Colloid Polym Sci 275:46–53 (1997)
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ORIGINAL CONTRIBUTION

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Development of positively charged colloidal drug carriers: chitosan-coated polyester nanocapsules and submicron-emulsions

Received: 17 May 1996
Accepted: 15 August 1996

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Abstract Positively charged colloidal drug carriers have shown interesting properties with respect to the negatively charged systems: they have improved stability in the presence of biological cations and their interaction with negatively charged biological membranes is facilitated. In the present work, a new approach in order to provide a positive charge to colloidal systems, i.e., poly-ε-caprolactone (PECL) nanocapsules and submicron emulsions, is presented. This is based on the coating of the colloidal droplets with the cationic polysaccharide chitosan (CS). An experimental factorial design 3^3 was used to investigate the influence of several factors (CS viscosity, PECL concentration and lecithin concentration) on the physicochemical properties of the systems. All the formulations displayed a particle size in the nanometer range (200–500 nm) and a high positive surface charge (from +30 up to +60 mV). The statistical analysis of these data (surface response methodology) indicated that both size and surface charge of the nanocapsules and submicron emulsions, were significantly affected by all factors under investigation, the CS viscosity being the most relevant factor. The CS coating of the nanocapsules was found to be efficient in preventing their destabilization in the presence of Ca^{2+}. Furthermore, the presence of CS permitted the adequate dispersion of the nanocapsules upon freeze-drying. Finally, using diazepam as model drug, it was observed that the encapsulation efficiency was, in all cases, higher than 90% irrespective of the presence of CS in the preparation. As expected, the diazepam release rate from the nanocapsules and submicron emulsions occurred rapidly and it was slightly slowed down due to the CS coating. These results clearly demonstrated that coating nanocapsules and submicron emulsion with CS increases their potential use as drug delivery systems.

Key words Nanocapsules – submicron emulsions – chitosan – colloidal drug carriers – drug delivery systems

Introduction

Nanoparticles, nanocapsules, emulsions and liposomes provide a range of highly attractive colloidal carriers for sules (oily droplets surrounded by a polymer coat) have been revealed as interesting systems for the administration of hydrophobic drugs by the ocular and oral routes [1–3]. On the other hand, submicron emulsions are gaining increasing importance as vehicles for the delivery of

hydrophobic drugs following intravenous [4], oral [5] and ocular administration [6]. Most of these colloidal drug carriers are characterized by a negative surface charge, which has been mainly attributed to the presence of the natural surfactant lecithin in their composition [7]. This important negative charge helps in preventing the coalescence of the droplets, however, it also has some negative consequences. On one hand, it promotes the adsorption of cationic proteins and sodium and calcium ions that are present in the biological fluids leading thereby to the neutralization of the surface charge, the breakdown of the system and the leakage of the entrapped agents. On the other hand, these negatively charged colloidal systems may suffer an electrostatic repulsion by the biological membranes since they are also negatively charged. In this sense, it is believed that positively charged drug carriers will be favorable due to their facilitated interaction with epithelia and improved capacity for the transport of drugs [8, 9]. In an attempt to provide a positive charge to the colloidal systems several authors have proposed the use of positive phospholipid derivates and other cationic surfactants in the preparation and stabilization of submicron emulsions and liposomes [10–12]. As an alternative, we describe in the present work a new approach based on the incorporation of the cationic polysaccharide chitosan (CS) at the oil–water interface. The efficacy of this new approach was applied not only to submicron emulsions but also to nanocapsules. The polysaccharide CS was chosen to coat these colloidal systems due to its cationic character and because it has some interesting features such as mucoadhesivity [13] and biocompatibility [14]. Furthermore, several authors have reported that there are no signs of toxicity upon oral and nasal administration [15, 16] and low toxicity following intravenous administration of CS [17]. Factors affecting the size and surface charge of the CS-coated colloidal systems were carefully investigated using an experimental factorial design. The stability of the nanocapsules during incubation with Ca^{2+} and also upon freeze-drying was studied. Finally, the interest of these new systems for entrapment of lipophilic compounds was assessed using diazepam as a drug model.

