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Preparation and evaluation of chitosan nanoparticles containing *Diphtheria* toxoid as new carriers for nasal vaccine delivery in mice

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ABSTRACT

The aim of the present work was to investigate the potential utility of nanoparticles made of chitosan (CS) and also CS chemically modified with polyethylene glycol (CS-PEG) as new vehicles for improving nasal vaccine delivery. For this purpose, diphtheria toxoid (DT) was chosen as a model antigen. DT was entrapped within nanoparticles made of CS of different molecular weight, and also made of CS-PEG, by an ionic cross linking technique. DT-loaded nanoparticles were characterized for their size, surface charge, loading efficiency and *in vitro* release of antigenically active toxoid. The nanoparticles were then administered intranasally to conscious mice in order to study their feasibility as vaccine carriers. The resulting nanoparticles had a size, which varied depending on the formulation conditions and on the PEG derivatization, between 100 and 500 nm. They exhibited a positive electrical charge (approx. +40 mV) which was substantially reduced for the PEGgylated CS nanoparticles (approx. +10 mV) and showed and excellent DT loading capacity (loading efficiency between 50-100% depending on the formulation). The results of the *in vitro* release studies displayed a biphasic release of antigenically active toxoid, the intensity of the first phase being less pronounced for CS-PEG nanoparticles than for CS nanoparticles. Following intranasal administration, DT-loaded nanoparticles elicited an increasing and enhanced humoral immunogenic response (IgG titers), as compared to the fluid vaccine. Similarly, the mucosal response (IgA levels) achieved at 70 days post-administration was significantly higher for the DT-loaded CS nanoparticles than for the fluid vaccine. Interestingly, this response was not affected by the CS molecular weight but it was positively influenced by the PEGylation of CS. CS and CS-PEG nanoparticles are promising carriers for nasal immunization with DT.

Keywords: Chitosan, Nanoparticles, Polyethylene glycol, Vaccines, Nasal administration

INTRODUCTION

Most human pathogens initiate their infection processes at mucosal surfaces; however, most of the conventional vaccines available against these agents are usually given parentally. It is well established that the mucosal administration of vaccines can provide effective humoral and cellular immuno responses, especially if the antigen is adjuvanted using an immunostimulator or a delivery system

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(Husband 1993, Lemoine *et al* 1998 and Partidos 2000). Among the delivery systems investigated until now, the use of micro and nanoparticles made of hydrophobic polymers such as the poly (lactic/glycolic acid) (PLGA) copolymers offer great promise for nasal vaccination (Almeida *et al* 1993, Ray *et al* 1993, Moore *et al* 1995, Shanin *et al* 1995 and Eyles *et al* 1998). Indeed, in these studies it has been shown that, following nasal administration, the encapsulated antigens elicit a higher immune response than fluid vaccines. More recent work carried out by our group has revealed that the size and surface composition play a significant role on the ability of these biodegradable particles to transport the associated antigen across the nasal mucosa and elicit an immune response. More specifically, it was reported that a hydrophilic PEG coating around PLA nanoparticles has a very positive effect in increasing the transport of the encapsulated antigen (tetanus toxoid) (Tobío *et al*. 1998) and, also, that the size of the PEG-coated particles affects the intensity of this transport (Vila *et al*. 2005). This was justified by the improved stability of the particles in contact with the mucus layer, followed by a greater transport of the antigen encapsulated in PEG-PLA nanoparticles as compared to that of antigen-loaded PLA nanoparticles (Vila *et al* 2002 and Vila *et al* 2004 a). Moreover, recently it was shown that this improved behavior of the PEG-coated particles following nasal administration has a remarkable consequence on the immune response of the encapsulated antigen (Vila *et al* 2004 b).

