Chitosan and Chitosan/Ethylene Oxide-Propylene Oxide Block Copolymer Nanoparticles as Novel Carriers for Proteins and Vaccines

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Purpose. The aim of this study was to investigate the interaction between the components of novel chitosan (CS) and CS/ethylene oxide-propylene oxide block copolymer (PEO-PPO) nanoparticles and to evaluate their potential for the association and controlled release of proteins and vaccines.

Methods. The presence of PEO-PPO on the surface of the nanoparticles and its interaction with the CS was identified by X-ray photoelectron spectroscopy (XPS). The mechanism of protein association was elucidated using several proteins, bovine serum albumin (BSA), and tetanus and diphtheria toxoids, and varying the formulation conditions (different pH values and concentrations of PEO-PPO), and the stage of protein incorporation into the nanoparticles formation medium.

Results. BSA and tetanus and diphtheria toxoids were highly associated with CS nanoparticles partly due to electrostatic interactions between the carboxyl groups of the protein and the amine groups of CS. PEO-PPO also interacted electrostatically with CS, thus competing with the proteins for association with CS nanoparticles. A visible amount of PEO-PPO was projected towards the outer phase of the nanoparticles. Proteins were released from the nanoparticles at an almost constant rate, the intensity of which was closely related to the protein loading. Furthermore, the tetanus vaccine was released in the active form for at least 15 days.

Conclusions. CS and CS/PEO-PPO nanoparticles prepared by a very mild ionic crosslinking technique are novel and suitable systems for the entrapment and controlled release of proteins and vaccines.

KEY WORDS: chitosan; ethylene oxide-propylene oxide block copolymer; nanoparticles; vaccine delivery; protein delivery; ionic gelation.

INTRODUCTION

The spectacular advance of genetic engineering and the recombinant DNA technology have been responsible for the current increase in the production of proteins for pharmaceutical use and the development of a new generation of recombinant vaccines. Unfortunately, despite the great potential of these new therapeutic and antigenic peptides and proteins only a low number has received approval by the FDA and other regulatory agencies. This has been, in general, a consequence of the lack of an appropriate form of administration which would permit the therapeutic potential of these molecules to be exploited. In fact, most of them still need to be administered repeatedly in an injectable form. Therefore, nowadays, the design of effective delivery systems and the search of new routes of administration for this new generation of drugs and vaccines are important challenges for pharmaceutical scientists. Alternative routes of administration that are being extensively explored include several mucosal surfaces such as the nasal, pulmonary, and peroral mucosae (1–4). In addition, of all the types of protein delivery systems currently under investigation, the transmucosal ones are showing the greatest promise, especially for mucosal immunization (5). It should be, however, kept in mind that the administration of macromolecules by these routes is also strongly hampered, not only by the chemical and physical instability of these molecules, but also by the high metabolic activity and the limited permeability of the mucosal barriers (6). These limitations have been overcome differently by using enzyme inhibitors, absorption enhancers and, more recently, colloidal carriers (7,8). For the latter, we have recently developed a new type of nanoparticle with interesting features which render them as promising protein carriers. These nanoparticles are composed of solely hydrophilic polymers and can be obtained spontaneously by a very mild preparation technique (9). More specifically, these particles can be made entirely of the polysaccharide chitosan (CS) or a combination of CS plus polyethylene oxide (PEO) or CS plus a diblock copolymer of ethylene oxide and propylene oxide (PEO-PPO). Besides the mildness of the preparation technique, the attractive features of these nanoparticles include: (i) they have shown a great capacity for the association of the model protein BSA, (ii) they have a very homogeneous and adjustable size (from 200 nm up to 1 μm), and (iii) they have a positive surface charge which can be conveniently modulated. Furthermore, the interest of these nanoparticles relies in the unique properties of their main component, CS. This cationic polysaccharide is a mucoadhesive, biocompatible, and biodegradable polymer (10) which has been shown to enhance the penetration of peptides and proteins across intestinal and nasal mucosa (11,12). All these features render CS nanoparticles potential vehicles for mucosal protein/vaccine delivery. In addition, it was expected that, by attaching PEO or PEO-PPO to CS nanoparticles, we could change their surface composition, thus making these nanoparticles more useful for parenteral administration.