Materials and methods

Materials

The polymer poly-ε-caprolactone (PECL) (MW: 40,000) was purchased from Aldrich Chemical Co. (Steinheim, Germany) and was used without further purification. CS of different viscosity (Seacure 123, 14 cps; Seacure 223, 100 cps and Seacure 320, 680 cps) were obtained from Pronova Biopolymer, A.S. (Drammen, Norway). The vegetable oil, propylene glycol dicaprylate/dicaprate (Migliol 840) was generously supplied by Lemmel (Barcelona, Spain). The diblock copolymer of ethylene oxide and propylene oxide, Poloxamer 188 (Synperonic F68) was a gift from ICI (Barcelona, Spain). The phospholipid mixture (soybean L-α-lecithin 40% phosphatidylcholine) and the cryoprotectans, glucose and dextran were supplied by Sigma Chemical Co. (St. Louis, MO). Diazepam was obtained from Laboratorios Roche (Madrid, Spain). All other reagents were of analytical grade.

Preparation and physicochemical characterization of CS-coated PECL nanocapsules and submicron emulsions

CS-coated PECL nanocapsules were prepared by the interfacial deposition technique [18] as previously reported but with an important modification: the introduction of the polysaccharide CS in the external aqueous phase. For this purpose, a number of preliminary experiments were performed, at first to determine the appropriate concentration of CS that allows the formation of colloidal systems. A variable amount of PECL and lecithin and 0.5 mL of Migliol 840 oil were dissolved in 25 mL acetone. Then, this organic solution was poured, under moderate magnetic stirring, into 50 mL of aqueous phase containing 125 mg of the non-ionic surfactant, Poloxamer 188 and different amounts of CS. The resulting mixed phase immediately turned milky as a result of the instantaneous formation of nanocapsules. The acetone was finally removed under vacuum.

CS-coated submicron emulsions were prepared using the same procedure, the only difference being that the polymer PECL was omitted.

The morphological examination of CS coated-PECL nanocapsules and submicron emulsions was performed using a transmission electron microscope (TEM), (Philips CM12, Eindhoven, Netherlands), following negative staining with an uracil acetate solution (0.2% w/v).

The mean particle size and size distribution of the colloidal systems were determined by photon correlation spectroscopy (PCS) using a Zetasizer III (Malvern Instruments, Malvern, UK). The determination of the zeta potential was performed using the technique of laser Doppler anemometry (Zetasizer III). The colloidal suspensions were diluted properly with NaCl 10^{-3} M and placed in the electrophoretic cell where a potential of $\pm$ 150 mV was established.

Statistical analysis

Particle size and Zeta potential data obtained from a factorial design 3^3 were analyzed statistically by an analysis

Colloid & Polymer Science, Vol. 275, No. 1 (1997)
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of variance (ANOVA) combined with a multiple regression analysis using the computer program *Statgraph 7.0*.

Particle size data were fitted to a second-order polynomial model that included only the terms with statistical significance determined by the ANOVA.

Evaluation of the diazepam encapsulation efficiency and in vitro release study

The diazepam-loaded nanocapsules and submicron emulsion were prepared, as described previously, by dissolving the drug in the oil before its incorporation into the acetone solution. The amount of diazepam encapsulated into nanocapsules and submicron emulsion was calculated by the difference between the total amount used to prepare the diazepam-loaded systems and the amount of the free diazepam in the aqueous medium. The aqueous phase was separated following the ultracentrifugation process at $120\,000 \times g$ for 1 h. The amount of free diazepam was determined by spectrophotometry ($\lambda = 240$ nm).