As an alternative approach for improving nasal vaccine delivery was designed as a new type of nanoparticles based upon the hydrophilic polysaccharide chitosan (CS) (Calvo *et al* 1997). This polymer was chosen because of its biodegradability, low toxicity, mucoadhesive properties and ability to enhance the penetration of drugs across mucosal barriers (Hirano *et al* 1990, Knapczzyk *et al* 1989, Artursson *et al* 1994 and Borchard *et al* 1996). Moreover, it has already been shown that CS in solution enhances the immune response to antigens administered intranasally, among them DT (Jabbal-Gill *et al* 1998, Bacon *et al* 2000 and McNeela *et al* 2001). Besides these interesting biological properties inherent to CS molecules, CS has the ability to gel upon interaction with specific anions leading to the formation of nanostructures which may exhibit additional advantages as compared to the CS solutions. For example, CS nanoparticles, which can be prepared according to a very mild technique, have shown a great capacity for the association of proteins as well as the ability to provide a favourable *in vitro* release of the entrapped protein (Calvo *et al* 1997 a, Calvo *et al* 1997 b). Moreover while the performance of CS solutions for nasal administration appears to be dependent on the use of a high molecular weight CS, the efficacy of a suspension of CS nanoparticles at improving the transport of macromolecules across the nasal barrier has not been limited by this property (Fernández-Urrusuno *et al* 1999). Furthermore, recent work from our group has shown that nanoparticles made of low molecular weight CS (less than 100 KDa) are able enhance and prolong the immune response elicited by the associated antigen (Vila *et al* 2004 c).

The increasing interest that CS is receiving in the pharmaceutical field has motivated its chemical modification with the purpose of improving its physicochemical and biomedical properties. For example, chemical modifications with carboxymethylene (Muzzarelli *et al* 1982) or Poly(ethylene glycol) (Sugimoto *et al* 1997) have been intended to increase the pH solubility range of CS. Moreover, its modification with PEG has been specially addressed to improve its biological performance (Mo *et al* 1997). Based on this information, the aim of the present work was to explore the potential of CS and CS-PEG nanoparticles as vehicles for the nasal administration of vaccines. More specifically, the studies were

aimed at evaluating the feasibility of nanoparticles made of CS of different molecular weight CS and also CS-PEG as carriers for nasal administration of diphtheria toxoid (DT). Following a rigorous *in vitro* characterization, the nanoparticles were administered intranasally to conscious mice and the systemic and mucosal antibody responses were evaluated at different times post-administration.

MATERIALS AND METHODS

Chemicals. Chitosan as a hydrochloride salt (Protasan Cl®110 and Protasan Cl®213 with a deacetylation degree of 86% respectively, was purchased from Pronova Biopolymer, S.A. (Norway)). According to the provider the apparent viscosity is 16 mPas for Protasan Cl®110 and 71 mPas for Protasan Cl®213. The molecular weight of Protasan Cl®110 calculated from viscosity measurements was ≈ 100 kDa. Chemical modified CS by polyethylene glycol (CS-110 PEG 1%) synthesis in our chemical lab. Purified Diphtheria Toxoid (DT, 1500 Lf/ml) in phosphate buffer saline pH 7.4, purified equine DT anti-toxin (100 Lf/ml) and purified mouse anti-DT IgG gifted kindly from Razi Institute for Serums and Vaccine Research, Tehran, Iran. A sample of DTP-alum (Diphtheria, Tetanus and pertussis, Infanrix, Smithkline Beecham, Madrid, Spain) was kindly donated by the Service of vaccines, SERGAS, Santiago de Compostela, Spain. Purified mouse immunoglobulin (reagent grade), Goat anti-mouse Ig G (whole molecule) peroxidase conjugate, goat anti-mouse immunoglobulin A (α-chain specific) peroxidase conjugate, o-phenylenediamine dihydrochloride, phenylmethyl sulfonyl fluoride (PMSF), sodium pentobarbital and pilocarpine were purchased from Sigma-Aldrich, Madrid, Spain. Pentasodium tripolyphosphate (TPP) and trehalose were supplied by Sigma Chemical (USA). Glucose was provided by Merck (Darmstadt, Germany). Ultrapure water (MilliQ Plus, Millipore Iberica S.A., Spain) was used throughout the study.

Male BALB/c mice (6 weeks old, 22-25 g), from the Central Animals House of the University of Santiago de Compostela (Spain), were used. They were kept in a-12 hour light-dark cycle and temperature of 20 ± 2 °C. The animals were allowed access to food and water during the study.

Preparation and characterization of CS nanoparticles. CS and CS-PEG nanoparticles were prepared base on the ionotropic gelation of CS upon contact with the TPP anions as described previously (Vila *et al* 2004 c). Briefly, the particles were formed spontaneously upon addition of different volumes of an aqueous solution of TPP (1 mg/ml) to 3 ml of a solution of CS or CS-PEG (1 mg/ml) under magnetic stirring. The volumes of the TPP solution were calculated in order to lead to CS:TPP ratios of 8:1 to 2:1. For the association of DT to CS nanoparticles, DT was incorporated in the TPP solution (300 µg of DT). CS nanoparticles were isolated by centrifugation at 10,000g on a glycerol bed, for 40 min at 5 °C. In the case of CS-PEG, the nanoparticles were collected over centrifugal ultrafilter (Amicon® ultra-4 100000 NMWL, Millipore, USA) at 3800g for 30 min. Supernatants were discarded and nanoparticles were resuspended in 5% glucose for lyophilized or phosphate buffer saline pH 7.4 for their administration to mice.