Taking this into account, in the present paper, we aimed to investigate in detail the physicochemical properties, surface composition, and mechanisms of protein association to CS and CS/PEO-PPO nanoparticles. Finally, we analyzed the in vitro release behavior of these novel particles in order to establish their utility for the delivery of proteins and vaccines.

MATERIALS AND METHODS

Materials

The polymer CS (Seacure® 123) (viscosity 14 cps) was purchased from Pronova Biopolymer A.S. (Norway). PEO/PPO, Synperonic® F68 (MW: 8,350) was generously supplied by ICI Iberica (Spain). Bovine serum albumin (BSA) and TPP were supplied by Sigma Chemical Co. (USA). Purified tetanus toxoid (TT) solution containing 8.500 Lf/ml and diphtheria toxoid (DT) solution containing 540 Lf/ml were provided by the

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Massachusetts Public Health Biologic Laboratories, MPHBL (Boston, USA). All other chemicals were reagent grade.

Methods

Preparation of CS or CS/PEO-PPO Nanoparticles: Association of BSA and Tetanus and Diphtheria Toxoids

CS or CS/PEO-PPO nanoparticles were prepared according to the procedure previously developed by our group (9). They are formed spontaneously upon addition of 2 mL of the TPP aqueous solution (0.1%) to 5 mL of the CS acidic solution (0.175%, 8.75 mg CS) under magnetic stirring. The pH of CS solutions varied between 3.0–5.0. In several preparations, a variable amount of PEO-PPO (10, 50 mg) was also dissolved in the CS solution either before or after the addition of the TPP solution. Nanoparticles were isolated by untracentrifugation (40.000 xg, 10°C, 30 min in the presence of 5% trehalose) and then resuspended in water by manual shaking.

BSA-loaded nanoparticles were obtained by dissolving the protein in the CS solution (1, 5, 10 mg) either before or after the incorporation of TPP solution, or by dissolving the protein in the TPP solution.

For the association of tetanus and diphtheria toxoids to the nanoparticles, they were incorporated (170–340 Lf) into 1 mL TPP solution (0.1%) and then added to 2.5 mL of CS solution pH 5.0 (0.175%, 4.37 mg CS).

Physicochemical Characterization of the Nanoparticles

The morphological examination of the nanospheres was performed by transmission electron microscopy (TEM) (CM12 Philips, Eindhoven, Netherlands)). The samples were stained with 2% (w/v) phosphotungstic acid and placed on copper grids with Formvar® films for viewing by TEM.

Measurements of particle size and zeta potential of the nanospheres were performed by photon correlation spectroscopy (PCS) and laser Doppler anemometry (LDA) respectively using a Zetasizer® III (Malvern Instruments, Malvern, UK). For the particle size analysis each sample was diluted to the appropriate concentration with filtered distilled water. Each analysis lasted 120 sec and was performed at 25°C with an angle detection of 90°. For the determination of the electrophoretic mobility, samples were diluted with NaCl 10^{-3} M and placed in the electrophoretic cell where a potential of ± 150 mV was established. The zeta potential values were calculated from the mean electrophoretic mobility values using the Smoluchowski's equation.

The chemical compositions of the surface of the nanoparticles was analyzed by X-ray photoelectron spectroscopy (XPS). Samples were placed on a glass slide and exposed to MGKa X-rays with a power of 300 W on a Perkin Elmer 5100 apparatus (Newton, MA).

Evaluation of Protein Loading Capacity of the Nanoparticles

The amount of protein entrapped in the nanoparticles was calculated by the difference between the total amount incorporated in the nanoparticles formation medium and the amount of non-entrapped protein remaining in the aqueous suspending medium. This latter amount was determined following the separation of protein-loaded nanoparticles from the aqueous suspension medium by ultracentrifugation at 40,000 xg and 10°C for 30 min. The amount of free BSA, DT or TT measured in the aqueous medium was performed using the Micro BCA protein assay (Pierce, USA).