The in vitro release studies were carried out by the bulk-equilibrium reverse dialysis bag technique, at 37 °C, as previously described [19]. Briefly, a volume of 1.5 mL of the diazepam loaded-colloidal suspensions was directly placed into 400 mL of a stirred sink solution (phosphate buffer 0.1 M, pH 7.4) where numerous dialysis sacs (cellulose membrane, M*r* cut off 12 000 D, Sigma Chemical Co. St. Louis, MO) containing 1.5 mL of the same sink solution were previously immersed. At given time intervals, a dialysis bag was withdrawn from the stirred release solution and the content of the dialysis bag was assayed for diazepam as described previously.

Freeze-drying studies

The suspensions of CS-coated PECL nanocapsules and submicron emulsions containing dextran (2%) and glucose (5%) as cryoprotectans were first frozen in liquid nitrogen. Then, these samples were freeze-dried for 24 h at a temperature of −30 °C followed by a drying period of 12 h at +20 °C at maximum vacuum (Labcomcon apparatus, UK). The reconstitution of the lyophilized products was performed by manual shaking.

Stability study in presence of Ca^{2+}

CS-coated and uncoated PECL nanocapsules and submicron emulsions were incubated with Cl_2Ca 0.2 M during 72 h. After that, the suspensions were visually analyzed in order to detect their coalescence, and their particle size was measured by PCS, as described previously.

Results and discussion

The main goal of the present work was to develop two types of positively charged colloidal systems, submicron emulsions and nanocapsules. Both systems consisted of an oily phase dispersed in an aqueous phase but the major difference between them is that nanocapsules have a PECL coat around the oily droplets. To achieve this goal our strategy was based on the adsorption of the cationic polysaccharide at the interface of the colloidal system.

Development of CS-coated PECL nanocapsules and submicron emulsion

The most relevant factors in the development of CS-coated colloidal systems were identified using a 3^3 factorial experimental design combined with a multiple regression analysis. The independent variables selected were the concentrations of the compounds which are located at the interface of the colloidal systems. These compounds include the polyester PECL (polymeric wall of the nanocapsules), the surfactant lecithin and the polysaccharide CS. The values of PECL concentration were fixed on the basis of the results of our previous work [20]. The levels of the variable lecithin and CS were decided following preliminary experiments. Results from these initial experiments indicated that, in order to obtain a colloidal system, the CS concentration in the aqueous solution should be not higher than 0.2%, while the minimum lecithin concentration in the acetone solution should be at least 0.1%. Based on this information, the following variables were selected for investigation:

- PECL concentration in the acetone solution: 0.0, 0.4 and 0.8% (w/v).
- Lecithin concentration in the acetone solution: 0.2, 0.4 and 0.6% (w/v).
- CS viscosity: 14, 100 and 680 cps (CS concentration 0.2%).

It should be noted that the formulations corresponding to 0.0% PECL concentration are submicron emulsions, since the only difference between nanocapsules and emulsions is the presence of the PECL coating around the oily nanodroplets.

The analysis of the particle size by PCS indicated that all the formulation designed displayed a particle size in the nanometer range and the size distributions were close to a monomodal distribution (polydispersion index ≤ 0.1)

Table 1 Mean particle size of CS-coated and uncoated PECL nanocapsules and submicron emulsions

CS viscosity (cps)	Mean Particle Size (nm)		
	Submicron emulsion	Nanocapsules	
		PECL 0.4%[a]	PECL 0.8%[a]
0[b]	216 ± 16 (0.10)[c]	232 ± 20 (0.09)	240 ± 10 (0.09)
14	324 ± 23 (0.14)	384 ± 60 (0.16)	313 ± 20 (0.16)
100	440 ± 27 (0.22)	445 ± 28 (0.16)	483 ± 17 (0.15)
680	429 ± 60 (0.14)	456 ± 21 (0.20)	509 ± 20 (0.20)

Data shown are mean ± standard deviation $n \geq 3$.
[a] PECL concentration in acetone phase.
[b] Uncoated colloidal systems.
[c] Polydispersity index: unimodal distribution ≤ 0.2.

(Table 1). These results were also confirmed by TEM (Fig. 1).