The particle size and zeta potential of nanoparticles were determined by photon correlation spectroscopy (PCS) and Laser Doppler Anemometry (LDA) using a Zetasizer®3000-HS (Malvern Instruments, United Kingdom) respectively. For the determination of the electrophoretic mobility, samples were diluted with 1 mM KCl and placed in the electrophoretic cell where a potential ±150 mV was established. Each batch was analyzed in triplicate. The morphology of the nanoparticles was examined by transmission electron microscopy (CM 12 Philips, Eindhoven, Netherlands).

Evaluation of DT encapsulation. The encapsulation efficiency and nanoparticle yield of the different formulations were determined by centrifugation of the samples at 16000g at 5 °C for 30 min. The sedimented nanoparticles were incubated at 80 °C overnight and weighed for the determination of the process yield. The amount of DT associated to the nanoparticles was calculated by the difference between the total amount used to prepare the particles and the amount of DT present in the aqueous phase determined using the micro-bichinchoninic acid (microBCA) protein assay (Pierce®, Rockford, USA) against a supernatant of blank nanoparticles. The encapsulation efficiency was calculated as follows:

$$\text{DT encapsulation efficiency} = \frac{\textit{Total}\ \text{DT} - \textit{Free}\ \text{DT}}{\textit{Total}\ \text{DT}}$$

Evaluation of *in vitro* DT release. Samples of 10 mg of nanoparticles were suspended in 1.5 ml of 5% w/v trehalose solution and incubated at 37 °C. At predetermined time intervals, the samples were centrifuged at 10,000g for 40 min at 5 °C. One ml of supernatant was removed and replaced by fresh release medium. The re-suspended particles were maintained under incubation at 37 °C. The amount of antigen DT released was determined by an indirect ELISA test as described previously (Muzzarelli *et al* 1982). The results are shown as the percentage of antigenically active DT released with respect to the total amount of DT associated in nanoparticles.

Immunization studies. Immunogenicity of the CS formulations was assessed in BALB/c mice following intranasal immunization. A dose (10 μg) of DT incorporated in 100 μg CS nanoparticles was given in 10 μl of PBS pH 7.4 (5 μl into each nostril) on days 0, 7 and 14 to conscious mice. For this study, five groups of mice (n = 6) were used. Two groups were treated with DT-loaded CS nanoparticles (CS-113 Cl, CS-213 Cl) one group with DT-loaded CS-PEG nanoparticles, and one group with the free toxoid (10 μg/mice) in PBS pH 7.4. In addition, as a control, one group received intra-peritoneally (i.p.) DTP vaccine (Diphtheria, Tetanus and Pertussis, Infanrix®, Smithkline Beecham, Madrid, Spain) adsorbed on Alum (10 μg/mice).

Blood and fluid body sampling. Blood samples were taken from the tail of animals on days 14, 28, 42, 56 and 70 after the administration of the first dose. The collected blood samples were stored at 4 °C and then centrifuged (3500g, 15 min, 5 °C). The resulting serum samples were maintained at –20 °C until analysis. Saliva, broncho-alveolar and intestinal lavage were collected on day 70, according to a previously described method (Sugimoto *et al*. 1997). Salivation was induced by i.p injection of pilocarpine (50 μl, 1 mg/ml). An aliquot of 100 μl of the initial flow of saliva was collected from each mouse. Then the mice were anaesthetised i.p with 1.8 mg of pentobarbital, and sacrificed. Broncho-alveolar lavages were obtained by injecting and aspirating 5 ml of ice-cold lavage medium (0.9% w/v NaCl, 0.05% w/v Tween 20 and 1mM PMSF) into the trachea to inflate the lunge by means of an intravenous cannula. Intestinal segments (duodenum, jejunum, ileum) were aseptically removed and homogenized (Ultra-Turrax, IKA-werkr, GmbH Co. Germany) in 4 ml solution of 1 mM PMSF, 1 mM iodoacetic acid and 10 mM EDTA. The samples were clarified by centrifugation (20000g, 30 min, 5 °C) and sodium azide, PMSF and bovine serum were added as preservative, protein inhibitor and alternate substrate for protease activity, respectively. All samples were stored at –20 °C until assayed for antibody concentration.

