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New surface-modified lipid nanoparticles as delivery vehicles for salmon calcitonin

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Abstract

The aim of the present work was to develop a new nanoparticulate system, consisting of lipid nanoparticles coated with chitosan (CS), intended for the oral administration of peptide drugs. These new nanoparticles were studied, and compared with the previously developed PEG-coated lipid nanoparticles, with regard to their ability to incorporate and deliver the model peptide salmon calcitonin (sCT). Moreover, the influence of the core composition, either a solid triglyceride (tripalmitin), or a mixture of a liquid and a solid triglyceride (Miglyol® 812 and tripalmitin) on the encapsulation and release of sCT was investigated. The results showed that a CS coating could be formed around the tripalmitin nanoparticles by simple incubation of the lipid cores in a CS solution, due to the high affinity of CS for the lipid core. In addition, sCT could be efficiently associated, irrespective of the core composition, to the nanoparticles. This important association was attributed to the marked affinity of sCT for the lipid cores, as confirmed by the adsorption studies. However, the nature of the coating affected the surface association of the peptide, which was less important for the nanoparticles coated with CS, than for PEG-coated nanoparticles. This was attributed to the displacement of the sCT molecules located on the surface of the nanoparticles by the positively charged CS molecules. This reduced surface association led to a decrease in the burst release effect, which was more pronounced for the nanoparticles coated with PEG than for those coated with CS. Following the initial burst, the systems provided a continuous and slow release of the associated peptide, independently of the nature of the coating. This slow release was attributed to the affinity of the peptide for the lipids and to the absence of degradation of the lipid matrix under the in vitro release conditions.

Keywords: Lipid nanoparticles; PEG coating; Chitosan coating; Salmon calcitonin

1. Introduction

Despite the important efforts dedicated to the design of oral peptide delivery systems, the oral administration of these sensitive molecules remains a challenge. Salmon calcitonin (sCT), as many other peptide drugs, is quickly degraded by peptidases in the gut and poorly

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transported through the intestinal epithelium. As a consequence of the extremely limited oral bioavailability (Lee and Sinko, 2000) the administration of this peptide is restricted to the parenteral route and, alternatively, to the nasal route where the absorption is still poor and highly variable (Lee et al., 1994). Among the approaches that have been explored so far to make feasible the oral administration of sCT, the use of colloidal carriers represents a promising strategy. The known ability of lipids to protect peptides from degradation has motivated the design of lipid-based colloidal delivery systems, such as nanoemulsions (Baluom et al., 1997), nanocapsules (Lowe and Temple, 1994), liposomes (Ebato et al., 2003) or complexes-containing nanoparticles (Yoo and Park, 2004), intended for the oral administration of sCT. A different approach towards the same goal has been based on improving the interaction of the colloidal carrier with the intestinal mucosa through the use of mucoadhesive polymers like Carbopol® (Baluom et al., 1997; Yoo and Park, 2004), chitosan (CS) (Kawashima et al., 2000; Takeuchi et al., 2003) poly(*N*-isopropylacrylamide) or poly(vinylamine) (Sakuma et al., 1997b, 1997a, 2002). Besides their mucoadhesive properties (Kawashima et al., 2000; Sakuma et al., 2002; Takeuchi et al., 2003), these hydrophilic coatings are supposed to provide protection for the peptide against proteolytic enzymes (Sakuma et al., 1997a) and/or to enhance the transport of the peptide through the intestinal mucosa (Artursson et al., 1994; Dodane et al., 1999; Lueben et al., 1995).

Nanoparticles made of solid lipids represent nowadays an alternative to polymeric nanoparticles. Due to their lipid nature, these nanoparticles were conceived as carriers for parenteral administration of hydrophobic drugs (Müller et al., 2000). However, as the potential of colloidal systems for transmucosal drug delivery has become evident, some authors have tested the utility of lipid nanoparticles for improving the oral absorption of drugs such as tobramycin (Cavalli et al., 2003), idarubicin (Zara et al., 2002), cyclosporine A (Zhang et al., 2000) or camptothecin (Yang et al., 1999a, 1999b). The overall conclusion from these studies was that the nanoencapsulation of these drugs in lipid nanoparticles improved their bioavailability, prolonged their blood residence time and/or modified their biodistribution.

Over the last few years, our group has dedicated important efforts to the development of surface-modified colloidal systems for transmucosal drug delivery. The results of these studies have shown that the coating of hydrophobic polymeric nanoparticles and nanocapsules with hydrophilic polymers such as CS and PEG has a clear benefit in their ability to enhance the transmucosal transport of the associated compounds, following either nasal, oral or ocular administration (Tobío et al., 1998; Tobío et al., 2000; Vila et al., 2002; De Campos et al., 2003). While the mechanisms responsible for this positive behavior are being investigated, the evidence from the experiments performed until now is that the hydrophilic coating improves the stability of the colloidal system in contact with the mucosal environment (Tobío et al., 2000; Vila et al., 2002) and, in some cases, favours the interaction with epithelia, as demonstrated in different cell culture models (Behrens et al., 2002).

Within this frame, we have recently reported the development of new lipid nanoparticles comprising a PEG stabilizing layer that rendered the system stable under the harsh conditions of the gastrointestinal tract. An additional goal of this previous study was to improve the capacity of the lipid nanoparticles to incorporate hydrosoluble peptides (Garcia-Fuentes et al., 2002). PEG-coated tripalmitin nanoparticles, prepared by the double emulsion solvent evaporation method, accomplished these two objectives, showing an improved stability in gastrointestinal fluids in comparison to other lipid carriers as well as the capacity to associate the model peptide insulin. In addition, we have recently reported a further modification of this system based on the co-encapsulation of the oil Miglyol® 812 (Garcia-Fuentes et al., 2004a). These particles containing a liquid lipid were conceived with the intention of creating small liquid reservoirs inside the solid matrix in order to facilitate the diffusion of the peptides associated to them, thus improving their release properties.

