Novel Hydrophilic Chitosan–Polyethylene Oxide Nanoparticles as Protein Carriers

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SYNOPSIS

Hydrophilic nanoparticulate carriers have important potential applications for the administration of therapeutic molecules. The recently developed hydrophobic–hydrophilic carriers require the use of organic solvents for their preparation and have a limited protein-loading capacity. To address these limitations a new approach for the preparation of nanoparticles made solely of hydrophilic polymers is presented. The preparation technique, based on an ionic gelation process, is extremely mild and involves the mixture of two aqueous phases at room temperature. One phase contains the polysaccharide chitosan (CS) and a diblock copolymer of ethylene oxide and propylene oxide (PEO–PPO) and, the other, contains the polyanion sodium tripolyphosphate (TPP). Size (200–1000 nm) and zeta potential (between +20 mV and +60 mV) of nanoparticles can be conveniently modulated by varying the ratio CS/PEO–PPO. Furthermore, using bovine serum albumin (BSA) as a model protein it was shown that these new nanoparticles have a great protein loading capacity (entrapment efficiency up to 80% of the protein) and provide a continuous release of the entrapped protein for up to 1 week.

INTRODUCTION

The development of hydrophilic nanoparticles as drug carriers has represented over the last few years an important challenge. Among the different approaches, polyethylene oxide (PEO)–polylactic acid (PLA) nanoparticles have been revealed as very promising systems for the intravenous administration of drugs.[1] Due to the creation of a PEO sterically stabilizing layer, these nanospheres avoid the rapid clearance from the blood stream, thus allowing an improved delivery of the drug. Besides this important feature, these hydrophobic–hydrophilic nanoparticles have a limitation in their preparation procedure which requires the use of organic solvents as well as sonication or homogenization. We present in this paper a new approach to create biodegradable nanoparticles made solely of hydrophilic polymers. The major components of these nanoparticles are PEO, the block copolymers polyethylene oxide–polypropylene oxide (PEO–PPO), and the polysaccharide chitosan (CS). PEO and PEO–PPO are nonionic polymers or diblock copolymers, respectively, which have been shown to be beneficial in improving the hemocompatibility of polymeric biomaterials.[2] On the other hand, the cationic polysaccharide CS has some important properties such as mucoadhesivity,[3] biocompatibility, and nontoxicity,[4,5] which render it an interesting biomaterial. From a physicochemical point of view, CS has the special quality of gelling upon contact with anions thus allowing the formation of beads under very mild conditions.[6,7] These CS beads showed a pH-dependent swelling behavior which makes them appropriate for the delivery of drugs in the gastric cavity. Nevertheless, due to their large size (1–2 mm), these beads are not appropriate for other routes of administration, such as those which require injection or deposition in mucosal surfaces (nasal, mucosal). The reduction of

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the size of CS particles down to the nanometers range was achieved by Ohya et al.[8] However, an important drawback of these nanoparticles is that they were prepared by a crosslinking reaction with the potentially antigenic agent glutaraldehyde. Furthermore, this procedure is not adequate for the incorporation of proteins or peptides within these nanoparticles since they could suffer from the covalent crosslinkage as well. Hence, the originality of the nanoparticles described in the present work relies on the absence of glutaraldehyde, on the incorporation of PEO in their structure, and on their capacity to entrap proteins. In this sense it should be mentioned that chitosan semi-interpenetrating (IPN) PEO networks were previously proposed as pH-sensitive drug delivery systems[9–11] and, also, as a way to improve the blood biocompatibility of CS.[12] However, these IPN-PEO networks also have the limitation of being prepared by covalent crosslinking with aldehydes. Furthermore, despite the interest of these hydrogels, the necessity of designing a suitable device for their proper exploitation has already been claimed.[12]

To summarize, the main goal of the present work was to create a new type of hydrophilic nanoparticles and to evaluate their efficacy for the entrapment and controlled release of proteins. Among the hydrophilic polymers we chose the cationic polysaccharide CS and the noncharged block copolymers PEO and PEO–PPO. The preparation of the nanoparticles was based on the ability of CS to undergo a liquid–gel transition due to the ionic interaction with a polyanion, such as TPP.