Evaluation of antibody responses in serum and mucosal tissues. An ELISA test was used for the evaluation of the antibody responses in serum and mucosal tissues of mice as describe previously. Briefly, the microplates (DYNEX, immulon®, USA) were coated with 100 μl DT and incubated overnight at 4 ºC. Between steps the wells were washed three times. Then the samples were diluted serially in two

fold steps in PBSTM (PBST containing 5% w/v dried skimmed milk powder and 0.1% w/v sodium azide as preservative) and the plates were incubated for another 2 h at 37 ºC. Then, 100 µl of goat anti-mouse IgG peroxidase conjugate or goat anti-mouse IgA peroxidase conjugate was added. The plates were washed and O-phenylenediamine dihydrochloride as substrate were added to the wells. Following color development plates were read at 450 nm on microplate reader (3550-UV, Biorad, Spain).

Statistical analysis. A General Linear Model (repeated measures) test ($p<0.05$) was applied to determine the significance of the difference between IgG level means and an ANOVA test was applied to IgA level means ($p<0.05$).

RESULTS

Preparation and characterization of CS nanoparticles. CS and CS-PEG nanoparticles were prepared base on the ionotropic gelation of CS upon contact with the TPP anions. Tables 1, 2 and 3 show different conditions for CS nanoparticles preparation. TEM micrographs of these nanoparticles are shown in Figure 1. In order to find the appropriate conditions for the association of DT to CS nanoparticles it was first evaluated the influence of two critical formulation parameters, such as the ratio of CS with respect to the ionic cross linking agent TPP and also the pH of the initial CS solution.

***In vitro* release behaviour.** The *in vitro* release of nanoprticles was carried out at 37 ºC for 192 hours in PBS (pH 7.2). Because of aggregation of nanoparticles during centrifugation/re-suspension cycles, the study was discontinued after this time. The results of the *in vitro* release experiments performed for the three selected formulations are shown in Figure 2.

***In vivo* evaluation of the immune response elicited by DT-loaded nanoparticles.** The *in vivo* evaluation of the CS formulations was assessed in BALB/c mice following intranasal immunization. The anti-diphtheria IgG levels elicited by the DT-loaded nanoparticles and the control DT solution following intranasal immunization are shown in Figures 3 and 4.

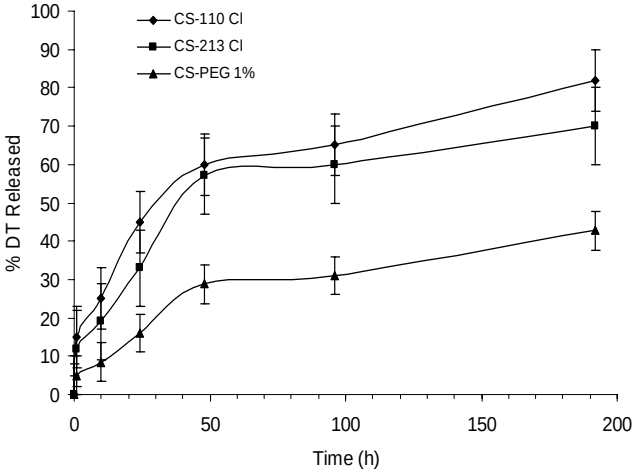

Figure 2: *In vitro* release of DT from nanoparticles made of CS and CS-PEG. (•) CS-110, (■) CS-213 and (▲) CS-PEG 1%. DT theoretical loading: 10%.

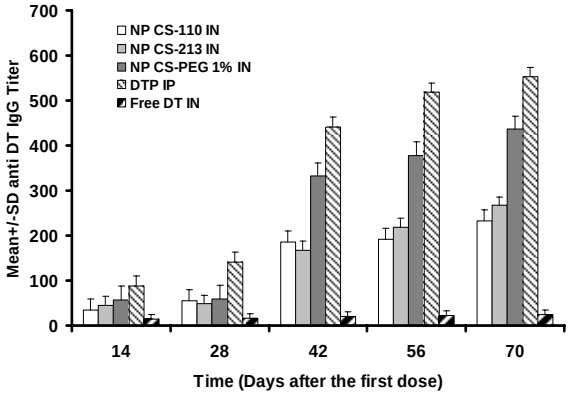

Figure 3. Anti-DT IgG end-point titers in mice serum after intranasal administration of 10 µg of DT incorporated into different formulations of CS and CS-PEG nanoparticles on day 0, 7 and 14 (n=6). IN: Intranasal; IP: Intraperitoneal.