Taking into account this information, the objectives of the present work were: (i) to develop a new nanoparticulate lipid carrier coated with CS and (ii) to study the ability of PEG- and CS-coated lipid NP to associate and deliver sCT. Therefore, three formulations, which differ either in their core or in their coating composition, were tested: (i) PEG-coated tripalmitin nanoparticles, (ii) PEG-coated tripalmitin/Miglyol® nanoparticles and (iii) CS-coated tripalmitin nanoparticles. The influence of the coating and core characteristics of NP on their in vitro properties was characterized.

2. Materials and methods

Tripalmitin [Dynasan® 116] (Condea, Germany) and medium chain triglycerides [Miglyol® 812] (Hülls, Germany) were the lipids selected for forming the cores. L-α-Lecithin from soya lecithin (Sigma, Spain), poloxamer 188 [Symperonic® F68] (ICI, Spain) and PEG-2000 stearate [Simulsol® M52], a kind gift from Seppic (France), were used as surfactants. Chitosan (CS) (specifications: 75–85% of deacetylation degree, viscosity 20–200 cP at 1% in 1% acetic acid) was purchased from Aldrich (Spain) and salmon calcitonin (sCT) was a kind donation from Almirall Prodespharma S.A. (Spain). Other reagents were analytical grade or better.

2.1. Preparation of polymer-coated lipid nanoparticles

Tripalmitin nanoparticles were prepared by the double emulsion-solvent emulsification method, according to the procedure previously developed (Garcia-Fuentes et al., 2002). Briefly, 50 μl of water were emulsified in a 0.5 ml methylene chloride solution containing 25 mg of lecithin and 50 mg of tripalmitin by sonication for 15 s (20 W). Then, 1 ml of water was quickly poured into this emulsion, and sonicated again for 60 s (20 W). Finally, the solvent was evaporated, first for 30 min at room temperature and then under vacuum.

PEG-coated tripalmitin nanoparticles were produced by an analogous procedure but adding 25 mg of PEG-2000 stearate to the organic phase. PEG-coated tripalmitin/Miglyol® nanoparticles were obtained similarly but substituting 12.5 mg of tripalmitin by the same amount of Miglyol® 812.

CS-coated tripalmitin nanoparticles were produced by simple adsorption of the polymer (CS chloride) to tripalmitin nanoparticles. CS chloride was obtaining from CS base according to the following procedure. A 2% (w/v) solution of CS in 0.1 M hydrochloric acid was prepared under stirring for 1 h. The solution was filtrated, and then washed by dialysis for a period of 3 days. After this procedure, no traces of residual hydrochloric acid were found. The CS solution was then centrifuged at 30 000 × *g* for 1 h and freeze-dried. The resultant CS powder was readily soluble in water.

The formulation conditions for producing CS-coated nanoparticles were optimized by evaluating the influence of CS and tripalmitin nanoparticle concentration on the size and polydispersity of the coated nanoparticles. The general method was as follows. A 2.5 ml tripalmitin nanoparticles suspension (0.42 or 1%, final concentration) was added to a 0.5 ml CS/poloxamer solution. Poloxamer final concentration was maintained at 1.5% (w/v) while CS final concentration was varied (0.01, 0.025 and 0.05%, w/v).

Nanoparticles were isolated by ultracentrifugation for 1 h (85 000 × *g* for PEG-tripalmitin nanoparticles, 150 000 × *g* for PEG-tripalmitin/Miglyol® nanoparticles and 50 000 × *g* for CS-tripalmitin nanoparticles). For CS-tripalmitin nanoparticles, a glycerol phase (20 μl) was placed in the distal end of the centrifugal tube in order to avoid any loss in the granulometry of the system in the isolation step.

2.2. Stability of CS-coated lipid nanoparticles in simulated gastrointestinal media

Isolated CS-coated nanoparticles were incubated at 37 °C either in simulated gastric or intestinal media (USP XXIV) at a final concentration of 0.5% (w/v). Samples were withdrawn immediately after mixing the nanoparticles with gastric or intestinal medium and after 1 h of incubation. Particles that interacted with the gastrointestinal fluids were separated by centrifugation at 3000 × *g* for 5 min. The size of the remaining nanoparticles in the supernatant was measured by photon correlation spectroscopy (PCS) and their concentration was determined by measuring the turbidity at λ = 450 nm, using the original suspension as a control.

2.3. Physicochemical and morphological characterization of polymer-coated lipid nanoparticles

Size and zeta potential were measured, respectively, by PCS and laser doppler anemometry (Zetasizer 3000HS, Malvern, UK) after isolation and resuspension of nanoparticles in NaCl 1 mM.

The transmission electron microscopy (TEM) (Philips CM12, Eindhoven, Netherlands) images were

taken following staining of the isolated nanoparticles with 2% (w/v) phosphotungstic acid.

2.4. sCT loading capacity of the polymer-coated lipid nanoparticles

sCT was associated to the lipid nanoparticles by incorporating 50 μl of a 10 mg/ml aqueous solution as the inner phase of the primary emulsion. The sCT encapsulation efficiency was determined by measuring the amount of sCT that was not associated to the nanoparticles and, therefore, remained in the supernatant upon isolation of the nanoparticles. Isolation procedures by means of a combined ultrafiltration–centrifugation technique (Contricom® YM-100, Millipore, Spain) in the case of PEG-coated nanoparticles, or by ultracentrifugation in the case of CS-coated nanoparticles, were optimized to minimize sCT loss due to adsorption to the glassware surfaces. The supernatant was diluted with phosphate buffer (pH = 4) containing poloxamer 188 (0.25%, w/v) and sodium azide (0.25%, w/v) and assayed for sCT content by HPLC at 220 nm, as described in the British Pharmacopoeia, 1998.

