Chitosan-based delivery systems for protein therapeutics and antigens

Maryam Amidi, Enrico Mastrobattista, Wim Jiskoot, Wim E. Hennink

Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, P.O. Box 80082, 3508 TB Utrecht, The Netherlands
Division of Drug Delivery Technology, Leiden/Amsterdam Center for Drug Research (LACDR), Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

Abstract

Therapeutic peptides/proteins and protein-based antigens are chemically and structurally labile compounds, which are almost exclusively administered by parenteral injections. Recently, non-invasive mucosal routes have attracted interest for administration of these biotherapeutics. Chitosan-based delivery systems enhance the absorption and/or cellular uptake of peptides/proteins across mucosal sites and have immunoadjuvant properties. Chitosan is a mucoadhesive polysaccharide capable of opening the tight junctions between epithelial cells and it has functional groups for chemical modifications, which has resulted in a large variety of chitosan derivatives with tunable properties for the aimed applications. This review provides an overview of chitosan-based polymers for preparation of both therapeutic peptides/protein and antigen formulations. The physicochemical properties of these carrier systems as well as their applications in protein and antigen delivery through parenteral and mucosal (particularly nasal and pulmonary) administrations are summarized and discussed.

© 2009 Elsevier B.V. All rights reserved.
1. Introduction

Recombinant DNA technology has resulted in the availability of a large number of therapeutic proteins and protein-based antigens, which are almost exclusively administered by parenteral injections or infusions. This is rather inconvenient for patients, usually requires medical personnel and/or hospitalization and is therefore expensive. Consequently, alternative routes for the administration of protein pharmaceuticals including oral, buccal, vaginal, nasal and pulmonary administration are presently explored. In particular, the respiratory tract including the nasal mucosa and pulmonary epithelium are attractive sites for the delivery of therapeutic proteins and antigens, because of their large absorptive surfaces, especially in the lungs, and low proteolytic activity [1–4]. Importantly, pulmonarily and intranasally administered antigens can induce both local and systemic immune responses. A local immune response can be induced by efficient (targeted) delivery of an antigen to the mucosal-associated lymphoid tissue (MALT) as well as antigen-presenting cell (APC) lining between the respiratory epithelial cells.

The inherent physical, chemical and proteolytic instability and the large size are major factors for poor absorption of therapeutic proteins and antigens across mucosal surfaces. Moreover, soluble antigens are hardly taken up by the MALT. The nasal mucosa and the central respiratory tract are covered by a mucous coat, which is constantly removed by mucociliary clearance. This in turn causes an additional obstacle for the mucosal delivery of proteins and antigens [3,5]. To overcome these barriers, protein therapeutics and antigens should be formulated with proper excipients that protect them against degradation, prevent their rapid elimination from administration sites and enhance their absorption and/or uptake across epithelial barriers. Because the nasal mucosa and the lungs have their own specific barriers in terms of accessibility, epithelial cell type and the presence or absence of a mucus layer in different parts of the respiratory tract, the properties of delivery systems for therapeutic proteins and antigens have to be tailored according to the route of administration.

In recent years, soluble and particulate carriers based on chitosan and its derivatives have received particular interest for the delivery of proteins via mucosal routes [6–17]. Chitosan-based polymers are mucoadhesive and are capable of opening the tight junctions between epithelial cells [6–17]. Both properties aid to stimulate the absorption of protein/antigen and/or uptake by epithelial cells and M-cells of the antigen-loaded chitosan-based nano and microparticles. Chitosan has functional groups (OH and NH₂) which allow chemical modification of the molecule and its physical properties. In recent years, many chitosan derivatives have been synthesized to enhance its solubility, mucoadhesiveness and/or its immunostimulatory properties. This article reviews chitosan-based systems for delivery of protein therapeutics and antigens via non-parenteral (in particular nasal and pulmonary) and through parenteral routes of administration, of which a few studies have been reported recently. An overview of mucosal tissues (nose and lungs), mechanisms involved in absorption and initiation of immune responses as well as general issues for delivery of therapeutic proteins and antigens are discussed. Our review further focuses on recent developments of chitosan-based systems for antigens and therapeutic proteins via mucosal and parenteral routes and protein/antigen formulation aspects. Oral delivery of proteins/antigens using chitosan-based polymers [18–21] and parenteral delivery of therapeutic proteins using chitosan-based hydrogels, discussed in the contribution of Bhattarai et al., are not addressed in this review article.

2. Non-parenteral protein and antigen delivery

Significant advances in biotechnology have resulted in the discovery and availability of therapeutic proteins as well as protein-based antigens. Most proteins and vaccines are delivered via parenteral routes, because of their low bioavailability and/or poor immunogenicity when administered via non-parenteral routes of administrations [22]. However, injections are inconvenient for patients and injectable formulations normally have high production costs. Therefore, in recent years considerable research has been focused on non-invasive routes, such as mucosal (oral, buccal, nasal, pulmonary and vaginal) and (trans)dermal, for delivery of proteins and vaccines [19–30]. However, the delivery of these macromolecules via these non-invasive routes remains a challenge because of their poor absorption and their susceptibility to enzymatic degradation. Because of the latter, both the nose and the lungs are particularly attractive sites as the local proteolytic activity is relatively low [2,23–25]. The nose is easily accessible but has a relatively small absorptive surface area (150 cm²), whereas the lungs have a large surface area (~75 m²), extensive vasculature and a thin membrane, but are less well accessible. Importantly, intranasally and pulmonarily administered vaccines can induce both systemic and local immune responses [2,26,27]. The potential of nasal and pulmonary delivery of macromolecular therapeutics and vaccines is very high, although, as pointed out in the next sections, a number of challenges still has to be overcome.

3. The respiratory epithelium and mucosal surfaces

The second barrier of the respiratory tract, after the mucus/surfactant layer, that proteins encounter is a monolayer of epithelial cells, comprising two completely different epithelia, namely, the airway and alveolar epithelium. The airway epithelium consists of pseudostratified columnar ciliated cells, basal cells and mucus-secreting goblet cells (Fig. 1). The epithelial cells are tightly sealed by intercellular junctions (named as the ‘tight junctions’), which make them essentially impermeable for macromolecules. This pseudostratified columnar epithelium is present in the nasal cavity, the trachea, the bronchi and the bronchioles, and is covered by a thick mucus layer, which is propelyl by beating cilia to the glottis and is removed from the airways [23]. The thickness of both the epithelium and the mucus layer decreases from the upper respiratory tract toward the lower part.

The epithelium at the mucosal surfaces of the respiratory tract is associated with the immunological active mucosal-associated lymphoid tissue (MALT) [28–30], which is an important site for antigen sampling and represents a major entry portal for pathogens. The mucosal lymphoid follicles in the nasal-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT) are homing several immunological cells such as dendritic cells (DCs), T and B lymphocytes, which contribute in induction of immune responses against pathogens that enter through the respiratory mucosa [27]. The epithelial cells covering the NALT and BALT, follicle-associated epithelium (FAE) are also equipped with micro-fold cells (M-cell) which are involved in uptake, transport and presentation of antigens present in the respiratory lumen. Transport of antigens to the subepithelial compartment (submucosa) within the MALT is a prerequisite for inducing local and subsequent systemic immune responses [31–33].

In the distal respiratory tract, the epithelium becomes less columnar and tight and the alveolar epithelial cells are rather thin (0.1–0.5 µm) and devoid of a mucus barrier. The respiratory epithelium at
the alveolar region comprises two cell types, namely extremely broad and thin cells (called Type I cells) and compact surfactant-secreting Type II cells [3,34] (Fig. 2). The alveolar epithelium of the lung is opposed to extensive capillaries where gas-exchange and absorption of molecules occur.

3.1. Absorption of proteins and antigens/vaccines through the respiratory epithelium

Macromolecules can pass the respiratory epithelia via two different pathways, namely, paracellularly through tight junctions between the cells or transcellularly by endocytosis [3,23] (Fig. 1). As pointed out in the previous section, the airway epithelium has firmly closed tight junctions, which makes them essentially impermeable for macromolecules [23]. Moreover, the mucus layer, which covers the upper respiratory (nasal cavity, conductive airways) and the central respiratory tract, as well as the clearance mechanisms in these regions are extra barriers that limit the uptake of proteins in the respiratory lumen. Therefore, agents, called ‘penetration enhancers’, that ideally reversibly open the tight junctions are added to the formulations to facilitate the transport of macromolecules across the epithelium to the sub-mucosa and subsequently to the systemic and/or the lymphatic circulation. Contrary to the upper airways epithelium, at the distal airways epithelium, just before the alveoli, the tight junctions between the epithelial cells are loose and macromolecules up to 22 kDa can passively diffuse via paracellular pathways [3,4,35–37]. Paracellular transport may also occur at the tricellular junctional complex (TJC), the specific spots on the circumference of both

Fig. 1. Schematic lateral view of airway epithelia at the upper/central respiratory tract.

Fig. 2. Schematic lateral (A) and surface view (B) of the alveolar epithelia at the distal respiratory tract (adapted from Patton et al.) [3]. (Reproduced with permission of Elsevier.)
endothelial and epithelial cells. It has been shown that at the TJC there is a gap, through which small macromolecules can diffuse to the systemic circulation. Moreover, there is no mucus barrier and consequently no mucociliary clearance occurs which is additionally advantageous for protein absorption.

Besides the paracellular route by which relatively small proteins are absorbed, larger proteins can be absorbed from the respiratory tract by the transcellular pathway, which includes both nonspecific and receptor-mediated endocytosis [4,37,38]. The transport of antibodies and plasma proteins, like albumin, across the epithelial barrier from the systemic circulation to the respiratory lumen and vice versa occurs by receptor-mediated endocytosis. On the other hand, it has been shown that macromolecular therapeutics pass the epithelial barrier by nonspecific endocytosis [3,39,40]. Both type I alveolar and endothelial cells contain vesicles, called caveolae, which are assumed to mediate transport of peptides and proteins. For small macromolecules (<22 kDa) it is uncertain whether they are absorbed primarily paracellularly or via transcytosis through caveoli [4,37].

3.2. Induction of immune responses in the respiratory tract

At the mucosal surfaces, M-cells of the FAE, the epithelium overlying NALT and BALT, are involved in the uptake and transport of antigens, especially in particulate form, to the sub-mucosa where antigen-presenting cells (APCs) and B- and T-cells are present [27,29,31–33,41]. This results in generation of IgG as well as secretory IgA antibodies. The latter cross the epithelial cells and contribute to protection of mucosal sites from further binding and entry of pathogens (Fig. 3). However, the exact mechanisms of immune induction, such as the function of BALT and antigen sampling by M-cells, are not fully unraveled, yet. The presence of BALT in humans is well established. BALTs are not well organized structures in human lungs and it is known that lungs can develop classical BALT structures when exposed to a high antigen load [42].

At the alveolar region, phagocytic cells like alveolar macrophages and DCs are crucial for protection and induction of immunity against particulate antigens [43–46]. They phagocytose, process, present and translocate the antigen to the lymph nodes and subsequently activate naïve T cells and B lymphocytes to induce immune responses [44–47].