The influence of the selected variables on the particle size was analyzed statistically by an analysis of variance (ANOVA, $p < 0.1$) combined with a multiple regression analysis. Results from this analysis indicated that the three independent variables have a statistically significant influence on the particle size. The particle size data showed a good fitting to the second-order polynomial equation (Fig. 2) (the lack of fit was not significant at 95% confidence level). The response surfaces obtained from this fit allowed the visualization of the simultaneous effect of two of the variables on the size, while keeping the third one constant. Figure 2 depicts the influence of the lecithin concentration and the CS viscosity on the particle size of nanocapsules and submicron emulsions. Similarly, the response surface in Fig. 3 illustrates the simultaneous influence of the PECL concentration and the CS viscosity on the particle size of nanocapsules elaborated with 0.4% of lecithin. From these graphs it is evident that the main factors that affect the particle size are the CS viscosity and the concentration of PECL. Concerning the influence of CS viscosity, the size of the systems become larger by increasing the CS viscosity from 14 to 100 cps, however further increments in the viscosity did not lead to significant changes in the size. In this sense, it should be noted that the differences in the CS viscosity are due to the differences in its molecular weight, consequently, the increase in the hydrodynamic size of the colloidal systems could be related to the attachment of CS molecules of a larger size to their surface. To further evaluate the role of the CS as a coating polymer we compared the size of the colloidal systems prepared with and without CS (Table 1). These data indicate that the presence of CS increases

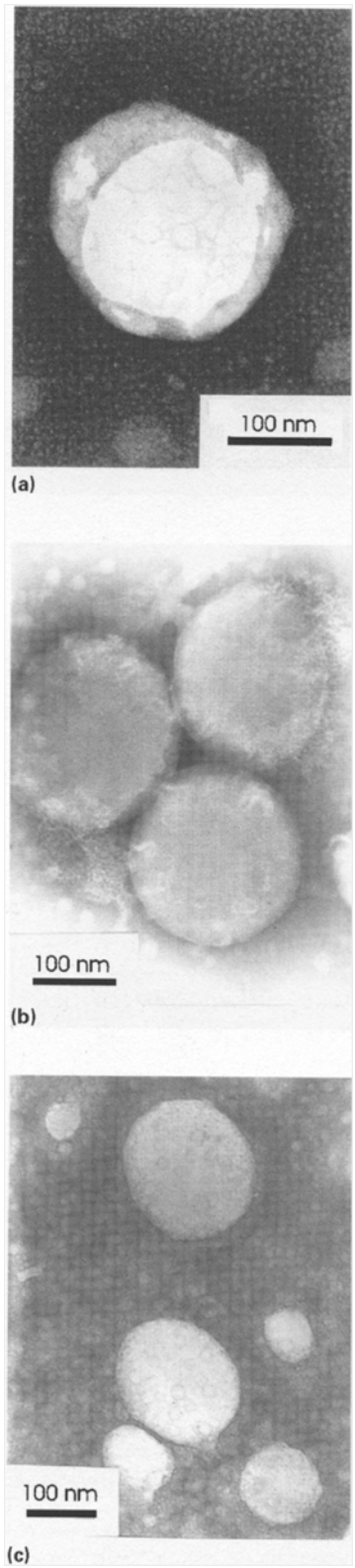

Fig. 1 Electron transmission microphotography of: **A** CS-coated PECL nanocapsules, **B** CS-coated submicron emulsion and **C** uncoated PECL nanocapsules