2.5. In vitro sCT release and sCT adsorption studies

For the release studies, 3 ml of the nanoparticles suspensions were incubated in phosphate buffer (pH = 4) containing Poloxamer 188 (0.25%, w/v) and sodium azide (0.25%, w/v) under horizontal agitation at 37 °C. The pH of the incubation medium was selected in order to preserve the stability of the sCT released from the nanoparticles. At times 0.25, 1 and 6 h, samples were collected and the nanoparticles were isolated by the same methods described for the encapsulation studies. The free (non-encapsulated) sCT in the supernatant of the samples was analyzed by HPLC.

On the other hand, in order to evaluate the affinity of sCT for the polymer-coated lipid nanoparticles, the surface association of the peptide to the nanoparticles was studied. A sCT solution of known concentration (100 μg/ml) was mixed with suspensions of lipid nanoparticles of different concentrations (0.2–5.5 mg/ml). After incubation for 1 h at 20 °C, the nanoparticles were isolated by the methods described above and free (non-associated) sCT was assayed in the supernatant by HPLC.

2.6. Statistical analysis

The statistical significance of the differences between the parameters analyzed for the different nanoparticle formulations was tested by the analysis of variance (ANOVA) together with the multiple comparison Student–Newman–Keuls method.

3. Results and discussion

As indicated in the introduction, the purpose of this work was to develop a new nanoparticulate carrier intended to the oral administration of peptides. The new carrier is composed of a lipid core aimed to protect and control the release of the peptide and a CS coating that was supposed to facilitate the interaction of the carrier with the intestinal mucosa and the further transport of the associated peptide. In addition, this work was aimed to compare the behavior of the newly developed CS-coated lipid nanoparticles with that of the previously reported PEG-coated lipid nanoparticles (Garcia-Fuentes et al., 2002, 2004a) in terms of their ability to associate and deliver the model peptide sCT. Moreover, an additional element, Miglyol® oil, was introduced in the PEG-coated formulation in order to modify the composition and structure of the lipid core and, to evaluate its influence on the association of the peptide.

3.1. Development of CS-coated lipid nanoparticles

The formation of the CS-coated nanoparticles occurred in two steps: first, we obtained the lipid cores by the double (water-in-oil-in-water) emulsion method using lecithin as a surfactant. The inner water phase of the double emulsion was intended to dissolve the peptide to be encapsulated. In a second step, the isolated lipid nanoparticles were incubated in a solution containing CS and Poloxamer 188. The results of the zeta potential of the nanoparticles before and after the incubation process evidenced the attachment of CS onto the surface of the nanoparticles. Indeed, as can be noted in Table 1, the zeta potential of the nanoparticles was inverted from negative (−50.3 mV) to positive values upon addition of increasing amounts of CS to the incubation medium. In the formulation prepared with 1% (w/v) of nanoparticles and 0.01% (w/v) of

Table 1
ζ Potential of CS-coated lipid nanoparticles prepared by incubation of lipid nanoparticles (1%, w/v) with CS solutions of different concentrations (mean ± S.D., n = 3)

CS concentration (w/v) (%)	ζ Potential (mV)
—[a]	−50.3 ± 1.8
0.010	−4.0 ± 0.4
0.025	+11.6 ± 5.3
0.050	+26.3 ± 7.6

[a] Tripalmitin nanoparticles before incubation with CS.

CS, the zeta potential was neutralized, whereas at a CS concentration of 0.025% (w/v), an inversion of the zeta potential was noted. This positive value was further increased after incorporation of a CS concentration of 0.050%. This trend of the zeta potential as a function of CS concentration should logically be attributed to the surface association of positively charged CS to the lipid nanoparticles. This surface association process is supposed to be driven by the electrostatic interaction between the negatively charged tripalmitin solid cores and the positively charged CS chains. The same phenomenon of surface attachment of CS to negatively charged cores has been previously observed by our group while developing CS-coated emulsions and CS-coated polymer nanoparticles (Calvo et al., 1997; Vila et al., 2002; De Campos et al., 2003).

The effect of the incubation of the lipid nanoparticles with different concentrations of CS is shown in the response surface in Fig. 1. The results indicate that both, the concentration of lipid nanoparticles and the concentration of CS had a significant influence on the particle size of the resulting formulations. More specifically, within the range of concentrations investigated, the size of the CS-coated nanoparticles became smaller by reducing the concentration of nanoparticles and increasing the concentration of CS. This observation was somehow surprising since, in agreement with the results reported by Kawashima et al. (2000) for CS-coated PLGA nanoparticles, one would expect an augmentation in the particle size upon addition of increasing amounts of CS. However, in the present study we have used low concentrations of CS and high concentrations of nanoparticles as compared to those previously reported. Consequently, it may be possible that in extreme conditions (low CS concentration and high nanoparticles concentration), the amount of CS adsorbed onto the particles was not sufficient to prevent particle interaction, thus resulting in the formation of aggregates. This interpretation agrees with the results of the zeta potential of nanoparticles discussed above. In fact, the formulation prepared with 1% (w/v) of nanoparticles and 0.01% (w/v) of CS, elicited an almost neutral zeta potential, which does not help to stabilize the particles. However, increasing the CS concentration up to 0.050% (w/v), an inversion of the zeta potential was noted, being this value high enough to prevent particle attraction. Because of these specific characteristics, the formulation prepared with CS at 0.05% (w/v) and a concentration of nanoparticles of