4. Practical issues for nasal delivery of therapeutic proteins and vaccines

The nasal cavity is an attractive route for administration of proteins and antigens but there are important issues to be taken into account for preparation of nasal formulations. Tightly impermeable epithelial cell layers in the nasal cavity and also covering NALT as well as the short residence time of formulations in the nasal cavity due to mucociliary clearance are severe limitations for protein/vaccine delivery in the upper respiratory tract [23,25,48]. Proper delivery systems are therefore needed to improve absorption/uptake of proteins/protein-loaded particles from the epithelium and prevent rapid elimination of the formulations from nasal cavity. Mucoadhesive delivery systems with and absorption-enhancing properties are needed to improve residence and absorption of therapeutic proteins without inducing undesired immunogenicity of protein drugs. Consequently, these delivery systems should facilitate passage of free peptides/proteins through paracellular pathways while uptake of the proteins by APCs should be avoided. For large proteins, which are not able to pass through paracellular pathways, particulate systems can facilitate their transport by transcytosis. Preferably, uptake of these particles by APCs should not occur.

For nasal delivery of antigens mucoadhesive micro- and nanoparticles, which can interact with the epithelial cells, have been applied to enhance antigen absorption/uptake [2,26,49–52]. These particulate carrier systems prolong the residence time of the proteins in the nasal cavity and significantly improve the uptake of antigen-loaded particles by epithelial cells, M-cells present APCs and epithelial cells. Intranasally delivered antigen-loaded nanoparticles are efficiently taken up by M- and epithelial cells. Subsequently they activate the immune cells in the NALT and drain lymph nodes [53].

5. Practical issues for pulmonary delivery of therapeutic proteins and vaccines

Despite the opportunities that pulmonary protein/antigen formulations offer, there is still a number of issues that have to be tackled. For pulmonary protein delivery, poor deposition of protein formulations at the alveoli (the absorption site), is a major limitation. Moreover, the central respiratory tract consisting of impermeable epithelial cells, which is covered by a thick mucus layer, forms a severe barrier for protein absorption. This implies that proper delivery systems are needed that efficiently deposit protein formulations at the absorption sites, facilitate the absorption of the proteins and prolong the residence time at the upper and central respiratory tract. To deposit protein formulations efficiently into the lungs, they preferably have aerodynamic diameters between 1.5–3 µm [4,45]. Mucoadhesive microparticles with permeation-enhancing properties and right aerodynamic diameters are therefore suitable systems for pulmonary delivery of macromolecules [26]. These particulate carrier systems prolong the residence time in the central respiratory tract and enhance the paracellular passage of loaded proteins through the respiratory epithelium at the central part of lungs and the alveoli. As mentioned earlier, for pulmonary immunization, the BALT located at the central respiratory tract and also the extensive network of APCs at the alveoli are responsible for local and systemic immune response induction. It has been demonstrated that mucoadhesive microparticles with proper aerodynamic diameters can significantly improve the uptake of antigen-loaded particles by epithelial cells, M-cells present in BALT [27,29,31–33,53] and alveolar DCs and macrophages [43–46]. The size of the particles, which can be controlled by the formulation conditions, is crucial for efficient pulmonary delivery of particles loaded with proteins or antigens [4,45].

6. Chitosan-based delivery systems

Chitosan and chitosan derivatives because of their excellent mucoadhesive and absorption-enhancing properties (further elaborated in the following sections) have been extensively studied for delivery of therapeutic proteins and antigens particularly via mucosal routes. Chitosan (derivatives) can interact with mucus and epithelial cells and induced a redistribution of cytoskeletal F-actin and the tight junction protein ZO-1 resulting in opening of cellular tight junctions and increasing the paracellular permeability of the epithelium [55–57]. Besides their charge, other structural elements of these polymers likely contribute to their penetration-enhancing activity, since cationic polysaccharides such as quaternized diethyl aminoethyl (DEAE)-dextran were ineffective as an enhancer [58]. In many studies, it has been demonstrated that chitosan-based formulations were superior in enhancing absorption of therapeutic proteins as well as induction of antibodies after mucosal vaccination [8,17,59,60].

6.1. Chitosan (derivatives)

Chitosan [α (1→4) 2-amino 2-deoxy β-D glucan], a copolymer of glucosamine and N-acetylg glucosamine (Fig. 4), is obtained by deacetylation of chitin, a naturally abundantly available polymer (e.g. in crustaceans).

Because of its favorable properties, as discussed in the present article as well as other articles in this issue, chitosan has been studied as a biomaterial and as a pharmaceutical excipient in drug formulations [55–57,61–63]. The primary amine groups introduce special
Fig. 3. Schematic representation of the induction of humoral immune responses at the mucosal lymphoid tissue (MALT) of the respiratory tract (A) and the alveolar region (B) (adapted from Neutral et al. [41]). A) At the mucosal sites, particulate antigens are transported by M-cells and delivered to sub-mucosa. Subsequently, dendritic cells (DCs) and macrophages process and present the antigen to naïve T-cells in the adjacent mucosal lymph nodes. Next, activated T-cells that are stimulated by DCs and macrophages induce IgG/IgA-committed B-cell development in the germinal center of the MALT. Finally, antigen-specific CD4+ T-cells and activated B-cells will migrate via the draining lymph nodes and the thoracic duct to the systemic circulation and other mucosal effector sites, where IgG/IgA-committed B-cells, after stimulation by CD4+ T-helper cells, differentiate to antibody secreting plasma cells, which produce IgG and IgA antibodies. B) At the alveoli, particulate antigens are taken up and processed by alveolar DCs and macrophages which migrate to the nearest draining lymph node to present the antigens to naïve T-cells. The activated T-cells and B-cells will migrate via the thoracic duct to the systemic circulation, where IgG-committed B-cells, after stimulation by CD4+ T-cells, differentiate to antibody secreting plasma cells, which produce IgG antibodies.
alkylated chitosan derivatives were used as antibacterial and antifungal agents using sodium borohydride as a reducing agent. These N-alkyl chitosans have mucoadhesive and penetration-enhancing properties due to the formation of inter- and intramolecular disulfide bonds at physiological pH [71–73]. The strong mucoadhesive properties of the thiolated chitosans make them particularly suitable carriers for prolonged protein delivery at the mucosal sites.

Mono-N-carboxymethyl chitosan (MCC) was synthesized by chemical modification of amine groups of chitosan with glyoxylic acid and sodium borohydride as a reducing agent [14]. In this way, even negatively charged chitosan derivatives were synthesized. Whereas chitosan forms precipitates with polyanions, MCC’s are compatible with anionic compounds. MCC showed a significant decrease of transepithelial electrical resistance (TEER) of Caco-2 cell monolayers when applied apically at concentrations of 3–5% (w/v) [14].

Quaternary chitosan derivatives are, because of their permanent cationic charge, soluble over a wide pH range. Importantly, these derivatives have mucoadhesive and penetration-enhancing properties also at neutral pH. The first quaternized chitosan was synthesized by alkylation of the primary amine groups of chitosan with various aldehydes using sodium borohydride as reducing agent. These N-alkylated chitosan derivatives were used as antibacterial and antifungal agents in the treatment of microbial infections [69–73].

Mono-N-carboxymethyl chitosan (MCC) was synthesized by chemical modification of amine groups of chitosan with glyoxylic acid and sodium borohydride as a reducing agent [14]. During this reaction some chain scission occurs which is likely due to the harsh reaction conditions (−4 M NaOH, a temperature of 60 °C and relatively long reaction time (2–3 h)) [79]. Also, this synthetic method is associated with partial methylation of the hydroxyl groups both at C3 and 6 (Fig. 5).

By varying the degree of methylation, the water-solubility of TMC can be tailored [80]. Soluble TMC has both mucoadhesive properties and excellent absorption-enhancing effects (the latter depending on its degree of quaternization (DQ)) even at neutral pH [81–83]. It has been shown in many studies that TMCs, depending on their degree of quaternization (DQ), enhance the permeation of hydrophilic macromolecules across the mucosal epithelia by opening the tight junctions [9,82–88]. At physiological pH, only TMC with a degree of quaternization above 36% increased the absorption of hydrophilic model materials [74,75]. N-trimethyl chitosan (TMC) is a partially quaternized and well water-soluble derivative of chitosan, which had been extensively studied for its mucoadhesive and absorption-enhancing effects for hydrophilic macromolecules in particular. The penetration-enhancing activity of TMC as well as other properties, among which is its biocompatibility, is discussed in more detail in the next section. Other quaternary chitosan derivatives have been synthesized by attaching a quaternary ammonium moiety to the amine groups of chitosan e.g. by reaction of N-chloroacyl-6-O-triphenylmethyl with chitosan [76]. Xu et al. synthesized a quaternary derivative of chitosan, N-(2-hydroxypropyl)-3-trimethyl ammonium chitosan chloride (HTCC), by reaction of chitosan with glycidyl trimethyl ammonium. This chitosan derivative was used to prepare albumin-loaded nanoparticles by ionic gelation with sodium tripolyphosphate (TPP). These nanoparticles had a size between 110–180 nm and their encapsulation efficiency for albumin was up to 90%. In vitro release studies showed a burst effect followed by a slow release. Addition of poly(ethylene glycol) (PEG) significantly decreased both the burst release and the encapsulation efficiency, whereas the addition of alginate reduced the burst release while protein loading remained high [77].

6.1.1. N-trimethyl chitosan (TMC)

In recent years much research has been done on cationic watersoluble chitosan derivatives in particularly N-trimethyl chitosan (TMC). This polymer is obtained by methylation of amine groups of chitosan with methyl iodide [78]. During this reaction some chain scission occurs which is likely due to the harsh reaction conditions (~4 M NaOH, a temperature of 60 °C and relatively long reaction time (2–3 h)) [79]. Also, this synthetic method is associated with partial methylation of the hydroxyl groups both at C3 and 6 (Fig. 5).

