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Research Paper

Efficacy and Mechanism of Action of Chitosan Nanocapsules for Oral Peptide Delivery

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Purpose. We have previously shown that high molecular weight (MW > 100 kDa) chitosan nanocapsules are efficient vehicles for improving the oral absorption of salmon calcitonin (sCT). In the present work, our objectives were, first, to investigate the influence of some formulation parameters on the efficacy of chitosan nanocapsules as carriers for the oral administration of sCT and, second, to elucidate the mechanism of interaction of chitosan nanocapsules with intestinal model cell lines.
Methods. sCT-loaded chitosan nanocapsules were prepared by the solvent displacement technique. They were characterized for their size, zeta potential, and sCT loading. The ability of chitosan nanocapsules to enhance the oral absorption of sCT was investigated in rats by monitoring the serum calcium levels. Finally, the mechanism of interaction of chitosan nanocapsules with the Caco-2 cell model or in the coculture of Caco-2 with HT29-M6 cells was investigated by confocal fluorescence microscopy.
Results. Chitosan nanocapsules presented a particle size in nanometer range, a positive surface charge, and an efficient encapsulation of sCT. Following oral administration to rats, all formulations of nanocapsules exhibited the ability to reduce calcemia levels; however, the intensity of the response varied depending on the formulation conditions. With regard to the mechanism of interaction of chitosan nanocapsules with cell culture, the *xz* images evidenced that chitosan nanocapsules interact and remain associated to the apical side of both model cell cultures. In addition, chitosan nanocapsules showed a preferable association to the mucus-secreting cells (HT29-M6).
Conclusions. Chitosan nanocapsules are able to enhance and prolong the intestinal absorption of sCT and this effect could be mainly ascribed to their mucoadhesive character and intimate interaction with the intestinal barrier.

KEY WORDS: chitosan; mucoadhesion; nanocapsules; oral peptide delivery.

INTRODUCTION

Peptide drugs are poorly absorbed after oral administration because of their susceptibility to enzymatic degradation and their low permeability across the intestinal epithelium. Being conscious of these important biopharmaceutical limitations, many pharmaceutical scientists have taken the challenge of designing new delivery strategies intended to enhance the oral absorption of these macromolecules. Among them, the encapsulation of macromolecular drugs in nanoparticles/nanocapsules is considered a promising approach toward overcoming the mentioned barriers. Indeed, nowadays it is known that these nanosystems can protect sensitive molecules against degradation in the gastrointestinal environment (1,2) and favor the interaction of the associated biomolecule with the intestinal epithelium (3–5). In addition, with respect to the factors that affect this interaction, it has been clearly demonstrated that the size of the particles has a critical role in their interaction with the intestinal barrier (6–8). Knowing this, recently, the challenge has been oriented towards elucidating how the surface properties and composition affect the interaction of the nanosystems with the intestinal epithelium (9).

Mucoadhesive polymers represent one class of biomaterials with an interesting potential for the design of transmucosal nanoparticulate carriers. These polymers offer the possibility to facilitate the interaction of the nanocarrier with the intestinal mucosa and, hence, its access to the underlying epithelium. Indeed, this mechanistic principle has been adopted to explain the efficacy of particles made of acrylic polymers (10,11), polyanhydrides (12,13), and chitosan (14,15) as carriers for the transmucosal delivery of peptides. More specifically, it has been suggested that, because of their composition, these particles are targeted to the mucus covering the intestinal epithelium. Once the particles reach the mucus blanket, they are able to diffuse through it and reach the underlying epithelium, where they may stay for extended periods. In fact, the possibility of the diffusion of nanoparticles into the mucus layer has already been demonstrated *in vitro* (16) and *in vivo* (17). Interestingly, the behavior of these particles is quite different compared to that

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of polymer solutions or large polymer devices. It was observed that chitosan solutions have poor diffusion through the mucus (18). However, when chitosan takes part as a carrier, the polymer can move in association with the vehicle and penetrate into the mucosa (19,20).

In recent years, we have particularly focused our work on designing and evaluating the potential of nanosystems based on the mucoadhesive polysaccharide chitosan. More specifically, we have observed that chitosan nanocapsules are able to enhance and prolong the systemic absorption of the model peptide, salmon calcitonin (sCT), administered orally (21). Additionally, recent experiments have indicated that the modification of chitosan molecular weight (within the range of 160–450 kDa) or the chitosan salt form (glutamate *vs.* hydrochloride) does not affect the efficacy of the nanocapsules as carriers for sCT (22). These nanocapsules consist of an oily core, conveniently stabilized with lecithin, and surrounded by a chitosan coating. The formation of this coating is mediated by the interaction between the negatively charged phospholipids and the positively charged chitosan molecules. Taking this previous information into account, we found it important to further evaluate the determinants of the *in vivo* performance of these nanocapsules by analyzing other experimental factors such as (i) the use of chitosan oligomers (MW < 10 kDa), (ii) the use of high lecithin concentration, which might affect the amount of chitosan associated to the surface of the nanocapsules, and (iii) the reduction of the administered dose of sCT in nanocapsules.