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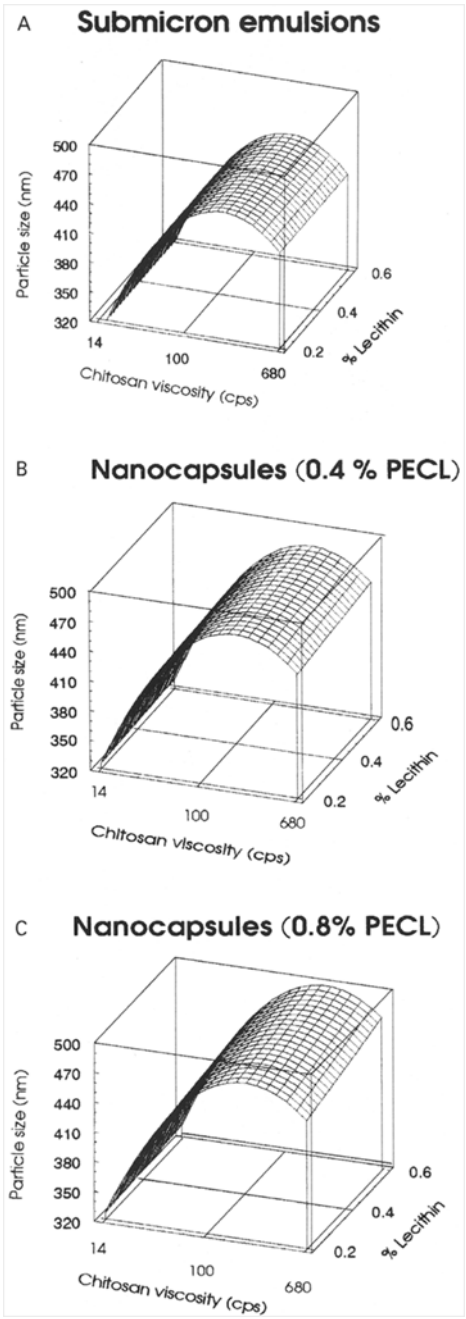

Fig. 2 Three-dimensional response surface showing the effect of the CS viscosity and the concentration of lecithin in the acetone phase on the mean particle size of **A** CS-coated submicron emulsions; **B** CS-coated PECL nanocapsules prepared with 0.4% of PECL in the acetone phase and **C** CS-coated PECL nanocapsules prepared with 0.8% of PECL in the acetone phase. The resulting second-order polynomial equation: Mean particle size $= 11.86 + 370.07X_1 + 31.017X_2 + 10.78X_2^2 - 78.25X_1^2 + 4.051X_1X_2X_3$. X_1 = CS viscosity, X_2 = PECL concentration in the acetone solution and X_3 = lecithin concentration in the acetone solution. $(r^2) = 0.8523$. The lack-of-fit was not significant (95% confidence level)

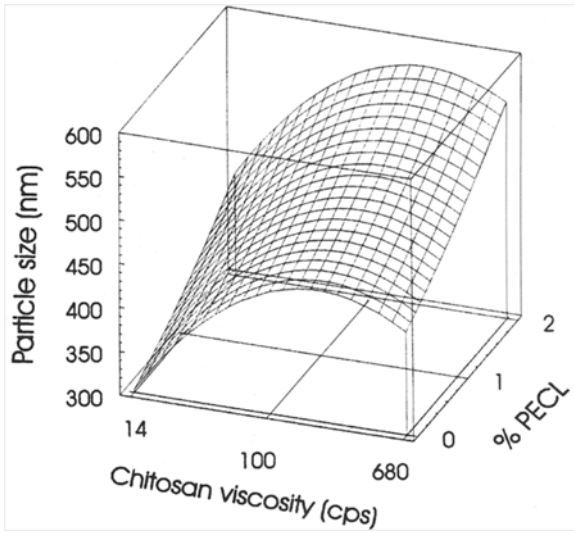

Fig. 3 Three-dimensional response surface showing the effect of the CS viscosity and PECL in the acetone phase on the mean particle size of the CS-coated PECL nanocapsules and submicron emulsions prepared with lecithin (0.4%)

significantly (ANOVA $p < 0.5$) the size of nanocapsules and submicron emulsions. The increased size could be either attributed to the presence of a CS coating around the colloidal systems, or to an enlarged size of the oily globules dispersed in a CS rich aqueous phase. In fact, it is known that the viscosity of the external phase affects the interfacial hydrodynamic phenomena responsible for the spontaneous emulsification of an oily solution into an aqueous phase [21]. Nevertheless, the first hypothesis seems to be more acceptable since TEM analysis of the CS coated and uncoated carriers (Fig. 1) permitted the identification of a CS coating around the oily globules of the nanocapsules and the submicron emulsions. Consequently, it could be expected that both the presence of the CS coating combined with the reduced dispersion of the oil in the aqueous phase are responsible for the increased size of the CS coated carriers.