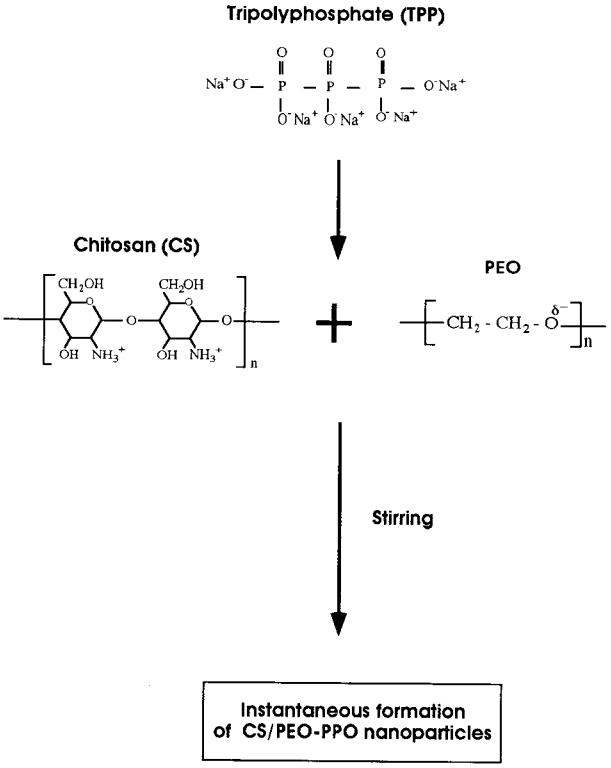

Figure 1 Scheme of the preparation of CS nanoparticles.

EXPERIMENTAL

Materials

The polymer chitosan (Seacure® 123) (viscosity 14 cps) was purchased from Pronova Biopolymer A.S. (Norway). The PEO/PPO, Synperonic® F38, Synperonic® F68, Synperonic® F88 (MW: 4800; 8350; 11,800, respectively) were generously supplied by ICI Iberica (Spain). Bovine albumin (BSA), TPP and PEG (MW: 4000 and 10,000) were supplied by Sigma Chemical Co. (USA). All other chemical were reagent grade chemicals.

Methods

Investigation of the Conditions for the Formation of CS Nanoparticles

The CS nanoparticles were obtained by inducing the gelation of a CS solution with TPP. The scheme for the preparation of nanoparticles is shown in Figure 1. Preliminary experiments were done in order to determine the production zone of the nanoparticles formation. For this purpose CS was dissolved in acetic acid aqueous solutions at various CS concentrations: 0.05%, 0.1%, 0.5%, and 1% (w/v). The concentration of acetic acid was, in all cases, 1.75 times higher than that of CS. Then, TPP was dissolved in purified water at the same concentrations as CS [0.05%, 0.1%, 0.5%, and 1% (w/v)]. Finally, a variable volume of the TPP solution (0.25, 1.0, 2.0, 2.5, and 3 mL) was added to 5 mL of the CS solution under magnetic stirring at room temperature. Then samples were visually analyzed and three different systems were identified: clear solution, opalescent suspension, and aggregates. The zone of the opalescent suspension, which should correspond to a suspension of very small particles, is illustrated in Figure 2(a) as a function of the CS and the TPP concentration in the final suspension. This opalescent suspension zone was further investigated by incorporating 2 mL of the TPP solution into 5 mL of the CS solution, thus achieving a final concentration of CS between 1–3 mg/mL and TPP between 0.2–1.0 mg/mL. The appearance of

