

Transmucosal macromolecular drug delivery

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Abstract

Mucosal surfaces are the most common and convenient routes for delivering drugs to the body. However, macromolecular drugs such as peptides and proteins are unable to overcome the mucosal barriers and/or are degraded before reaching the blood stream. Among the approaches explored so far in order to optimize the transport of these macromolecules across mucosal barriers, the use of nanoparticulate carriers represents a challenging but promising strategy. The present paper aims to compare the characteristics and potential of nanostructures based on the mucoadhesive polysaccharide chitosan (CS). These are CS nanoparticles, CS-coated oil nanodroplets (nanocapsules) and CS-coated lipid nanoparticles. The characteristics and behavior of CS nanoparticles and CS-coated lipid nanoparticles already reported [A. Vila, A. Sánchez, M. Tobío, P. Calvo, M.J. Alonso, Design of biodegradable particles for protein delivery, *J. Control. Rel.* 78 (2002) 15–24; R. Fernández-Urrusuno, P. Calvo, C. Remuñán-López, J.L. Vila-Jato, M.J. Alonso, Enhancement of nasal absorption of insulin using chitosan nanoparticles, *Pharm. Res.* 16 (1999) 1576–1581; M. Garcia-Fuentes, D. Torres, M.J. Alonso, New surface-modified lipid nanoparticles as delivery vehicles for salmon calcitonin (submitted for publication).] are compared with those of CS nanocapsules originally reported here. The three types of systems have a size in the nanometer range and a positive zeta potential that was attributed to the presence of CS on their surface. They showed an important capacity for the association of peptides such as insulin, salmon calcitonin and proteins, such as tetanus toxoid. Their mechanism of interaction with epithelia was investigated using the Caco-2 model cell line. The results showed that CS-coated systems caused a concentration-dependent reduction in the transepithelial resistance of the cell monolayer. Moreover, within the range of concentrations investigated, these systems were internalized in the monolayer in a concentration-dependent manner. This uptake was slightly enhanced by the presence of the CS coating but, as compared with previously published results [M. Garcia-Fuentes, C. Prego, D. Torres, M.J. Alonso, Triglyceride-chitosan nanostructures for oral calcitonin delivery: evaluation in the Caco-2 cell model and in vivo (submitted for publication)], highly dependent on the nature of the lipid core. Nevertheless, these differences in the uptake of the CS-coated systems (solid lipid core or oily core) by the Caco-2 cells did not have a consequence in the in vivo behaviour. Indeed, both CS-coated systems (nanocapsules and CS-coated nanoparticles) showed an important capacity to enhance the intestinal absorption of the model peptide, salmon calcitonin, as shown by the important and long-lasting decrease in the calcemia levels observed in rats.

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Keywords: Chitosan; Nanoparticles; Nanocapsules; Transmucosal transport; Peptide/protein carriers

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1. Introduction

Over the last decades significant efforts have been dedicated to explore new routes, alternative to injection, for the administration of macromolecules such as peptides and proteins. Among them, the transmucosal routes such as the nasal, pulmonary and oral routes, are those which have received the most important deal of attention [1–5]. As a consequence of this activity, the pulmonary administration of the peptide insulin has recently become a reality in clinical practice [5]. In addition, the number of peptides that are being administered intranasally increases year by year. However, the possibility to administer large macromolecules by the oral route needs still further investigations. The harsh conditions of the gastrointestinal tract and the limited permeability of the intestinal barrier make the situation very complex, and the solution to these problems a great challenge [6]. Nevertheless, despite the past pessimistic view, the knowledge accumulated during recent years regarding the mechanisms of interaction between nanostructured biomaterials and biological surfaces, as well as the discovery of new nanotechnologies and characterization approaches, has led to a new promising perspective on the use of nanocarriers for transmucosal macromolecular drug delivery [7–9]. The hypothesis behind this strategy has been that nanosystems, due to their colloidal size, are able to cross and, hence, transport the associated drug through the mucosal barrier, thus acting as transmucosal macromolecular nanocarriers. Indeed, at present, there is no doubt on the fact that the size is a critical parameter for the nanosystems to cross biological barriers [10,11]. However, the question that remains to be answered is how the ability of these systems to overcome these barriers can be optimized.

By accepting the premise that not only the size but also the surface properties and composition of the nanocarriers may affect their stability in biological fluids and their interaction with mucosal surfaces, we have focused our activity to the design of new types of nanocarriers especially adapted for transmucosal drug delivery [12–16]. The first relevant conclusion from this work was that a hydrophilic polyethylene glycol (PEG) coating around the nanosystem has a very positive effect on its *in vivo* success as a macromolecular drug carrier. More specifically, using