A similar, but less important effect in the particle size was observed for the parameter: PECL concentration. In this case, the increased size observed for the preparations containing PECL compared to submicron emulsions is related to the presence of a PECL coat in the nanocapsules, the thickness of which is dependent on the amount of PECL used in their preparation [20].

On the other hand, although statistically significant, the influence of the lecithin concentration on the particle size of nanocapsules and the emulsion was not remarkable. In fact, only an increase of 30 nm in the size of the nanocapsules prepared with the highest CS viscosity (680 cps) was observed when the lecithin concentration varied from 0.2 up 0.6%.

Table 2 Zeta potential of CS-coated and uncoated PECL nanocapsules and submicron emulsions

CS viscosity (cps)	Lecithin[a] (%)	Zeta potential (mV)		
		Submicron emulsion	Nanocapsules PECL 0.4%[b]	Nanocapsules PECL 0.8%[b]
0[c]	0.2	− 42.32 ± 11.50	− 41.94 ± 8.65	–
14	0.2	+ 42.15 ± 1.93	+ 37.14 ± 1.89	–
100	0.2	+ 57.72 ± 0.34	+ 55.79 ± 2.88	–
680	0.2	+ 52.62 ± 1.72	+ 60.70 ± 1.01	+ 60.14 ± 1.24
680	0.4	+ 60.23 ± 0.82	+ 61.30 ± 1.06	+ 60.56 ± 0.07
680	0.6	+ 59.44 ± 0.37	+ 58.81 ± 1.82	+ 60.91 ± 0.38

Data shown are the mean ± standard deviation $n \geq 3$.
[a] Lecithin concentration in the acetone phase.
[b] PECL concentration in the acetone phase.
[c] Uncoated colloidal systems.

In order to investigate the ability of CS of attaching itself at the interface of the colloidal system, we evaluated the zeta potential of some selected formulations. Table 2 shows the inversion of the surface charge of the colloidal particles from highly negative to highly positive values due to the presence of CS. Consequently, these results suggest that CS forms a coating around the dispersed phase. Furthermore, a statistically significant influence (ANOVA $p < 0.5$) of the CS viscosity on the zeta potential was observed. Keeping in mind that the CS viscosity correlates with its molecular weight it may be accepted that the high positive charge observed with the high CS viscosity could be a consequence of a larger number of amino groups attached to the surface of the colloidal particle. This interpretation agrees with the enlarged size of the systems prepared with high molecular weight CS.

On the other hand, it was observed that the concentrations of PECL and lecithin had no significant influence on the zeta potential (Table 2). These results allow us to assume that the entrapment of CS was not determined by the presence of PECL and that the increased size of the nanocapsules prepared with a high PECL concentration was solely related to the thicker PECL wall around the oily droplets. It should be also mentioned that, despite the negligible effect of the lecithin concentration on the zeta potential, a minimum amount of lecithin was required to obtain the colloidal suspensions. The requirement of lecithin suggests that the ionic interaction between the CS and the negatively charged lecithin is the driving phenomenon which explains the attachment of CS to the surface of the colloidal systems. The same explanation has been already presented by a few authors who succeeded in improving the stability of liposomes [22, 23] and emulsions [24] using CS and its derivatives. However, the mechanism of interaction of CS with the liposomes has not been fully understood yet [25].