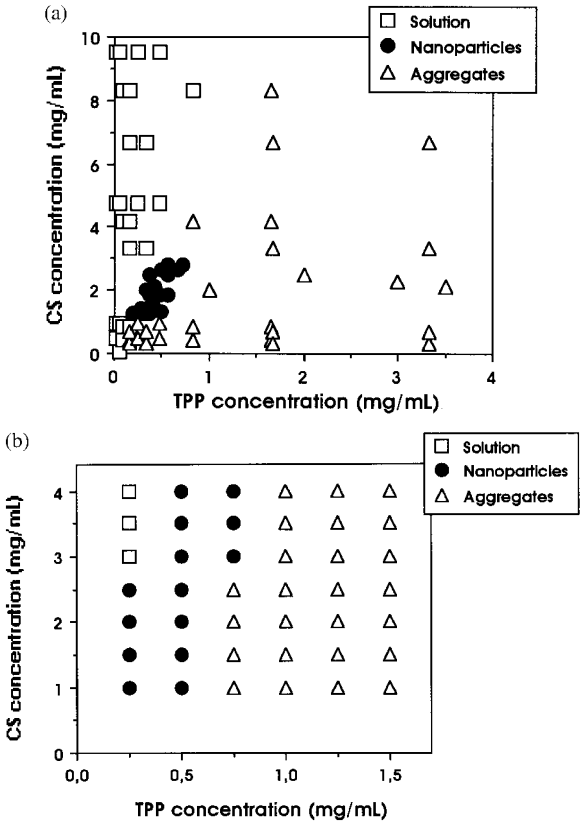

Figure 2 Identification of CS nanoparticles domain formation. (a) 1st step; (b) 2nd step.

these preparations was observed microscopically and samples classified in aggregates and nanoparticles, as shown in Figure 2(b).

Preparation of CS/PEO and CS/PEO-PPO Nanoparticles

Nanoparticles were formed spontaneously upon incorporation of 2 mL of the TPP aqueous solution (1 mg/mL) to 5 mL of the CS acidic solution (1.75 mg/mL) containing various concentrations of PEO or PEO–PPO (10, 50, and 100 mg/mL) under magnetic stirring. In several control experiments the PEO–PPO was incorporated following the gelation with TPP. Various molecular weights of PEO and PEO–PPO (PEO: 4000 and 10,000 PEO–PPO: 4800, 8350, and 11,800) were also introduced into the preparation medium.

Preparation of BSA-Loaded Nanoparticles

The BSA-loaded nanoparticles were formed spontaneously upon incorporation of 2 mL of the TPP aqueous solution (1 mg/mL) to 5 mL of the CS acidic solution (1.75 mg/mL) containing various concentrations of BSA (0.2, 0.5, 1.0, 2.0 mg/mL), under magnetic stirring. In several preparations a variable amount of PEO–PPO (10, 50 mg/mL) was also dissolved in the CS solution either before or after the incorporation of the TPP solution.

Physicochemical Characterization of the Nanoparticles

The morphological examination of the nanospheres was performed by transmission electron microscopy (TEM) (CM12 Philips). 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, UK).

Evaluation of BSA-Loading Capacity of the Nanoparticles

The BSA-loaded nanoparticles were separated from the aqueous suspension medium by ultracentrifugation at 40,000 × *g* and 10°C for 30 min. The amount of free BSA was measured in the clear supernatant by UV spectrophotometry at $\lambda = 280$ nm.

The BSA-loading capacity (LC) of the nanoparticles and the BSA encapsulation efficiency (AE) of the process were calculated from eq. (1) and (2) indicated below:

$$\mathrm{LC} = \frac{\text{Total amount BSA} - \text{Free amount BSA}}{\text{Nanoparticles weight}} \times 100 \qquad (1)$$

$$\mathrm{AE} = \frac{\text{Total amount BSA} - \text{Free amount BSA}}{\text{Total amount BSA}} \times 100 \qquad (2)$$

Evaluation of BSA In Vitro *Release from the Nanoparticles*

The BSA-loaded CS and CS/PEO–PPO nanoparticles were placed into test tubes and incubated at 37°C, in 6 mL of an aqueous solution of trehalosa (5%). At appropriate 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 Micro BCA protein assay (Pierce, USA). A calibration curve was made at each time

Table I Mean Particle Size of CS Nanoparticles

TPP Concentration[a] (mg/mL)	Chitosan Concentration[a] (mg/mL)			
	1	1.25	1.42	2.8
0.21	263.8 ± 23.6[b]	307.6 ± 14.6	353.5 ± 19.3	743.9 ± 153.4
0.28	282.7 ± 9.4	304.7 ± 24.9	336.4 ± 11.0	682.9 ± 16.6
0.43	408.1 ± 105.2	411.0 ± 27.1	387.6 ± 40.0	745.5 ± 74.6

[a] Final concentration in nanoparticle suspensions.
[b] Mean particle size (nm). Data shown are the mean ± standard deviation ($n = 4$).

interval using nonloaded nanoparticles in order to correct the absorbency due to the CS.

