measured and compared to the chitosan, TMC and triethyl chitosan (TEC). The results indicate that the newly synthesized polymers have higher zeta potential and antibacterial activities in comparison with the original chitosan, TMC or TEC. Hence, these

polymers may be good candidates as permeation enhancer for peptide oral drug delivery.

However latest research results indicate despite the highest zeta potential values of these double quaternized chitosan compounds a reduced ability to open the tight-junctions in comparison with TMC.

Chapter 3 describes the synthesis and characterization of yet another chitosan derivative: Dimethylethyl chitosan (DMEC) using a nucleophilic base substitution method. The new polymer was characterized using ^1H -NMR and FTIR techniques and the substitution degree was calculated to be $50\pm 5\%$.

Part II: Preparation and characterization of insulin nanoparticles using chitosan and its derivatives

Chapter 4 describes the insulin nanoparticle preparation using chitosan and two of its derivatives, namely TMC and Diethylmethyl (DEMC). Nanoparticles were prepared using both the ionotropic gelation and polyelectrolyte complexation (PEC) methods. The nanoparticles were fully characterized for their shape, size, zeta potential and loading efficiency by SEM, zeta sizing and HPLC, respectively. Moreover, the insulin release from the nanoparticles was studied at different pH values of 1.2, 3.0 and 6.8. The antibacterial activity of the nanoparticles was compared with the free polymers using gram positive staphylococcus aureus bacteria. It was concluded in this chapter that the nanoparticles made by the PEC method were

more suitable in comparison with the ionotropic gelation technique with respect to insulin loading and zeta potential.

Part III: In vitro Caco-2 cell studies

The insulin permeation across the Caco-2 cell monolayer was investigated in chapter 5. In this study insulin was used both as nanoparticulate system made by the PEC method and as free form in the presence of free soluble polymer. The insulin transport across the monolayer was measured using HPLC. Transepithelial electrical resistance (TEER) studies revealed that chitosan derivatives and to a lesser degree chitosan, all of them in free soluble form were able to reduce the TEER to about 40% of their initial values. TMC was shown to have the highest reduction in TEER in comparison with other derivatives and resulted in 8% increase in insulin transport to the basolateral side. On the other hand, the nanoparticles had no significant effect in reducing TEER and showed less insulin permeation across the Caco-2 cell monolayer. It was concluded in this chapter that although the nanoparticles had more success in inducing insulin permeation in comparison with the free insulin, the free soluble polymers with their high positive zeta potential were more successful in facilitating insulin permeation across the monolayer. Mass balance studies also indicate that nanoparticles were taken up by the Caco-2 cells but not transcytosed into the basolateral compartment. Finally, trypan blue studies have shown that the cells excluded trypan blue dye after incubation with the polymers and the number of viable cells was estimated to be higher than 95% which

indicates that the mitochondrial activity of the cells was preserved. Hence it was concluded that the enhancing effect of the polymers was not due to the damage to the Caco-2 cell monolayer.

Part IV: The development and characterization of a novel Gas Empowered Drug Delivery (GEDD) system the in-vitro, ex-vivo and in-vivo studies

Chapter 5 deals with the development of a novel peroral drug delivery system for the absorption of peptides in the small intestine. The system was developed using a 2^3 factorial design approach. The delivery system develops CO_2 as power generating force, and contains the mucoadhesive PEO polymer as well as TMC as permeation enhancer. The preparation and enteric coating of the system is described in detail in this chapter. The in-vitro insulin release was studied and it was shown that while the dosage form was acid labile, the insulin was released completely after 30 minutes at pH 6.8. The ex-vivo studies were done in sheep's intestine and revealed that the GEDD system containing TMC as an enhancer showed an increased insulin permeation of about 7% in comparison with the free insulin. Furthermore, studies in the intestine of rabbits were performed ex-vivo to show that the presence of CO_2 gas by itself has a mechanical effect on opening the tight junctions and increased the insulin permeation up to 3 times in comparison to the free insulin. Finally, the results of the in vivo studies in rabbits are presented in this chapter. Accordingly, 3 different GEDD formulations of F1: GEDD system without PEO or TMC; F2: GEDD system with mucoadhesive polymer (PEO); F3: GEDD system with mucoadhesive

polymer (PEO) and the permeation enhancer (TMC) (complete GEDD system) were compared to the s.c. insulin injection. The relative bioavailability values of 0.2 ± 0.1 (S.D.), 0.6 ± 0.2 (S.D.) and 1.0 ± 0.4 (S.D.) were obtained with formulations F1, F2 and F3, respectively in comparison with the s.c. injection. It was concluded from the results that the enhancing activity of the GEDD system is due to a synergistic effect of the CO₂, PEO and TMC. Once in the small intestine the enteric coat is dissolved, the released CO₂ gas pushes the system towards the intestinal wall and the mucoadhesive PEO aid in attaching the system to the lumen. The TMC can then act as the permeation enhancer to open the tight junction and increase the paracellular transport of insulin.