tetanus toxoid as a model of a large protein, we showed that its transport across the nasal and intestinal barriers was greatly improved following its nanoencapsulation into PEG-coated poly(lactic acid) (PLA) nanoparticles [12,13]. More importantly, we observed that the positive behavior of the nanoparticles was mainly due to the presence of a PEG coating around them. The efficacy of these PEG-coated nanoparticles as nasal protein and DNA carriers was further corroborated by studying the immune response generated by the nanoencapsulated protein and plasmid DNA [14–16]. The positive effect of the hydrophilic coating around the nanosystems was not exclusive of PEG. In fact, another early discovery from our work was that a coating consisting of the hydrophilic polysaccharide CS significantly improved the ability of polyester nanocapsules to transport drugs across the corneal epithelium [17]. More recently, we have compared the effect of a CS vs. a PEG coating on the transport of these nanocapsules across the corneal epithelium [18]. While the mechanistic details need to be further investigated, the results indicated that both types of coatings affected significantly the interaction of these nanosystems with the ocular mucosa. Therefore, despite the different nature of the two hydrophilic coatings, PEG and CS, these initial studies led us to the overall conclusion that an adequate selection of the biomaterials and a specialized design of the nanosystems are required in order to optimize the capacity of nanocarriers to transport drugs across mucosal surfaces.

The purpose of the present report was to compare the characteristics and potential of different nanostructures having as a common material the mucoadhesive polysaccharide CS. These nanosystems are CS nanoparticles, CS-coated oil nanodroplets (nanocapsules) and CS-coated lipid nanoparticles. By comparing these systems, the idea was to evaluate the role of the CS alone or in combination with lipids, and also the importance of the structural organization of these materials, in their behavior as transmucosal drug carriers.

The selection of CS was based on its excellent properties including biocompatibility, mucoadhesion [19] and ability to reversibly alter the tight junctions [20]. On the other hand, for the selection of the lipid cores we have taken into account the promising results obtained for solid lipid nanoparticles [21] as well as

for oily polyisobutylcyanoacrylates nanocapsules with regard to their use as transmucosal drug carriers [22].

2. Materials and methods

2.1. Chemicals and animals

The materials and methods used for the preparation and evaluation of CS nanoparticles have been described in Refs. [14,23]. Similarly, the materials and methods used for the preparation and evaluation of CS-coated lipid nanoparticles have been described in Refs. [24–26]. The chemicals and animals used for the preparation and evaluation of the CS-coated oil nanodroplets are reported below.

CS with 85% degree of deacetylation and a viscosity of 16 mPa was purchased from FMC Biopolymer/ Novamatrix, (Norway). Miglyol 812®, a triglyceride formed from medium chain fatty acids was supplied by Lemmel (Spain). The surfactant soybean L- α -lecithin and Poloxamer 188 (Pluronic F-68®) were supplied from Sigma-Aldrich (Spain). Salmon calcitonin (sCT) was kindly donated by Almirall Prodesfarma (Spain). Rhodamine was purchased from Sigma (Spain).

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC, UK). Minimum Eagle Medium (MEM), fetal bovine serum (FBS), non-essential amino acids, L-glutamine, 100 UI/ml Penicillin/100 μ g/ml Streptomycin solution, 0.05% Trypsin/0.02% EDTA solution, Dulbecco's phosphate buffered saline (DPBS) and Hank's basal salt solution (HBSS) were purchased from Sigma (Spain).

Male Sprague–Dawley rats (225–275 g), from the Central Animals House of the University of Santiago de Compostela (Spain) were used. They were kept in a 12-h light–dark cycle and at a temperature of 20 ± 2 °C. The animals were allowed access to food and water ad libitum.

2.2. Preparation of CS nanocapsules

CS-coated oily nanodroplets (here called CS nanocapsules) were prepared according to the procedure previously described by our group, in two steps. More specifically, firstly, we prepared a nanoemulsion using

a solvent displacement technique [27]. For this purpose, 125 μ l of Miglyol 812 were added to an organic phase composed of 40 mg of lecithin dissolved in 0.5 ml of ethanol and 9.5 ml of acetone. This organic phase was added to an aqueous phase containing Poloxamer 188 (0.25% w/v). The mixture immediately turned milky due to the formation of the nanoemulsion. Then, the solvents were evaporated under vacuum. Secondly, this nanoemulsion was coated with CS by simple incubation in the polymer solution [28,29]. More specifically, 5 ml of the control nanoemulsion were incubated with 1 ml of CS aqueous solution (0.5% w/v) for 1 h, leading to the formation of CS nanocapsules.

2.3. Physicochemical characterization of CS-coated systems

The different colloidal carriers were characterized with regard to morphology, particle size, zeta potential and encapsulation efficiency. The association efficiency of sCT to the nanosystems was calculated from the concentration of the non-encapsulated peptide. The separation of the nanocapsules from the non-encapsulated peptide was performed by ultrafiltration (Centricom® YM-100, Millipore, Spain) for CS nanocapsules. The concentration of sCT was determined by HPLC as described (BP 1998).

2.4. Cell culture experiments

Caco-2 cells were cultivated in 80 cm² flasks (Nunc, Denmark) using MEM supplemented with 10% FBS, 1% L-glutamine, 1% nonessential amino acids and Penicillin/Streptomycin solution. Cells were maintained in a controlled atmosphere at 37 °C with 95% of relative humidity and 5% CO₂. The culture medium was changed every two days for approximately 5–6 days until cells reached approximately 80–90% confluency. After the passage operation, cells were seeded approximately at 2.5×10^5 cells per flask. For the experiments, cells with passage numbers between 30 and 35 were used.