Another important goal of the present work was to elucidate the mechanism of action of chitosan nanocapsules. In previous studies in Caco-2 cells, we have observed that the association of chitosan nanocapsules to the monolayer is low and similar to that observed for the control noncoated nanoemulsion (21). This lack of evidence of the role of the chitosan coating led us to consider the necessity of selecting a different model cell line to investigate the mechanism of action of chitosan nanocapsules. Indeed, Caco-2 cells undergo spontaneous differentiation, forming a monolayer of polarized enterocytes that possess morphologic and functional similarities to the small intestine (23). However, a disadvantage of this well-established cell culture model is the lack of the mucus layer. Thus, in this study we analyzed and compared the behavior of chitosan nanocapsules upon incubation with the Caco-2 monolayer and with the coculture of Caco-2 and mucus-secreting cells (HT29-M6).

Therefore, the aims of the present work were, first, to investigate further the determinants of the efficacy of chitosan nanocapsules as carriers for the oral administration of peptides and, second, to elucidate the mechanistic events of chitosan nanocapsules responsible for the enhancement of the oral peptide delivery.

MATERIALS AND METHODS

Materials

sCT was kindly donated by Almirall Prodesfarma, S.A. (Spain). 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 by Sigma-Aldrich (Spain). Chitosan chloride (deacetylation degree, 85%; viscosity, 16 mPa) was purchased from FMC Biopolymer/Novamatrix with the name of Protasan Cl 113 (Norway). *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt, was purchased from Molecular Probes Europe BV (the Netherlands).

Preparation of Chitosan Oligomers

Chitosan oligomers were prepared from medium molecular weight chitosan by oxidative degradation using sodium nitrite ($NaNO_2$) according to the procedure described by Janes and Alonso (24). For this purpose, 0.1 mL of $NaNO_2$ (0.1 M) was added to 2 mL of a chitosan solution (1% w/v) at room temperature under magnetic stirring. The reaction was left overnight to ensure completion of the degradation. Finally, the resulting chitosan solution was freeze-dried.

The molecular weight of chitosan was estimated from viscosity values, measured in a capillary viscosimeter (Automated Microviscometer, Anton Para GmbH, Austria) at 25°C. The capillary diameter used was 1.6 mm. To measure the viscosity of the polymer, chitosan solutions were prepared at concentrations lower than 1% (w/v). The solvent used was 2% acetic acid with 0.2 M sodium acetate. The intrinsic viscosity was determined by linear extrapolation to infinite dilution and used with the appropriate Mark–Houwink parameters (25) to obtain the molecular weight of the original and the resulting chitosan oligomers.

Preparation of Chitosan Nanocapsules

Chitosan nanocapsules were prepared according to the procedure previously described by our group (21). First, we prepared a lecithin-stabilized nanoemulsion (26,27) as a reference formulation by solvent displacement technique. Briefly, an organic phase composed of 125 μL of Miglyol, 40 mg of lecithin dissolved in 0.5 mL of ethanol, and 9.5 mL of acetone was added to 20 mL of an aqueous phase containing Poloxamer 188 (0.25% w/v) under magnetic stirring. The mixture turned milky immediately because of the formation of the nanoemulsion. Then, the solvents were evaporated under vacuum to a final volume of 10 mL. In a second step, this nanoemulsion was coated with chitosan by simple incubation in the polymer solutions. More specifically, 4 mL of the control nanoemulsion was incubated with 1 mL of chitosan aqueous solution (chitosan oligomers or medium molecular weight chitosan, Cl 113) (0.5% w/v) for 1 h, leading to the formation of chitosan nanocapsules.

In a different experiment, chitosan nanocapsules were prepared according to the conditions indicated above but increasing the amount of lecithin up to 120 mg.

sCT-loaded chitosan nanocapsules were obtained following the procedure described above but with the addition of 50 μL of an aqueous solution of sCT (20 mg/mL) to the organic phase containing the oil.

For the *in vitro* cell culture studies, fluorescent nanostructures were prepared. In this case, 1 mg/mL of fluorescein–phosphoethanolamine (λ_{ex} 496 nm/λ_{em} 519 nm) was added to the organic phase and, then, the procedure was followed as indicated above.

Characterization of Chitosan Nanocapsules

The size and polydispersity index of the nanocapsules were analyzed by photon correlation spectroscopy (PCS) after appropriate dilution with ultrapure water. Each analysis was performed in triplicate at 25°C with an angle detection of 90°. The zeta potential was determined by laser Doppler anemometry (LDA). For the determination of electrophoretic mobility, samples were diluted with 1 mM KCl and placed in the electrophoretic cell where a potential of ± 150 mV was established. The PCS and LDA analysis were performed using a Zetasizer 3000 HS (Malvern Instruments, Malvern, UK). Each batch was analyzed in triplicate.