By varying the degree of methylation, the water-solubility of TMC can be tailored [80]. Soluble TMC has both mucoadhesive properties and excellent absorption-enhancing effects (the latter depending on its degree of quaternization (DQ)) even at neutral pH [81–83]. It has been shown in many studies that TMCs, depending on their degree of quaternization (DQ), enhance the permeation of hydrophilic macromolecules across the mucosal epithelia by opening the tight junctions [9,82–88]. At physiological pH, only TMC with a degree of quaternization above 36% increased the absorption of hydrophilic model materials [74,75]. N-trimethyl chitosan (TMC) is a partially quaternized and well water-soluble derivative of chitosan, which had been extensively studied for its mucoadhesive and absorption-enhancing effects for hydrophilic macromolecules in particular. The penetration-enhancing activity of TMC as well as other properties, among which is its biocompatibility, is discussed in more detail in the next section. Other quaternary chitosan derivatives have been synthesized by attaching a quaternary ammonium moiety to the amine groups of chitosan e.g. by reaction of N-chloroacyl-6-O-tri phenylmethyl with chitosan [76]. Xu et al. synthesized a quaternary derivative of chitosan, N-(2-hydroxypropyl)-3-trimethyl ammonium chitosan chloride (HTCC), by reaction of chitosan with glycidyl trimethyl ammonium. This chitosan derivative was used to prepare albumin-loaded nanoparticles by ionic gelation with sodium tripolyphosphate (TPP). These nanoparticles had a size between 110–180 nm and their encapsulation efficiency for albumin was up to 90%. In vitro release studies showed a burst effect followed by a slow release. Addition of poly(ethylene glycol) (PEG) significantly decreased both the burst release and the encapsulation efficiency, whereas the addition of alginate reduced the burst release while protein loading remained high [77].
compounds such as mannitol and poly(ethylene glycol) 4000 across intestinal epithelia and nasal mucosa. The permeation-enhancing effect of TMC increases with an increasing degree of quaternization [9,87,89]. It has been shown that TMC can also enhance the permeation of hydrophilic high molecular weight compounds (dextran Mw 4400 Da) across stratified epithelia such as buccal mucosa which lack tight junctions [90]. In these studies, TMC with higher DQs showed stronger mucoadhesive and penetration-enhancing properties. In several studies, it has been shown that TMC with a DQ of 40–60% showed the best absorption-enhancing properties for small proteins and peptides and a further increase in DQ of TMC did not considerably improve its absorption properties but increased its toxicity. In contrast to TMC as a potent absorption enhancer, fully quaternized diethyl aminoethyl (DEAE)-dextran was ineffective as an enhancer [58]. This indicates that the other properties of TMC rather than solely its charge, contribute to its role as absorption enhancer. In a recent study, a variety of quaternized derivatives of chitosan (TMC, dimethyl ethyl chitosan (DMEC), diethyl methyl chitosan (DEMC) and triethyl chitosan (TEC)) had been synthesized. The effects of these cationic polymers on opening tight junctions of and transport of insulin across Caco-2 cells were in the following order TMC>DMEC>DEMC>TEC>chitosan. The authors stated that due to steric hindrance the alkyl groups likely shield the positive charge of the quaternary amines, which plays a role on interaction of these polymers with cell membranes and their potency to act as absorption enhancers [91]. Recently, Verheul et al. developed a new mild method to synthesize TMC, in which side reactions associated with the synthesis of TMC using the conventional CH3I method, O-methylation of hydroxyl groups and chain scission of chitosan, did not occur, while the DQ could be tailored [80]. These O-methyl free TMCs were synthesized using a two-step reaction. First, chitosan was fully dimethylated using formic acid and formaldehyde. Then, in the presence of an excess CH3I, TMC with different DQs were obtained by varying the reaction time. The O-methyl free TMCs had stronger membrane permeability activity as demonstrated by a larger decrease of the TEER of Caco-2 cells as compared to O-methylated TMCs synthesized by the conventional method (Fig. 6). Also, a higher in vitro cytotoxicity for the O-methyl free TMCs was observed which indicates that partial O-methylation of TMC reduces the cytotoxicity of cationic polymer [80]. In a follow-up paper, the same authors synthesized a variety of TMC’s with a degree of acetylation (DA) ranging from 11 to 55%. TMC (DQ 40%) with high DA (~50%). They showed that highly reacetylated TMC (DQ 40%) was not able to open tight junctions as measured using a TEER assay in contrast to TMCs with similar DQ but lower DA [92] (Fig. 7).

Jintapattanakit et al. investigated the effect of DQ and the degree of dimethylation (DD) on cytotoxicity and physicochemical properties of TMCs synthesized using the conventional method [93]. It was found that an increase in the number of reaction steps increased DQ and decreased DD, whereas with longer reaction times both DQ and DD increased. TMC with DQ of 40% showed maximum water-solubility regardless of polymer molecular weight and DD. TMC with a DQ lower than 24% and high DD (60%) showed lower solubility, mucoadhesiveness and cytotoxicity than TMC with the same DQ and low DD (20%). This indicates that DD affected solubility and toxicity of TMC with DQ lower than 24% [93]. This is in agreement with the study of Verheul et al. that O-methyl free TMC with a high DD and a DQ lower than 20% showed lower solubility and toxicity than TMC with the same DQ and lower DD [80]. The mucoadhesive properties of TMC decreased with an increasing ratio of DD/DQ, because a high number of methyl groups shield the positive charges of TMC which in turn reduces the interaction between the polymer and mucin. The mucoadhesive properties of TMC with different DDs were investigated by Sandri et al. The authors found an increase in mucoadhesiveness of TMC with increasing DQ and they showed that TMC with a low molecular weight and a high DQ showed the best mucoadhesive and absorption-enhancing properties as compared to high molecular weight TMCs [90]. From the studies mentioned above it can be concluded that other properties of chitosan derivatives (in particular TMC) such as their Mw, DA and DD rather than solely their charge (DQ) influence their interactions with epithelial cell membranes and contribute to their role as absorption enhancers.

For the practical use of TMC as a mucoadhesive and permeation enhancer, safety studies are required to guarantee the absence of cytotoxicity and tissue damage [6,94–97]. Thanou et al. incubated monolayers of Caco-2 cells with different TMC solutions also containing the fluorescent probe YO-PRO-1, which is able to stain nuclei if the cell membrane damage occurs. Confocal scanning microscopy (CLSM) analysis showed no staining of nuclei after 4 h incubation with TMCs [97], which indicates that TMCs did not induce cell membrane damage. In other studies, the viability of Calu-3 cell monolayers, as a model for mucus-secreting respiratory epithelial cells, COS-7 (monkey kidney fibroblast) and MCF-7 (epithelial breast cancer) cells after exposure to various soluble TMCs and TMC nanoparticles was examined by
measuring the mitochondrial dehydrogenase activity of the cells [6,80,84,92,95,96]. These studies showed that TMCs with a low DQ were non-toxic whereas with increasing DQ the cytotoxicity increased. However, the cytotoxicity of the investigated TMCs was substantially less than that of linear polyethylene imine (PEI), a polymer frequently used for gene delivery purposes [95,98]. Ciliary beat frequency (CBF) of chicken embryo trachea has proven to be a valuable ex vivo model to evaluate the safety of nasal drug formulations, but because this excised tissue is devoid of a protective mucus layer, it may result in an overestimation of the in vivo ciliotoxicity. Several studies have shown that high molecular weight TMCs with a high DQ decreased the CBF more than low molecular weight TMCs with a high DQ. Importantly, the cilio-static effect is mostly reversible [6,86,94,97]. The toxicity of TMC formulations has also been investigated in vivo. Haffegee et al. showed that intranasally administered TMC solutions did not cause tissue damage in rats’ nasal mucosa [94]. Furthermore, histological evaluation of rats’ lungs after intratracheal instillation of TMC, differing in DQ, solutions [84] and pulmonary administration of TMC powder formulations [99] did not show tissue damage and/or infiltration of immune cells such as neutrophils. From these studies it can be concluded that TMC’s with a high charge density (due to a high DQ) interact safely with cell membranes and induce paracellular permeabilization of the epithelium. Taken together, from the penetration and toxicity studies it can be concluded that TMCs are safe mucosalhesives and absorption enhancers for hydrophilic macromolecules across respiratory and other mucosal sites. The biodistribution and toxicity of chitosan-based polymers is discussed in more detail in the contribution of Kean et al. in this chitosan issue (reference to be included later).

6.2. Techniques for the preparation of chitosan-based micro/nanoparticle formulations

Chitosan-based particles loaded with proteins can be prepared by both chemical and physical methods. However major drawbacks are associated with the use of chemical crosslinking methods. Firstly, organic solvents used to make w/o emulsions may adversely affect the stability of proteins and, more importantly, the applied crosslinking agents can chemically modify proteins [100]. Secondly, complete removal of the unreacted and often toxic crosslinker is difficult to achieve. Consequently, methods by which chitosan and its derivatives are crosslinked by physical methods to yield particles are preferred. Spray drying is a relatively protein friendly technique that has been applied for the preparation of protein-loaded chitosan microparticles and nanoparticles suitable e.g. for pulmonary delivery [101,102]. Also other techniques, such as ionic crosslinking methods and drying processes have been used for the preparation of protein-loaded chitosan-based particles. These techniques are discussed in more detail in the following sections.

6.2.1. Chemical crosslinking methods

Chitosan-based particles can be formed by chemical processes, e.g. by reacting the primary amine groups of chitosan with an di-aldehyde (mostly glutaraldehyde) crosslinker. Here, first a water-in-oil (w/o) emulsion of chitosan with the drug in a water-immiscible solvent (e.g. liquid paraffin) is formed, after which glutaraldehyde is added to crosslink chitosan to yield drug-loaded microspheres [103]. In another study, insulin-loaded chitosan microspheres were prepared by dissolving the protein and the polymer in an acetic acid solution. This solution was subsequently emulsified in mineral oil and chitosan was chemically crosslinked with ascorbyl palmiate or dehydroascorbyl palmiate. This preparation method yielded microparticles characterized by high loading levels of insulin, and they completely released the drug in an active form in about 80 h at an almost constant release rate [104]. In this study, mineral oil was used as oil phase to make a w/o emulsion. Ascorbyl or dehydroascorbyl palmiate, as interfacial crosslinkers, resides at the water/oil interface of the emulsion and might protect the protein from the high interfacial surface tension. Moreover, as compared to glutaraldehyde, ascorbyl or dehydroascorbyl palmiate has substantially lower toxicity. It should be mentioned that the authors did not discuss a possible chemical modification of the proteins by the crosslinking agents [104]. Wang et al. prepared uniform-sized protein-loaded chitosan microspheres by a step-wise crosslinking [105]. First, a w/o emulsion of chitosan/insulin in paraffin/petroleum ether mixture was prepared and extruded through a membrane with uniform pores. Then, tripolyphosphate (TPP) was added as an ionic crosslinker followed by the addition glutaraldehyde to stabilize the microspheres (Fig. 8).

It was shown that at pH 3.5–4.0, the ionic gelation with TPP was optimal in such a way that drug leakage was prevented and chemical crosslinking mainly occurred between the amine groups of chitosan and glutaraldehyde resulting in spherical microspheres with an average diameter of 6 µm, a high insulin loading and stability. To improve stability of insulin, different excipients were added to the aqueous phase. It was shown that in particular gelatin improved insulin encapsulation, its release kinetics and stability. Likely gelatin and TPP strongly interact at the selected pH, which might prevent drug leakage from the microspheres. Also, chemical reaction between amine groups of gelatin and the crosslinker might protect insulin against chemical modification during the preparation process [105]. However, it should be emphasized that chemical modification cannot be avoided and protein stability may be compromised using such techniques. In another study, monodisperse microspheres of chitosan and/or a quarternized chitosan derivative, N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HTCC), were prepared by a one-step or a two-step chemical crosslinking method using p-phthaldehyde and/or glutaraldehyde as crosslinker. BSA was post-loaded into the formed microspheres [106]. Microspheres with different properties, such as protein encapsulation efficiency and release profiles were obtained (Fig. 9). Particles made from a mixture of chitosan and HTCC and prepared by a single step crosslinking showed high porosity and large cavities (Fig. 9C) allowing a high protein encapsulation. Interestingly, these loaded particles showed a slower release kinetic and a lower burst as compared to the other particles prepared by a double crosslinking method or made from chitosan alone (Fig. 9A,B,D). The microspheres prepared by a double crosslinking were either hollow or...
porous in which BSA was loaded into their outer shell and into the channels (Fig. 9). Consequently, these particles showed different loading efficiencies and different release profiles.