2.5. Cytotoxicity studies

The effect of the different nanosystems on their *in vitro* biocompatibility was investigated at different

concentrations using Caco-2 cell cultures. Cells were seeded on 96 well plates with a cell density of 10^4 cells/cm² for 7 days until cell monolayer was obtained. After 7 days, the medium was replaced by the nanocapsule suspensions at concentrations between 0.01 and 2 mg/ml in an appropriate volume to obtain a density of nanocapsules between 6 and 1212 $\mu\text{g}/\text{cm}^2$. As a positive control we used HBSS and as a negative control 2% sodium dodecyl sulfate (SDS). Nanocapsules and the controls were in contact with the monolayers for 90 min, at 37 °C and 5% CO₂. After this time, the nanocapsule suspensions were removed. The cell viability was determined using a colorimetric method (MTS, Promega), which determines the intracellular dehydrogenase activity. The absorbance was measured at 490 nm.

2.6. Measurement of the transepithelial electrical resistance

The measurement of the transepithelial electrical resistance (TEER) was performed on Transwell® plates with a cell density of 5.5×10^4 cells 21 days post-seeding. The integrity of the monolayers was checked on the 21st day in MEM. The TEER measurements were performed with a Millicell®-ERS connected to a pair of chopstick electrodes (Millipore, Spain). After these measurements, the medium was changed by HBSS (HBSS pH 6 in the apical side in the case of CS nanocapsules) in order to perform the experiments in buffer. After 1 hour of equilibrium, the apical solutions were replaced by the nanocapsule suspensions and the respective controls (HBSS or HBSS pH 6). The concentration of nanocapsules was 250 $\mu\text{g}/\text{ml}$ in two different cell growing areas, 1.1 and 4.5 cm² leading to a nanocapsule dose of 204.5 and 80 $\mu\text{g}/\text{cm}^2$, respectively. Nanocapsule suspensions were incubated for 100 min and TEER measurements were performed every 25 min. After this time, the solutions were removed and replaced by MEM in order to check the recuperation of the TEER values at 24 h.

2.7. Quantitative uptake studies

Caco-2 cells were seeded with a density of 5.5×10^4 cells/cm² on 12-well plates with an area of 3.5 cm² and left to grow until full differentiation

(19–21 days). The culture medium was replaced by rhodamine-loaded nanostructures in HBSS (or HBSS pH 6) at concentrations of 0, 250, 500 and 1000 $\mu\text{g}/\text{ml}$ (the corresponding dose per surface area values were: 0, 71.43, 142.86 and 286.71 $\mu\text{g}/\text{cm}^2$, respectively). The suspensions were incubated for 1 h. After incubation, the nanocapsule suspensions were removed and the monolayers washed five times with ice-cold DPBS. Monolayers were finally incubated for 15 min with 5 mM EDTA in DPBS adjusted to pH=5 (“acid wash”), a medium intended to detach the nanocapsules associated to the monolayer surface. Caco-2 monolayers were dried overnight and dissolved in a 2% SDS/50 mM EDTA/ DPBS at pH=6 (“lysis medium”). The nanocapsules-associated fluorescence internalized by the monolayers was quantified by fluorimetry. The calculation of the amount of fluorescent carriers taken up by the cells was performed using the corresponding calibration curves (different concentrations of fluorescence nanocapsules incubated in the lysis medium). The fluorescence of monolayers without nanocapsules (0 mg/ml samples) in lysis medium was subtracted from the experimental values.

2.8. In vivo studies

Male Sprague–Dawley rats (225–275 g) were fasted for 12 h before experiments, but allowed water ad libitum. Animals were kept conscious during the experiments.

The following formulations were administered intragastrically to rats: (1) sCT aqueous solution, (2) nanoemulsion, and (3) CS nanocapsules. The dose at sCT administered was 500 IU/kg in a volume of 0.5 ml. Blood samples were collected from the tail vein 30 min prior to the oral administration to establish the baseline calcium levels and at different times after dosing. The serum was separated by centrifugation at $3000 \times g$ for 5 min. Hypocalcemic effects were determined in serum samples by a colorimetric method at 570 nm (Kit OR-cresolphthalein v/v, Spinreact, Spain).

2.9. Statistical analysis

The statistical significance of the differences between parameters of the oily-based nanosystems

was tested by the analysis of variance (ANOVA) together with the multiple comparison Student–Newman–Keuls method.

The mean serum calcium levels determined in samples collected before sCT administration was taken as the baseline levels. Using these values, the statistical comparison of the percentage of calcium reduction at each time after dosing was performed by the one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons between treatments. Differences were considered to be significant at a level of $p < 0.05$.