In Vitro *Release from CS Nanoparticles of BSA or TT*

The BSA-loaded CS or CS/PEO-PPO nanoparticles obtained from 8.75 mg of CS were placed into test tubes and incubated at 37°C, in 6 mL of an aqueous solution of trehalosa (5%). At appropriate time intervals, samples were centrifuged and 5 mL of the supernatant were taken and replaced by fresh medium. The amount of BSA released from the nanoparticles was evaluated by the MicroBCA protein assay (Pierce, USA). A calibration curve was made at each time interval using non-loaded nanoparticles in order to correct the intrinsic absorbance of CS.

The amount of immunochemically detected TT released from the nanoparticles over the time was determined by incubation at 37°C in 4 mL of an aqueous trehalose solution (5%, w/v) of the TT loaded-nanoparticles. At appropriate time intervals, nanoparticles suspensions were centrifuged and 3 mL of the supernatant was taken out and replaced by fresh medium. The amount of TT in the supernatant was determined by enzyme-linked immunosorbent assay (ELISA).

RESULTS AND DISCUSSION

In a very recent paper we described a new approach for the preparation of a new type of nanoparticles made of solely hydrophilic polymers (9). These nanoparticles are composed of the polysaccharide CS and the polyether PEO or PEO-PPO. The manufacturing technique involves the mixture of two aqueous phases at room temperature. One phase contains CS and PEO-PPO and the other contains the polyanion sodium tripolyphosphate (TPP). The CS or CS/PEO-PPO nanoparticles are obtained spontaneously due to the interaction between CS and the counterion TPP. In this sense, it is known that the inter- and intramolecular linkages created between the negative groups of the TPP and the positively charged amino groups of CS are responsible for the success of the process (13–16). An interesting particularity of this procedure is that the size and zeta potential of nanoparticles can be conveniently modulated by varying the ratio CS/PEO-PPO (from 200 nm up to 1 μm and from +20 up to +60 mV respectively). The increased particle size and the reduced ζ potential observed for the nanoparticles elaborated in the presence of PEO-PPO suggested that this block copolymer was incorporated into the nanoparticle's structure. Furthermore, TEM photographs evidenced that CS nanoparticles have a solid and consistent structure, whereas CS/PEO-PPO nanoparticles exhibited a compact core surrounded by a thick but fluffy coat presumably consisting of PEO-PPO (Fig. 1).

CS and CS/PEO-PPO nanoparticles were analyzed by XPS in an attempt to evaluate their surface chemical groups. This is a non-destructive analysis which provides a quantitative determination of the elemental surface composition at a depth in the range of 1–10 nm. Fig. 2a shows the XPS spectra of the polymers CS and PEO-PPO, and CS or CS/PPO-PPO nanoparticles. The C1s and O1s binding energies do not permit the identification of PEO-PPO since both CS and PEO-PPO have

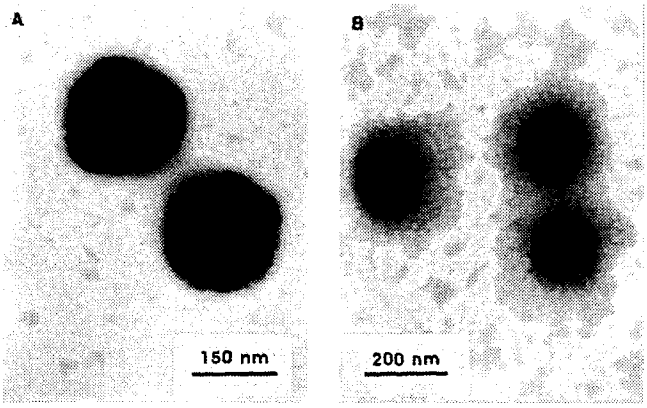

Fig. 1. Electron transmission microphotography of: (A) CS nanoparticles, and (B) CS/PEO-PPO nanoparticles.