6.2.2. Ionic crosslinking methods

The complexation between chitosan-based polymers and oppositely charged macromolecules can be exploited to prepare micro/nanoparticles suitable for drug delivery. The particles are prepared by ionic crosslinking through self-assembly of chitosan/chitosan derivatives and oppositely charged macromolecules or by addition of a low molecular weight anionic crosslinker, such as tripolyphosphate (TPP), sodium sulfate or cyclodextrin (CD) derivatives to chitosan solutions.

The ionic crosslinking methods mentioned above have received much attention in recent years for the preparation of protein formulations because the used processes are simple and mild to proteins, as they do not involve the use of chemical crosslinkers and avoid the use of organic solvents and high temperatures [11,77,107]. Coacervation/precipitation has been used to prepare a great variety of protein-loaded chitosan microparticles. In these methods, a coacervate, e.g. sodium sulfate, is added dropwise to an acidic solution of chitosan under stirring and sonication to prepare ionically crosslinked particles.

Self-assembled polyelectrolyte complexes (PECs) have been recently investigated for protein delivery [11,109–114]. Oppositely charged polyelectrolytes can form stable intermolecular complexes [111]. These PEC nanoparticles are either positively or negatively charged, and they show a pH-dependent destabilization. Schatz et al. synthesized a partially N-sulfated chitosan. Upon acidification of an aqueous solution of this amphoteric chitosan, nanoparticles were formed by electrostatic interactions between the non-sulfated protonated amine groups of chitosan and the negatively charged N-sulfated chitosan amines. These polyelectrolyte complexes can be used for encapsulation of macromolecules but loading and releases studies have not been reported [114].

Glucomannan (GM) binds to mannose receptors on M-cells and macrophages and consequently has been used for the targeted delivery of mucosal vaccines. *Amorphophallus konjac* glucomannan- and phosphorylated glucomannan–chitosan nanoparticles were prepared by ionic crosslinking with and without using (TPP) as a crosslinker. These nanoparticles exhibited high loading efficiencies for insulin and the immunomodulatory protein P1. Moreover, the release of the proteins could be modulated by the composition of the nanoparticles (Fig. 10) [109]. The protein release was slower from the formulations containing non-phosphorylated (KGM) as compared to those without GM or containing phosphorylated GM.

Chitosan-based nanoparticles were prepared by electrostatic complexation of poly-γ-glutamic acid (γ-PGA) and chitosan. The particle size and zeta potential of the formed nanoparticles were mainly dependent on the volumes and concentration of the γ-PGA solution added to the chitosan solution. γ-PGA was selected as a negatively charged crosslinking agent because it has been shown that nanoparticles containing this polymer have the capacity to target hepatocytes. [115]. In another approach, Mao et al. prepared polyelectrolyte complexes (PEC) formed from chitosan, (pegylated)-TMC and insulin. Complexation of the polymers and insulin occurred only above the pI (5.3) of insulin. PEC nanoparticles of insulin and chitosan were spherical, had a smooth surface and their size was in the range of 200–500 nm. The characteristics of PEC nanoparticles were unaffected...
after lyophilization [11]. In other studies, chitosan–dextran sulfate PEC nanoparticles were prepared for delivery of insulin [110] or vascular endothelial growth factor (VEGF) [112]. Insulin-loaded PEC nanoparticles had an average size of ∼500 nm, a negative surface charge and a loading efficiency of 85% [110]. VEGF nanocomplexes were prepared by ionic crosslinking with dextran sulfate [112]. The average size of the nanocomplex was ∼250 nm and they had a negative surface charge. The nanoparticles showed a sustained release of the protein for 10 days. Cell proliferation studies revealed that cells incubated with the VEGF-loaded PEC nanoparticles had a constant VEGF concentration in their medium as compared to cells incubated with a VEGF solution, which showed a rapid decrease in protein concentration in time. This constant level of VEGF in medium containing VEGF-loaded PEC nanoparticles resulted in cell proliferation [112].

Boddohi et al. prepared PEC nanoparticles based on chitosan–heparin (chi-hep) and chitosan–hyaluronan (chi-ha) polycation–polyanion pairs [111]. The nanoparticles were spontaneously formed by addition of anionic polyelectrolytes to chitosan or vice versa. The authors observed different colloidal characteristics of negatively and positively charged PEC nanoparticles. For chi-hep particles, the breadth of size distribution and mean particles size increased significantly (from 350 to 660 nm) as the charge-mixing ratio approached one. Most likely, the size increased because the net particle charge was minimal at this polymer ratio. At charge ratios far above one, chi-hep nanoparticles showed a smaller size (450 nm) which can be attributed to a higher particle charge and thus better colloidal stability. Positively charged chi-hep nanoparticles tended to aggregate with increasing amount of heparin. Schatz et al. reported that chitosan–dextran sulfate (DS) PEC nanoparticles prepared reduced in size after addition of extra DS to positively charged chi-DS PEC nanoparticles [116]. The authors attributed this to the physical characteristic of heparin and DS [111].

Ionic gelation of chitosan with TPP has been extensively used for the preparation of protein and antigen-loaded nanoparticles [6–8,17,107,117,118]. In this process, an aqueous solution of TPP is added dropwise to an aqueous solution of chitosan at ambient temperature under stirring. Due to complexation of the oppositely charged components, chitosan nanoparticles are formed [8,17]. Using this method, chitosan nanoparticles loaded with insulin and tetanus toxoid have been prepared and investigated as nasal delivery vehicles [8,17]. Fernandez-Urrusuno et al. prepared insulin-loaded chitosan nanoparticles with a size 300–400 nm and a positive surface charge. Insulin release in vitro occurred in less than 3 h. However, this relatively fast release is not a disadvantage since the average residence time of nasally administered formulations never exceeds few hours [8]. In another study, tetanus toxoid (TT)-loaded chitosan nanoparticles, with an average size about 350 nm and a positive surface charge, showed a high loading efficiency (around 50–60%). In vitro release studies showed an initial burst followed by a sustained release of antigenically active toxoid for 16 days [17]. Recently, there have been many studies focusing on the mucosal delivery of proteins and vaccines using TMC nanoparticles prepared by ionic gelation [6,117,119–121], which are further discussed in Section 6.3.6.

Sun and Wan synthesized O–(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (O-HTCC), which carries two quaternary amines. Both Chitosan and O-HTCC nanoparticles were prepared by ionic gelation with TPP. O-HTCC nanoparticles showed a decrease in size (from 500 to 100 nm) with increasing TPP concentration (from 1 to 4 mg/ml). This was in contrast to chitosan nanoparticles, which showed an increase in size from 10 to 50 nm with increasing TPP concentration, indicating a different colloidal behavior of these two nanoparticle formulations. This was explained by competition between three kinds of electrostatic forces that exists during gelation process: electrostatic attraction between the protonated amine of chitosan and/or −N+(CH3)3 of O-HTCC with the TPP anion and repulsion forces between −N+(CH3)3 groups of the polymer. At low TPP concentration the repulsion forces exceed the attraction forces yielding ‘loose’ nanoparticles with a relatively large size (500 nm). At high TPP the attraction forces are stronger than the repulsion forces and relatively small and stable particles (100 nm) are formed. Importantly, the O-HTCC nanoparticles had substantially higher loading efficiency and capacity for BSA than the chitosan nanoparticles, which might be due to the higher charge density of O-HTCC, as compared to chitosan [122].

In other studies, negatively charged cyclodextran (CD) derivatives have been used as crosslinker or co-crosslinker to prepare chitosan nanoparticles [123,124]. These negatively charged CD derivatives can also complex and stabilize proteins and have the capacity to enhance nasal absorption of peptide drugs [123,125]. The potential of these nanoparticles, as nasal protein delivery, is further discussed in Section 6.3.1.

6.2.3. Drying techniques used for the preparation of protein-loaded chitosan-based particles

There is a need for particle-formation processes which are simple and protein friendly, can easily be scaled, offer the possibility to produce formulations with different particle sizes and yield particles with a good life.

Different drying processes have been recently used for preparation of chitosan–protein powder formulations [102,126–130]. Spray
drying is the most commonly used drying method for preparation of protein microparticles. It is efficient process and particles are formed in a one-step process. Protein-loaded chitosan microparticles were prepared by spraying of an aqueous solution of chitosan and a protein into a drying chamber, yielding protein–chitosan microparticles (size 4–7 µm) [102,129]. In another study, mannitol microspheres containing protein-loaded chitosan nanoparticles suitable for pulmonary delivery were prepared by spray drying of a chitosan–FITC-BSA nanoparticles suspension in an aqueous mannitol solution [128]. Mannitol stabilizes the protein structure and improves aerosolization of protein drugs into lungs. FITC-BSA loaded chitosan nanoparticles (300–400 nm) were homogenously encapsulated in mannitol microparticles particles with a mean aerodynamic diameter of 2.7 µm, adequate for pulmonary delivery. However, the structural integrity of the encapsulated protein of these particles was not reported [128].

Supercritical fluid (SCF) drying has been recently investigated as an alternative process for producing powder formulations [131–135]. SCF drying is a fast and mild process, is cost effective and offers the possibility to produce small microparticles suitable for inhalation [131–135]. Above the critical points (temperature and pressure), a SCF has liquid-like viscosity and density, and gas-like diffusivity properties, and can therefore easily penetrate into substances like a gas and dissolve materials like a liquid [136].

The most widely used SCF for pharmaceutical applications is carbon dioxide (CO2) because it has a low critical temperature (31.2 °C) and pressure (75.8 bar), and it is non-flammable, non-toxic and inexpensive [133]. Because proteins have a very low solubility in supercritical CO2 (SC-CO2), this fluid has been used as an antisolvent to precipitate proteins from their aqueous solutions [137]. It is however possible to modify the solvent power of SC-CO2 by adding volatile co-solvents such as ethanol [138].

Pérez de Diego et al. prepared protein-loaded TMC microparticles by spraying a water/DMSO solution of albumin/polymer into SC-CO2 as an antisolvent. Adding water to DMSO/CO2 phase was necessary to dissolve both albumin and TMC in the mixture. The experimental conditions resulted in protein–TMC spherical non-agglomerated microspheres with a size between 1–10 µm, potentially suitable for inhalation. No stability studies of the dried protein have been reported, yet [131].

6.3. Chitosan-based nanoparticles and microparticles for protein delivery

The mucoadhesive and/or absorption-enhancing properties of chitosan and its derivatives are important factors for enhancing protein absorption/uptake across epithelial barriers [50,55–57,64,65]. Moreover, particulate carrier systems intensify the interaction of proteins with epithelial cell membranes and/or mucus, increase the residence time of formulations at the site of administration, protect labile proteins from enzymatic degradation and promote the absorption of the free protein via the paracellular pathway as well as transcytosis of the encapsulated proteins by cells.

The effects of molecular weight and degree of deacetylation (DD) of chitosan on cellular uptake of nanoparticles prepared from these polymers were studied using A549 cells. The uptake of nanoparticles was a saturable event and importantly, cell-associated chitosan nanoparticles were internalized, but not the cell-associated chitosan polymers. It was shown that nanoparticles prepared at pH 6.2 using chitosan with a low DD contain more primary amines available for protonation) had a higher positive charged and were more extensively taken up by the A459 cells than particles prepared with chitosan with a higher DD [139].