The morphological analysis was performed by transmission electron microscopy (Philips CM12, Eindhoven, Netherlands). Samples were stained with phosphotungstic acid solution (2% w/v) for 1 min and then dried overnight.

Salmon Calcitonin Encapsulation Efficiency and *In Vitro* Release Studies

sCT was associated to chitosan oligomer nanocapsules. The amount of peptide encapsulated was determined indirectly in the supernatant following separation of nanocapsules from aqueous medium by a combined ultrafiltration–centrifugation technique (Centricon YM-100, Millipore, USA) at 1000 × *g* for 1 h. The supernatant was diluted with acetate buffer (pH 4) and assayed for sCT content by HPLC at 220 nm (Agilent Technologies, Germany), as described in the 1998 British Pharmacopoeia (column Vidac 218TP). A calibration curve was made with solutions of sCT in acetate buffer (pH 4.0) at concentrations ranging from 5 to 100 μg/mL. Each sample was assayed in triplicate.

The *in vitro* release studies of sCT from chitosan nanocapsules were performed by incubating the loaded nanocapsules in acetate buffer (pH 4) under horizontal stirring at 37°C. At appropriate time intervals, individual samples were ultrafiltered at 1000 × g for 1 h. The sCT released was determined by HPLC.

In Vivo Efficacy of Chitosan Nanocapsules

To perform the *in vivo* studies, male Sprague–Dawley rats (225–275 g) from the Central Animals House of the University of Santiago de Compostela (Spain) were fasted for 12 h before experiments, but were allowed access to water *ad libitum*. Animals were kept conscious during the experiments. All animal experiments were approved by the Ethical Committee of the Faculty of Medicine of the University of Santiago de Compostela.

In this study, we analyzed the effects of some experimental variables on the efficacy of chitosan nanocapsules to induce a pharmacological effect after oral administration: (i) the effect of the nature of the coating, using chitosan oligomers (10 *vs.* 100 kDa); (ii) the effect of the nature of the coating, using a high amount of the surfactant lecithin (120 *vs.* 40 mg); (iii) the effect of the dose of sCT-containing nanocapsules (250 *vs.* 500 IU/kg).

Blood samples were collected from the tail vein 30 min prior to the oral administration of the different formulations to establish the baseline calcium levels, and at different times after dosing. The serum was separated by centrifugation at 3000 × *g* for 5 min. Hypocalcemic effect was determined in serum samples by a colorimetric method at 570 nm (Kit OR-cresolphtalein v/v, Spinreact, Spain).

Cell Culture Experiments

Caco-2 (enterocyte-like cells) and HT29-M6 (mucus-secreting cells) were cultivated in 80-cm^2 flasks (Nunc, Denmark) using minimum Eagle's medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% nonessential amino acids, and 1% penicillin–streptomycin solution (10,000 U/mL and 10 mg/mL, respectively). Cells were maintained in a controlled atmosphere at 37°C with 95% relative humidity and 5% CO_2. The culture medium was changed every second day for approximately 5–6 days until cells reached approximately 80–90% of confluence. After the passage operation, cells were seeded at approximately 2.5 × 10^5 cells per flask. For the experiments, Caco-2 cells at passage number 67 and HT29-M6 at passage number 11 were used.

Qualitative Study of the Interaction of Chitosan Nanocapsules with the Caco-2 and HT29-M6 Cells

The study of the interaction of chitosan nanocapsules with the cell cultures was performed on Transwell filter inserts (0.4-μm pore size, area 0.33 cm^2). For this purpose, Caco-2 (enterocyte-like cells) and HT29-M6 (mucus-secreting cells) model cell lines were seeded with a density of 6 × 10^4 cells/cm^2, either as a single-cell type (Caco-2) or as coculture (Caco-2/HT29-M6 at a 1:1 ratio). Cells were left to grow for 21 days until differentiation and the barrier integrity was checked by TEER measurement and Lucifer yellow paracellular permeability.

