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Covalently stabilized trimethyl chitosan-hyaluronic acid nanoparticles for nasal and intradermal vaccination

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ABSTRACT

The physical stability of polyelectrolyte nanocomplexes composed of trimethyl chitosan (TMC) and hyaluronic acid (HA) is limited in physiological conditions. This may minimize the favorable adjuvant effects associated with particulate systems for nasal and intradermal immunization. Therefore, covalently stabilized nanoparticles loaded with ovalbumin (OVA) were prepared with thiolated TMC and thiolated HA via ionic gelation followed by spontaneous disulfide formation after incubation at pH 7.4 and 37 °C. Also, maleimide PEG was coupled to the remaining thiol-moieties on the particles to shield their surface charge.
OVA-loaded TMC/HA nanoparticles had a size of around 250–350 nm, a positive zeta potential and OVA loading efficiencies up to 60%. Reacting the thiolated particles with maleimide PEG resulted in a slight reduction of zeta potential (from +7 to +4 mV) and a minor increase in particle size. Stabilized TMC-S-S-HA particles (PEGylated or not) showed superior stability in saline solutions compared to non-stabilized particles (composed of nonthiolated polymers) but readily disintegrated upon incubation in a saline buffer containing 10 mM dithiothreitol. In both the nasal and intradermal immunization study, OVA loaded stabilized TMC-S-S-HA particles demonstrated superior immunogenicity compared to non-stabilized particles (indicated by higher IgG titers). Intranasal, PEGylation completely abolished the beneficial effects of stabilization and it induced no enhanced immune responses against OVA after intradermal administration. In conclusion, stabilization of the TMC/HA particulate system greatly enhances the immunogenicity of OVA in nasal and intradermal vaccination.

1. Introduction

Most vaccines under development today are subunit vaccines based on highly purified and well-characterized antigens derived from the respective pathogens against which one wants to protect. Although favorable because of their safety profile, these purified proteins generally show reduced immunogenicity compared to inactivated or attenuated pathogens [1–3]. Therefore, subunit vaccines have to be formulated with adjuvants, i.e. delivery systems and/or immune potentiators that improve the immunogenicity of the antigens to elicit adequate, protective immune responses [4]. For instance, when co-formulated in micro- or nanoparticles, foreign proteins are much more effective in eliciting immune responses than as plain protein solution [5–9], most likely because of the particle's resemblance to the original pathogen, their multimeric antigen presentation and improved protection of the antigen against degradation [10]. Furthermore, particles are better taken up by antigen presenting cells (APCs), they may prolong the residence time of the antigen at the site of action and can co-deliver antigen and adjuvant to the same cell [8,11,12]. As a consequence, several types of particulate systems have been studied for vaccine delivery, including liposomes, oil-in-water emulsions, virus like particles, ISCOMs and polymeric carriers [1,8,9,13].

Alternative vaccine administration routes to conventional intramuscular immunization have been widely studied [2,3,5,10,14]. Mucosal vaccination offers several advantages over invasive (intramuscular or subcutaneous) immunization routes, like needle-free administration, potentially less adverse effects and the induction of local mucosal immune responses [15]. However, adequate antigen delivery via the nasal route is challenging because of intranasal degradation and poor antigen uptake through the nasal epithelium. As trimethyl chitosan (TMC, a quaternized, water-soluble derivative of chitosan) and hyaluronic acid (HA) have muco-adhesive properties [16,17], both polymers have been investigated in particulate form in mucosal vaccine delivery [5,18–23]. In nasal vaccine delivery, TMC nanoparticles have proven to have excellent adjuvant properties, most likely due to improved antigen delivery [5,24], but also immunostimulatory effects of TMC on monocyte derived dendritic cells (DCs) were observed [12,23]. In these studies tripolyphosphate (TPP) was used as a physical crosslinker to form TMC nanoparticles via ionic gelation. Interestingly, results by Sayın et al. [25] showed that nasal immunization with tetanus toxoid loaded TMC:mono-*N*-carboxymethyl chitosan nanoparticles

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resulted in superior antibody titers compared to the TMC/TPP particles, indicating that combining TMC with an anionic polymer may further improve the adjuvant activity. However, the physical stability of such polyelectrolyte complexes may be limited in physiological conditions [26,27] or at low pH [28].