3. Results and discussion

The main goal of the present report was to provide an overview of the potential of different nanosystems consisting of solely CS or CS in combination with solid and liquid lipids as carriers for the transmucosal (nasal and oral) transport of macromolecules. The common feature of these nanosystems is the presence of CS in their composition. As illustrated in Fig. 1, the key differences between these systems are the following: (i) CS is in the form of solid nanoparticles (nanogel network) or as a soluble coating around lipid cores, (ii) the lipid core of the CS-coated systems is either an oil (Miglyol® 812) or a solid lipid (tripalmitin). Here, we describe the physicochemical properties of these systems (size and zeta potential) as well as their ability to associate and release macromolecules. In addition, we provide information about the *in vitro* viability of Caco-2 cells exposed to CS nanocapsules and about their mechanism of interaction with this cell model and compared with that

reported for CS-coated lipid nanoparticles and CS nanoparticles. Finally, we present the efficacy of the CS-based systems as transmucosal macromolecular drug carriers.

3.1. Preparation and physicochemical characterization of the CS-based nanosystems

We have previously reported [14,27–30] the optimal conditions for the formation of CS nanoparticles and CS nanocapsules. The formation of the nanoparticles was achieved according to an ionic gelation process of CS upon contact with a counter anion, i.e. sodium tripolyphosphate. On the other hand, the formation of CS-coated oily droplets (nanocapsules) was achieved by means of an ionic complexation process between the negatively charged lecithin and the positively charged CS. This polymer layer was found to be responsible for the stabilization of the oily core of the nanoemulsions [27,28]. The same approach was recently applied to the formation of CS coated-lipid nanoparticles [25,26]. In this latter case, the coating was formed by simple incubation of the lipid nanoparticles with an aqueous solution of CS. The attachment of CS was due to its interaction with lecithin and probably with other negative lipids that are present in the core lipid nanoparticles.

Table 1 shows the physicochemical properties (size and zeta potential) of the different systems we have designed and those of the corresponding control nanoemulsion. In addition, the table presents some macromolecules that have been associated to the systems and the modality of administration for which we have tested them as carriers for transmucosal macromolecular drugs. These results indicate that,

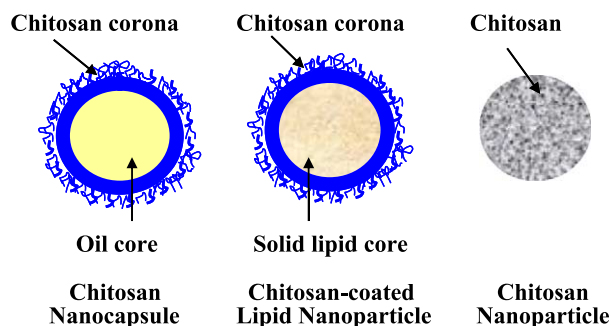


Fig. 1. Illustration of the different chitosan-based nanosystems described in the present report.

Table 1

Physicochemical properties and encapsulation efficiency of different macromolecules into the control nanoemulsion, chitosan-coated lipid nanoparticles chitosan nanocapsules and chitosan nanoparticles as well as the route of administration of the nanosystems (mean $\pm$ S.D.; $n=3$)

Carrier	Size (nm)	ζ Potential (mV)	Encapsulated peptide	Administration route	Encapsulation efficiency (%)
Nanoemulsion	195.8 $\pm$ 1.1	−52.0 $\pm$ 1.1	Salmon calcitonin	Oral	>98
Chitosan nanocapsules	266.6 $\pm$ 7.6	+34.8 $\pm$ 0.6	Salmon calcitonin	Oral	44.1 $\pm$ 3.2
Chitosan-coated lipid nanoparticles ^a	537 $\pm$ 16	+29.2 $\pm$ 6.2	Salmon calcitonin	Oral	30.7 $\pm$ 2.3
Chitosan nanoparticles ^b	337 $\pm$ 14	+36.9 $\pm$ 0.3	Insulin	Nasal	94.7 $\pm$ 2.1

^a Data from Ref. [26,33].

^b Insulin/chitosan ratio (w/w): 0.4. Data from Ref. [23].

despite their different composition, all CS-based systems have a colloidal size and a positive zeta potential. Moreover, it can be noted that the zeta potential was inverted from highly negative values for the uncoated nanoemulsion (about −52 mV) to highly positive values for CS nanocapsules (about +34 mV). The positive zeta potential can be logically explained by the presence of the cationic polysaccharide CS on the surface of these nanosystems. Simultaneously to the inversion of the zeta potential, we observed an increase in the size that was attributed to the presence of a CS coating. The presence of this coating could also be visualized by transmission electron microscopy (Fig. 2). It can be seen that both, the control nanoemulsion and CS nanocapsules, have a spherical shape. However, the nanocapsules show a corona that was attributed to the CS molecules attached to the surface of the oily droplets. Interestingly, besides the potential mucoadhesive characteristics that CS confers to the nanosystems, it was recently observed that this CS corona enhanced the stability of the oily nanodroplets in the gastric fluids [31].