Prior to the experiment, cells were rinsed with Hank's balanced salt solution (HBSS)–5% glucose (pH 6.5) and allowed to equilibrate at 37°C. After this time, the apical medium was removed and cells were incubated with fluorescent chitosan Cl 113 nanocapsules in HBSS–5% glucose (pH 6.5) for 1 h. After incubation, the suspensions were removed and the epithelium was thoroughly washed with PBS–CaMg (in mM: 140 NaCl, 2.68 KCl, 1.76 KH_2PO_4, 10 Na_2HPO_4, 1.12 $CaCl_2$, 0.49 $MgCl_2$). The samples were fixed with 3% paraformaldehyde in MTSB buffer (80 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$, adjusted to pH 6.8 with KOH) and 3% paraformaldehyde with 0.2% saponin from quillaja bark (sapogenin content ~25%, Sigma-Aldrich) for 5 min. After permeabilization, the cells were washed and the intercellular protein, E-cadherin, was stained using the immunofluorescence method. The cells were incubated with the primary antibody for 1 h and then, after extensive washing with PBS, with the secondary antibody, an anti-mouse IgG antibody, covalently attached to Alexa 546 (λ_{ex} 550 nm/λ_{em} 580 nm) together with Hoechst reagent (λ_{ex} 350 nm/λ_{em} 461 nm) at 1 μg/mL for nuclear labeling for 45 min. After labeling, the cells were thoroughly washed with PBS, filters were cut out and directly embedded in one drop of the mounting medium (Dako). Preparations were examined under a confocal laser scanning microscope (Leica TCS-SP2, Leica Microsystems).

The same protocol was followed for the experiments in Caco-2 cells and in the coculture (Caco-2 and HT29-M6 cells).

RESULTS AND DISCUSSION

As indicated in the "Introduction," we have previously shown that chitosan nanocapsules are able to enhance the oral absorption of the peptide sCT, leading to an important and long-lasting hypocalcemic effect (21,22). Interestingly, the uncoated formulation, a nanoemulsion, led to an insignificant response—a result that evidenced the important role of the chitosan coating for the successful delivery of the associated peptide. In the present work, our first goal was to evaluate the effect of some technological factors inherent to the formation of the chitosan nanocapsules, i.e., the use of chitosan oligomers and the amount of lecithin used to prepare nanocapsules, as well as the effect of the dose of sCT-loaded nanocapsules on their *in vivo* efficacy. In the second part of this work, our aim was to elucidate the mechanism of action of chitosan nanocapsules as potential nanocarriers for oral peptide delivery.

Production and Characterization of Chitosan Oligomers

Chitosan is commercially available with a molecular weight of about 100 kDa and higher. Therefore, to obtain chitosan oligomers, an initial step of this work was to depolymerize chitosan by oxidative degradation using sodium nitrite. With this procedure, the degree of depolymerization of chitosan can be accurately controlled because the number of glycosidic linkages broken is stoichiometric with the moles of sodium nitrite added. After incubation of the polymer solutions with sodium nitrite and using the parameters previously reported (25), straight-line fits were obtained for chitosan oligomers and commercially available chitosan (Protasan Cl 113) ($R^2 > 0.9$). The molecular weights calculated for chitosan oligomers and the commercially available chitosan Cl 113 were 10 and 100 kDa, respectively.

Production and Characterization of Chitosan Nanocapsules

Chitosan nanocapsules were obtained by attaching chitosan onto preformed nanoemulsions. The attachment of chitosan was mediated by the ionic interaction between the negatively charged phospholipids and the positively charged chitosan molecules. The evidence of the coating was obtained from the analysis of the size and zeta potential of the different formulations (Table I). The results show that the size of chitosan nanocapsules is significantly larger than that of the control nanoemulsion and that it decreases with the molecular weight of chitosan. These results could be well explained by the fact that the thickness of the chitosan coating is expected to be dependent on the chain length of chitosan molecules. These differences in the coating were not translated into differences in the surface charge. Indeed, both formulations of nanocapsules exhibited very similar zeta potential values, thus confirming the effective coating of the nanoemulsion irrespective of the chitosan molecular weight. On the other hand, with respect to the effect of the amount of lecithin, it was found that an increase in this amount from 40 to 120 mg led to a significant enlargement of the nanocapsules (from 266 to 333 nm), which was also accompanied by a reduction of the surface charge from +34.8 to +28 mV (27). These results suggest the formation of a larger coating caused by the interaction of lecithin and chitosan. Additionally, the presence of a greater amount of lecithin at the interface would justify the reduction of the zeta potential.

The morphological appearance of the nanocapsules prepared with oligomers and medium molecular weight chitosan are shown in Fig. 1. Both formulations presented a spherical morphology; however, their appearance was drastically different: medium molecular weight chitosan nanocapsules exhibit a thick coating, whereas chitosan oligomer nanocapsules do not show an apparent coating. These results agree with those of the particle size, which indicated that the thickness of the coating is greatly dependent on the chitosan molecular weight.

On the other hand, the preparation of fluorescent nanocapsules, using FITC–phosphoethanolamine as a marker, for *in vitro* cell culture experiments did not lead to any change in the physicochemical properties of the nanostructures (particle size and superficial charge) as compared to the blank formulations.

