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Effect of Chitosan-Coated Alginate Microspheres on the Permeability of Caco-2 Cell Monolayers

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ABSTRACT Alginate microspheres were prepared by emulsification/internal gelation and coated with chitosan. The ability of chitosan-coated alginate microspheres to increase the paracellular transport across Caco-2 cell monolayers was evaluated in comparison to uncoated microspheres and chitosan solutions. Transport studies were performed by using a permeability marker, Lucifer Yellow (LY), and by measuring the transepithelial electric resistance (TEER) variations. Furthermore, the occurrence of cytotoxic effects was assessed by evaluating neutral red uptake in viable cells and lactate dehydrogenase (LDH) release from damaged cells. A 3-fold increase on LY permeability was obtained for coated microspheres when compared to chitosan solutions. TEER variations were in agreement with permeability results. Chitosan solutions exhibited a dose-dependent toxicity, but coated microspheres did not decrease the viability of cells. Chitosan-coated alginate microspheres have potential to be used as carriers of poorly absorbable hydrophilic drugs to the intestinal epithelia and possibly increase their oral bioavailability.

KEYWORDS alginate, Caco-2, chitosan, microspheres, paracellular transport

INTRODUCTION

Therapeutic compounds that are characterized by poor permeability across mucosal membranes are ineffective when administered orally because only a limited portion of the dose reaches the plasma to exert its pharmacological effect. Obvious disadvantages of parenteral drug delivery are the low patient compliance, septic risk, and high costs due to both manufacturing of sterile products and the need for qualified personnel to administer the drug. The per-oral administration and absorption to therapeutic levels of such compounds may be improved by the design of pharmaceutical dosage forms comprising excipients that provide the drug, in its active form, to the specific site of absorption, increase its residence time, reversibly increase the permeability of the mucosal epithelium, and do not show adverse systemic effects, damage, or exfoliation of the epithelium (Thanou et al., 2001). Oral absorption of drugs across mucosal tissues by a passive mechanism involves mainly either trans- or

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paracellular diffusion through the intestinal epithelium. The transcellular route is restricted mainly to small lipophilic compounds able to pass through the lipophilic cell membrane. Hydrophilic compounds may be absorbed by paracellular route; however, this route comprises only a small part of epithelium, and passage of molecules is limited by tight junctions (Zhou, 1994).

Chitosan [(1→4)-2-amino-2-deoxy-β-D-glucan] is a cationic polysaccharide chemically derived from chitin by deacetylation and has been reported to enhance absorption of various compounds across the mucosal barrier via the paracellular transport pathway. Illum et al. (1994) first reported that chitosan is able to enhance the absorption of insulin across the nasal mucosa of rat and sheep, causing minimal membrane and cellular damage in a rat nasal perfusion model (Aspden et al., 1996). Artursson et al. (1994) reported that chitosan could increase the paracellular permeability of ^{14}C-mannitol, a hydrophilic marker for paracellular routes, across Caco-2 intestinal epithelia. Later, the transport of the peptide drug 9-desglycinamide, 8-L-arginine vasopressin across Caco-2 cell monolayers was seen to be increased by coadministration of chitosan glutamate (Lueßen et al., 1997). Chitosan hydrochloride has also been used to improve the bioavailability of buserelin in rats after intraduodenal administration (Lueßen et al., 1996) and of transforming growth factor-b (TGF-b) across porcine oral mucosa (Senel et al., 2000). The mechanism of action of chitosan was suggested to be a combination of mucoadhesion by increasing the contact time of the drug with the mucosa and an induction of a transient opening of epithelial cell tight junctions (Artursson et al., 1994; Illum et al., 1994; Lueßen et al., 1994). The interaction of chitosan with the cell membrane results in a structural reorganization of tight junction-associated proteins and is mediated through its positive charges (Schipper et al., 1997). At molecular level, chitosan-mediated tight junction disruption is caused by a translocation of tight junction proteins from the membrane to the cytoskeleton (Smith et al., 2004, 2005). Chitosan increases cell monolayer permeability in a reversible manner and viability of cells is not affected (Dodane et al., 1999). Besides, due to their high molecular weight, the polymers themselves are not absorbed and are therefore not expected to have any undesirable systemic effect.

However, using an in situ perfusion model of rat ileum, chitosan solutions did not seem to influence the bioavailability of atenolol, as a result of the mucus barrier in this tissue (Schipper et al., 1999). It was suggested that this effect could be overcome through formulation of chitosan into a particulate dosage form, which could increase the local concentrations of chitosan.