RESULTS AND DISCUSSION

In the present study we describe a novel nanoparticulate system which is composed of solely hydrophilic polymers such as CS, PEO, and PEO–PPO, and has the following interesting features: i) it is obtained spontaneously under exceptionally mild conditions without involving high temperatures, organic solvents, or sonication; ii) it has a surface charge which can be modulated from high to low positive values; iii) it has a great protein loading capacity and provides a continuous release of the entrapped protein for several days; and iv) it has a pH-dependent dissolution behavior. All these special properties rend these nanoparticles as very promising protein carriers with a great potential for the parenteral or mucosal administration of macromolecules, such as peptide and protein drugs, genes, and vaccines.

For the development of this new protein carrier we first conducted a preliminary study focused on the production of CS nanoparticles; secondly, we investigated the feasibility of incorporating hydrophilic diblock copolymers in the nanoparticulate structure, and third, we evaluated the ability of the system to entrap and release proteins.

Conditions for the Formation of CS Nanoparticles

In order to investigate the feasibility of preparing CS nanoparticles by ionotropic gelation, we adopted a protocol similar to the one previously established for the development of alginate nanoparticles by ionic gelation with calcium.[13] Given that CS is a cationic polyelectrolite, our study was based on inducing its gelation by controlling its interaction with the counterion TPP. In this sense, it is known that the inter and intramolecular linkages created between TPP and the positively charged amino groups of CS are responsible for the success of the gelation process.[6,7,14,15] Results of this study are represented in Figures 2(a) and 2(b), in which it can be observed that the formation of nanoparticles is only possible for some specific concentrations of CS and TPP. The final CS concentration can be up to 4 mg/mL while the maximum TPP concentration is only 0.75 mg/mL. Furthermore, in Table I, it can be noted that the particle size is dependent on both CS and TPP concentrations, the minimum size (260 nm) being obtained for the lowest CS and TPP concentrations. Further experiments were conducted using a TPP final concentration of 0.28 mg/mL and CS final concentration of 1.25 mg/mL, therefore the ratio CS/TPP is 5/1.

Incorporation of PEO and PEO–PPO in CS Nanoparticles

The incorporation of PEO and PEO–PPO in the CS nanoparticles was simply achieved by dissolving these block copolymers in the CS solution either before or after the incorporation of the ionic

Table II Mean Particle Size of CS/PEO Nanoparticles

PEO Concentration[a] (mg/mL)	MW PEO	
	4,000	10,000
0	304.7 ± 24.9[b]	304.7 ± 24.9
10	413.3 ± 157.0	484.2 ± 80.9
50	540.0 ± 134.5	813.3 ± 243.7
100	1049.7 ± 91.9	1348.1 ± 193.9

[a] PEO initial concentration in the chitosan solution.
[b] Mean particle size (nm). Data shown are the mean ± standard deviation, ($n = 4$).

Table III Mean Particle Size of CS/PEO–PPO Nanoparticles

PEO–PPO Concentration[a] (mg/mL)	MW PEO–PPO		
	4,800	8,350	11,800
0	304.7 ± 24.9[b]	304.7 ± 24.9	304.7 ± 24.9
10	417.8 ± 160.8	360.5 ± 93.7	585.1 ± 334.1
50	470.6 ± 114.3	647.2 ± 199.0	975.1 ± 614.2
100	741.8 ± 261.4	1002.0 ± 105.7	1498.0 ± 698.7

[a] PEO–PPO initial concentration in the chitosan solution.
[b] Mean particle size (nm). Data shown are the mean ± standard deviation, (n = 4).

crosslinker TPP. The increased size and reduced zeta potential (Tables II–IV) of these nanoparticles is a good indication of the incorporation of PEO–PPO in the nanoparticle's structure. It has been previously reported that the incorporation of PEO in the gel system is through intermolecular hydrogen bonding between the electro-positive amino hydrogen of CS and electro-negative oxygen atom of polyethers, thus forming a CS/PEO semi-interpenetrating network.[9] Consequently, it is not surprising that the increase in the concentration and molecular weight of PEO and PEO–PPO leads to an augmentation of the size and a reduction of the positive charge of the nanoparticles. On the other hand, the same increase in the size was observed when the PEO–PPO was incorporated after the formation of the nanoparticles, a fact that suggests that PEO–PPO preferably attaches itself to the nanoparticle's surface.