This novel delivery system has promising prospects for the development of a suitable oral peptide drug delivery system. While preliminary studies on the system were done, more formulation studies on industrial scale are required and bioavailability studies have to be done in larger animals like pigs or humans to confirm the results of this investigation. Especially the shape of the delivery system, its residence time in the stomach and how it can be influenced, the homogenous coating with suitable enteric coating materials and how it can be influenced and the drug delivery at the optimal target site has to be further investigated. As insulin was used in these studies as a model drug, this delivery system is not restricted to peptide or protein drugs but can be further developed for the improved permeation of other hydrophilic drugs with poor gut

permeation characteristics like the bisphosphonates or Low Molecular Weight Heparin (LMWH) and other molecules which in many aspects are easier to handle than insulin with regard to their stability and lower molecular weight.

Samenvatting

Geneesmiddelenafgifte via de orale route is de gemakkelijkste en meest geliefde toedieningsroute. Nieuwe ontwikkelingen op het gebied van de biotechnologie en de moleculaire biologie hebben geleid tot het op de markt brengen van een aantal via natuurlijke en synthetisch weg bereide farmaceutische peptiden voor de behandeling van chronische ziekten.

Insuline is het bekendste farmaceutische peptide vanwege het grote aantal diabetes patiënten dat wereldwijd nog steeds toeneemt. Insuline is een hydrofiel macromolecuul dat bestaat uit zes polypeptideketens en veelvuldig gebruikt is als modelgeneesmiddel ter bestudering van perorale geneesmiddelafgiftesystemen, zoals blijkt uit recente publicaties. Veelal zijn dergelijke toedieningssystemen slechts ontworpen voor de afgifte van het peptide. Echter, niet de peptideafgifte is de primaire uitdaging van een dergelijk systeem, maar het bereiken van een reproduceerbare absorptie in de systemische circulatie. Derhalve moet een afgiftesysteem, wil het succesvol zijn, voldoen aan de volgende criteria:

- 1) bescherming van het geneesmiddel tegen afbraak in de zure omgeving van de maag en de proteolytische enzymen in de darm;
- 2) inhibitie van de “brush border” proteases;
- 3) aanhechting aan de darmwand en daarmee verlengen van de verblijftijd van het afgiftesysteem in de darm;

- 4) het openen van de “tight-junctions” en het zorgen voor paracellulair transport over de darmwand op het juiste moment.

Om aan zoveel mogelijk van bovenstaande criteria te kunnen voldoen, zijn verschillende afgiftesystemen ontworpen. Dit proefschrift beschrijft de ontwikkeling van een nieuw door gas aangedreven geneesmiddelafigftesysteem (Gas Empowered Drug Delivery: GEDD), dat bestaat uit het mucoadhesivum polyethyleenoxide (PEO) en de permeatiebevorderaar trimethylchitosan (TMC). TMC is een polymeer dat in staat is om specifiek de tight-junctions tussen enterocyten te openen. Het GEDD-systeem biedt nieuwe mogelijkheden voor de absorptie van het modelpeptide insuline via de darmwand. De belangrijkste voordelen van dit systeem zijn: i) de bescherming van het hydrofiele peptide tegen de zure omgeving van de maag en de proteolytische enzymen in de darm via respectievelijk enterische coating van het afgiftesysteem en de vorming van CO₂ in de darm, ii) de directe afgifte van het peptide op de gewenste plaats in de darm middels het vrijgekomen CO₂, dat het peptide, PEO en TMC naar het absorptieoppervlak drijft, iii) verlenging van de passagetijd van het afgiftesysteem in de darm door middel van een stevige aanhechting aan de darmwand, iv) het door de permeatiebevorderaar TMC geïnduceerde openen van de tight-junctions en v) toename van het insulinetransport via de

paracellulaire route in de darm. Voorts kan dit afgiftesysteem gemakkelijk op grote schaal worden geproduceerd.