The three CS-based systems showed a great capacity to associate macromolecules. For example, CS nanoparticles have a high loading capacity for acidic proteins such as insulin (I.P.=5.4) and tetanus toxoid (I.P.=4.4–5.9), reaching association efficiencies as high as 97% and 55%, respectively [14,23]. The reason for this high association was related to the affinity of these negatively charged molecules with the positively charged CS molecules. The association of other types of macromolecules such as the lipophilic peptide cyclosporin A has also been possible through the adjustment of the formulation conditions. On the other hand, the CS-coated lipid systems have also shown an ability to associate insulin as well as

the cationic peptide sCT (I.P.=10.4) [25,26,29]. The association of insulin was attributed to its affinity for lipid materials and also for CS, whereas the associ-

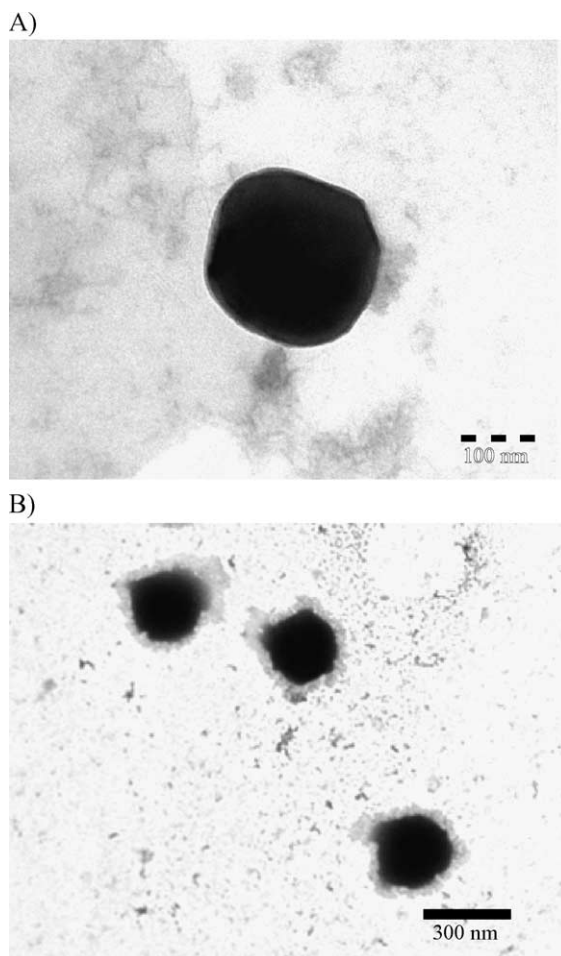


Fig. 2. Transmission electron micrographs of (A) the control nanoemulsion and (B) chitosan nanocapsules.

ation of sCT was facilitated by the lipids but slightly inhibited by CS. This inhibition of sCT association was ascribed to the positive character of both sCT and CS, that may compete for the anionic binding sites of the lipid cores. Overall, these results indicated that a variety of macromolecules, either hydrophilic or lipophilic, can be efficiently associated to these CS-based nanosystems.

With respect to the *in vitro* release behavior, our previous work on CS nanoparticles led us to the conclusion that the chemical structure of the protein, and its interaction with CS in the release medium conditions, were major factors governing the release process. For example, in the case of insulin, we observed a fast release that was associated to its easy detachment from CS in buffered conditions [23]. In contrast, in the case of tetanus toxoid-loaded CS nanoparticles, we noted an initial burst of release followed by a slow release that was dependent on the CS molecular weight [32]. This slow release was justified by the affinity of the toxoid for CS and the characteristics of the release medium. Similarly, the release of the peptide sCT from the CS-coated lipid systems followed a biphasic pattern consisting of an initial burst followed by a continuous and slow release [26,29]. The burst of release was attributed to the surface-associated peptide whereas the second slow phase corresponds to the release of the well-entrapped peptide molecules. These results suggest that more studies are needed in order to fully understand the factors that govern the release from the different nanostructures. However, we must keep in mind that the physiological conditions under which these nanosystems release their content *in vivo* are expected to be quite different from those *in vitro* and, consequently, *in vitro*–*in vivo* correlations should not be expected.

3.2. Cytotoxicity of CS-based nanosystems in *Caco-2* cells

In order to investigate the cytotoxicity of CS associated to a nanosystem, we determined the intracellular dehydrogenase activity in *Caco-2* cells exposed to different concentrations of CS nanocapsules and to the control nanoemulsion. Fig. 3 shows that, as it was the case for the lipid nanoparticles [33], the control nanoemulsion did not affect

cell viability in the range of concentrations assayed. On the other hand, following exposure of *Caco-2* cells to CS nanocapsules, we observed a dose-dependent cell viability [34]. More specifically, the cellular viability decreased when the concentration varied between 250 $\mu\text{g/ml}$ (152 $\mu\text{g/cm}^2$) and 1000 $\mu\text{g/ml}$ (606 $\mu\text{g/cm}^2$), being the media lethal concentration (LC50) around 1000 $\mu\text{g/ml}$ of nanocapsules suspension (606 $\mu\text{g/cm}^2$), a value that corresponds to approximately 80 μg of chitosan/ml (48.49 $\mu\text{g/cm}^2$). A similar dependence of cell viability with CS dose was observed for CS-coated lipid nanoparticles [33] and CS [35]. A conclusion that can be drawn from this study is that the toxicity values are not clearly dependent on the nanocarrier structure (polymer solution, or CS-coated nanosystems). This conclusion is also corroborated by a recent study that showed similar cellular toxicity for CS nanoparticles and CS solutions [36]. On the other hand, from the perspective of the potential *in vivo* toxicity, it is worthwhile to mention that the toxicity of these new formulations is low if we take into account the surface area of the intestinal barrier (1 m^2 for rats, 200 m^2 for humans) [37].