the same functional groups (C-C, C-H, C-O). However, the ratios C/O and C/N permitted us to distinguish between PEO-PPO (higher C/O ratio) and CS (higher C/N ratio). Table I shows the experimental and stoichiometrical C/O and C/N ratios for both independent polymers and also for CS and CS/PEO-PPO nanoparticles. Given that the agreement between the experimental and stoichiometrical data was good, the similar C/O ratio obtained for PEO-PPO and CS/PEO-PPO nanoparticles can be taken as a good indication of the presence of PEO-PPO on the surface of the nanoparticles. On the other hand, the nitrogen signal in CS/PEO-PPO nanoparticles is smaller, although still visible, than in CS nanoparticles (Fig. 2a and Table I). This suggests that CS nanoparticles are not completely coated by PEO-PPO. The interaction between CS and PEO-PPO was further investigated by analyzing the nitrogen peak at a higher resolution. Fig. 2b shows the N1s binding energy of CS, CS nanoparticles and CS/PEO-PPO nanoparticles. The comparison of the binding energy of the ammonium group before and after reticulation of CS with TPP in both, CS and CS/PEO-PPO, nanoparticles revealed an additional peak. This binding energy modification can be attributed to the ionic interaction between the CS ammonium group and phosphate groups of TPP. Nevertheless, no modifications in the binding energy profiles were observed when PEO-PPO was associated to the CS nanoparticles, thus indicating the absence of ionic or covalent linkages involved in the process. These results, and also the fact that weak forces do not alter the electron distribution in an atom to the extent of changing the binding energy (17), led us to assume that the hydrogen bonding between CS and PEO-PPO is the most realistic interaction mechanism. The same conclusion was drawn by other authors, who have previously reported the formation of CS/PEO-PPO gel systems (18). These authors observed the formation semi-interpenetrating networks through intermolecular hydrogen binding between the electro-negative oxygen of polyethers and the electro-positive amino hydrogen of CS.

Association of BSA to CS and CS/PEO-PPO Nanoparticles In Vitro *Release Studies*

We have previously reported that BSA can be very efficiently associated to CS nanoparticles, reaching protein loading values as high as 50% (50 mg of BSA per 100 mg of nanopar-