The surface attachment was clearly illustrated by TEM (Fig. 3). The CS nanoparticles have a solid and consistent structure, whereas CS/PEO–PPO nanoparticles exhibit a compact core surrounded by a thick but fluffy coat presumably made of PEO–PPO. It can be also noted that the size of the core of the CS/PEO–PPO nanoparticles is similar to the size of CS nanoparticles. Consequently, it could be assumed that the increased size of the CS/PEO–PPO nanoparticles is due to the hydrophilic PEO–PPO coating.

The preparation of these interpenetrating networks was recently reported by several authors,[9–12] nevertheless, previously, the crosslinking of CS was made by covalent linkage with an aldehyde. In addition, the main advantage of our system is the fact of being presented in a nanoparticulate form. On the other hand, it is important to mention that the hydrogels, previously reported, swelled when exposed to an acidic medium, whereas our nanoparticles are stable in water but dissolve in a few minutes in HCl 0.1N. This particular behavior indicates that CS and CS–PEO nanoparticles are pH-sensitive delivery systems, a quality that gives these particles new prospects in the field of gene therapy. In fact, this novel application of positively charged CS particles as nonviral gene transfer carriers was recently reported by Mumper et al.[16]

Entrapment of BSA within CS and PEO/PPO Nanoparticles

We selected BSA as a model protein in order to investigate the feasibility of using CS and CS–PEO/PPO nanoparticles as protein carriers. Re-

Table IV Zeta Potential of CS/PEO–PPO and CS/PEO Nanoparticles

PEO–PPO or PEO Concentration[a] (mg/mL)	MW PEO–PPO		MW PEO
	4,800	8,350	10,000
0	+43.30 ± 1.37[b]	+43.30 ± 1.37	+43.30 ± 1.37
10	+39.69 ± 1.84	+38.35 ± 0.95	+39.35 ± 1.08
50	+34.84 ± 1.11	+26.75 ± 1.33	+33.26 ± 0.33
100	+29.09 ± 0.70	+17.69 ± 1.49	+26.10 ± 0.31

[a] PEO–PPO or PEO initial concentration in the chitosan solution.
[b] Zeta potential (mV). Data shown are the mean ± standard deviation, (n = 4).

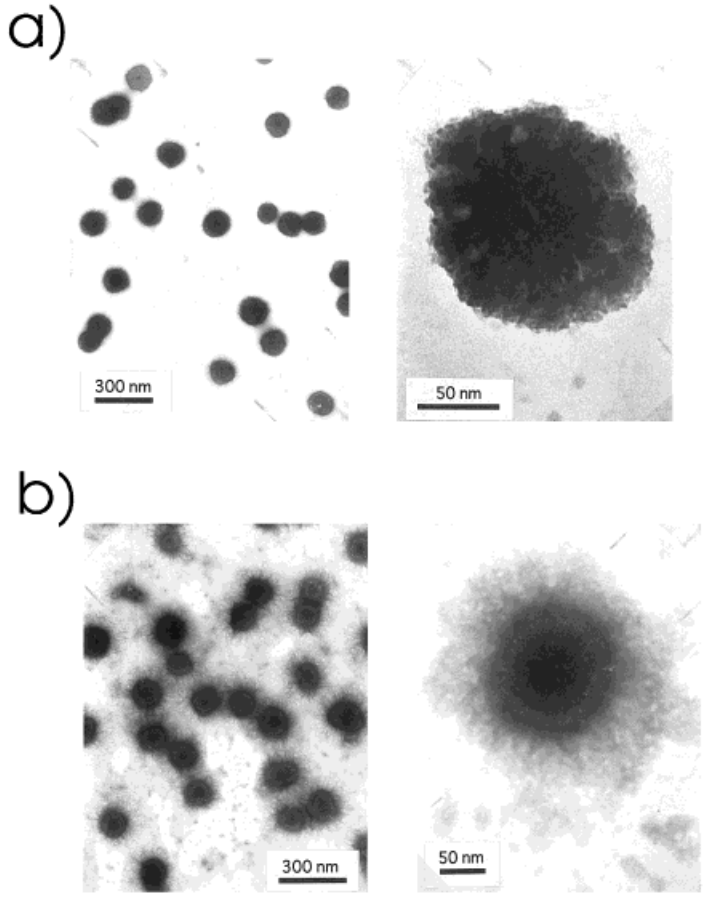

Figure 3 Electron transmission microphotography of: (a) CS nanoparticles; (b) CS/PEO–PPO nanoparticles (concentration of PEO–PPO in the chitosan solution: 10 mg/mL).