Encapsulation Efficiency and *In Vitro* Release Studies of Salmon Calcitonin

We have already shown that the encapsulation efficiency of sCT into chitosan nanocapsules was reduced when compared to that attained in the noncoated nanoemulsion (21). This effect was attributed to a competition between the peptide and the chitosan, both positively charged, in their association to the surface of the nanoemulsion. In the present study, we observed that the encapsulation efficiency was also dependent on the chitosan molecular weight. In fact, as shown in Table I, chitosan oligomer nanocapsules showed slightly higher encapsulation efficiencies (60%) than medium molecular weight chitosan nanocapsules (44%). This indicates that the chitosan oligomer coating was not able to displace sCT to the same extent as medium molecular weight

Table I. Physicochemical Properties and Encapsulation Efficiency of Different Nanocarriers

Nanocarrier	Polymer MW (kDa)	Lecithin (% w/v)	Size (nm)	ζ Potential (mV)	Encapsulation efficiency (%)
Nanoemulsion	–	0.4	195.8 ± 1.1	−52.0 ± 1.1	>90
Medium MW chitosan nanocapsules	100	1.2	333.0 ± 6.1	+28.3 ± 2.1	51.9 ± 1.9
Medium MW chitosan nanocapsules	100	0.4	266.6 ± 7.6	+34.8 ± 0.6	44.12 ± 3.24
Chitosan oligomer nanocapsules	10	0.4	202.1 ± 1.3	+30.7 ± 1.9	60 ± 2.65

Values are given as mean ± SD; $n = 3$.

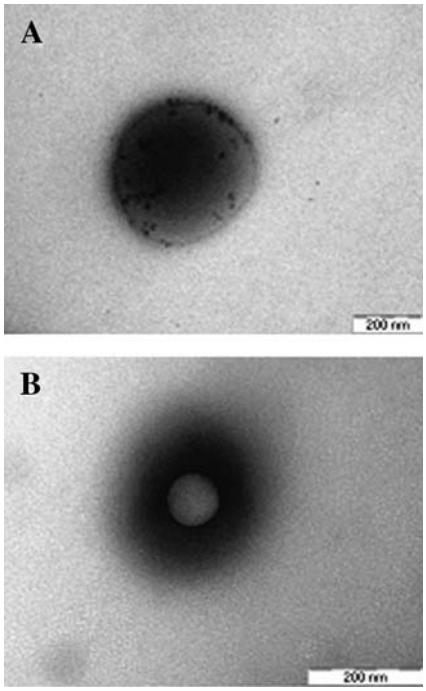

Fig. 1. Transmission electron micrographs of (A) chitosan oligomer nanocapsules and (B) medium molecular weight chitosan nanocapsules.

chitosan coating. This result could be an indirect indication of the reduced amount of chitosan oligomers forming the coating as compared to that corresponding to the medium molecular weight chitosan. Unfortunately, the experimental approaches aimed at quantifying the chitosan coating were unsuccessful. However, the indirect observation of the lower amount of chitosan oligomers forming the coating is in agreement with the smaller size of the resulting chitosan nanocapsules.

The results of the *in vitro* release studies of sCT from chitosan oligomer nanocapsules indicated that the amount of peptide released from the carrier was negligible for up 6 h. These results are comparable to those obtained for the control nanoemulsion; however, they differ from those observed for medium molecular weight chitosan nanocapsules. In fact, this latter formulation released 20% of the encapsulated sCT very rapidly (21). This fast release was attributed to the amount of peptide that was expelled from the oily cores due to the competition with chitosan molecules. Consequently, the lack of this fast release phase in the chitosan oligomer nanocapsules could be, as suggested above, in relation with the reduced amount of chitosan on the coating of these nanocapsules.

In Vivo Efficacy of Chitosan Nanocapsules

We have previously shown that chitosan nanocapsules are able to enhance the oral absorption of sCT (21,22). In the present work, one of the modifications we have introduced into the formulation is an increase in the amount of lecithin from 0.4 to 1.2% w/v. The idea behind this modification was that the use of a greater amount of lecithin would probably lead to a denser coating and, as a consequence, to a different *in vivo* behavior. This was assumed based on the mechanism of coating formation due to the complexation of chitosan and lecithin at the interface of the emulsion. In agreement with this hypothesis, we observed an increase in the size and a reduction in the zeta potential of the nanocapsules (as previously discussed). However, as shown in Fig. 2, this change in the formulation did not have a consequence on the *in vivo* performance of the nanocapsules. Indeed, the response elicited by the new formulation was not significantly different from the one corresponding to the chitosan nanocapsules already described (21). In both cases, an important and long-lasting reduction of the serum calcium levels was also observed after oral administration of the sCT-loaded nanocapsules.