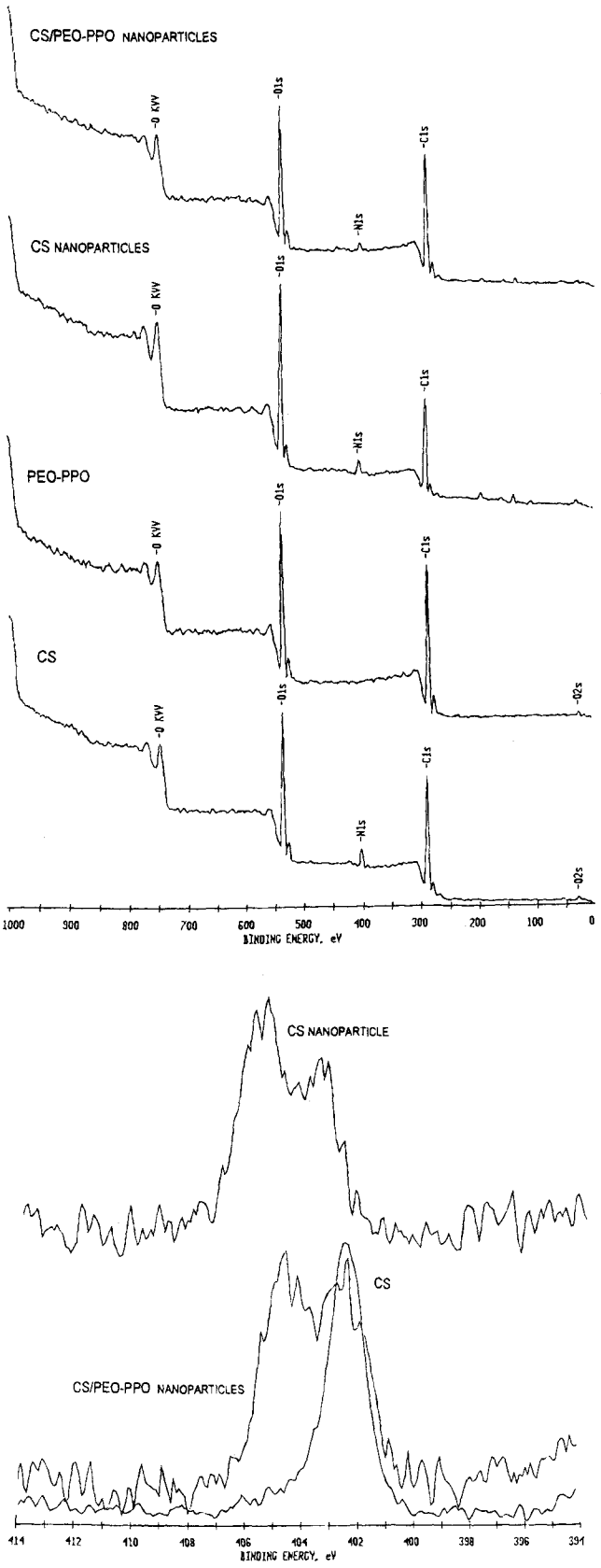

Fig. 2. (a) XPS spectra of CS and PEO-PPO polymers, and CS and CS/PEO-PPO nanoparticles. (b) Shape of the nitrogen peaks in the XPS spectra: CS polymer, CS/PEO-PPO and CS nanoparticles.

Table I. C/O and C/N Ratio of CS and PEO-PPO Polymer and Nanoparticles Determined by XPS

	C/O ratio		C/N ratio	
	ESCA	Stoichiometry	ESCA	Stoichiometry
CS	1.6	1.7	9.2	6
PEO-PPO	2.3	2.5	—	—
CS NP	1.4	—	10.9	—
CS/PEO-PPO NP	2.1	—	24.8	—

Table III. Influence of the BSA Incorporation Medium on the Entrapment Efficiency of BSA into CS Nanoparticles

BSA conc.[a] (mg/mL)	BSA entrapment efficiency (%)		
	CS nanoparticles	CS solution	TPP solution
0.2		91.79 ± 3.63	100 ± 2.50
1	14.64 ± 5.71	39.08 ± 2.42	60.57 ± 5.62
2		35.51 ± 5.10	44.61 ± 2.54

Note: Data shown are the mean ± standard deviation (n = 3).
[a] BSA initial concentration in the CS solution.

ticles) (9). In the present paper we aimed to investigate the mechanism of association of several proteins to this new type of nanoparticle. For this purpose, we analyzed the BSA entrapment efficiency as a function of the pH and PEO-PPO concentration of the CS solution and the stage of the BSA incorporation during the nanoparticles formation processes.

Results in Table II show the values of the BSA entrapment efficiency obtained when nanoparticles were prepared at different pH values and using various amounts of BSA. These results indicate that the BSA entrapment efficiency decreases as the initial BSA concentration increases. However, the total amount of protein entrapped per weight of nanoparticles (BSA loading) was related to the BSA concentration. On the other hand, Table II shows that the maximum BSA association efficiency was achieved when nanoparticles were prepared at pH 5. For the interpretation of these data it should be taken into account that, at all the pH values investigated, CS (pKa = 6.5) is positively charged, whereas the BSA (isoelectric point (pI) = 4.8) will be negatively charged at pH 5, but predominantly positively charged at pHs 4 and 3. Consequently, it seems reasonable to conclude that the electrostatic interactions between the acidic BSA groups and the amine CS groups play a role in the association of BSA to the chitosan nanoparticles. The same conclusion was drawn by Yoshida et al. who studied the adsorption of BSA onto crosslinked CS (19). However, since the BSA entrapment was significant at all pH values investigated, it should also be accepted that other forces such as hydrogen bonding and hydrophobic forces may account for the entrapment of the BSA into the nanoparticles as well.

Following this, we analyzed the effect of the stage and medium in which the BSA was dissolved on its encapsulation efficiently. Thus, BSA was incorporated into the nanoparticles formation medium in different ways: (i) dissolved in the CS solution (pH 5.00), (ii) dissolved in the TPP solution (pH 8.00), (iii) dissolved in the nanoparticles suspension (after the incorporation of the TPP to the CS solution). In Table III it can be observed that the maximum association efficiency was obtained when BSA was dissolved in the TPP solution, whereas a minimum amount of BSA was entrapped following its incorporation into the nanoparticles suspension. These results could be easily explained by the fact that at pH 8 the BSA is highly negatively ionized and therefore its interaction with the ammonium groups of CS is favored. On the other hand, when BSA is added to the previously formed nanoparticles the association efficiency is then limited by the reduced number of groups of amino of the CS available on the nanoparticles surface, for their interaction with BSA.