3.3. Interaction of CS-based nanosystems with the *Caco-2* cells monolayer: measurement of the trans-epithelial electrical resistance (TEER)

It is well known that CS solutions cause a significant and dose-dependent decrease of the TEER of the *Caco-2* cell monolayers. The first evidence of this phenomenon was reported in the early 1990s [20]. These initial studies intended to explore the mechanism of interaction between CS and the intestinal epithelium concluded that the loss of tight junction integrity was caused by a reorganization of the actin rings [20]. However, a more recent hypothesis points to an interaction between chitosan and the tight junction protein ZO-1, leading to its translocation to the cytoskeleton [38]. In addition to these mechanistic details, some studies have shown that the ability of CS to modify the TEER of *Caco-2* monolayers is dose-dependent [38,39]. Unfortunately, quantitative comparisons between studies are difficult due to the different experimental conditions used and also to the different types of CS tested (different deacetylation degrees and molecular weights). For example,

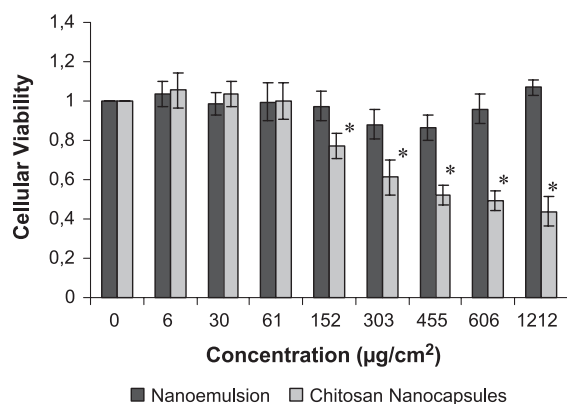


Fig. 3. Cytotoxicity profiles of chitosan nanocapsules and the control nanoemulsion in Caco-2 cell monolayer (Mean $\pm$ SD, $n=4-8$). *Statistically significant differences from the nanoemulsion ($p<0.01$).

very marked TEER reductions (up to 80%) were observed for high CS concentrations (5 mg/ml) [38]. However, significant reductions in the TEER values after prolonged exposure to low CS concentrations 0.5 mg/ml were also reported [39].

Based on this information, we found it important to evaluate if the association of CS to a colloidal carrier would modify its interaction with the intestinal epithelial cells. With this purpose in mind, we have measured the TEER of the monolayer exposed to different doses of CS nanocapsules and to the corresponding controls. In Fig. 4 we can notice that neither the controls (HBSS and HBSS pH 6) nor the control nanoemulsion influenced the TEER values after incubation for 100 minutes. On the other hand, following exposure of the cells to CS nanocapsules, we detected a reduction in the TEER values of the monolayer, however, the extent of this decrease was remarkable only for high concentrations of CS nanocapsules. More precisely, when the dose of CS nanocapsules was 80 $\mu\text{g}/\text{cm}^2$ the TEER value was slightly reduced (10%), and a decrease of around 35% was only observed for a high dose of CS nanocapsules (204.5 $\mu\text{g}/\text{cm}^2$). Higher doses were not tested since the dose of 204.5 $\mu\text{g}/\text{cm}^2$ led to a decrease in cell viability of 20–30%. An additional observation was that in all the cases, the TEER values returned gradually to the normal values (total recuperation after 24 h in MEM). A comparable decrease in TEER was observed with similar CS doses in the form of

CS-coated lipid nanoparticles [33]. Despite the difficulties to compare experiments only on the basis of the polymer concentrations, globally CS nanosystems and solutions seem to have similar dose-TEER reduction profiles [39]. This indicates that CS-coated lipid systems mostly maintain the intrinsic permeabilizing properties of the CS polymer solutions. In contrast, preliminary data using CS nanoparticles suggested that these nanosystems do not display this permeabilizing property at least at concentrations below 250 $\mu\text{g}/\text{ml}$. At this point it is tempting to speculate that the different presentation of CS, in the form of a soluble coating or as solid CS nanoparticles, may be responsible from this different behaviour.