Om de beste op chitosan gebaseerde permeatiebevorderaar te kunnen selecteren, is een serie chitosanderivaten gesynthetiseerd, welke zijn geëvalueerd voor wat betreft een optimale chemische polymeerstructuur ter bevordering van de absorptie van peptiderge farmaca via de paracellulaire route. Verder is er een vergelijkende studie uitgevoerd tussen insuline, dat als nanopartikels gecomplexeerd is met de quaternaire chitosanderivaten, en insuline in combinatie met vrij, niet-gebonden chitosanderivaten. Het doel van deze studie was het optimaliseren van de insulineafgifte en absorptie over de darmwand. De resultaten van de bovengenoemde studies zijn beschreven in de volgende hoofdstukken.

Deel I. Algemene inleiding en fysich-chemische aspecten

Dit hoofdstuk bespreekt de principes van perorale systemen voor de afgifte van peptiderge farmaca. Verder wordt er een overzicht gegeven van de meest relevante orale afgiftesystemen die in de afgelopen jaren ontwikkeld zijn, waaronder “gel state” en “liquid state” liposomen, micropartikels, nanopartikels, super-poreuze hydrogelen en mucoadhesieve nano-afgiftesystemen. Tenslotte passeren de voor- en nadelen van dergelijke afgiftesystemen de revue, wat leidt tot de conclusie dat het door gas aangedreven geneesmiddelafigftesysteem (GEDD) het meest geschikte systeem is,

dat bovendien eenvoudiger te produceren is dan de voornoemde systemen.

De synthese en karakterisatie van twee nieuwe chitosanderivaten, namelijk C₂-C₆ trimethyl 6-amino-6-deoxychitosan en C₂-C₆ triethyl 6-amino-6-deoxychitosan, worden besproken in hoofdstuk 2 van dit proefschrift. De gesynthetiseerde polymeren zijn gekarakteriseerd met behulp van de analysetechnieken ¹H Nucleaire Magnetische Resonantie (NMR) en Fourier Transform Infraroodspectroscopie (*FTIR*). De berekende substitutiegraad van beide polymeren is 50±5%. Zowel de gemeten zèta-potentiaal als de anti-microbiële activiteit van de betreffende polymeren bleek hoger te zijn dan die van chitosan, TMC en triethylchitosan (TEC). Derhalve zijn deze twee polymeren goede potentiële kandidaten als permeatiebevorderaars voor de afgifte van peptiderge farmaca via de orale route.

Spijts de hoge zèta-potentiaal van deze dubbel gekwaterniseerde chitosanverbindingen, hebben zeer recente onderzoeksresultaten erop gewezen dat ze in vergelijking met TMC minder goed in staat zijn om tight-junctions te openen. In hoofdstuk drie wordt de synthese en karakterisatie van een ander chitosanderivaat, dimethylethylchitosan (DMEC), beschreven. DMEC werd gesynthetiseerd middels de nucleofiele base-substitutiemethode. Dit polymeer is eveneens gekarakteriseerd met behulp van ¹H-NMR en FTIR en de berekende substitutiegraad bleek ook 50±5% te zijn.

Deel II. Bereiding en karakterisatie van insuline-nanopartikels gebaseerd op chitosan en chitosanderivaten

Hoofdstuk 4 beschrijft de bereiding van insuline-nanopartikels door gebruik te maken chitosan en -derivaten (TMC en DEMC). De gebruikte methoden ter bereiding van de nanopartikels waren ionotrope gelering en polyelectrolietencomplexatie (PEC). De verkregen nanopartikels zijn volledig gekarakteriseerd wat betreft hun vorm, grootte, zèta-potentiaal en beladingsefficiëntie met behulp van respectievelijk Scanning Electronen Microscopie (SEM), Zètasizer en Hoge Druk Vloeistofchromatografie (HPLC). Tevens is de insulinevrijgifte uit de nanopartikels bestudeerd bij verschillende pH-waarden (1,2, 3,0 en 6,8). De anti-bacteriële activiteit van de nanopartikels voor de Gram positieve bacterie *Staphylococcus aureus* is vergeleken met die van de vrije polymeren (chitosan, TMC en DEMC). Op basis van de uitkomsten van de zèta-potentiaalmetingen en insulinebelading, is geconcludeerd dat de PEC-methode meer geschikt is voor de bereiding van insuline-nanopartikels dan de ionotrope geleringsmethode.