An alternative for modifying the coating of the nanocapsules was based on the use of chitosan oligomers. Fig. 3 depicts the pharmacological response attained after oral administration of chitosan oligomer nanocapsules (MW = 10 kDa) and medium molecular weight chitosan nanocapsules (MW = 100 kDa). The results indicate that chitosan oligomer nanocapsules led to an enhancement of the hypocalcemic effect when compared to the peptide solution. However, the improvement of the response associated to this formulation only lasted 4 h, whereas that corresponding to medium molecular weight chitosan nanocapsules was maintained for at least 24 h. This reduction in the efficacy of the formulation could be related with the limited amount of chitosan around these nanocapsules, as suggested above. In fact, in a previous work we observed that the performance of chitosan nanoparticles as nasal vaccine carriers was not significantly affected by the molecular weight of chitosan (28).

Finally, we found it important to determine the relationship between the dose of sCT (250 *vs.* 500 IU/kg) and, consequently, of chitosan nanocapsules administered orally and the resulting pharmacological response. As shown in Fig. 4, there was a clear effect of this parameter, the

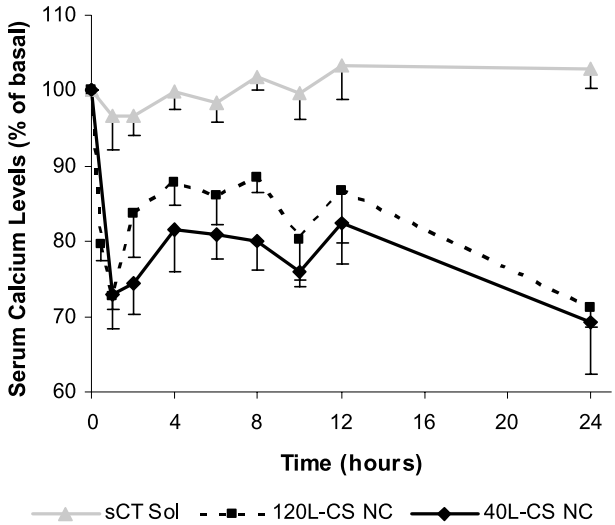

Fig. 2. Hypocalcemic effect after oral administration to rats of chitosan nanocapsules prepared with 0.4 and 1.2% w/v of lecithin (40L-CS NC and 120L-CS NC, respectively) and an sCT aqueous solution (sCT Sol). The dose administered in all the cases was 500 IU/kg (mean ± SE, $n = 6$).

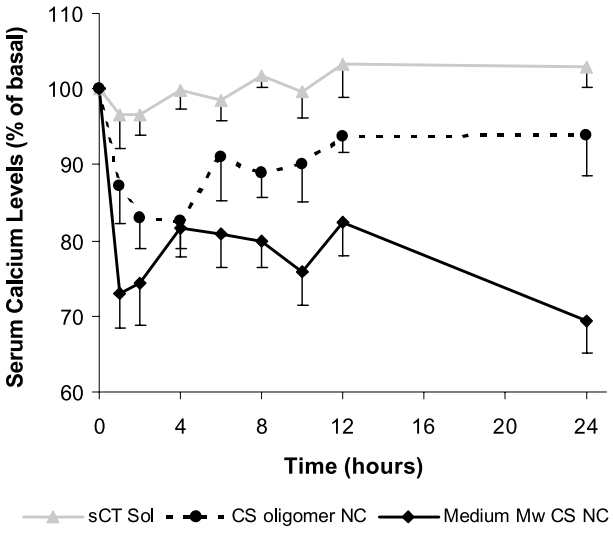

Fig. 3. Hypocalcemic effect after oral administration to rats of chitosan oligomers nanocapsules (CS oligomer NC), medium molecular weight chitosan nanocapsules (medium Mw CS NC), and an sCT aqueous solution. The dose of sCT was 500 IU/kg (mean ± SE, $n = 6$).

response being greatly reduced for the lowest dose of sCT (250 IU/kg). This was expected, as most of the oral administration studies performed with this peptide have used doses of 500 IU/kg or higher. However, the fact that the low dose led to a pulsatile pharmacological profile, characterized by one peak at 1–2 h and a secondary response between 12 and 24 h, was surprising. This kind of profile could be related to the release behavior of the nanocapsules. We could speculate that there is a certain amount of sCT retained in the chitosan coating that would be available for a fast release, whereas most of the well-encapsulated peptide will require a certain time to be released from the carrier and then absorbed. This long-term controlled delivery hypothesis would only be acceptable if we could assume that the nanocapsules are associated to the intestinal mucosa. The following experiments were intended to clarify this end.