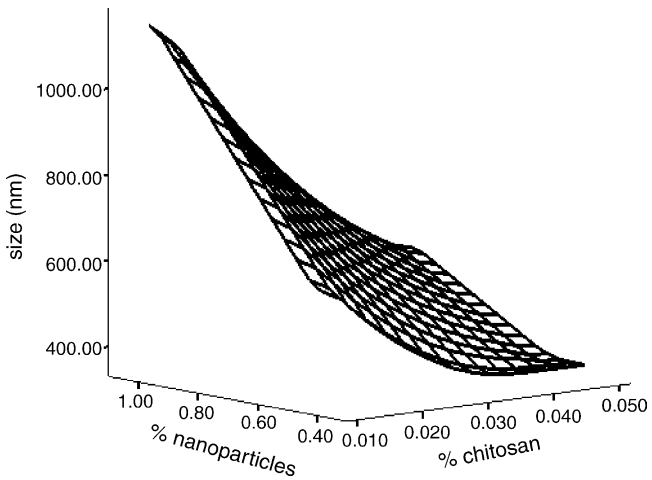

Fig. 1. Size of CS-coated tripalmitin nanoparticles as a function of CS and nanoparticle concentration.

Table 2
Relative turbidity and particle size of CS-coated tripalmitin nanoparticles incubated in simulated gastric and intestinal media

Time	Simulated Gastric Medium		Simulated Intestinal Medium	
	Relative turbidity	Size (nm)	Relative turbidity	Size (nm)
Initial	1.00 ± 0.14	509 ± 43	1.00 ± 0.14	509 ± 43
0 h	1.17 ± 0.18	527 ± 20	1.07 ± 0.03	447 ± 46[a]
1 h.	1.07 ± 0.15	546 ± 65	1.07 ± 0.04	608 ± 8[a]

Turbidity is expressed in relation to initial values (nanoparticles in water) (mean ± S.D., $n = 3$).

[a] Significantly different from the initial value of particle size ($\alpha < 0.01$).

1% (w/v), was selected for the following studies of stability and sCT encapsulation.

3.2. Stability of CS-coated lipid nanoparticles in simulated digestive fluids

The assessment of the stability of colloidal carriers in gastrointestinal fluids is essential in order to predict their suitability for oral administration. Indeed, it is known that the size is a critical parameter for the interaction of the particles with the intestinal mucosa. Consequently, if the particles aggregate, they will have a limited access to the absorptive epithelium (Jani et al., 1990). Lipid nanoparticles are an example of a colloidal system that undergoes this aggregation process upon contact with gastrointestinal fluids, being also susceptible to degradation by the intestinal enzymes (Müller et al., 1996). In a recent report we showed that the surface modification with PEG-stearate led to a significant improvement in the stability and resistance to lipolytic enzymes (Garcia-Fuentes et al., 2002). In the present work, one of the objectives was to evaluate whether or not a CS coating could improve the stability of tripalmitin nanoparticles in gastric and intestinal simulated fluids.

The results showed that CS-coated nanoparticles remained practically unaltered in both gastric and intestinal media (Table 2). No significant changes in the particle size were detected in gastric medium, thus indicating that CS did not detach from the nanoparticle surface upon exposure to acidic pH. Interestingly, upon contact with simulated intestinal medium, a 50 nm size reduction compared with the initial value was detected. This result can be interpreted as a consequence of the polymer coating shrinkage in a thermodynamically less compatible medium, whose pH (6.8; USP XXIV) is above the solubility pH of CS (pH = 6.5) (Hejazi and Amiji, 2003). This size reduction was followed by an increase of around 100 nm after 1 h of incubation, which could be attributed to a slight particle aggregation. This small size modification agrees with the unchanged turbidity of the suspension in both gastric and intestinal media. Therefore, these results support the suitability of the CS-coated nanoparticles for oral administration.

3.3. Morphological and physicochemical characterization of PEG- and CS-coated lipid nanoparticles

In the present study, the microscopical appearance of CS-coated tripalmitin nanoparticles was compared with that of non-coated and PEG-coated nanoparticles, using TEM (Fig. 2). In general, the particles showed a compact and spherical structure. The particles coated with PEG exhibit a less stained outer layer that could be attributed to the presence of a PEG coating around the nanoparticles. This appearance is consistent with

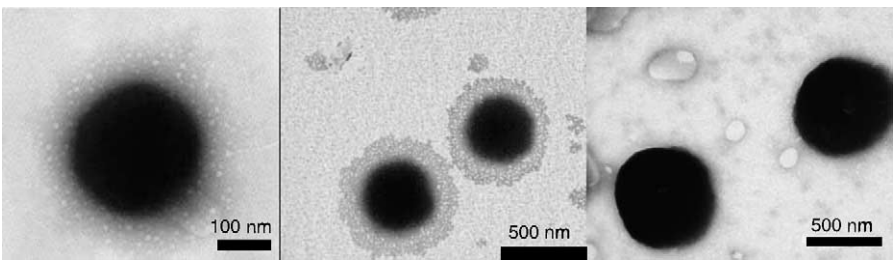

Fig. 2. Transmission electron micrographs of PEG-coated tripalmitin nanoparticles (left), PEG-coated tripalmitin/Miglyol® nanoparticles (center) and CS-coated tripalmitin nanoparticles (right).

Table 3
Physicochemical properties (particle size, polydispersity index and ζ potential) and sCT encapsulation efficiency (EE) of the different lipid nanoparticles (NP) formulations containing salmon calcitonin (mean ± S.D., n = 3)

Formulation	Size (nm)	Poly. index	ζ Potential (mV)	EE (%)
Uncoated tripalmitin NP	200.0 ± 2.3	0.29 ± 0.09	−50.3 ± 1.8	>90
PEG-coated tripalmitin NP	226.4 ± 7.5	0.25 ± 0.07	−34.8 ± 2.8	>90
PEG-coated tripalmitin/Miglyol® NP	207.4 ± 19.1	0.18 ± 0.08	−36.6 ± 2.5	>90
CS-coated tripalmitin NP	537.7 ± 16.6	0.43 ± 0.14	+29.2 ± 6.7	30.7 ± 2.3

the results of the NMR studies indicating the presence of PEG chains as a coating surrounding the particles (Garcia-Fuentes et al., 2004b). However, in the case of CS-coated nanoparticles, the polymer coating could not be clearly identified in the TEM images. This could be due to the close and longitudinal attachment of the CS chains during the drying process required for TEM visualization. In fact, the changes on particle size (observed by TEM and PCS) and the inversion of the surface charge provide a clear evidence of the polymer coating.