6.3.1. Chitosan particles for nasal delivery of peptides/proteins

Several studies of chitosan particles or polyelectrolytes complexes for nasal delivery of therapeutic proteins have been done [8,123, 140,141]. It has been shown that insulin-loaded chitosan nanoparticles enhanced nasal absorption of proteins to a greater extent than chitosan solutions [8,141]. Wang et al. showed that insulin-loaded thiolated chitosan nanoparticles substantially improved absorption of insulin across nasal mucosa as compared to non-thiolated chitosan nanoparticles as well as soluble chitosan. Likely, thiolated chitosan nanoparticles have higher mucoadhesion properties and thus a longer residence time in nasal cavity. Moreover, thiolated chitosan nanoparticles showed a faster swelling and release as compared to chitosan nanoparticles, which might facilitate diffusion of the encapsulated drug. In vivo evaluations showed that after intranasal administration of the insulin-loaded thiolated nanoparticles to rats, the blood glucose levels of the animals rapidly decreased. The glucose levels of these animals were similar to those that received insulin subcutaneously [140].

In another study nanoparticles consisting of chitosan and negatively charged cyclodextrin (sulfobutylether-β-CD (SBE-β-CD) or carboxymethyl-β-CD (CM-β-CD) derivatives were prepared and characterized [123]. It was demonstrated that chitosan–SBE-β-CD–TPP nanoparticles induced lower TEER values of Calu-3 cells than chitosan–CM-β-CD–TPP nanoparticles. However, both insulin-loaded nanoparticles showed similar effects on reduction of rats’ plasma glucose levels upon intranasal administrations. It should be noted that, the plasma insulin concentrations of the treated animals which may give better indications in absorption enhancement properties of the formulations, were not determined [123].

6.3.2. Chitosan particles for pulmonary delivery of peptides/proteins

Powder formulations of protein-loaded chitosan nanoparticles suitable for pulmonary delivery were prepared by spray drying [101, 142,143]. Insulin-loaded nanoparticles were obtained by ionic gelation of a chitosan solution with a TPP solution also containing insulin. The nanoparticles were suspended in a solution of mannitol and lactose. Spray drying yielded microparticle powders with a suitable aerodynamic diameter (1–3 µm) for alveolar deposition. The insulin-loaded chitosan nanoparticles had a good loading capacity (65–80%) and were fully recovered from the powder formulations after contact with an aqueous medium, and showed a fast release of insulin [101]. However, no in vivo pulmonary delivery data with these powder formulations have been published so far. In another study by the same authors, the biocompatibility and penetration-enhancing effects of their chitosan powder formulations were examined in vitro using A549 and Calu-3 cells as models for alveolar and respiratory epithelial cells, respectively [144,145]. The formulations exhibited a very low cytotoxicity in both cell lines, but no effects on opening of tight junctions of the cells were reported. Further, CLSM studies did not reveal internalization of nanoparticles which contrasts previously reported studies [142]. The authors speculated that the total amount of chitosan used in their study was lower than that used in other publications. Moreover, Lim et al. used a different chitosan salt (glutamate) than ‘normal’ chitosan, which probably did not lose its charge after dispersing the particles in buffer [142].

Yang et al. prepared an inhalable chitosan-based powder formulation of salmon calcitonin-containing mannitol (as a cropyrotection agent) using a spray drying process. The effect of chitosan on the physicochemical stability of the protein was investigated with chromatographic and spectrometric techniques. The dissolution rate of the protein decreased when formulated with chitosan, which might be due to an irreversible complex formation between the (aggregated) protein and chitosan during the drying process [102]. Yamamoto et al. showed that chitosan-coated PLGA nanoparticle suspensions improved the absorption of calcitonin after pulmonary administration. A chitosan-coated PLGA nanoparticle suspension was aerosolized with a nebulizer. The elimination of the chitosan-coated nanoparticles from the lungs was retarded as compared to non-coated particles, most likely due to the mucoadhesive properties of chitosan. It was shown that after pulmonary administration of the chitosan-
coated particles the pharmacological action of calcitonin was prolonged as compared to that of the protein loaded in the non-coated nanoparticles [146]. In another study, the potential of chitosan oligomers and polymers for pulmonary delivery of proteins was studied. The absorption of interferon-α (INF) in rats was improved after pulmonary administration of aqueous solutions of the oligomers and INF. Among various oligomers, glucoseamine hexamers at a concentration of 0.5% (w/v) showed the highest efficacy. Chitosan polymers were less efficient than the studied oligomers in increasing the systemic level of INF, likely due to their lower solubility in lung fluids [147].

Amidi et al. prepared N-trimethyl chitosan (TMC) powder formulations using a SC-CO₂ drying process for pulmonary delivery of insulin [126]. The particles had an average volume aerodynamic diameter of 4 µm suitable for peripherally pulmonary deposition. In the freshly prepared dried insulin powders, no insulin degradation products were detected by HPLC and GPC chromatographic analysis. Moreover, the secondary and tertiary structure of insulin as determined by circular dichroism and fluorescence spectroscopy was preserved in all formulations. After one-year storage at 4°C, the particle characteristics were maintained and the insulin structure was largely preserved [126]. In a follow-up study, the potential of the TMC (as a mucoadhesive and permeation enhancer) and dextran (as a non-mucoadhesive and non-permeation enhancer) powder formulations, for pulmonary delivery of insulin was evaluated in diabetic rats. Pulmonary administration of TMC60–insulin microparticles as compared to dextran- and TMC20–insulin microparticles, significantly enhanced the systemic absorption of insulin, with a bioavailability of about 95% relative to subcutaneously (SC) administered insulin. TMC20–insulin powder showed an extended release of insulin (Fig. 11), which may result from differences in dissolution and diffusion properties of insulin [148].

6.3.3. Chitosan particles for parenteral delivery of peptides/proteins
Kim et al. synthesized hydrophobically modified glycol chitosan with 5-(3-cholanic acid (HCG) groups, which subsequently was used to prepare nanoparticles by a solvent evaporation technique. The particles are hold together by hydrophobic interactions of the HCG groups, whereas the glycol groups contribute to the stability of the particles in aqueous environment. The anti-angiogenic RGD peptide (Arg-Gly-Asp) was efficiently encapsulated in nanoparticles (loading efficiency >85%). The nanoparticles had an average size of 230 nm and showed a prolonged release of RGD for 1 week. Intratumoral administration of RGD–HCG nanoparticles demonstrated a substantially decreased tumor growth as compared to the RGD peptide administered intravenously (i.v.) or intratumorally (i.t.) [149] (Figs. 12 and 13).

6.3.4. Chitosan-coated particles for delivery of peptides/proteins
Chitosan polymers have been used for coating polymeric particles, emulsions and liposomes. Core–shell nanoparticles prepared via a layer-by-layer (L-b-L) self-assembly technique has been prepared by Hadar et al., where alginate and chitosan were alternately adsorbed onto the surface of cationic liposomes. The L-b-L disposition resulted in spherical nanoparticles with a size of 380 nm and a positive surface charge. BSA encapsulation was high (>-80%) and by increasing the coating thickness, the protein slowly released from the particles in about 30 days. This controlled release system may have potential for the sustained delivery of therapeutics proteins [150]. Ye et al. prepared a multilayer chitosan/alginate self-assembled coating on melanime formaldehyde (MF) microparticles, of which after deposition of the multilayer the MP template was removed by dissolving the core at low pH. Insulin was post-loaded into the hollow chitosan/alginate microparticles. To slow down the release kinetics, crosslinked particles were prepared by adding a calcium chloride (CaCl₂) solution to the insulin-loaded particles. The chitosan/alginate particles showed a sustained release of insulin of 80% in 15 h, which was decreased to 50% for the crosslinked particles [151].

6.3.5. Pegylated chitosan particles for delivery of peptides/proteins
Poly(ethylene glycol)-grafted-chitosan copolymers have been synthesized to increase chitosan’s solubility and to improve its the biocompatibility [141,155]. Pegylated chitosan nanoparticles, prepared by reacting PEG-aldehyde with amine groups of chitosan were studied by Zhang et al. Furthermore, insulin-loaded PEG–chitosan nanoparticles improved the mucosal absorption of salmon calcitonin in rats [152,153].

Nanocapsules with either a solid lipid or an oil core and coated with chitosan have shown interesting features as mucoadhesive delivery systems for peptides and proteins. The chitosan-coated nanoemulsions and lipid nanoparticles had a size of about 300–500 nm with a positive surface charge and released a model protein, salmon calcitonin, for 6 h. In vivo studies showed that these nanocapsules improved the mucosal absorption of salmon calcitonin in rats [152,153].

Chitosan-coated gold nanoparticles have been investigated for mucosal protein delivery [154]. Chitosan was used as a reducing agent in the synthesis of gold nanoparticles and also as a mucoadhesive and penetration enhancer. Insulin was efficiently adsorbed (~50%) through electrostatic interaction onto the surface of the coated nanoparticles and they were coeloidally stable for 6 months. Intranasal administration of these nanoparticles in diabetic rats showed an improved pharmacodynamic effect as evidenced by higher reduction in blood glucose levels as compared to insulin-loaded sodium borohydride gold nanoparticles [154].

Fig. 11. Plasma insulin concentration-time curves (A) and plasma glucose level-time curves (B) of rats that received pulmonarily TMC60–insulin microparticles (○), TMC20–insulin microparticles (●), dextran–insulin microparticles (△), or SC free insulin in PBS (◆). The insulin dose was 1.25 IU. Data are expressed as mean±SD (n = 4). Glucose plasma concentrations are normalized to baseline levels prior to insulin administration [148]. (Reproduced with permission of Elsevier.)
nanoparticles were prepared by ion ionic gelation of PEG–chitosan with TPP. The molecular weight (Mw) of chitosan and PEG affected the release kinetics of insulin (Fig. 14). Formulations made of higher Mw chitosan showed a faster and higher burst release than those made of a lower Mw chitosan, which has higher chain flexibility to yield a tighter network [141]. Pegylated chitosan nanoparticle formulations showed faster insulin release as compared to insulin–chitosan nanoparticles [155]. With increasing Mw of the PEG grafts the insulin release was retarded (Fig. 14). The pegylated formulations with fastest insulin release in vitro, showed the highest insulin plasma concentrations and a rapid reduction in blood glucose levels [141].

6.3.6. TMC particulate systems for delivery of peptides/proteins

As pointed out in the Section 6.1.1., TMC, a partially quaternized chitosan derivative, is an attractive alternative for chitosan for the design of protein-loaded particles. Although mucosal delivery of

Fig. 12. In vivo quantification of local concentrations of RGD peptide in nude mice bearing s.c. tumors after i.v. or i.t. injection of RGD peptide or i.t. injection of RGD–HGC nanoparticles. (A) The fluorescence intensity for the region-of-interest (tumor) was recorded as total photon counts per tumor. (B) Tumor contrast (tumor-to-normal tissue ratio) measured as a function of time post-injection. (●) RGD–i.v., (▲) RGD–i.t., and (■) RGD–HGC nanoparticles–i.t. [149]. (Reproduced with permission of Elsevier.)