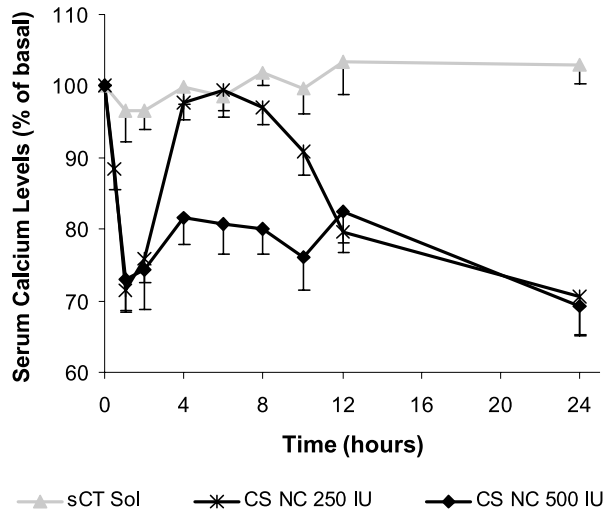

Fig. 4. Hypocalcemic effect after oral administration to rats of chitosan nanocapsules prepared with 0.4% w/v of lecithin with two different doses of sCT: 250 IU/kg (CS NC 250 IU) and 500 IU/kg (CS NC 500 IU). As a control, we have included an sCT aqueous solution of 500 IU/kg (sCT Sol) (mean ± SE, $n = 6$).

Qualitative Study of the Interaction of Chitosan Nanocapsules with the Caco-2 and HT29-M6 Cells

We have previously performed some mechanistic studies on Caco-2 cells aimed at understanding the greater *in vivo* performance of chitosan nanocapsules compared to the control nanoemulsion (21). An observation from this previous study was that chitosan nanocapsules are able to open the tight junctions only when applied at a very high dose. Therefore, we concluded that such a situation is not likely to happen *in vivo* and, consequently, the slight increase in the permeability cannot be taken as the mechanism to explain the *in vivo* efficacy of the nanocapsules. On the other hand, we performed a quantitative analysis of the association of fluorescent nanocarriers to the Caco-2 monolayer and observed a very similar association of chitosan nanocapsules and the control nanoemulsion (21). Thus, we concluded that the chitosan coating did not affect the association of the nanocarrier with the cells.

Taking these previous results into account, our goal in this study was to further elucidate the mechanism of action of chitosan nanocapsules by comparing their qualitative interaction with the Caco-2 cell monolayer and also with a coculture of enterocytes and mucus-secreting cells (Caco-2/HT29-M6). Using confocal laser scanning microscopy, we could visualize the intercellular unions of cells after immunostaining of the intercellular protein E-cadherin, as well as the nucleus labeled with Hoechst. The localization of the nanocapsules was possible after labeling the formulations with phosphoethanolamine covalently attached to fluorescein. The incorporation of the marker to the nanocapsules did not modify their physicochemical properties.

The results of the study with the Caco-2 cell monolayer indicated that chitosan nanocapsules (green spots) were able to interact with the cells, showing a random distribution (Fig. 5A1). In addition, the images of consecutive cross sections suggest that the nanocapsules are able to enter the cells by a transcellular pathway. In fact, the lack of colocalization of the nanocapsules (green) with the E-cadherin marker (red), led us to exclude the presence of the nanocapsules in the paracellular region (which should appear in yellow). Furthermore, the analysis of the *xz* section of the monolayer indicated that after 1 h of incubation, the nanosystems have not crossed the monolayer, but rather they remained at the apical side of the monolayer (Fig. 5A2). These observations corroborate those from our previous studies that suggested the intracellular presence of chitosan nanocapsules (21) and also those reported about the interaction of chitosan nanoparticles with Caco-2 cells (4,29). Nevertheless, an additional conclusion from this study is that despite their apparent intracellular localization, the nanocapsules do not exhibit a capacity to cross the epithelium. The reason for their permanence at the upper level of the monolayer is still unknown and requires further investigation.

Interestingly, the images obtained upon exposure of the formulations with the coculture Caco-2/HT29-M6 are slightly different. First, we have evidenced a great interaction of chitosan nanocapsules with the cells (Fig. 5B1), which was

not seen for the nanoemulsion control. In addition, we have observed that the association pattern of chitosan nanocapsules to both cell types was quite different. Indeed, there was a remarkable and preferable association of the nanocapsules with one cell type, HT29-M6 cells. Figure 5B2 shows an *xz* image of a region consisting of a multilayer of HT29-M6 cells (nuclei labeled in blue). As in the case of the Caco-2 monolayer, the nanocapsules (green) remained associated to the apical side of the coculture, and the paracellular transport of the carriers was excluded. Unfortunately, it was not feasible to take an *xz* confocal image showing the Caco-2 cells in alternation with the HT29-M6 cells due to their different organizational depth (Caco-2 in monolayer and HT29-M6 in multilayer). However, it was possible to observe the surface of the coculture by fluorescent microscopy (Fig. 6). This image shows an important number of fluorescent chitosan nanocapsules specifically located on the top of Goblet islets, thus evidencing the mucoadhesive character of the nanocapsules.