3.4. Interaction of chitosan-based nanosystems with the Caco-2 cells monolayer: quantitative uptake studies

We have previously investigated the mechanism of interaction of CS nanoparticles with the Caco-2 cells [40]. The results of this study made clear that CS nanoparticles were internalized by the cells and also that this internalization process was saturable (30 min approx.) as well as energy and temperature-

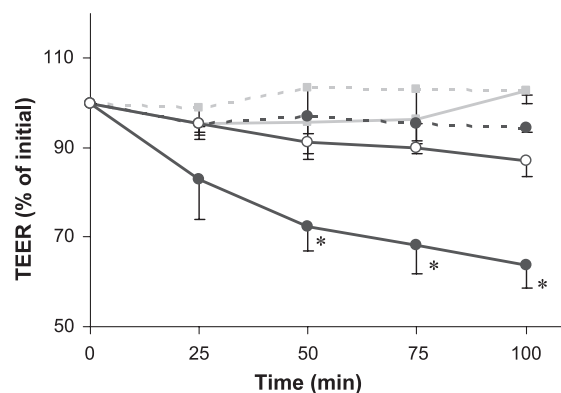


Fig. 4. Transepithelial electric resistance (TEER) of Caco-2 monolayer exposed to chitosan nanocapsules with two doses of formulations per surface area, 80 $\mu\text{g}/\text{cm}^2$ (solid black line with open circles, ○) and 204.5 $\mu\text{g}/\text{cm}^2$ (solid black line with close circles, ●), the nanoemulsion (solid grey line, —), or their respective controls of Hanks balanced salt solution (HBSS) (HBSS pH 7.4; dotted grey line, ---, and HBSS pH 6; dotted black line, - - -). (Mean $\pm$ SD, $n=3$). *Statistically significant differences from chitosan nanocapsules with the lowest dose per surface area, the nanoemulsion and both HBSS ($p<0.01$).

dependent. Moreover, these previous results showed that the presence of mucus in the monolayers (MTX-E12 cells) strongly increased the association of the nanoparticles.

In the study reported here, we aimed to investigate the behavior of CS nanocapsules and, hence, to elucidate if the composition of the carrier would affect the interaction of CS-based systems with the Caco-2 cells monolayer (Fig. 5). For this purpose, we used fluorescent rhodamine-loaded CS nanocapsules and quantified the percentage of internalized fluorescence as a function of the dose per area of nanocapsules added to the incubation medium. The results showed a linear dose-dependent uptake for the range of concentrations between 250 and 1000 $\mu\text{g}/\text{ml}$ (71–286 $\mu\text{g}/\text{cm}^2$). It was also noted that the uptake was low and similar for the nanocapsules (maximum value of uptake of 7 $\mu\text{g}/\text{cm}^2$) than for the corresponding nanoemulsion (5 $\mu\text{g}/\text{cm}^2$). Interestingly, a much higher association was observed for both CS-coated lipid nanoparticles and the corresponding control lipid nanoparticles as compared to that observed for the nanocapsules [33]. Consequently, these results suggest that, not only the CS coating but mainly the nature of the core (either oil or tripalmitin) influences the systems uptake. On the other hand, we observed that the low association values of CS nanocapsules to the Caco-2 cells monolayer are also comparable to

those we have observed for CS nanoparticles [40]. The differences observed for the various nanosystems in the Caco-2 cells association profiles, as well as the suggested dependence on the structure of the system, underline the necessity of additional studies in order to gain a better understanding about the role of CS in the interaction of nanosystems with the intestinal epithelium.

3.5. *In vivo* efficacy of CS-based nanosystems as transmucosal macromolecular drug carriers

Bearing in mind that the main goal of our work has been to design new transmucosal macromolecular drug carriers, we have tested different CS-based nanosystems for their ability to increase the transport of peptides and proteins across the nasal and intestinal mucosa. We showed the first evidence of this capacity for CS nanoparticles containing insulin. Indeed, these nanoparticles led to a significant decrease of the glycemia levels, as compared to a control solution of insulin and CS, following intranasal administration to rabbits [23]. Moreover, we observed that this increased absorption was maintained after freeze-drying and further resuspension of the nanoparticles [41]. Recently, we also showed that chitosan nanoparticles were able to increase the nasal transport of antigens, i.e. tetanus toxoid [32]. More importantly, the results of the increasing and long-lasting IgG levels generated following intranasal administration of tetanus toxoid-loaded CS nanoparticles led us to hypothesize that the nanoparticles facilitated the delivery of the antigen to the immunocompetent cells.

As an alternative vehicle for nasal administration, we have also evaluated the efficacy of CS nanocapsules for enhancing the systemic absorption of sCT [29]. With this purpose in mind, we determined the hypocalcemic effect of sCT in aqueous solution or sCT associated to a control nanoemulsion and to CS nanocapsules after nasal administration to rats. The results showed a negligible response for the controls (either the aqueous sCT solution or the nanoemulsion) but a significant reduction in the calcemia levels for the nanocapsules.