Chitosan-coated alginate microspheres have been used for the encapsulation of a wide variety of biologically active agents including proteins (Onal & Zihnioglu, 2002), enzymes (DeGroot & Neufeld, 2001), antibodies (Albarghouthi et al., 2000), cells (Klinkenberg et al., 2001), and DNA (Quong et al., 1999). Alginate and chitosan were reported to be bioadhesive (Lehr et al., 1992; Chickering et al., 1997); therefore, a more intimate contact with the intestinal mucosa will be expected with smaller microspheres, due to an increase on surface area.

Alginate gel microparticles have been prepared conventionally using extrusion by dropping an alginate solution through a syringe needle into a calcium chloride solution (external gelation), but this approach has a limitation in reducing the diameter of the microspheres. The use of an emulsification/internal gelation method could permit the reduction of the diameter of the microparticles (Poncelet et al., 1992). In this method, an alginate solution containing an insoluble calcium salt is dispersed in an oil, and gelation is achieved by gentle acidification with an oil-soluble acid.

The most commonly used cell line in gastrointestinal drug absorption studies is Caco-2 because, unlike alternative cell lines, it forms confluent monolayers and differentiates to cells with an enterocyte-like morphology using standard cell culture conditions (Artursson, 1993; Pinto et al., 1983). Moreover, it represents a valuable model system for the investigation of drug transport across the small intestinal epithelium (Hidalgo et al., 1989).

Lucifer Yellow (LY) is a hydrophilic molecule (MW 457 Da) that does not permeate the membrane and has been used as a marker to evaluate paracellular permeability and junctional integrity (Hidalgo et al., 1989; Konishi, 2003; Tsukazaki et al., 2004).

The purpose of this study was to evaluate the capacity of chitosan solutions and chitosan-coated alginate microspheres prepared by emulsification/internal gelation to enhance the permeability of Lucifer Yellow, used as a model compound, across Caco-2 cell monolayers and to evaluate its safety toward the cells. The TC-7 clone was used, which proved to be a valuable alternative to the use of parental Caco-2 cells (Gres et al., 1998).

MATERIALS AND METHODS

Materials

Sodium alginate SG300 was supplied by SKW Biosystems (Paris, France). This alginate exhibits an average molecular weight of 694 kDa and guluronic content of 60% (Quong et al., 1998). Chitosan having a low molecular weight (150 kDa; degree of deacetylation near 85%) and sorbitan monooleate (Span® 80, HLB 4.3 ± 1) were purchased from Fluka Biochemika (Steinheim, Germany). Ultrafine calcium carbonate (Setacarb), having 97% of particles with less than 2 μm, was supplied by Omya (Orgon, France), and paraffin oil was supplied by Vaz Pereira (Lisbon, Portugal). All culture media were obtained from Invitrogen Life Technologies (Merelbeke, Belgium). Lucifer Yellow CH, neutral red, and 4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol (Triton® X100) were obtained from Sigma (Steinheim, Germany). All other chemicals were of analytical reagent grade.

Preparation of Chitosan Solutions

Stock solutions of chitosan were prepared at 0.4, 1.0, and 2.0% (w/v) in 1% v/v acetic acid. After dissolution, pH was adjusted to 6.1 with NaOH 5 M. These solutions were then diluted to 0.10%, 0.25%, and 0.50%, respectively, in Hank's' balanced salt solution (HBSS) buffered to pH 6.1 with 10 mM 2-(N-morpholino)ethanesulfonic acid (MES).

Preparation of Microspheres

Microspheres were prepared by emulsification/internal gelation (Poncelet et al., 1992). A suspension of 5% w/v $CaCO_3$ was added into a 2% w/v sodium alginate solution. After homogenization, the mixture was dispersed into paraffin oil (water/oil 30:70) containing 1% Span® 80 (v/v), by stirring at 400 rpm with an Ika-Eurostar mixer (Staufen, Germany) with a marine impeller. After emulsification (15 min), glacial acetic acid in 20 mL of paraffin oil was slowly added to the w/o emulsion during 60 min to allow $CaCO_3$ solubilization. An acetic acid/calcium carbonate molar ratio of 3.5:1 was used. Uncoated microspheres (UM) were recovered from the oily phase by using acetate buffer at pH 4.5 (USP XXVIII) and successively washed with this buffer until no more oil was detected by optical microscope observation. Microspheres were then frozen in an ethanol bath (Benchtop shell freezer, Freezone® model 79490; Labconco, Kansas City, MO, USA) at –40°C and freeze-dried (Lyph-lock 6 apparatus, Labconco) at 0°C for at least 48 h, using validated conditions.