On the other hand, based on the above mentioned interaction between PEO-PPO and CS, we decided to study the influence of both, PEO-PPO and BSA concentrations on the BSA entrapment efficiency. Results in Fig. 3 indicate that the entrapment of BSA is limited by the presence of PEO-PPO in the CS solution, thus suggesting that BSA and PEO-PPO compete in their interaction with CS. This is not surprising if we take into account that some ammonium groups of CS are blocked by their interaction with the electro-negative oxygens of the PEO-PPO and, therefore, are not available for their interaction with the BSA. In addition, it is possible that the polyether in

Table II. Influence of the pH of the CS Solution on the Entrapment Efficiency of BSA into CS Nanoparticles

BSA conc.[a] (mg/mL)	BSA entrapment efficiency (%)		
	pH 3	pH 4	pH 5
0.2	66.84 ± 7.2	80.44 ± 3.20	91.79 ± 3.6
1	25.77 ± 1.4	26.80 ± 0.69	39.08 ± 2.4
2	19.44 ± 3.6	21.63 ± 2.04	35.51 ± 5.1

Note: Data shown are the mean ± standard deviation (n = 3).
[a] BSA initial concentration in the CS solution.

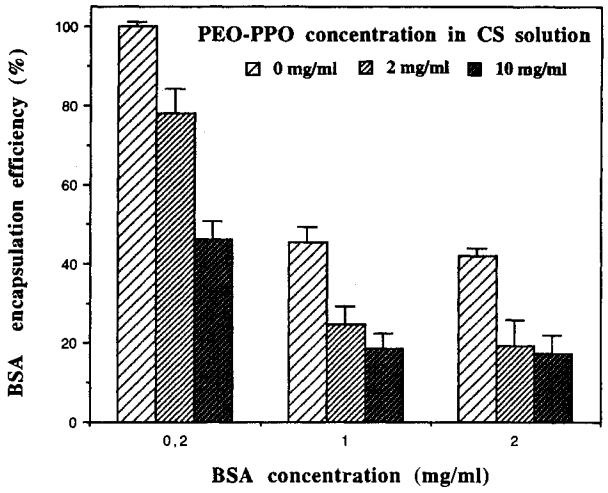

Fig. 3. Influence of the PEO-PPO concentration on the BSA entrapment efficiency. Nanoparticles prepared at pH 5 incorporating the BSA in the TPP solution. ▨ CS nanoparticles ▨ CS/PEO-PPO nanoparticles (2 mg/ml CS solution. formulation 3). ■ CS/PEO-PPO nanoparticles (10 mg/ml CS solution CS/PEO-PPO nanoparticles (loading 20%, formulation 3, figure 3).

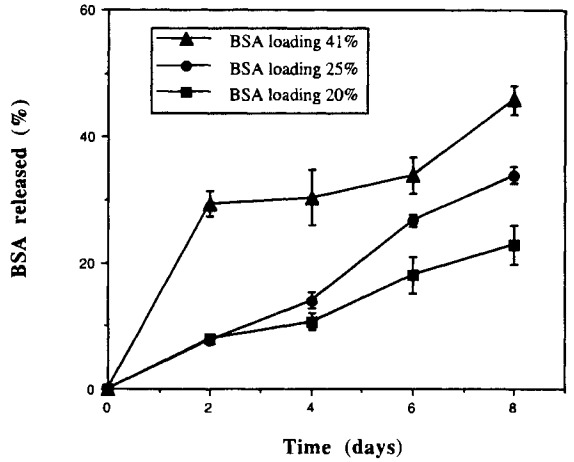

Fig. 4. BSA release profiles from: ▲ CS nanoparticles (loading 41%). ● CS nanoparticles (loading 25%). ■ CS/PEO-PPO nanoparticles (loading 20%).