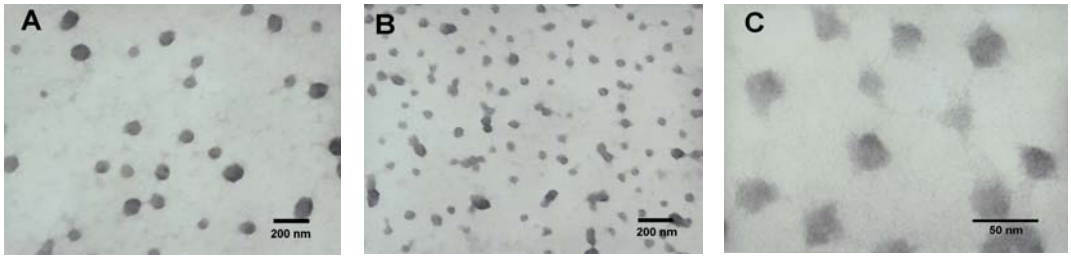

Figure 1. TEM micrographs of CS nanoparticles (A) CS-110 (CS:TPP 4:1), B) CS-213 (CS:TPP 3:1) and C) CS-PEG 1% (CS:TPP 3.1).

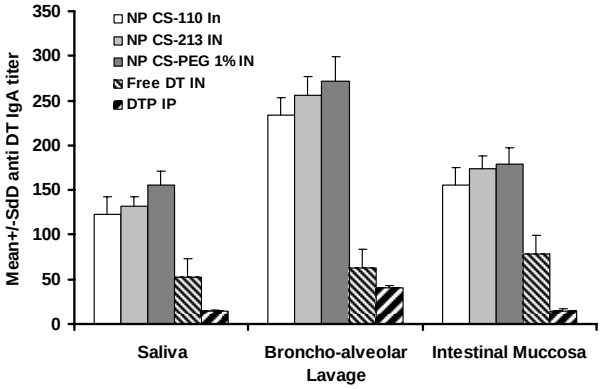

Figure 4. Anti DT IgA end-point titers in mice tissues after intranasal administration of 10 µg of DT incorporated incorporated into different formulations of CS and CS-PEG nanoparticles on day 0, 7 and 14 (n=6). IN: Intranasal; IP: Intraperitoneal.

DISCUSSION

As indicated in the introductory section, they were previously shown that CS nanoparticles, administered as an aqueous suspension, are able to enhance the transport of macromolecular drugs, i.e. insulin, and antigens such as tetanus toxoid across the nasal mucosa (Fernández-Urrusuno *et al* 1999 and Vila *et al* 2004c). This special ability has also been reported for CS solutions and CS powders (Illum *et al* 2001). However, while the performance of CS solutions has been limited to high molecular weight CS (apparent viscosity> 10 mPa, estimated molecular weight higher than 100 KDa), that of CS nanoparticles is not limited by this feature. Besides, the association of antigens to nanoparticles offer the association of antigens to nanoparticles offer some potential additional advantages such as (i) the concentration of both, antigen and CS, in association in some specific regions of the nasal mucosa (ii) the possibility to convert soluble antigens into well-controlled particulate material, which are more easily recognized by the antigen presenting cells (iii) the ability to deliver the associated antigen for extended periods of time. On the other hand, CS powders have also been considered as a candidate carrier for vaccine delivery (Illum *et al* 2001). However, a limitation of this form of presentation could be that related to the important water uptake of CS powders and the subsequent dehydration of the nasal mucosa.

Based on the above comments, we found it important to investigate whether or not the positive results that we have already observed for tetanus toxoid-loaded CS nanoparticles could be extrapolated to other protein antigens such as DT. In addition, our interest was to evaluate if the molecular weight of CS could affect the performance of the resulting nanoparticles as DT nasal carriers. Finally, taking into account the beneficial effect that the chemical modification of polymers with PEG has impaired to nanoparticulate vaccine carriers (Vila *et al* 2005, Vila *et al* 2002 and Vila *et al* 2004a), the value of this modification was also assayed.