Consequently, from this study we can infer that the mucoadhesive character of chitosan nanocapsules is a determinant of their ability to interact with the intestinal mucosa and to facilitate the intestinal absorption of sCT. Theoretically, this mechanistic behavior would also be applicable to other chitosan-coated nanostructures such as lipid nanoparticles (30) or PLGA nanoparticles (14). In fact, Kawashima *et al.* (14), using the everted rat intestinal sac model, found that the mucoadhesive properties of chitosan-coated PLGA nanoparticles were responsible for the intimate contact of the nanosystem with the intestine and, thus, for the improvement on the peptide absorption.

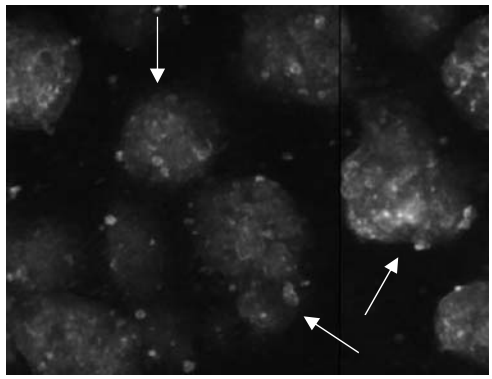

Fig. 6. Fluorescent microscopy image of the coculture Caco-2/HT29-M6 showing chitosan nanocapsules labeling specifically on top of goblet islets (white arrows).

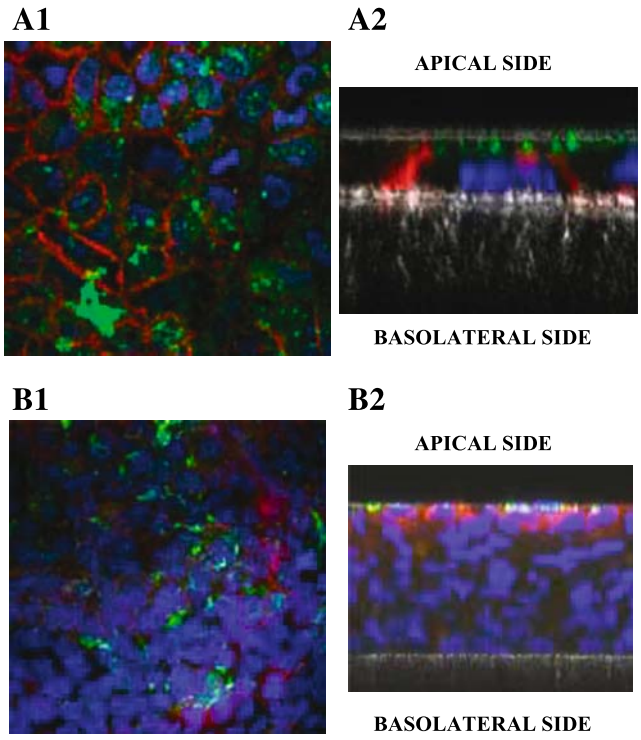

Fig. 5. Confocal scanning microscopy images showing the association of fluorescent nanocapsules (green) to Caco-2 cells and to the coculture Caco-2/HT29-M6 (E-cadherin in red and nucleus in blue). Caco-2: (A1) Montage of 24 horizontal cross sections illustrating the interaction of fluorescent chitosan nanocapsules to the cells (step size in *z* axis of 0.5 μm.); (A2) confocal *xz* section showing the accumulation of fluorescent chitosan nanocapsules in the apical side of the monolayer. Caco-2/HT29-M6 coculture: (B1) Montage of microscopy images showing the association of fluorescent chitosan nanocapsules to the coculture Caco-2:HT29-M6. (B2) Confocal scanning microscopy *xz* section showing the accumulation of fluorescent chitosan nanocapsules (green) in the apical side of the HT29-M6 cells.

These mucoadhesive properties apply not only to chitosan nanocarriers but also to chitosan solutions. However, as previously noted, the ability of chitosan to enhance the absorption of drugs is greatly reduced in mucus-covered cultures (18). Additionally, the concentration of both chitosan and the drug into a nanoparticulate form could help favor the absorption of the drug. Moreover, the oral administration of chitosan in solution is not a viable option because it precipitates upon reaching the intestinal region and, consequently, it is not expected to enhance drug absorption.

Finally, it should be emphasized that despite this first evidence of the mucoadhesive properties of chitosan nanocapsules, further studies are required to fully elucidate the mechanism of action of chitosan nanocapsules.

CONCLUSIONS

In the present report we observed that the *in vivo* efficacy of chitosan nanocapsules as carriers for sCT can be modulated by adjusting the formulation parameters. In addition, the mechanistic studies performed in Caco-2 cells and in a coculture with mucus-secreting cells have revealed that the mucoadhesive properties of chitosan nanocapsules may represent a key factor for their ability in improving the peptide absorption after oral administration.

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