Freeze-drying studies

Freeze-drying is one of the best ways of preserving colloidal suspensions with the potential to yield products which are stable as well as convenient to ship and handle. Freeze-drying and further resuspension of nanoparticles can be properly achieved using acceptable concentrations of cryoprotective agents (< 10% w/v) [26]. However, to date, adequate freeze-drying of nanocapsules has been only achieved by incorporating cryoprotective agents at extremely high concentrations (> 20%) [26, 27]. Bearing this limitation in mind, we investigated the possibility of perserving the stability of the nanocapsules and submicron emulsions upon freeze-drying, by coating them with CS and with the help of a low concentration of cryoprotective agents (7%). The size of some selected formulations of CS-coated nanocapsules, before and after freeze-drying and further resuspension in water, is presented in Table 3. Results show that the suspensions of nanocapsules coated with CS recover their homogeneous aspect and initial particle size following freeze-drying. Therefore, it could be stated that the combined coat of PECL-CS around the oily core of the nanocapsules plays an important protective role during freeze-drying.

Diazepam loading and release studies

The ability of the CS-coated colloidal systems to entrap and release drugs was evaluated using diazepam as a model compound. The efficiency of encapsulation of this drug within the nanocapsules and submicron emulsions

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Table 3 Particle size of lyophilizable CS-coated PECL nanocapsules. Lecithin concentration: 0.2%; PECL concentration: 1%

CS viscosity (cps)	Particle size (nm)	
	Initial	After lyophilization
100	472 ± 80	462 ± 19
680	461 ± 13	505 ± 16

Data shown are the mean ± standard deviation $n \geq 3$.

and its subsequent release from these systems are shown in Table 4 and Fig. 4, respectively. Results indicate that more than 80% of diazepam was encapsulated irrespective of the carrier composition and no influence of the CS coat was observed. In addition, the encapsulation of diazepam had no significant effect on the size and zeta potential of the colloidal systems. On the other hand, the similarity of the release profiles obtained for the nanocapsules and the emulsions evidenced that the PECL coating of nanocapsules has no effect on the release process. The same conclusion has been pointed out by our group using various drug molecules [1, 2, 28]. In contrast, the CS coating of the nanocapsules significantly slowed down the release of diazepam during the first hour (Fig. 4a). This effect was not, however, observed for submicron emulsions (Fig. 4b). These results suggest that the combined CS-PECL coating of the nanocapsules has a retarding effect in the drug release process; a fact that was previously observed for polysaccharide-coated liposomes [21, 29].

Stability in presence of Ca^{2+}

To investigate the capacity of the CS coating to prevent the coalescence of the colloidal systems by the adsorption of cations, CS-coated and uncoated nanocapsules and emulsions were incubated in presence of Ca^{2+} ions. Results in Table 5 indicate that after 72 h of incubation,