Preparation and characterization of CS nanoparticles. The results presented in Table 1,

indicate that nanoparticles consisting of CS Cl-110 can be obtained using CS: TPP ratios of 4:1, 5:1 and 6:1. In addition, these nanoparticles can be formed from CS solutions at either pH 4.28 or 5.50. An overall conclusion from these results is that, irrespective of the formulation conditions, the size of the particles varies between 268 and 475 nm and their zeta potential between +30 and +45 m*V*. A greater dependency on the formulation conditions was observed in the nanoparticles production yield and also in the association efficiency. In particular, the nanoparticles recovery was remarkably higher when the pH of the CS solution was raised to 5.5 and also for the 4:1 CS:TPP ratio (highest TPP content). These results are logical if we take into that the more important amount of TPP permits the cross-linking of a more important number of CS molecules and, hence, the formation of a larger population of nanoparticles (Fernández-Urrusuno *et al* 1999). In addition, it may change the conformation of the CS molecules and decrease its solubility by increasing the pH (CS, pKa=6.2), therefore, favouring its tendency to nanoaggregate. On the other hand, in Table 1 it can also be noted that, as expected, the formulations with a more important recovery are those which exhibit an optimum association efficiency. Obviously, as the number of particles increases, there is a greater possibility for the protein molecules to associate to them. This important DT association efficiency could be interpreted by its low solubility and favourable interaction with CS. In fact, DT is an acidic protein (*I*p= 4.2-4.8) that it is negatively ionized at the pH of the TPP solution and, thus prone to interact ionically with the positively charged CS molecules. The same explanation was used to explain the important association of TT and BSA to these nanoparticles (Calvo *et al* 1997). However, there is possibility of hydrogen bonding and hydrophobic interactions between the protein and CS.

Following the observation of the positive effect of increasing the amount of TPP a formulation containing CS:TPP 3:1 was prepared. Unfortunately, the DT loaded particles prepared in these conditions suffered and aggregation process, a result that was attributed to the excessive neutralization of the positive charges of CS due to its interaction with TPP and also with DT.

In a second step of the formulation process, a high molecular weight CS (CS-213) and the most favourable conditions defined in the previous step (CS:TPP ratio 4:1 and pH of CS solution: 5.50) was selected. In addition, it was attempted to reduce the CS:TPP ratio down to 3:1. As expected, the recovery was increased by raising the amount of TPP and the DT association efficiency was accordingly enhanced (Table 2). Under these conditions, the size varied between 244 and 288 nm and the zeta potential between +25 and +35 m*V*. This slight reduction in the zeta potential as compared to the values observed for the lower molecular weight CS (CS-110) is in good agreement with the previously reported values (Fernández-Urrusuno *et al* 1999). Although there were not found a clear explanation for this result, it could be speculated that the length of the CS chains affect their cross-linking and entanglement during the formation of the nanoparticles. In a third step, for the preparation of CS-PEG nanoparticles, the CS:TPP ratio of 3:1 were selected and evaluated the effect of the pH (5.50 and 4.28). Interestingly, the size of CS-PEG nanoparticles was significantly reduced (between 80-150 nm) and the zeta potential importantly neutralized (between + 8 and +12 m*V*), as compared to those of CS nanoparticles (Table 3). Probably, the PEG*ylation* of CS limits the intermolecular cross-linking of the polymer molecules, thus resulting in nanoparticles of very small size. In addition, the PEG*ylation* implies the consumption of some amine groups and, thus, the reduction of the CS electrical charge. It could also be understood that, the presence of PEG molecules on the surface of the particles would lead to a displacement of the shear plain and, hence, to a reduction of their surface charge (data not shown).

The TEM images illustrated in Figure 1 show the size and physical appearance of some selected formulations of DT-loaded CS nanoparticles. These formulations, which were selected for further *in vitro* and *in vivo* evaluation, exhibited a very small size (between 50 and 200 nm) and a dense and spherical structure. In addition, the nanoparticles made of CS-PEG showed a less defined surface which appears to be partially covered by a sort of fluffy material. This could be assigned to the presence of PEG on the surface of the particles.