sults of the encapsulation efficiency and protein loading of these nanoparticles are displayed in Figure 4(a) and Figure 4(b). The BSA encapsulation efficiency was affected by the initial BSA concentration, the lower the concentration, the higher the encapsulation efficiency. However, the protein loading was enhanced by increasing the initial BSA concentration, reaching a maximum of 51 mg of BSA entrapped in 100 mg of nanoparticles. This is, to the best of our knowledge, the greatest loading capacity ever reported for a nanoparticulate protein carrier. In order to investigate the mechanism of protein-carrier association we performed a number of control experiments. We observed that the incorporation of a BSA aqueous solution into an acidic medium (pH 4.8) (BSA concentration 2 mg/mL) led to a perfectly clear solution, however, the preparation become cloudy when CS was previously dissolved in the acidic medium. Furthermore, we observed that the higher the pH, the more intense is the opacity of the CS-BSA preparation. Taking into account that the isoelectric point of BSA is 4.8, it is understandable that, at pHs higher than 4.8, the BSA will be negatively charged and interact with the CS amine groups. The pH of the nanoparticles formation medium was between 4.5–5.0, a pH value that favors the interaction of BSA and CS, thus leading to the entrapment of high amounts of BSA within the nanoparticles. Consequently, it could be stated that the main leading factor of the protein association to the nanoparticles is the protein–polysaccharide electrostatic interaction. This conclusion is also supported by the results previously reported by Yoshida, Nishilara, and Kataoka.[17] These authors studied the adsorption of BSA onto basic crosslinked CS and indicated that the acid groups of BSA interact, by electrostatic attraction, with the positively charged ammonium groups of the basic CS.

On the other hand, in Figure 4(a) and Figure 4(b), it can be also seen that the entrapment of

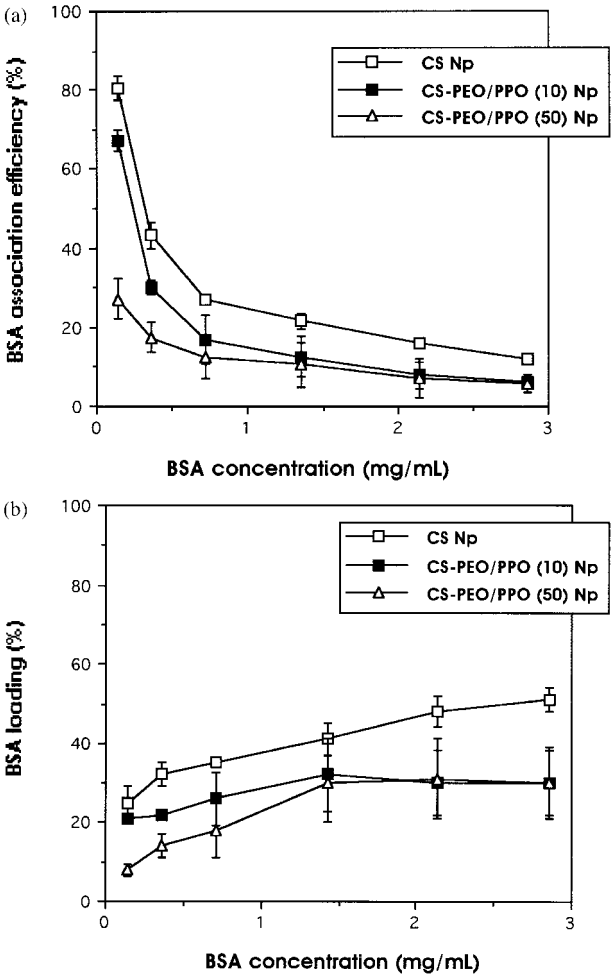

Figure 4 BSA encapsulation efficiency (a) and BSA loading (b) of the CS nanoparticles and CS/PEO–PPO nanoparticles. Data shown are the mean ± standard deviation ($n = 3$).

the protein is limited by the presence of PEO–PPO. These data allow us to accept the fact that BSA and PEO–PPO compete in their interaction with CS. Hence, since PEO–PPO was incorporated into the CS solution before BSA, some of the CS amine groups were already occupied and, therefore, the possibilities of an interaction between the BSA and the CS were reduced. This conclusion was corroborated by the observed fact that the BSA loading was not modified when PEO–PPO was incorporated subsequently to the protein entrapment in the nanoparticles (results not shown).