Intradermal (ID) immunization is another interesting immunization route. While muscular and subcutaneous tissues contain only limited numbers of APCs [29], skin tissue is abundant in DCs that play a central role in eliciting an immune response [12]. Recently, TMC nanoparticles were studied as intradermal vaccine carrier system, showing superior antibody titers against ovalbumin and diphtheria toxoid (DT) compared to the plain antigens. For DT encapsulated in TMC nanoparticles comparable immunopotency as subcutaneously administered DT-alum was observed [12].

Recently, we developed covalently stabilized nanoparticles made from two oppositely charged, partially thiolated polymers, namely, thiolated trimethyl chitosan (TMC-SH) and thiolated hyaluronic acid (HA-SH) [30]. Polyelectrolyte complexes prepared with these polymers had a size of about 200–300 nm, a positive zeta potential and showed ovalbumin (OVA) encapsulation capacity up to 30%. The intermolecular disulfide bonds resulted in increased stability of the TMC-S-S-HA particles in saline as compared to particles made with their nonthiolated counterparts (TMC/HA particles), which were kept together only by electrostatic interactions. Importantly, these covalently stabilized particles still allowed simple, aqueous and low stress preparation conditions as used with the preparation of conventional polyelectrolyte complexes [20,27].

It can be expected that, with both nasal and ID immunization, antigen-loaded covalently stabilized TMC-S-S-HA particles may show enhanced immunogenicity compared to non-stabilized particles because superior particle integrity in the external environment may result in improved antigen delivery to, and activation of, APCs.

Furthermore, remaining thiol groups present on the surface of TMC-S-S-HA particles allow post-particle modifications [31,32]. Selective PEGylation of the free thiol moieties with PEG-maleimide may prove an interesting strategy as PEGylation of chitosan led to improved antibody titers in a nasal vaccination study with diphtheria toxoid possibly due to improved stability [1]. Furthermore, shielding of cationic charges may be beneficial in ID vaccination by limiting interactions with the negatively charged extracellular matrix. Therefore, PEGylation may result in increased mobility and uptake by APCs [33].

In the present study we investigated covalently stabilized TMC-S-S-HA nanoparticles, with and without PEG coating, as potential nasal and intradermal vaccine delivery systems and compared them with non-stabilized TMC/HA nanoparticles. The particles contained ovalbumin as a model antigen. The formulations were physico-chemically characterized and their stability in buffered saline and in the presence or absence of a reducing agent was studied. Furthermore, the extent and type of immune response elicited after nasal and intradermal administration of the formulations in mice was determined.