***In vitro* release behaviour.** The *in vitro* releases of CS nanoparticles are shown in Figure 2. These results, which correspond to the amount of antigenically active toxoid released over the time, indicate that DT is continuously released from the nanoparticles in its active form for a long time. Moreover, under the present experimental conditions, the DT release was not affected by the molecular weight of the polymer, however, it was clearly dependent on the chemical derivatization of CS with PEG. These results provide some insights about the mechanism of release. Under the present experimental conditions, the polymer is not expected to degrade and, thus, the release of the associated protein will be a result of two simultaneous processes: (i) the disassociation of the CS chains, which will may lead to the fragmentation of the nanoparticles and, consequently, to the release of the entrapped protein, (ii) the disassociation of the protein from the CS polymer chains. Therefore, in agreement with this hypothetical mechanism, the disassociation process of the protein is not affected by the CS chain length but, it is importantly influenced by the copolymer composition. Accordingly, one could speculate that, either the interaction forces of DT with CS-PEG are more important than those with CS, or the PEG chains located not only inside but also within the nanoparticle structure hinders the diffusion of the protein through the gel towards the external aqueous environment. The first possibility does not agree with the protein-rejecting properties normally ascribed to PEG, although, one would expect that the protein-PEG forces are very much dependent on the protein. However, both possibilities agree well with the more important protein association efficiency observed for the CS-PEG nanoparticles.

It is note worthy to mention that these results are similar to those previously reported for bovine serum albumin (Calvo *et al* 1997). The similarity may come from the fact that both DT and BSA have a comparable size and isoelectric point, and, consequently, the interaction forces between the protein and the polymer might also be comparable. A very different profile was observed for the release of TT from CS nanoparticles (Vila *et al* 2004 c). In this case, the release was very slow; a result that may be related to the large size of this protein (150 kDa) and its interaction forces with CS.

***In vivo* evaluation of the immune response elicited by DT-loaded nanoparticles.** The anti-diphtheria IgG levels elicited by the DT-loaded nanoparticles and the control DT solution following intranasal immunization are shown in Figure 3. In this figure, the results corresponding to the marketed formulation (Aluminum Phosphate-Adsorbed DT) administered intraperitoneally was represented. Overall, the results indicate that, after the first month, the IgG levels observed for the DT-loaded nanoparticles were significantly higher than those corresponding to the fluid vaccine ($p<0.05$). In fact, these values are comparable to those elicited by the adjuvanted formulation (Aluminum Phosphate-Adsorbed DT) administered parenterally. Consequently, these results clearly reflect the adjuvant effect of the nanoparticulate formulations. Another remarkable observation was the long-lasting and increasing immune response over the time. Finally, with respect to the influence of the nanoparticles composition, it was interesting to note that the molecular weight of CS did not have an effect on the immune response achieved for CS nanoparticles; however, the PEG*ylation* of CS had a

remarkable consequence on the efficacy of the nanoparticles. Indeed, after the first month, the IgG levels were significantly higher for CS-PEG nanoparticles than for CS nanoparticles.

The conclusions that can be drawn from the anti-diphtheria IgA titers detected in saliva, broncho-alveolar and intestinal lavages at 70 days post the first dose administered (Figure 4) are slightly different from those of the IgG titers. As it was the case for the humoral response, the mucosal response elicited by the DT-loaded particles was significantly higher than that of the free toxoid. However, in this case, the response was also remarkably higher that corresponding to the alum-adsorbed DT administered intra-peritoneally ($p<0.05$). This could be explained by the different presentation of the antigen, following both modalities of administration. In fact, it is known that the mucosal vaccination is the most effective way of inducing a protective mucosal immune response (Holmgren *et al* 1996).

There are several previous observations that could help to understand the possible mechanisms by which, CS and CS-PEG, nanoparticles enhance the immune response to nasally applied DT. First, taking into account the dose-dependent decreases in the trans-epithelial resistance of the Caco-2 cell mono-layers caused by CS (Dodane *et al* 1999), and the facilitated interaction CS nanoparticles with the mucus (Behrens *et al* 2002). It was assumed that the nanoparticles could reach the underlying epithelium increasing its permeability and simultaneously delivering the associated toxoid. This penetration enhancement mechanism was previously proposed to explain the adjuvant effect of CS solutions following intranasal administration of *Bordetella pertussis* proteins (Jabbal-Gill *et al* 1998), influenza virus (Bacon *et al* 2000), and diphtheria toxoid (McNeela *et al* 2001). Secondly, there is also the possibility that these CS molecules, and even entire nanoparticles, will cross the nasal mucosal and, hence, exhibit the immunoadjuvant properties, classically attributed to CS (Nishimura *et al* 1984, Nishimura *et al* 1985 and Iida *et al* 1987). This activity, which would necessarily imply the internalization of CS, agrees with the reported ability of CS to increase cell permeability by affecting both paracellular and intracellular pathways (Dodane *et al* 1999). The fact that CS can enter mucosal barriers has been shown to some extent for CS solutions, CS nanoparticles and CS microparticles (Van del Lunbe *et al* 2001). For example, it was previously visualized the transport of soluble CS and also nanoparticulated CS by confocal microscopy, while crossing the well organized rabbit corneal epithelium (De Campos *et al* 2004).