In Vitro Release of BSA from the Nanoparticles

Figure 5(a) shows the BSA *in vitro* release behavior of CS and CS/PEO–PPO nanoparticles. Results indicate that BSA releases at a constant but at a different rate from both types of nanoparticles. Thus, CS/PEO–PPO nanoparticles release their content faster than CS nanoparticles. Furthermore, the higher the concentration of PEO–PPO in the nanoparticles preparation medium was, the faster was the BSA release from the nanoparticles.

Consequently, these results indicate that there are possibilities of modulating the release rate of the BSA by adjusting the composition of the nanoparticles or their BSA loading. On the other hand, Figure 5(b), which shows the *in vitro* release of BSA from CS nanoparticles containing different BSA loading, depicts that the BSA release rate is highly influenced by the amount of protein entrapped. In addition, these data suggest that BSA releases from the carrier by a simple desorption process mechanism; nevertheless, more experiments need to be done (i.e., investigation of the nanoparticles modifications during the *in vitro* re-

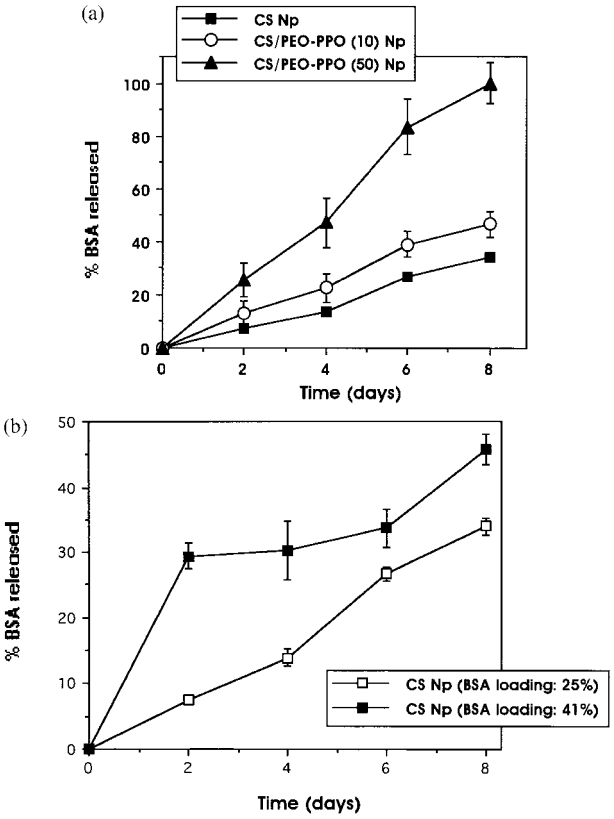

Figure 5 BSA release profiles from: (a) BSA loaded CS and CS/PEO–PPO nanoparticles [BSA loading: CS Np: 25%; CS/PEO–PPO (10) Np: 21%; CS/PEO–PPO (50) Np: 8%]. (b) CS Np, (BSA loading: 25% and 41%, respectively). Data shown are the mean ± standard deviation ($n = 3$).

lease process) to further interpret the mechanism of protein release.

CONCLUSIONS

This paper describes the preparation of a novel hydrophilic protein delivery system consisting of a suspension of nanoparticles. The system is made of hydrophilic polymers, i.e., chitosan and polyethylene oxide, which are nontoxic and biocompatible. A major advantage of these nanoparticles is that they are prepared under extremely mild conditions and in a very short time. Furthermore, they have an excellent capacity for the entrapment of proteins and provide a continuous protein release for extended periods of time. All these interesting features make this novel system a very promising vehicle for the administration of therapeutic proteins, genes, and antigens by various routes.

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