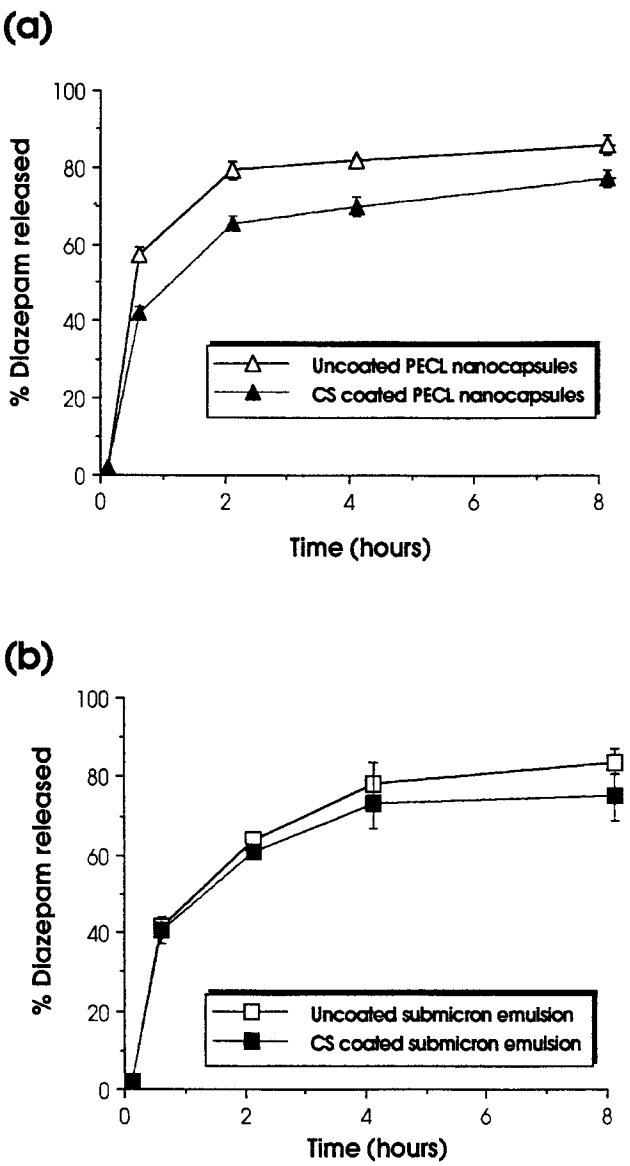

Fig. 4 Diazepam release profiles from **A** uncoated and CS-coated PECL nanocapsules and **B** uncoated and CS-coated submicron emulsion

Table 4 Mean particle size, zeta potential and percentage of diazepam encapsulated into CS-coated and uncoated PECL nanocapsules and submicron emulsion

Formulation	Particle size (nm)	Zeta potential (mV)	% DZM encapsulated
DZM-uncoated NC	225 ± 10	− 49.60 ± 1.90	92.15 ± 0.46
DZM-CS-coated NC	466 ± 12	+ 58.51 ± 1.99	94.87 ± 0.31
DZM-uncoated SE	211 ± 60	− 48.22 ± 1.91	92.82 ± 0.82
DZM-CS-coated NE	427 ± 70	+ 52.71 ± 0.01	90.89 ± 0.77

Data shown are the mean ± standard deviation $n \geq 3$.
DZM = Diazepam.
NC = Nanocapsules. CS (680 cps) 0.2% in aqueous phase; PECL 0.4% and lecithin 0.2% in acetone phase.
SE = Submicron emulsion. CS (680 cps) 0.2% in aqueous phase; lecithin 0.2% in acetone phase.

Table 5 Particle size of uncoated and CS-coated PECL nanocapsules after incubation with Ca^{2+} ions

Formulation	Particle size (nm)*	
	Initial	After incubation with Ca^{2+}
Uncoated NC	-	coalescence
CS-coated NC	461 ± 14	470 ± 29

Data shown are the mean ± standard deviation $n \geq 3$.
NC = Nanocapsules. CS (680 cps) 0.2% in aqueous phase; PECL 0.4% and lecithin 0.2% in acetone phase.

CS-coated nanocapsules remained unchanged while uncoated nanocapsules suffered coalescence. On the other hand, Ca^{2+} incubation induced coalescence on both coated and uncoated emulsions. Hence, as mentioned above, the combination of a PECL-CS coat stabilizes the oily globules to a greater extent than either a PECL or a CS coat.

Conclusions

In this paper we describe the development of new positively charged drug carriers, CS-coated nanocapsules and CS-submicron emulsions. To the best of our knowledge, this is the first report that describes the preparation of nanocapsules made of a combination of hydrophobic (PECL) and hydrophilic (CS) polymers. The double polymer coating composed of CS and PECL confers to the nanocapsules the following advantages: 1) a positive charge which prevents the destabilization by cations adsorption, 2) the possibility of freeze-drying, and 3) a better controlled release of drugs. Furthermore, these systems are expected to favorably interact with negatively charged mucosa and epithelia. Therefore, these new drug delivery systems may be accepted as very promising drug carriers for various administration routes.

Acknowledgements This work was supported by a grant from the Spanish Commission of Sciences and Technology, (CICYT, FAR94-0579).

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