As it can be seen in Table 3, the physicochemical characteristics of the coated nanoparticles were mainly dependent on the type of polymer coating. PEG-coated formulations showed a size around 200 nm and moderate polydispersity indexes. CS-coated nanoparticles showed a 100% increase in size in comparison to those uncoated, this being related to the deposition of CS layers on the surface of the preformed nanoparticles. In addition, the polydispersity index of this formulation was larger than that of the PEG-coated particles but similar to the uncoated particles.

As expected, the differences in the zeta potential values of the formulations were remarkable. The zeta potential of the PEG-coated lipid nanoparticles showed a less negative value than that of the particles without a PEG coating. The presence of PEG on the surface of nanoparticles partially masked the negative charge of the uncoated particles, an effect which is common for PEG-coated nanoparticles, due to an extension of the plane of shear of the nanoparticles (Gref et al., 2000). The similarity between the zeta potential values of both PEG-coated formulations indicates that the incorporation of Miglyol® within the core does not affect the characteristics of the nanoparticles. In contrast, after the coating of the tripalmitin nanoparticles with CS, the zeta potential underwent an inversion from highly negative to positive values due to the deposition of the cationic polymer CS on their surface. This value, and the increase in particle size, reflected the successful coating with CS.

3.4. sCT encapsulation efficiency and release properties of PEG- and CS-coated lipid nanoparticles

Lipid nanoparticles made of solid lipids are highly hydrophobic matrixes that solidify in crystalline form. The most popular technique for the production of lipid nanoparticles is the homogenization of melted lipids, a technique particularly suited for the encapsulation of hydrophobic drugs (Müller et al., 2000). In these formulations, the compatibility between the drug and the lipid crystal matrix is the main factor governing the encapsulation efficiency. In fact, due to this reason, the number of studies on the incorporation of peptides into these nanoparticles is very limited (Almeida et al., 1997; Hu et al., 2004). As an alternative, we have previously explored the feasibility of the double emulsion-solvent evaporation method for the production of lipid nanoparticles (Garcia-Fuentes et al., 2002). The results showed that, using this approach, it is possible to efficiently encapsulate hydrophilic peptides such as insulin. In the present work, this technique was applied to the encapsulation of the peptide sCT in the three types of coated-lipid nanoparticles, with the final purpose of designing a formulation intended for the oral administration of this peptide.

As can be seen in Table 3, the encapsulation efficiency of the studied formulations varied markedly depending on the type of polymer coating. In the case of PEG-coated formulations, no exact values are given because the amount of free (non-encapsulated) sCT in all the samples investigated was below the limit of detection (10% of the total amount of sCT). Consequently, this undetectable amount of free sCT, which represents an association efficiency higher than 90%, is a proof of the remarkable affinity of this peptide to the lipid

core of the nanoparticles, irrespective of the presence of Miglyol® reservoirs in the core. Since sCT has an isoelectric point of 10.4 (Epand et al., 1983), the most probable association mechanism is the electrostatic interaction between the positively charged peptide and the negatively charged lipids forming the nanoparticles (tripalmitin and lecithin). The same explanation was given to justify the high association of sCT to other lipid structures (Law and Shih, 2001; Takeuchi et al., 2003; Yoo and Park, 2004). On the other hand, in Table 3 it can be noted that the amount of peptide associated was significantly reduced for the CS-coated lipid nanoparticles. This result was unexpected given the fact that sCT was incorporated in the inner aqueous phase of the first emulsion and, hence, associated to the nanoparticles prior to the formation of the CS coating. Therefore, the reduction of the sCT association due to CS could only be explained on the basis of the displacement of the sCT molecules, located on the surface of the nanoparticles by the positively charged CS molecules, as a consequence of a competition process in their binding to the lipids. This surface association of sCT to the lipid nanoparticles was further investigated as discussed in the last section of this article.

The results of the in vitro release studies showed that a certain amount of sCT was readily released from the lipid nanoparticles immediately after their dilution in pH = 4 buffer (Fig. 3). The intensity of this initial burst was slightly affected by the nature of the coating being it more important for the PEG-coated formulations than for the CS-coated nanoparticles. This difference could be simply related to the lower amount of sCT located at the surface of the CS-coated nanoparticles, as discussed above. After the initial burst, a slow release was observed, reaching the value of 40% of sCT released after 6 h. This second-phase release was not affected by the incorporation of the Miglyol® oil into the nanoparticles structure. This low and continuous release of sCT from the lipid nanoparticles, irrespective of the lipid composition, could be justified by the important affinity of the peptide for the lipids and, thus, by its tendency to remain associated to the particles. In this regard, it is worthwhile to mention that besides the solid and liquid triglycerides, the lecithin used to obtain the particles contains high percentages of phosphatidyl glycerol, a compound that is known to form complexes with sCT (Epand et al., 1983). These results, illustrative of the affinity of sCT for the lipid core, are in good agreement with the previous observation regarding the competition of sCT and CS in their association with the lipids. Hence, these results motivated us to study the relevance of the lipid–sCT interaction and its implication in the mechanism of association and release of sCT from the nanoparticles.