2. Materials and methods

2.1. Materials

Chitosan with a residual degree of acetylation (DAc) of 17% (determined with ^{1}H-NMR) and a number average (M_n) and weight average molecular weight (M_w) of 28 and 43 kDa, (determined with GPC-TD as described in Section 2.2), respectively, was purchased from Primex (Siglufjodur, Iceland). Sodium borohydride, formaldehyde 37% (stabilized with methanol), glyoxilic acid monohydrate, cystamine dihydrochloride, dithiotreitol (DTT), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC), L-cysteine HCl monohydrate, hen egg-white ovalbumin (OVA, grade V), deuterium oxide, sodium acetate, acetic acid (anhydrous), sodium hydroxide and hydrochloric acid were obtained from Sigma-Aldrich Chemical Co. Fluorescently labeled ovalbumin (OVA-FITC) was obtained from Invitrogen (Breda, The Netherlands). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (γ chain specific), IgG1 (γ1 chain specific) and IgG2a (γ2a chain specific) were purchased from Southern Biotech Birmingham, USA. Chromogen 3, 3′, 5, 5′-tetramethylbenzidine (TMB) and the substrate buffer were purchased from Invitrogen. Iodomethane 99% stabilized with copper was obtained from Acros Organics (Geel, Belgium). 5,5-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent) was purchased from Pierce (Rockford, IL, USA). Hyaluronic acid (HA, molecular weight 17 kDa as determined by manufacturer) was obtained from Lifecore (Chaska, USA). Methoxy polyethylene glycol (mPEG) maleimide (M_w 2000) was purchased from JenKem Technology (Beijing, China). TMCs with degrees of quaternization (DQs) of 30% (M_n 33 kDa, M_w 59 kDa) and 56% (M_n 37 kDa, M_w 78 kDa) were synthesized and characterized as described previously [34]. Thiolated hyaluronic acid (HA-SH) with a M_n of 15 kDa, PDI of 2.6 and a degree of thiolation of 21% was synthesized and characterized according to the procedure by Shu et al. [35,36]. All other chemicals used were of analytical grade.

2.2. Synthesis and characterization of O-methyl free, trimethylated, partially thiolated chitosan (TMC-SH)

Thiolated TMCs with different degrees of quaternization (DQ) were synthesized as described before [30]. The degree of thiolation (D_{thiol}) and the total thiol content (free thiol moieties and disulfides) of the TMC-SHs were quantified with 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) as described elsewhere [30,37]. The M_n and M_w of the TMC-SHs were determined, as described previously [38], by GPC on a Viscotek system detecting refractive index, viscosity and light scattering. A Shodex OHPak SB-806 column (30 cm) was used with 0.3 M sodium acetate pH 4.4 (adjusted with acetic acid) as running buffer.

2.3. Preparation of covalently stabilized nanoparticles with or without post-PEGylation

Covalently stabilized nanoparticles loaded with ovalbumin (OVA) were prepared with TMC-SH and HA-SH essentially as described before [30]. TMC-SH with a DQ 25% and D_{thiol} 5% or a DQ of 54% and D_{thiol} 6% were dissolved in 10 mM HEPES pH 7.4 at 1 mg/ml and subsequently mixed with 2.5 mg/ml ovalbumin in 10 mM HEPES pH 7.4 at a weight ratio of 10:1 (TMC-SH:OVA) under magnetic stirring. Then, HA-SH (0.5 mg/ml in 10 mM HEPES pH 7.4) was added drop-wise yielding an opalescent nanoparticle dispersion which was incubated at 37 °C for 3 hours to allow disulfide formation.

In case of PEGylated particles, after adding the HA-SH solution to the TMC-SH/OVA mixture, the formed particles were incubated for 30 min at 37 °C for spontaneous disulfide formation. Then mPEG maleimide (M_w 2000 Da) was added to the mixture in a TMC:PEG w/w ratio of 2/1 and particles were incubated for an additional 2.5 hours at 37 °C. In this second incubation step, next to disulfide formation, the remaining thiol-moieties on the surface of the particles were used to react with the maleimide group on the PEG at pH 7.4 (post PEGylation of the TMC-S-S-HA particles). Also, nanoparticles with non-thiolated TMC (DQ 30% or 56%), OVA and hyaluronic acid (HA) were prepared in a similar way to obtain 'conventional' particles only kept together by charge interactions.

After incubation, the nanoparticle dispersions were centrifuged for 10 min at 10000 rpm (Biofuge Pico, PP1/97 #3324; Heraeus Instruments, Osterode, Germany) to remove the free polymers and unbound OVA. The obtained nanoparticle pellets were resuspended in 10 mM HEPES pH 7.4 and diluted to obtain a final OVA concentration of 0.5 mg/ml. An overview of the different formulations is presented in Table 2.

2.3.1. Physical characterization of prepared nanoparticles

Particles ($n=3$) were diluted in 10 mM HEPES until a slightly opalescent dispersion was obtained. Particle size was measured by