Deel III. *In-vitro* Caco-2-celstudies

De permeatie van insuline over een monolaag van Caco-2-cellen is onderzocht in hoofdstuk 5. In deze studie zijn insuline nanopartikels, bereid via de PEC-methode, gebruikt, alsook insuline in aanwezigheid van polymeren, beide in vrije oplosbare vorm. HPLC is gebruikt om het transport van insuline over de monolaag te analyseren. Studies van de trans-epithele elektrische weerstand (“transepithelial electrical resistance”, TEER) wijzen uit dat

chitosanderivaten en in mindere mate chitosan, beide in vrije vorm, in staat bleken om de TEER met 40% te doen verlagen ten opzichte van de uitgangssituatie. Voorts is voor het derivaat TMC aangetoond dat het de grootste verlaging van de TEER kan bewerkstelligen, wat heeft geresulteerd in een toename van 8% van het insulinetransport naar de basolaterale zijde van de monolaag. Echter, de nanopartikels bleken de TEER niet significant te verlagen en lieten een verminderd insulinetransport over de Caco-2-cellaag zien. Op basis van deze resultaten is geconcludeerd dat, alhoewel de nanopartikels een hogere permeatie van insuline induceerden dan vrij insuline, de vrij oplosbare polymeren met hun hoge positieve zèta-potentiaal meer succesvol bleken in het faciliteren van insulinetransport over de monolaag. Massabalansstudies duiden er ook op, dat nanopartikels weliswaar werden opgenomen door de Caco-2-cellen, maar niet via transcytose in het basolaterale compartiment terechtkwamen. Tenslotte hebben studies met trypaanblauw aangetoond dat deze kleurstof niet in de Caco-2-cellen terechtkwam na incubatie met de polymeren en dat het aantal levende cellen groter dan 95% was. Dit is een indicatie voor het behoud van de mitochondriale activiteit van de cellen. De conclusie was dan ook, dat het permeatiebevorderende effect van de polymeren niet te wijten is aan beschadiging van de monolaag van Caco-2-cellen.

Deel IV. De ontwikkeling en karakterisatie van een nieuw door gas aangedreven geneesmiddelaafgifte (GEDD) systeem middels *in-vitro*, *ex-vivo* en *in-vivo* studies

Hoofdstuk 5 gaat in op de ontwikkeling van een nieuw peroraal geneesmiddelaafgiftesysteem voor de absorptie van peptiden in de dunne darm. Het betreffende systeem is ontwikkeld door gebruik te maken van een 2^3 "factorial design" methode. Het systeem vormt CO₂ als drijvende kracht en bevat zowel het mucoadhesieve polymeer PEO als de permeatiebevorderaar TMC. Dit hoofdstuk beschrijft in detail de enterische coating van het systeem. Zoals uit de *in-vitro* afgiftestudies blijkt, is het insuline volledig afgegeven na 30 minuten bij een pH van 6.8, terwijl het systeem niet stabiel is in zuur milieu,. De *ex-vivo* studies zijn uitgevoerd in een geïsoleerde darm van een schaap. Deze studies wezen uit dat het TMC bevattende GEDD-systeem leidde tot een toename van de insulineopname van 7% ten opzichte van vrij insuline. Tevens zijn er *ex-vivo* studies in een konijnendarm gedaan om aan te tonen dat de aanwezigheid van CO₂-gas op zichzelf reeds een mechanisch effect heeft op de opening van de tight-junctions en een drievoudige toename van het insulinetransport over de darmwand te zien geeft in vergelijking met vrij insuline. Tenslotte worden de *in-vivo* studies in ratten gepresenteerd in dit hoofdstuk. Drie verschillende GEDD-formuleringen (F1: GEDD-systeem zonder PEO en TMC; F2: GEDD-systeem met PEO; F3: GEDD-systeem met PEO en TMC (compleet systeem)) werden vergeleken met een subcutane (s.c.) injectie van insuline. De relatieve biobeschikbaarheidswaarden

verkregen voor de verschillende systemen ten opzichte van s.c. insuline zijn (gemiddelde $\pm$ standaarddeviatie): $0,2 \pm 0,1$ (F1), $0,6 \pm 0,2$ (F2) en $1,0 \pm 0,4$ (F3). Op basis van deze resultaten is geconcludeerd dat het absorptiebevorderende effect van het GEDD-systeem toegeschreven kan worden aan een synergistisch effect van CO₂, PEO en TMC. Op het moment dat de enterische coating in de dunne darm opgelost is, komt het CO₂ gas vrij, waardoor het systeem naar de darmwand gedreven wordt, met als gevolg dat het mucoadhesivum PEO met de darmwand in aanraking komt, zodat het systeem aan het lumen blijft kleven. Vervolgens kan het TMC als penetratiebevorderaar fungeren en de tight-junctions openen, wat leidt tot een toename van het paracellulaire transport van insuline.