3.5. sCT adsorption to polymer-coated tripalmitin nanoparticles

For the evaluation of the adsorption of sCT onto the nanoparticles surface, free sCT in solution was mixed with known concentrations of nanoparticles. The results fit an equation corresponding to a Langmuir-type isotherm with the difference that the variable investigated was the concentration of adsorbent

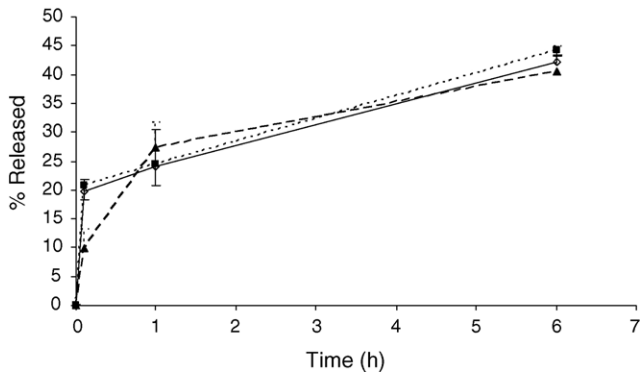

Fig. 3. Release profiles of salmon calcitonin from the different lipid nanoparticles: (◊) PEG-coated tripalmitin nanoparticles (lower continuous error bars), (■) PEG-coated tripalmitin/Miglyol® nanoparticles (upper continuous error bars), (▲) CS-coated tripalmitin nanoparticles (upper dashed error bars) (mean ± S.D., $n = 3$).

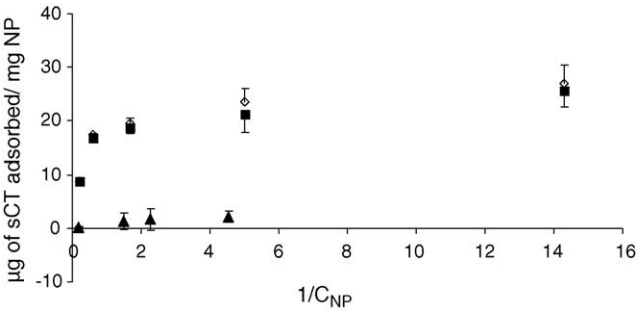

Fig. 4. Adsorption isotherms of salmon calcitonin (sCT) onto the different lipid nanoparticles: (◊) PEG-coated tripalmitin nanoparticles, (■) PEG-coated tripalmitin/Miglyol® nanoparticles, (▲) CS-coated tripalmitin nanoparticles (mean ± S.D., $n = 3$).

(nanoparticles) instead of the concentration of sCT:

$$A = \frac{K_1 K_2 \left(\frac{1}{C_{NP}}\right)}{1 + K_1 \left(\frac{1}{C_{NP}}\right)} \quad (1)$$

or, in a linearized form:

$$\frac{1}{A}\frac{1}{C_{NP}} = \frac{1}{K_1 K_2} \frac{1}{K_2 C_{NP}} \quad (2)$$

where A represents the mass of sCT adsorbed per mass of nanoparticles, K_1 is the affinity constant, K_2 the plateau concentration (capacity constant) and C_{NP}, the concentration of nanoparticles. The adsorption isotherms and linearized plots representing $(1/A)(1/C_{NP})$ versus $1/C_{NP}$ are shown in Figs. 4 and 5, respectively. The linear regression analysis confirmed the goodness of the fitting ($R^2 > 0.99$) and indicated that, under the experimental conditions of this study, sCT adsorbs forming monolayers around the particles. These results are different from those obtained by Calis and coworkers who showed that sCT adsorbs to PLGA microspheres according to a mixed Langmuir and Freundlich isotherm (Calis et al., 1995). These authors argued that sCT tends to form monolayers (Langmuir phase) or multilayers (Freundlich phase) depending on its concentration in the incubation medium. In the present study, the adsorption values of sCT onto coated-lipid nanoparticles did not fit the Freundlich equation and, therefore, a multilayer configuration could not be deduced.

The calculated parameters, illustrative of the adsorption of sCT onto the coated-lipid nanoparticles

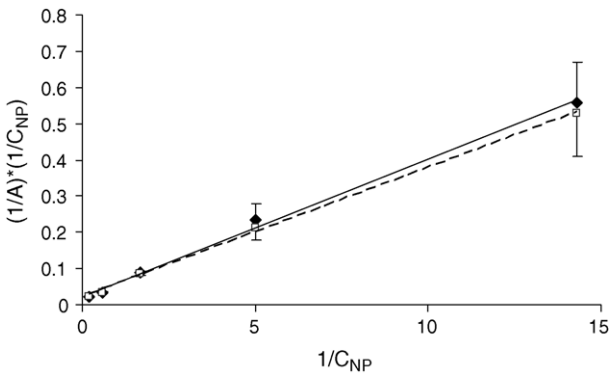

Fig. 5. Langmuir type linear adsorption fitting obtained with the different lipid nanoparticles (◊) PEG-coated tripalmitin nanoparticles, (■) PEG-coated tripalmitin/Miglyol® nanoparticles. CS-coated tripalmitin nanoparticles fitting is no represented because it is off-scale with the other plots (mean ± S.D., $n = 3$). A represents µg of salmon calcitonin (sCT) adsorbed per mg of formulation and C_{NP} is nanoparticle concentration in mg/ml. Regression equation values were: $y = 0.038x + 0.0229$, $R^2 = 0.9961$ for PEG-coated tripalmitin NP $y = 0.0359x + 0.0207$, $R^2 = 0.9983$ for PEG-coated tripalmitin/Miglyol® NP $y = 0.3503x - 0.6706$, $R^2 = 0.9959$ for CS-coated tripalmitin NP.