However, despite the ability to cross mucosal barriers, the explanation of the immunoadjuvant effect of CS does not seem to apply since the co-administration of CS and an antigen (at different times) has not led to an enhancement of the immune response. Nevertheless, what it could be well assumed is that, at least, some particles will cross the mucosa, thereby transporting the associated antigen and delivering it for extended periods of time. Therefore, it is the carrier effect and possibly the penetration enhancement ability, the mechanisms which could explain the increasing and long lasting responses observed in our study. In addition, besides the transport of the nanoparticle-associated antigen across the regular nasal epithelial cells, CS nanoparticles could also be taken up by the NALT cells.

In fact, some preliminary studies using fluorescent microscopy revealed an intense fluorescence in this specific region following nasal administration of CS fluorescent nanoparticles to mice. However, more detailed studies need to be carried out in order to further corroborate the preferable uptake of the particles by the NALT cells. The same mechanisms have been postulated to explain the efficacy of CS-based colloidal systems as nasal vaccine carriers either for model protein (Nagamoto *et al* 2004) or DNA vaccines (Xu *et al* 2004). The similar responses observed for CS nanoparticles

irrespective of the CS molecular are in good agreement with a previous work that showed that the CS molecular weight has only a minor effect on the efficacy of CS nanoparticles as carriers for tetanus toxoid (Vila *et al* 2004 c). In this sense, it is important to call the attention to the fact that the effect of the CS molecular weight on the permeability enhancing properties of CS solutions has not been completely identified. For example, Shipper *et al* (Shipper *et al* 1996) observed that the effect of the molecular weight on the ability of CS to increase membrane permeability on Caco-2 cell cultures was dependent on the deacetylation degree. In contrast, this effect was not observed when studying the performance of CS nanoparticles and CS nanocapsules as nasal and intestinal macromolecular drug carriers (Fernández-Urrusuno *et al* 1999 and Prego *et al* 2005). Therefore, at present it seems reasonable to accept that the importance of CS molecular weight may vary depending on multiple parameters such as the CS type, its deacetylation degree and its presentation form (solution vs. Particles).

Finally, the explanation to the positive effect of the PEGylation on the humoral responses (IgG titers) elicited by DT-loaded CS nanoparticles (Figuer 3) could be founded upon different basis. One could be related to the smaller size of the CS-PEG nanoparticles (100 nm approx.) as compared to CS nanoparticles (200-300 nm approx.). However, given the small difference in size, this hypothesis does not seem to be plausible. In fact, previous works with PEG-modified polyester nanoparticles (Vila *et al* 2004 b) used for nasal vaccination have agreed on the negligible effect of the particle size (as far as it is within the manometer range) on the immune response. On the other hand, CS-PEG nanoparticles displayed a slower antigen release as compared to CS nanoparticles (Figure 2). This ability of CS-PEG nanoparticles to further sustain the release of the associated DT could be related to the more important response elicited at late times (after 42 days). However, this interpretation should be taken cautiously given the fact that the *in vitro* release was monitored for a short time and also the remarkable difference between the *in vitro* experimental conditions and the *in vivo* release environment. Finally, the enhanced *in vivo* response assigned to the Peg*ylated* particles could be in relation with the greater ability of these particles to overcome the nasal barrier. This hypothesis is supported by the reported positive effect of PEG in the interaction and transport of PEG-coated polyester nanoparticles across the nasal mucosa (Vila *et al* 2004a). However, due to the different nature of both types of nanoparticles, it is obvious that this latter hypothesis requires further corroboration.

CS and CS-PEG nanoparticles were able to efficiently associate diphtheria toxoid and deliver it adequately following intranasal administration, eliciting high and long lasting mucosal and humoral immune responses. Given the versatility of these nanoparticles and the facility for the association of macromolecules to them, they could be considered as promising new nasal vaccine delivery systems.

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