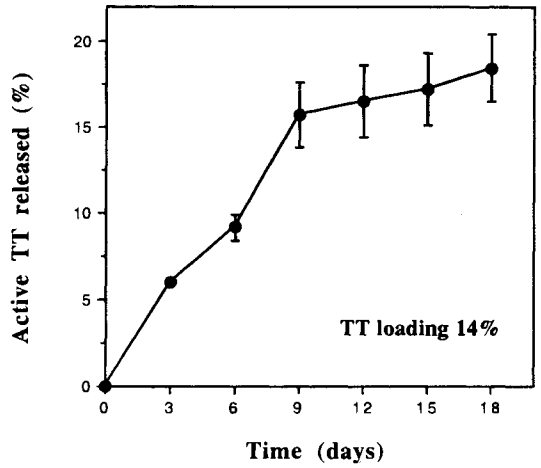

Fig. 5. TT release profile from CS nanoparticles prepared at pH 5 incorporating the TT in the TPP solution (loading 14%).

the nanoparticles could mask, by steric effect, the ammonium groups of CS, thus hindering the attachment of the BSA.

Following the observation that CS nanoparticles have a great capacity for the association of the model protein BSA, it was of major interest to determine how the entrapped protein can be conveniently released from the nanoparticles. Fig. 4 depicts the in vitro release behavior of three formulations of CS nanoparticles containing different BSA loadings. The results show that the nanoparticles with a 20–25% BSA loading release the entrapped protein, with no burst effect, at a fairly constant rate, whereby the release rate depends on the BSA loading. As expected, the higher the loading the faster BSA released from nanoparticle. For the nanoparticles containing 41% BSA the release profile become biphasic. This fact could be explained by the presence of an important amount of BSA on the surface of these nanoparticles. Consequently, these results indicate that there are possibilities of modulating the release rate of BSA by adjusting the composition and the BSA loading of the nanoparticles.

Association of Tetanus and Diphtheria Toxoids to CS Nanoparticles In Vitro *Release Studies*

The suitability of the CS nanoparticles for the entrapment of vaccines and their subsequent release in their active form was studied using tetanus and diphtheria toxoids. Table IV compares the association of BSA and TT and DT to CS nanoparticles prepared at a pH 5. In all cases, the proteins were dissolved in the TPP solution and then incorporated into the CS solution. The results show that the toxoids became associated to the nanoparticles very efficiently, however the association extent of BSA was higher than that of the toxoids. Bearing in mind the above-discussed binding mechanism of BSA with CS, the different attachment of the three proteins could be explained on the basis of their different isoelectic points. The pI of BSA is 4.8, however, the toxoids are a mixture of proteins with different pI from 4 to 6. Consequently, the differences in their association to nanoparticles could be partially attributed to the different ionization of the toxoids. However, it should taken into consideration that other mechanisms of interaction (hydrogen bonding, hydrophobic forces, etc.) may also be responsible for the different extent of association.

Table IV. Entrapment Efficiency of BSA and Tetanus and Diphtheria Toxoids into CS Nanoparticles

	M.W (daltons)	Protein/ Chitosan (%)	Isoelectric point	Association efficiency (%)
BSA	69,000	12	4.5 – 4.8	100 ± 2.5
Tetanus toxoid	150,000	12	4.4 – 5.9	56.73 ± 2.7
		6		53.30 ± 4.2
Diptheria toxoid	62,000	6	4.1 – 6.0	55.10 ± 5.5

Note: Data shown are the mean ± standard deviation, (n = 3).

On the other hand, the in vitro release studies (Fig. 5) of TT from CS nanoparticles clearly revealed the possibility of providing an extended release of the antigenically active toxoid. The slower second phase of release profile could be due to the aggregation of the particles or to the inactivation of the toxoid following the incubation of the particles for periods longer than 9 days.

CONCLUSIONS

The CS and CS/PEO-PPO nanoparticles described in this paper showed interesting features as protein delivery systems. These hydrophilic particles are formed under very mild conditions and they have an excellent capacity for the association of proteins. The association of proteins and vaccines is mediated by electrostatic interactions between the acid groups of proteins and the positive ammonium group of CS. These systems provide an extended release of the entrapped protein, the protein released being in the active form. Another interesting feature of these nanoparticles is the presence of PEO/PPO on their surface, a fact that renders them interesting carriers for parenteral administration. To summarize, these hydrophilic systems are very promising carriers for the administration of therapeutic proteins and other macromolecules which are susceptible to interaction with CS (i.e. genes or oligonucleotides.)

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