Table 4
Parameters calculated from a Langmuir equation for the adsorption of the salmon calcitonin (sCT) onto nanoparticles: affinity constant of sCT for the nanoparticles (K1) and adsorption capacity constant of the nanoparticles (K2)

Formulation	K_1	K_2 (μg/mg)
PEG-coated tripalmitin NP	1.66	26.3
PEG-coated tripalmitin/Miglyol® NP	1.73	27.9
CS-coated tripalmitin NP	0.52	2.9

are shown in Table 4. These results evidence the important interaction between sCT and PEG-coated nanoparticles and its low affinity for the CS-coated nanoparticles. More specifically, the maximum amount of sCT associated was 23.4 μg/mg for PEG-coated tripalmitin nanoparticles, 25.6 μg/mg for PEG-coated tripalmitin/Miglyol® nanoparticles and 1.9 μg/mg for CS-coated tripalmitin nanoparticles. Consequently, these results corroborate the competition between the sCT and CS molecules for their association with the particles and support the hypothesis that a fraction of the sCT molecules (those located at the surface of the nanoparticles) may be displaced by the CS molecules forming the coating. Furthermore, these results provided us with a better understanding of the surface-coated systems in terms of their ability to associate and deliver sCT. Therefore, it can be deduced that in both systems (PEG-coated and CS-coated) the association of the peptide to the particles is governed not only by the incorporation approach (physical entrapment), but also by the great affinity of the peptide for the lipids (physicochemical association). In addition, it can be understood that the surface-located sCT molecules will be detached in the CS coating process but will remain surface-associated in the case of the PEG-coated nanoparticles. This different association to both systems would also explain the greater burst observed for the PEG-coated particles as compared to those CS-coated. Besides, the affinity of the peptide for the lipid core and the absence of degradation of the lipid matrix would justify the slow release of the peptide after the initial burst and the negligible effect of the co-encapsulation of the Miglyol® oil.

4. Conclusions

We have developed a new nanoparticulate delivery system, consisting of lipid nanoparticles coated with CS, with potential application for the oral administration of peptides. Furthermore, using sCT as a model peptide, we have compared the association and release characteristics of sCT from this new vehicle, with those of PEG-coated lipid nanoparticles. The results showed that the nature of the coating may affect the surface association and, hence, the immediate release of the peptide, being this effect reduced for the nanoparticles coated with CS as compared to those coated with PEG. Finally, both coated nanoparticulate systems were able to provide a continuous delivery of the associated peptide. Current studies are aimed at evaluating the in vitro (Caco-2 model cell line) and in vivo efficacy of these newly developed formulations.

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References

Almeida, A.J., Runge, S., Müller, R.H., 1997. Peptide-loaded solid lipid nanoparticles (SLN): influence of production parameters. Int. J. Pharm. 149, 255–265.

Artursson, P., Lindmark, T., Davis, S.S., Illum, L., 1994. Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). Pharm. Res. 11, 1358–1361.

Baluom, M., Friedman, D.I., Rubinstein, A., 1997. Absorption enhancement of calcitonin in the rat intestine by carbopol-containing submicron emulsion. Int. J. Pharm. 154, 235–243.

Behrens, I., Vila Pena, A.I., Alonso, M.J., Kissel, T., 2002. Comparative uptake studies of bioadhesive and non-bioadhesive nanoparticles in human intestinal cell lines and rats: the effect of mucus on particle adsorption and transport. Pharm. Res. 19, 1185–1193.

Calis, S., Jeyanthi, R., Tsai, T., Mehta, R.C., DeLuca, P.P., 1995. Adsorption of salmon calcitonin to PLGA microspheres. Pharm. Res. 12, 1072–1076.

Calvo, P., Remuñán-López, C., Vila-Jato, J.L., Alonso, M.J., 1997. Development of positively colloidal drug carriers: chitosan-coated polyester nanocapsules and submicron-emulsions. Colloid. Polym. Sci. 275, 46–53.

Cavalli, R., Bargoni, A., Podio, V., Muntoni, E., Zara, G.P., Gasco, M.R., 2003. Duodenal administration of solid lipid nanoparticles loaded with different percentages of Tobramycin. J. Pharm. Sci. 92, 1085–1094.

De Campos, A.M., Sánchez, A., Gref, R., Calvo, P., Alonso, M.J., 2003. The effect of a PEG versus a chitosan coating on the inter-

action of drug colloidal carriers with the ocular mucosa. Eur. J. Pharm. Sci. 20, 73–81.

Dodane, V., Khan, M.A., Merwin, J.R., 1999. Effect of chitosan on epithelial permeability and structure. Int. J. Pharm. 182, 21–32.

Ebato, Y., Kato, Y., Onishi, H., Nagai, T., Machida, Y., 2003. In vivo efficacy of a novel double liposome as an oral dosage form of salmon calcitonin. Drug Develop. Res. 58, 253–257.

Epand, R.M., Epand, R.F., Orlowski, R.C., Schlueter, R.J., Boni, L.T., Hui, S.W., 1983. Amphipathic helix and its relationship to the interaction of calcitonin with phospholipids. Biochemistry 22, 5074–5084.

Garcia-Fuentes, M., Torres, D., Alonso, M.J., 2002. Design of lipid nanoparticles for the oral delivery of hydrophilic macromolecules. Coll. Surf. B: Biointerf. 27, 159–168.

Garcia-Fuentes, M., Torres, D., Alonso, M.J., 2004a. Design and characterization of a new drug nanocarrier made from solid-liquid lipid mixtures. J. Coll. Int. Sci. 292, 63–73.

Garcia-Fuentes, M., Martin-Pastor, M., Torres, D., Alonso, M.J., 2004b. Application of NMR spectroscopy to the characterization of PEG-stabilized lipid nanoparticles. Langmuir 20, 8839–8845.