Dit nieuwe GEDD-systeem is een veelbelovende kandidaat voor de ontwikkeling van een geschikt oraal afgiftesysteem van peptiderge farmaca. Alhoewel er voorstudies op het systeem zijn gedaan, zijn uitgebreidere formuleringsstudies op industriële schaal noodzakelijk. Tevens dienen er biobeschikbaarheidsstudies in grotere dieren, zoals varkens, alsook in mensen uitgevoerd te worden om de in dit proefschrift beschreven resultaten te bevestigen. Met name de vorm van het afgiftesysteem, de verblijftijd in de maag, een homogene coating met geschikte enterische coatingsmaterialen en de beïnvloeding van deze parameters, alsmede het afgeven van de werkzame stof naar de optimale plaats van werking, moeten verder worden bestudeerd. Hoewel insuline is gekozen als modelfarmacon in deze studies, blijft dit geneesmiddelafigftesysteem niet beperkt tot eiwitten en peptiden, maar kan verder ontwikkeld worden om de

penetratie te bevorderen van andere hydrofiele farmaca, zoals bifosfonaten of laag-moleculair heparine, die in vele opzichten gemakkelijker te formuleren zijn dan insuline, omdat ze stabiel zijn en een lager molecuulgewicht hebben.

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List of Publications

Scientific Articles

1. A.M.M.Sadeghi, M.Amini, M.R.Avadi, F.Seidi, M.Rafiee-Tehrani, H.E. Junginger, Synthesis, characterization and antibacterial effects of trimethylated and triethylated 6-NH₂-6-Deoxy Chitosan; J. Bioact. & Compat. Polym. 23(2008) 262-275.
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A. Abstracts

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Curriculum Vitae

Assal M.M. Sadeghi was born on the 23rd of March of 1972 in Tehran, Iran. She was awarded an Honours High School Diploma from Carson Graham High School in Vancouver, Canada in 1990. She was accepted in the Faculty of Science of the University of British Columbia (U.B.C.) where she studied in the field of Microbiology and has conferred a degree of Bachelors of Science in May of 1994. She was then accepted in the Faculty of Graduate studies in the department of Pathology and Laboratory Medicine, University of British Columbia for the Masters programme in September of 1994. The title of her thesis was A Biological Mutant of Cocksackievirus B3 and its Pathogenesis in Mice. She has taken the defence examination on June of 1997 and obtained an A standing on the exam. She has conferred the degree of Masters of Science in November of 1997. The result of her study was published in the Journal of Virology in 2004.

She has then started to work at Hakim Pharmaceutical Company in Tehran, Iran as a consultant in the department of Microbiology for 15 months. She was then moved to the R&D department and worked as an assistant manager on numerous projects. In September of 2006 she was also given the title of project manager of the company where she had to closely follow all the projects and parties involved in the company.

In December 2005, she was accepted as a Ph.D. researcher in Leiden University, Leiden, The Netherlands. She has done most of her research in Tehran, Iran under close supervision of her promoters Prof. H.E. Junginger and Prof. M. Rafiee-Tehrani. She has presented her PhD project in the area of peroral peptide delivery at Controlled release symposium (CRS) in July 2006 (Vienna, Austria), July 2007 (Long Beach, U.S.A.) and July 2008 (New York, U.S.A.); Pharmaceutical Sciences World Congress (PSWC)

(Amsterdam, The Netherlands) in April of 2007; the 5th International Postgraduate research symposium on pharmaceutics (IPORSIP) (Istanbul, Turkey) in September of 2007; and the 6th world meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology (PBP) in Barcelona, Spain April 2008. She was awarded with the best poster presentation in Sep 2007 at IPORSIP conference.

A patent for her studies in developing a novel peroral delivery system has been filed by Lupin Company (Pune, India) in June 2008.

Assal M.M. Sadeghi will continue with her scientific carrier with Hakim Pharm. Co. as part of a new research group on drug research and development.