Fig. 13. The inhibition of tumor growth and microvessel density in tumors. (A) Mice were given s.c. injections of B16F10 cells and when tumor volumes were ca. 100 mm³, the mice received saline (●), HGC nanoparticles (■; 100 mg/kg/over 2 days, i.t.), cyclic RGD peptide (◆; 10 mg/kg/over 2 days, i.v.), cyclic RGD peptide (▼; 10 mg/kg/over 2 days, i.t.), or RGD–HGC nanoparticles (★; 10 mg RGD/kg/over 2 days, i.t.). Data are means ± SEMs (n = 10) [149]. (Reproduced with permission of Elsevier.)

Fig. 14. In vitro release of insulin from PEG-g-chitosan nanoparticles: A) PEG350–chitosan 2 kDa (molar ratio: 8); B) PEG350–chitosan 2 kDa (molar ratio: 4); C) PEG750–chitosan 6 kDa (molar ratio: 4); D) PEG350–chitosan 6 kDa (molar ratio: 4); E) chitosan 2 kDa [155]. (Reproduced with permission of Springer Standard Collection.)

peptides/proteins formulated with soluble TMC has been extensively studied [9,10,50,70,81–83,88,90,156], there are only a few studies that report mucosal delivery of proteins administered in the form of particulate systems. Amidi et al. prepared TMC nanoparticles, as a carrier system for the nasal delivery of proteins, by ionic crosslinking of TMC (DQ 20%) in the presence of ovalbumin as model antigen with TPP. The nanoparticles had an average size of about 350 nm and a positive surface charge. They showed a loading efficiency up to 95% and a loading capacity up to 50% (w/w). The integrity of the entrapped ovalbumin was preserved as demonstrated by Western blot analysis. Ovalbumin–TMC nanoparticles showed a burst release of 30% and the rest of the protein remained associated with the TMC nanoparticles for at least 3 h during incubation in PBS (pH 7.4) at
37 °C. Cytotoxicity tests with Calu-3 cells, as a model for respiratory epithelial cells, showed no toxic effects of TMC nanoparticles, whereas a partially reversible cilio-inhibiting effect on the ciliary beat frequency of chicken trachea was observed. FITC-albumin-associated TMC nanoparticles were efficiently taken up by the nasal epithelial cells and NALT of rats [126].

Mao et al. grafted poly(ethylene glycol) (PEG, Mw 5 and 550 kDa) on TMC polymers (DQ 40%) with molecular weights ranging from 5 to 400 kDa to improve the biocompatibility of TMC. It was shown that pegylation substantially reduced the in vitro cytotoxicity of particularly low molecular weight TMCs. Complexation of pegylated-TMC with insulin further masked the toxicity of the polymers [96]. In another study, polyelectrolyte nanocomplexes (PEC) consisting of chitosan or TMC and insulin were prepared and characterized. The stability of nanocomplexes was dependent on the molecular weight of chitosan/TMC and stable particles were obtained when the molecular weight of the polymers was above 25 kDa; particles prepared with lower molecular weights of TMC showed severe aggregation. Pegylated-TMC protected insulin from chemical degradation even at 50 °C. All complexes could be lyophilized without affecting the particle size and stability of insulin. These PECs were aimed for nasal protein delivery, however, no in vivo studies have been reported [11].

The effect of TMC with various DQ on nanoparticle characteristics, protein loading and release of two model proteins with different isoelectric points (pl), bovine serum albumin (pl 4.8) and bovine hemoglobin (pl 6.8), were studied by Chen et al. The TMC nanoparticles had a low loading efficiency for hemoglobin, presumably because of the weak negative charge of the protein at neutral pH. The loading efficiency of the nanoparticles with BSA was substantially better than that of hemoglobin (95 and 30%, respectively). Nanoparticles of TMC with a lower DQ showed slower release kinetics for both proteins, because its lower charge density and lower hydrophilicity, swelling of the particles and consequently diffusion of the protein through the nanoparticle network are slower. The burst release was reduced by incorporating alginate (a negatively charge polysaccharide) during preparation, probably by forming a polyelectrolyte barrier on the particle surface [119]. Sandri et al. investigated TMC nanoparticles, prepared using TMC polymers with different DQs, for mucosal peptide delivery. The permeation-enhancing properties of the TMC nanoparticles and chitosan nanoparticles (as a control) were studied in an in vitro Caco-2 cell model and an ex vivo rat jejunum model. All TMC nanoparticles enhanced the absorption of a model compound, fluorescein isocyanate dextran, comparable or superior to that of chitosan nanoparticles except TMC particles with a high DQ (90%), which were internalized and remained entrapped in the cells. Similar results were seen with ex vivo absorption enhancement studies. The higher mucoadhesion and internalization of TMC nanoparticles as compared to chitosan nanoparticles make TMC nanosystems, in particular those made with intermediate DQ (35%), suitable carriers for mucosal peptide/protein delivery [120].

A folate conjugated TMC was synthesized by Zheng et al. to enhance the intracellular delivery of macromolecules. Folate decorated nanoparticles can bind to folate receptors on cells and are subsequently internalized by endocytosis. Folate–TMC nanoparticles loaded with FITC-BSA were prepared by ionic crosslinking using alginate. Folate functionalized nanoparticles showed a ~4-fold higher uptake by SKOV3 cells (folate receptor overexpressing cells) than non-functionalized TMC nanoparticles. The folate receptor-mediated uptake could be inhibited by the presence of 1 mM folate in the culture medium demonstrating that the uptake of the targeted particles was indeed via the folate receptor [157].

Mi et al. prepared and characterized self-assembled TMC (DQ 40%–poly(γ-glutamic acid) nanoparticles for mucosal delivery of insulin. These nanoparticles showed a loading efficiency of 74% for insulin. TMC–γ-PGA nanoparticles had superior colloidal stability in a wide pH range, as compared to insulin–chitosan nanoparticles. At the physiological pH, insulin–TMC–γ-PGA nanoparticles showed a sustained release of insulin for 12 days (Fig. 15). Staining for ZO-1 tight junction proteins showed a discontinuous ring between adjacent cells after incubation with the TMC nanoparticles (Fig. 16), indicating opening of cell tight junctions. After removal of the nanoparticles, the discontinuous staining for ZO-1 proteins disappeared, proving the recovery of cell tight junctions [158].

6.4. Chitosan-based formulations for vaccine delivery

Chitosan-based carriers have been extensively studied for parenteral and mucosal delivery of antigens [15–17,51,117,159–172]. In these studies mucosal and parenteral immunizations with various antigen co-administered with soluble chitosan, antigen-loaded chitosan powders/ micro/nanoparticles and chitosan-coated poly(lactic acid) nanospheres demonstrated various levels of both systemic and local immune responses. Moreover, in a phase I clinical study, intranasal immunization with influenza vaccine formulated with soluble chitosan glutamate showed positive effects of the polymer on the immune responses raised in the vaccinees [173].

6.4.1. Adjuvant activity and immunostimulatory effects of chitosan and its precursor polymer, chitin

The adjuvant activities of chitosan and its precursor chitin with degrees of deacetylation (DD) of 30 and 70%, after intraperitoneal administration in mice and guinea pigs, were studies in terms of induction of cytokines, long-lasting circulating antibodies and cell-based immunity against bacterial alpha-amylase and an Escherichia coli infection [174,175]. In another study, chitosan (DD 70%) showed induction of cytokines, interleukin (IL)-1 and colony-stimulating factor (CSF) in macrophages in vitro [176]. In a later study Shibata et al. demonstrated that intravenous administration of phagocytosable chitin microparticles (1–10 μm) resulted in activation of alveolar macrophages which showed upregulation of the expression of IL-12, tumor necrosis factor (TNF)-α and IL-18, leading to production of INF-γ by NK cells. Subsequent studies by the same group showed that cytokines production was mediated by a mannose-receptor-dependent phagocytic process and that the mannose receptors mediated the internalization of the chitin particles [177,178]. It has been demonstrated that chitin microparticles and also water-soluble hydroxypropyl chitosan are strong Th1-type adjuvants, which downregulate allergic responses [179–182]. It is important to point that chitin can also augment Th2 responses [183,184]. These studies demonstrate that chitin is a size-dependent pathogen-associated molecular pattern (PAMP) that interacts with or activates different macrophages TRLs.
and dectin-1 (a C-type lectin receptor) resulting in production of both pro-inflammatory (TNF) and anti-inflammatory (IL-10) cytokines [183,185] (Fig. 17). Both small chitin microparticles (1–10 µm) and, to a larger extent, intermediate chitin particles (40–70 µm) stimulated TNF production but only the small ones stimulated formation of the anti-inflammatory cytokine IL-10. These effects are mediated by pathways that involve TLR2, dectin-1 and mannose receptors [183].

Zaharoff et al. showed that chitosan dissolved in buffer pH 6.2 enhanced the immunoadjuvant properties of cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), when co-administered subcutaneously. Likely, chitosan prolonged dissemination of GM-CSF at the site of injection resulting in prolonged exposure of immune cells to this cytokine and enhancing the immunoadjuvant properties of GM-CSF. After single subcutaneous injection of GM-CSF/chitosan solution, the cytokine expanded lymph nodes about 5-fold higher than GM-CSF alone, which was injected four times. It was also suggested that chitosan could enhance the antigen-presenting capability of DCs and induced greater allogeneic T-cell proliferation. The number of MHC class II expressing dendritic cells was increased up to ∼7-fold after single subcutaneous injection of chitosan/GM-CSF [186]. In a subsequent paper, the same authors demonstrated that soluble chitosan admixed with inactivated influenza virus increased cytotoxic activity of splenic NK T-lymphocytes and enhanced the proliferative activity of mononuclear lymphocytes in the spleen. Moreover, it was shown that the number of CD3, C3/NK and CD25 T-cells also increased. The authors suggested that chitosan activates cell immunity because of its proliferation activity, which is initiated through receptor complex TCR–CD3 as well as activation signals linked with lectin receptors [189,190]. In these studies, it was shown that none of the animals immunized with chitosan formulations showed an anti-chitosan and IgE antibody response. This is opposite to alum adjuvant, which is a strong inducer of IgE antibodies [189].

6.4.2. Chitosan-based polymers for mucosal and parenteral vaccination

Chitosan (derivatives) have been investigated as immunoadjuvants and antigen delivery systems for mucosal and parenteral vaccinations [159,160]. Svirshchevskaya et al. investigated the adjuvant activity of water-soluble lipid- and different glyco-conjugated chitosans after nasal and subcutaneous vaccinations [160]. Unexpectedly, chitosan conjugated with mannose, galactose, glucose, lactobionic acid or glucoseamine, as targeting ligands for DCs, did not induce higher antibody responses than non-targeted chitosan upon subcutaneous immunization. Intranasal vaccination with lipid (palmitic or oleic)-conjugated chitosan, as mucosal adjuvant, did not enhance humoral immune responses as compared to unmodified chitosan as well. The authors did not comment on the ineffectiveness of their targeted systems. It should be noted that firstly, a low Mw chitosan with a degree of deacetylation DD of 80%, to which mono or disaccharides were conjugated, was used. It has been shown that chitosans with such a high DD cannot activate APCs. Secondly, only mono and disaccharides were used as targeting ligands. It can be speculated that most likely longer sugar units are needed to properly bind to APCs mannose receptors and induce their activation [174–176].