Gref, R., Lück, M., Quellec, P., Marchand, M., Dellacherie, E., Harnisch, S., Blunk, T., Müller, R.H., 2000. 'Stealth' corona-core nanoparticle surface modified by polyethylene glycol (PEG): influence of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsortion. Coll. Surf. B: Biointerf. 18, 301–313.

Hejazi, R., Amiji, M., 2003. Chitosan-based gastrointestinal delivery systems. J. Control Release 89, 151–165.

Hu, F.Q., Hong, Y., Yuan, H., 2004. Preparation and characterization of solid lipid nanoparticles containing peptide. Int. J. Pharm. 273, 29–35.

Jani, P.U., Halbert, G.W., Langridge, J., Florence, A.T., 1990. Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency. J. Pharm. Pharmacol. 42, 821–826.

Kawashima, Y., Yamamoto, H., Takeuchi, H., Kuno, Y., 2000. Mucoadhesive DL-lactide/glycolide copolymer nanospheres coated with chitosan to improve oral delivery of elcatonin. Pharmaceut. Develop. Technol. 5, 77–85.

Law, S.L., Shih, C.L., 2001. Characterization of calcitonin-containing liposome formulations for intestinal delivery. J. Microencaps. 18, 211–221.

Lee, W.A., Ennis, R.D., Longenecker, J.P., Bengtsson, P., 1994. The bioavailabililty of intranasal salmon calcitonin in healthy volunteers with and without permeation enhancer. Pharm. Res. 11, 747–750.

Lee, Y.-H., Sinko, P.J., 2000. Oral delivery of salmon calcitonin. Adv. Drug Deliv. Rev. 42, 225–238.

Lowe, P.L., Temple, C.S., 1994. Calcitonin and insulin in isobutilcyanoacrylate nanocapsules: protection against proteases and effect on intestinal absorption. J. Pharm. Pharmacol. 46, 547–552.

Lueben, H.L., Verhoef, J.C., Borchard, G., Lehr, C.-M., de Boer, A.G., Junginger, H.E., 1995. Mucoadhesive polymers in peroral peptide drug delivery. II. Carbomer and polycarbophil are potent inhibitors of the intestinal proteolytic enzyme trypsin. Pharm. Res. 12, 1–6.

Müller, R.H., Mäder, K., Gohla, S., 2000. Solid lipid nanoparticles (SLN) for controlled drug delivery – a review of the state of the art. Eur. J. Pharm. Biopharm. 50, 161–177.

Müller, R.H., Rühl, D., Runge, S., 1996. Biodegradation of solid lipid nanoparticles as a function of lipase incubation time. Int. J. Pharm. 144, 115–121.

Sakuma, S., Ishida, Y., Sudo, R., Suzuki, N., Kikuchi, H., Hiwatari, K., Kishida, A., Akashi, M., Hayashi, M., 1997a. Stabilization of salmon calcitonin by polystyrene nanoparticles having surface hydrophilic polymeric chains, against enzymatic degradation. Int. J. Pharm. 159, 181–189.

Sakuma, S., Sudo, R., Suzuki, N., Kikuchi, H., Akashi, M., Ishida, Y., Hayashi, M., 2002. Behavior of mucoadhesive nanoparticles having hydrophilic polymeric chains in the intestine. J. Control Release 81, 281–290.

Sakuma, S., Suzuki, N., Kikuchi, H., Hiwatari, K., Arikawa, K., Kishida, A., Akashi, M., 1997b. Oral peptide delivery using nanoparticles composed of novel graft copolymers having hydrophobic backbone and hydrophilic branches. Int. J. Pharm. 149, 93–106.

Takeuchi, H., Matsui, Y., Yamamoto, H., Kawashima, Y., 2003. Mucoadhesive properties of carbopol or chitosan-coated liposomes and their effectiveness in the oral administration of calcitonin to rats. J. Control Release 86, 235–242.

Tobío, M., Gref, R., Sánchez, A., Langer, R., Alonso, M.J., 1998. Stealth PLA-PEG nanoparticles as protein carriers for nasal administration. Pharm. Res. 15, 270–276.

Tobío, M., Sánchez, A., Vila, A., Soriano, I., Evora, C., Vila-Jato, J.L., Alonso, M.J., 2000. The role of PEG on the stability in digestive fluids and in vivo fate of PEG-PLA nanoparticle following oral administration. Coll. Surf. B: Biointerf. 18, 315–323.

Vila, A., Sánchez, A., Tobío, M., Calvo, P., Alonso, M.J., 2002. Design of biodedegradable particles for protein delivery. J. Control Release 78, 15–24.

Yang, S., Zhu, J., Lu, Y., Liang, B., Yang, C., 1999a. Body distribution of camptothecin solid lipid nanoparticles after oral administration. Pharm. Res. 16, 751–757.

Yang, S.C., Lu, L.F., Cai, Y., Zhu, J.B., Liang, B.W., Yang, C.Z., 1999b. Body distribution in mice of intravenously injected camptothecin solid lipid nanoparticles and targeting effect on the brain. J. Control Release 59, 299–307.

Yoo, H.S., Park, T.G., 2004. Biodegradable nanoparticles containing protein-fatty acid complexes for oral delivery of salmon calcitonin. J. Pharm. Sci. 93, 488–495.

Zara, G.P., Bargoni, A., Cavalli, R., Fundarò, A., Vighetto, D., Gasco, M.R., 2002. Pharmacokinetics and tissue distribution of Idarubicin-loaded solid lipid nanoparticles after duodenal administration to rats. J. Pharm. Sci. 91, 1324–1333.

Zhang, Q., Yie, G., Li, Y., Yang, Q., Nagai, T., 2000. Studies on the cyclosporin A loaded stearic acid nanoparticles. Int. J. Pharm. 200, 153–159.