The potential of CS nanoparticles for oral peptide administration has also been reported [42]. The results of this previous study, which was performed in rats, showed that CS nanoparticles were efficient in

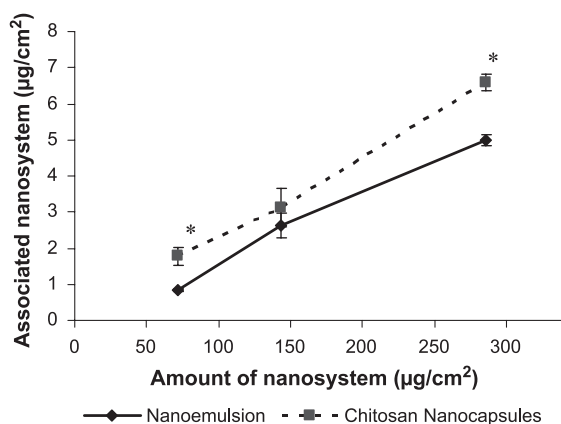


Fig. 5. Percentages of fluorescent nanoemulsions and chitosan nanocapsules associated to the Caco-2 monolayer following their incubation for 1 hour at different concentrations (Mean $\pm$ SD, $n=3$). The dose of the incubated formulations per surface area values were: 0, 71.43, 142.86 and 286.71 $\mu\text{g}/\text{cm}^2$. *Statistically significant differences from the nanoemulsion ($p<0.01$).

improving the response of insulin administered orally. The authors speculated that the hypoglycemic response obtained with CS nanoparticles could be attributed to the protection of the peptide by the formulation and to the potential mucoadhesive and absorption enhancing properties of CS.

Very recently, we have evaluated the potential of CS-coated lipid systems (CS nanocapsules and CS-coated lipid nanoparticles) for oral administration of sCT, using a rat animal model [25,28,33]. In order to elucidate the role of the CS coating we have compared the behavior of the CS-coated systems with that of the control formulations (nanoemulsion and lipid nanoparticles). In Fig. 6 it is shown that the reduction of the serum calcium levels following oral administration of sCT in the form of an aqueous solution or a nanoemulsion was insignificant. However, the hypocalcemic response was greatly enhanced for CS nanocapsules. More importantly, the percentage of reduction of the serum calcium levels (27% for CS nanocapsules reached at 1 h post-administration) was maintained for 24 h. This long-lasting hypocalcemic response was similar to the one observed for sCT-containing CS-coated lipid nanoparticles [25,33]. Given the lack of efficacy of the controls (non-coated nanoemulsion and lipid nanoparticles), the important hypocalcemic effect elicited by the new formulations was, logically, attributed to

the presence of the CS corona around the nano-systems. These results agree with those previously reported for CS-coated poly(lactic acid/glycolic acid) nanoparticles and CS-coated liposomes containing sCT [43,44] and underline the positive effect of a CS coating around the colloidal peptide carriers.

The overall conclusion from these studies is that CS-based nanosystems are efficient vehicles for improving the transmucosal transport of peptides. The explanation to this positive effect of CS could be found at different levels: (i) first, as suggested by Kawashima et al. [43] this effect could be related to the mucoadhesive properties of CS and, hence, to its positive interaction with the negatively charged mucosal surfaces; (ii) there is also the possibility that some of these nanosystems interact with the underlying epithelium entering the cells and also opening the tight junctions; (iii) the peptide, either in a free form or associated to the nanosystem, reaches the blood or lymphatic vessels. If we take into account that the quantity of particles that enter the Caco-2 cells (with no mucus layer) was low for both, uncoated and CS-coated systems, while only those coated with CS were effective *in vivo*, we could speculate that, indeed, the mucoadhesion properties of CS might be determinant in the efficacy of these nanosystems. Nevertheless, more detailed studies will have to be performed in order to fully understand the *in vivo* behavior of these novel nanocarriers.

4. Conclusions

The present study reports the efficacy of three different CS-based nanosystems, i.e., CS nanoparticles, CS nanocapsules and CS-coated lipid nanoparticles, as carriers for nasal/oral administration of macromolecules. Moreover, it gives some mechanistic details that help to understand the mechanism of action of these new systems. The presence of CS on the systems provides a modification of the trans-epithelial resistance and enhances slightly the uptake of the nanosystems by the Caco-2 model cell line. However, the great enhancement of the *in vivo* response to a model peptide, sCT, associated to the nanosystems suggests that additional mechanisms, which remain to be elucidated, are responsible for this positive behavior.

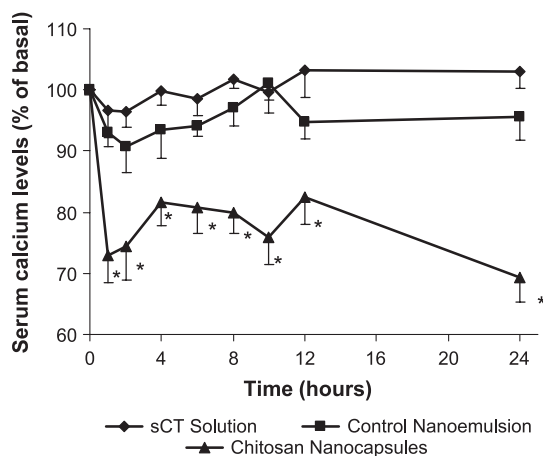


Fig. 6. Hypocalcemic effect observed following oral administration of chitosan nanocapsules, a control nanoemulsion and an aqueous solution of sCT to rats (mean $\pm$ s.e.; $n=6$). The dose of sCT administered was 500 IU/kg. *Statistically significant differences from sCT solution ($p<0.05$).

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