To increase the water-solubility of the lipid conjugates, they were succinylated. However, the succinyl derivatives were significantly less active in inducing humoral immune responses than non-succinylated
lipid–chitosan derivatives. Boonyo et al. evaluated the adjuvant effects of soluble chitosan of different molecular weights and TMC of various DQ (20, 40 and 60%) [159]. It was demonstrated that antigen admixed with either high or low molecular weight chitosan elicited similar systemic and local immune responses after intranasal administration, but the responses were lower than observed with the TMC40 formulation which showed the highest response of the different investigated TMCs after prime vaccination. However, after booster administrations there were no significant differences between the different TMC formulations. In a recent study, it was shown that whole inactivated virus (WIV) coated with TMC of various DQ (37 and 15%) substantially enhanced systemic/local antibody responses in mice as compared to non-coated WIV after intranasal immunization. Intranasal administration of a TMC solution prior to WIV did not result in significant immune responses, indicating that the immunostimulatory effect of TMC is primarily caused by improved i.n. delivery of TMC and TMC 37 [170].

6.4.3. Chitosan-based micro- and nanoparticles for parenteral and mucosal vaccination

Particulate chitosan systems can protect antigens from degradation and enhance the uptake of the particles by APCs, macrophages and M-cells at mucosal sites and other sites of administration. Nishimura et al. have shown that porous chitosan (DD 80%) microspheres with a mean diameter of 2.5 µm enhanced the cytolytic activity of peritoneal macrophages resulting in the production of CSF both in vitro and in vivo. On the other hand, porous chitin microspheres (size 2.5 µm) did not activate peritoneal macrophages, but slightly enhanced the production of CSF in vivo [191].

Shibata et al. showed that chitin and chitosan particles have to be phagocytosed by alveolar macrophages in order to upregulate the expression of interferon gamma (INF-γ) [192].

Van der Lubben et al. prepared ovalbumin-loaded chitosan microparticles and their uptake by murine Peyer’s patches was shown by confocal microscopy [15]. Furthermore, they investigated the potential of chitosan particles loaded with diphtheria toxoid (DT) after nasal and oral immunization. After both administration routes, the DT chitosan formulation induced both high neutralizing antibody levels and secretory IgA antibody levels [16]. In another study, intranasal immunization with DT-loaded TMC (DQ 20%) microparticles induced the formation of both IgG and IgA similar to that of soluble TMC20 co-administered with the DT antigen [193]. Contrary, in a study by Amidi et al. it was demonstrated that influenza hemagglutinin–loaded TMC (DQ 20%) nanoparticles elicited much stronger systemic and local immune responses than those obtained after intranasal administration of soluble TMC20 and antigen, or after intramuscular immunization with influenza antigen (Fig. 18). In this study, both intranasally and intramuscularly administered vaccines showed mixed Th1/Th2-type responses, of which the Th2-type response was predominant [117].

The potential of TMC (DQ 60%) as an adjuvant for mucosal vaccination was shown in guinea pigs after pulmonary administration of diphtheria toxoid (DT)-loaded TMC microparticles. The animals that received TMC–DT microparticle powders showed comparable or superior systemic and local immune responses compared to the animals that received a subcutaneously administered alum-adsorbed DT vaccine [130]. After a single pulmonary immunization with DT–TMC powder, the immune response shifted towards a strong Th1-type response compared to the subcutaneously administered vaccine. This is in contrast to the previous study by Amidi et al., in which intranasal immunization with influenza antigen–loaded TMC nanoparticles elicited more a Th2-type response [117]. Sayin et al. prepared negatively charged mono-N-carboxymethyl chitosan (MCC) and positively charged TMC (DQ 57%) nanoparticles for nasal vaccination [162]. Both tetanus toxoid (TT) loaded TMC and MCC/chitosan particles were efficiently taken up by murine macrophages regardless of their surface charge. Mice that received either TT–chitosan or TT–TMC nanoparticles intranasally developed higher serum antibodies than those that received intranasally TT–MCC particles, and comparable to the control mice vaccinated subcutaneously with free TT. Antigen formulated with soluble TMC or MCC induced similar antibody responses as compared to the nanoparticle formulations [162]. Positively and negatively charged polyelectrolyte complexes (PECs) made by electrostatic interaction of positively charged chitosan and negatively charged dextran sulfate, were investigated by Drogoz et al. for delivery of the capsid protein of HIV-1 virus (p24) [194]. Loading was done by incubation of preformed nanoparticles with the antigen and it was found that negatively charged PECs showed higher antigen binding capacities, faster sorption kinetics and better stability of the adsorbed antigen as compared to positively charged particles. The observed serum antibody titers after subcutaneous administration of the antigen associated with either cationic and anionic PECs in mice were similar to those found after administration of the Freund adjuvanted vaccine [194].
upon immunizations as compared to free antigens [163]. Antigen-loaded alginate–chitosan nanoparticles did not enhance CTL and humoral immune responses as compared to soluble antigen. This is contrary to many other studies, in which chitosan particles were shown to be superior to the soluble antigen in inducing immune responses after intranasal immunization [161,170,172]. Borges et al. speculated that the weak mucosal immune responses might be due to the weak bio/mucoadhesive properties of alginate-coated chitosan particles as compared to chitosan particles and also to the poor immunogenicity of the Hepatitis B antigen. Interestingly, it was shown that when the antigen and the adjuvant were loaded in separate nanoparticles and co-administered intranasally, the formulation was poorly immunogenic [163]. This indicates that co-delivery of antigen and adjuvant to APC are likely necessary for a proper activation of APCs.

In another study, Pluronic® F127 was used as a mucosal adjuvant in chitosan particles loaded with Bordetella bronchiseptica antigens [164]. The adjuvant formulations remarkably improved systemic IgG and especially S-IgA antibodies as compared to control free antigen after intranasal administrations, but they induced lower serum IgG titers than the intramuscularly administered vaccine [164]. It should be noted, however, that the animals vaccinated with the adjuvanted chitosan particles, were better protected after nasal challenge with B. bronchiseptica bacteria than those that received the B. bronchiseptica antigens intramuscularly. Similar results were reported by Hagenaaars et al. [170]. Unlike to intramuscular immunization with TMC-coated WIV, intranasal vaccination, which induced lower serum IgG titers, could completely protect vaccinated mice against a viral challenge [170]. These observations indicate that mucosal immunity plays a crucial role in protection against mucosal pathogens.

Zhu et al. prepared chitosan microparticles loaded with a tuberculosis subunit antigen for subcutaneous immunization. The loading efficiency was almost quantitative and the particles released the antigen slowly over a period of 16 days. Upon subcutaneous immunization, the chitosan formulations elicited higher serum IgG, IgG1 and IgG2a antibodies as well as INF-γ levels than free antigen in PBS [195].

6.4.5. Chitosan-coated nanoparticles for parenteral and mucosal vaccination

Many studies have been carried out in which antigen-loaded chitosan particles were combined with other immunoadjuvants [163–166,195,196]. Antigen–chitosan particulate formulations showed enhanced antigen uptake and also induced elevated local and/or systemic antibody responses as well as induction of CTL cytokines upon immunizations as compared to free antigens [163–166,195,196]. Combination of TMC and LTK63, a non-toxic mucosal adjuvant, was investigated by Baudner et al. Intranasal immunizations with a group C meningococcal conjugate vaccine (CRM-Menc) formulated with soluble TMC and LTK63, and with a suspension of TMC microparticles loaded with CRM-Menc antigen and LTK63, induced bactericidal antibodies in mice superior to those after parenteral immunizations of the same formulations [197]. Borges et al. vaccinated mice with alginate-coated chitosan nanoparticles loaded with hepatitis B antigen and CpG oligodeoxynucleotide (ODN) as adjuvant. Only alginate–chitosan/CpG-ODN nanoparticles enhanced systemic IgGs and mucosal S-IgA antibody levels after intranasal vaccination as compared to the nanoparticles without adjuvant and soluble antigen in PBS [163]. Antigen-loaded alginate–chitosan nanoparticles did not enhance CTL and humoral immune responses as compared to soluble antigen. This is contrary to many other studies, in which chitosan particles were shown to be superior to the soluble antigen in inducing immune responses after intranasal immunization [161,170,172]. Borges et al. speculated that the weak mucosal immune responses might be due to the weak bio/mucoadhesive properties of alginate-coated chitosan particles as compared to chitosan particles and also to the poor immunogenicity of the Hepatitis B antigen. Interestingly, it was shown that when the antigen and the adjuvant were loaded in separate nanoparticles and co-administered intranasally, the formulation was poorly immunogenic [163]. This indicates that co-delivery of antigen and adjuvant to APC are likely necessary for a proper activation of APCs.

In another study, Pluronic® F127 was used as a mucosal adjuvant in chitosan particles loaded with Bordetella bronchiseptica antigens [164]. The adjuvant formulations remarkably improved systemic IgG and especially S-IgA antibodies as compared to control free antigen after intranasal administrations, but they induced lower serum IgG titers than the intramuscularly administered vaccine [164]. It should be noted, however, that the animals vaccinated with the adjuvanted chitosan particles, were better protected after nasal challenge with B. bronchiseptica bacteria than those that received the B. bronchiseptica antigens intramuscularly. Similar results were reported by Hagenaaars et al. [170]. Unlike to intramuscular immunization with TMC-coated WIV, intranasal vaccination, which induced lower serum IgG titers, could completely protect vaccinated mice against a viral challenge [170]. These observations indicate that mucosal immunity plays a crucial role in protection against mucosal pathogens.

Jiang et al. developed adjuvanted chitosan microparticles functionalized with mannose ligands to target mannose receptors on APCs. Fluorescently labeled mannosylated-chitosan particles (MCMs) loaded with B. bronchiseptica antigens containing dermonecrototoxin (BBD) bound efficiently to the mannose receptor of murine macrophages and subsequently were internalized via receptor-mediated endocytosis. Moreover, they showed much higher immunostimulatory effects on macrophages than non-targeted BBD-loaded chitosan microparticles (CMs). A weak immunostimulatory cytokine response was detected after incubation of empty chitosan or mannosylated-chitosan particles with macrophages indicating that the presence of antigen together with the adjuvant is necessary to obtain good immunostimulatory effects on APCs. Mice vaccinated intranasally with the mannosylated and non-functionalized chitosan nanoparticles induced similar S-IgA antibody levels in the nasal lavages, but higher saliva serum S-IgA levels were observed in mice that received the mannosylated-chitosan nanoparticles (Fig. 19) [165].

6.4.5. Chitosan-coated particles for parenteral and mucosal vaccination

Chitosan and its derivatives have been extensively used as a coating for negatively charged particles loaded with an antigen for mucosal and parenteral vaccinations. Nagamoto et al. prepared chitosan particles and chitosan-coated emulsions with different particle diameters. A model antigen, albumin, and cholera toxin, as an adjuvant, were loaded into the particles and they were administered either intranasally (i.n.) or intraperitoneally (i.p.). The chitosan-coated emulsion droplets and nanoparticle formulations induced high serum IgG and mucosal IgA antibody titers in rats [172]. In another study, chitosan-coated liposomes were prepared by a reverse evaporation method and these coated liposomes showed a lower burst release than unmodified DT–liposomes. Mice subcutaneously vaccinated with the DT-loaded liposomes coated with chitosan showed mix Th1 and Th2-type immune responses [198] which indicates that chitosan not only enhances mucoadhesivity and immunogenicity of vaccines, but also improves...
encapsulation efficiency of antigens as well as modulate the release of the antigens.

Chitosan-coated PLGA/PLA particles in which various antigens were encapsulated, have been studied for mucosal and parenteral vaccinations [168,199,200]. Jaganathan and Vyas prepared mucoadhesive chitosan-coated PLGA microparticles loaded with hepatitis B surface protein (HBsAg). Intranasally administered chitosan-coated PLGA particles elicited comparable systemic but stronger local and cell-mediated immune responses against HBsAg, as compared with subcutaneously administered alum-adsorbed HBsAg and unmodified PLGA particles with CTB as mucosal adjuvant (Fig. 20) [168,200].

In another study, HBsAg and HBsAg–alum loaded PLA micro-particles were prepared by a double emulsion solvent evaporation technique using polyvinyl alcohol (PVA) or chitosan as a stabilizer. The PLA–chitosan particles had a positive surface charge and a high HBsAg encapsulation efficiency (86% (w/w)). After subcutaneous administration in mice, both the antigen-loaded PLA–alum and antigen-loaded PLA–chitosan particles induced HBsAg specific IgG antibody levels that were significantly higher than those observed after administration of HBsAg–PLA particles [199]. Cytokine production (IL4, IL6 and INF-γ) observed after subcutaneous administration of all formulations demonstrated a Th2 based response. The INF-γ production by splenocytes of mice immunized with chitosan formu-

lations was remarkably higher than that observed after administration of the other formulations, which indicates induction of a stronger CTL response with the chitosan-modified particles. It was further found that 300 days post immunizations, the IgG antibody titers of mice immunized with PLA–alum and PLA–chitosan formulations were 8–10 higher than IgG titers in mice vaccinated with HBsAg loaded PLA particles, alum-adsorbed HBsAg or HBsAg in chitosan solution [199].

Chitosan-coated poly-ε-caprolactone (PCL) micro- and nanoparticles were investigated as vaccine against Streptococcus equi subsp. [169,201]. Unmodified and surface modified PCL particles were prepared by a double emulsion solvent evaporation technique and compared to PCL particles containing other absorption enhancers such as spermine and oleic acid. In the first study, S. equi bacterial extract containing antigenic proteins was post-loaded into the empty PCL and PCL–chitosan microparticles by incubating the particles with the antigen. The loading efficiency and capacity of S. equi extract for the negatively (PCL) and positively (PCL–chitosan) charged particles were similar. Single subcutaneous injection of the above mentioned formulations resulted in a strong mix of Th1- and Th2-type responses that peaked on day 90 post immunizations. PCL–chitosan induced substantially higher IgG1 and IgG2a antibody titers than those of the other formulations. Moreover, PCL–chitosan particles induced higher levels of INF-γ and IL-2, Th1-dependant cytokines, whereas PCL
S-IgA antibody levels in mice treated with the modulation. However, only intranasal immunizations induced elevated booster vaccination, which were maintained for 12 weeks post immunization. PCL formulations induced high antibody titers after or/without CTB (as a mucosal adjuvant and absorption enhancer). All higher titers than free antigen or antigen-loaded formulations with efficiencies of the investigated particles under investigation have limited colloidal stability particularly in biological fluids. As a consequence, they dissociate and/or aggregate at mucosal lumens before reaching the absorption site (the mucosal epithelial cells). Therefore, strategies to stabilize chitosan particles need further attention.

Fig. 20. Interferon-γ and interleukin-2 levels in the spleen of mice immunized with PLGA-based formulations. Values are expressed as mean±S.D. (n = 6). The alum formulation was given subcutaneously, whereas the PLGA formulations were administered intranasally. The modified PLGA microspheres with or without CTB (booster dose) showed significant differences from alum-HBsAg (control) (p<0.05) (UNPLGA, unmodified PLGA microspheres; MPLGA, chitosan-modified PLGA microspheres) [168]. (Reproduced with permission of Elsevier.)

Chitosan particles have been formulated with other polymers e.g. alginate to increase both the mucoadhesion properties of the vaccine and the colloidal stability of the particles as well as to improve the release characteristics of a loaded antigen. An alginate-coated chitosan microparticulate system was reported by Li et al. Alginates as a mucoadhesive and a release modifier decreased the burst release of a loaded antigen (BSA) [203].

Chitosan has been used as a stabilizer for vaccine formulations. Norwalk virus-like particles (VLPs) showed increased thermal stability in the presence of chitosan glutamate, which was ascribed to stabilization of the secondary and tertiary structures of the VLPs, particularly the P-domain of VP1 protein, a constructive unit of Norwalk VLPs [204].

7. General discussion and conclusions

This paper reviews chitosan-based systems for delivery of therapeutic proteins/peptides and antigens, particularly after administration particles via mucosal (nasal and pulmonary) and parenteral routes. In many studies, it has been shown that bio/mucoadhesive chitosan (derivatives) can prolong the residence time of formulations at the mucosal sites, fairly protect the peptide/protein of interest from degradation and enhance its absorption across epithelial barriers. However, systematic studies on the bioavailability of proteins formulated with chitosan are lacking. It might be argued that the bioavailability of therapeutic proteins formulated with chitosans are low because of their poor transport to the sub-mucosal sites, and degradation of the protein either loaded into particles or in its soluble form. Further, it has been shown that chitosan-based nanoparticles are efficiently taken up by the epithelial cells, but not necessarily transported across the cells. Finally, most of the chitosan-based nanoparticles under investigation have limited colloidal stability particularly in biological fluids. As a consequence, they dissociate and/or aggregate at mucosal lumens before reaching the absorption site (the mucosal epithelial cells). Therefore, strategies to stabilize chitosan particles need further attention.

Another aspect, hardly addressed in drug delivery literature so far is the potential immunogenicity of particulate drug delivery systems such as chitosan-based carriers [205]. As described in this review, chitosan polymers have adhesive properties, particularly in particulate form. After parenteral or mucosal administration of protein-loaded chitosan particles, they can be taken up and subsequently processed by APCs, which may initiate an immune response against the therapeutic protein. It is important to note that most of therapeutic proteins have to be repeatedly administered to patients that may increase the potential risk of antibody formation against the formulated therapeutic proteins and pose a safety concern [206]. A possible way to avoid these unwanted immune reactions is to prepare large uniform chitosan-based particles (>10 µm), which are hardly taken up by epithelial cells and APCs. Such large sized mucoadhesive particles can interact with epithelial cells and facilitate the absorption of the released protein via paracellular pathway. Moreover, such relatively large chitosan-based particles with sustained protein release properties may be suitable for parenteral administrations.

For large therapeutic proteins, which have lower paracellular absorption than small proteins the major route of transport is through particle transcytosis by epithelial cells to reach the sub-mucosa and systemic circulation. However, as mentioned, there is a risk that internalized particles are intercellularly degraded. To prevent possible
degradation of the particles and the loaded proteins and further facilitate their transport across the cells, small targeted-nanoparticles bearing a specific ligand for transcytosis can be used. Ligands such as anti-intercellular adhesion molecule-1 (ICAM-1) antibodies (mAb F10.2) can interact with ICAM-1 receptors inside the cells and facilitate transcytosis of the internalized particles. In recent years it has been demonstrated that chitosan derivatives such as thiolated or quaternized chitosan have superior mucoadhesive and absorption-enhancing properties. These chitosan derivatives might be efficient both in solubile and particulate form. It can be concluded that so far chitosan-based particles do not fulfill all criteria needed for delivery of therapeutics. Items such as poor efficacy and avoidance of possible immunogenicity of therapeutic proteins as well as possible long-term toxicity of chitosan-based polymers need further investigation.

Chitosan-based systems for mucosal vaccine delivery have entered clinical trials and shown encouraging results [51,173,207,208]. Although in preclinical studies it has been shown that chitos/antigen formulations give both a strong mucosal and systemic immune response, only restricted local immune response in humans were found. Therefore, further optimization of these vaccines is required to improve the local immune responses. Preclinical in vivo studies have shown that chitosan-loaded particles can be taken up by APCs, epithelial cells and M-cells (at mucosal sites) and be transported into lymphatic tissues, where an immune response is initiated. Generally speaking, the strength and type of immune responses are strongly dependent on the physicochemical characteristics of the vaccine formulations such as charge and size, as well as the biochemical properties and the dose of the antigen. Also, the route of administration is a dominant factor that determines the efficacy of a vaccine. It is clear from the studies discussed in this review that chitosan and its soluble derivatives have strong mucoadjuvant activity and enhance local and systemic immunity upon mucosal immunization. Moreover, upon parenteral administration, particulate chitosan vaccines are also taken up by APCs and induced high systemic immune responses in vaccinated animals. It is known that chitosan as well as its precursor, chitin, activate both macrophages and NK cells to express cytokines such as CSF, IL-1β and INF-γ [176,178,192,209]. Moreover, vaccination studies with chitosan have proven its adjuvant activities in terms of induction of CTL related cytokines such as IL-6 and INF-γ [165,202]. It is important to note that in the absence of antigen, chitosan and its derivatives hardly show immunostimulating effects [195,199,200]. Upon nasal administration of chitosan formulations, a mixed Th1/Th2 response is induced, which is more towards a Th2 response, whereas pulmonary administration of chitosan-based vaccines induces stronger Th1-type responses [117,130]. Type and immunogenicity of antigens as well as the type and adjuvant activity of chitosan also play an important role in type of immune responses and adjuvant activity of chitosan formulations. To evaluate the efficacy of targeted chitosan-based vaccines, booster immunizations and dose–response vaccination studies are highly recommended. The differences in physicochemical properties such as charge density and molecular structure of chitosan derivatives have a great impact on their interaction with APCs and their adjuvant activity, but the mechanisms involved need further investigations.

To achieve clinical significance of chitosan-based systems for vaccination, the bio/mucoadhesive and colloidal stability of the particles need optimization. Further, to enhance immune responses, the new generations of chitosan derivatives, such as partly N-acetylated quaternary chitosan, with better mucoadjuvant properties and safe adjuvants have to be likely co-formulated. Also, active targeting strategies, using ligands that are recognized by APCs, have to be explored. It is likely that these actively targeted particles are taken up by endocytosis and it is therefore recommended, as demonstrated for liposomal vaccines [210–212] to decorate the particles with a ligand (e.g. a pH-dependent fusogenic peptide (dILINF–7)) that facilitates endosomal escape. In conclusion, chitosan-based systems offer great opportunities for delivery of protein therapeutics and antigens. To come to clinical exploitations of chitosan-based formulations of therapeutics some important hurdles need to be taken, as pointed out in this review. Chitosan-based vaccines have shown excellent potency in preclinical models and promising results in clinical trials but also for these systems further optimizations are necessary for clinical approval.

References