Chitosan-coated microspheres (CM) were prepared by using a continuous coating procedure (Ribeiro et al., 2005). An emulsion of 0.3% w/v chitosan in 1% (v/v) acetic acid, at pH 6.4 (adjusted with NaOH 5 M), in paraffin oil (50:50, v/v) was added to the oily-dispersed gelled microspheres. The mixture was magnetically stirred for 30 min. Coated microspheres were recovered as described above.

Morphological and Particle Size Analysis

The morphology of the microspheres was monitored by optical microscopic observation. Granulometric size distribution was determined in washing medium by laser diffractometry (Fraunhofer model) using a Coulter LS130 particle analyzer, with a size range from 0.1 to 1000 μm. Particle size is expressed as volume mean diameter (μm) ± SD values of the mean. Measurements were made in triplicate for each batch.

Cell Cultures

Caco-2/TC7 cells were kindly provided by Dr. Sweibaum and Dr. M. Rousset (INSERM U710, Villejuif, France) at passage 8. Cells (0.5×10^6) were seeded on 75-cm^2 plastic flasks and the medium was completely changed every day with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 1% nonessential amino acids. Caco-2 cells were incubated in a controlled atmosphere at 37°C, with 95% relative humidity and 10% CO_2 and reached confluence after 7 days. Cells of passages 28–34 were used.

Permeability Studies

Cells were seeded on polycarbonate filters (Costar® Transwell 6-well plate inserts, pore size 0.4 μm, Costar, Acton, MA, USA) at a density of 1.5×10^6 cells per well. The cells were allowed to grow and differentiate during 21 days in complete medium supplemented with penicillin (110 IU/mL) and streptomycin (110 μg/mL). The cells were used for experiments 21 to 28 days after seeding.

The apical transport medium was HBSS containing 10 mM MES at pH 6.1. HBSS containing 25 mM N-(2-hydroxyethyl)piperazine-N''-(2-ethanesulfonic acid) (HEPES) at pH 7.4 was added to the basolateral compartment of the cells monolayer. After removal of culture medium, cells were preequilibrated for 20 min with the transport media. Then, the apical fluid was replaced by 1.5 mL of working solution. Chitosan solutions at 0.1%, 0.25%, and 0.5% (w/v) in HBSS/MES at pH 6.1 and uncoated or coated microspheres (4 and 8 mg/mL) dispersed in apical transport medium, both containing solubilized Lucifer Yellow (LY) at 300 μg/mL, were tested. The apical transport medium containing LY at 300 μg/mL was used as control. At time zero, samples (500 μL) from the apical compartment were withdrawn to determine the initial concentration. Monolayers were incubated at 37°C under controlled atmosphere (10% CO_2, 95% relative humidity). Every hour, during 3-h, samples (1 mL) were withdrawn from each basolateral compartment and immediately replaced by fresh HBSS/HEPES, also incubated at 37°C. All experiments were performed in triplicate. Results are expressed as apparent permeability coefficient (P_{app}), calculated according to Eq. (1).

$$P_{app}(cm\ \ s^{-1}) = \left(\frac{dQ}{dt}\right) \times \left(\frac{1}{A \cdot C_0}\right) \qquad (1)$$

where dQ/dt is the flux of LY across the monolayer (mol/sec), C_0 is the initial concentration of LY in the apical compartment (mol/mL), and A is the surface area of the monolayer (4.71 cm^2). The Papp values are expressed as $cm.sec^{-1}$. The Papp values were calculated for each time point separately and, in addition, a mean Papp was calculated for the 3-h period of the experiment.

Recovery rates were calculated as controls of compound adsorption to cells and/or support, according to Eq. (2), to verify the mass balance of each experiment:

$$\%\ Recovery = \frac{\underline{Q}basolateral_{t180} + \underline{Q}apical_{t180}}{\underline{Q}apical_{t0}} \qquad (2)$$

where $\underline{Q}basolateral_{t180}$ is the total amount of LY in the basolateral compartment after 180-min incubation, $\underline{Q}apical_{t180}$ is the total amount of LY remaining in the apical compartment, and $\underline{Q}apical_{t0}$ is the total amount of LY initially added to the apical compartment.

Measurement of the Transepithelial Electrical Resistance (TEER)

To verify the monolayers confluence and integrity, the transepithelial electrical resistance (TEER) values were measured using a Millicell®-ERS epithelial voltohmmeter fitted with planar electrodes (Millipore Co., Bedford, MA, USA) and calculated according to Eq. (3).

$$TEER(\Omega \cdot cm^2) = (R_{total} - R_{filter}) \cdot A \qquad (3)$$

where R_{total} (Ω) is the measured resistance, R_{filter} (Ω) is the resistance of the filter without cells, and A is the surface area (cm^2).

TEER values were measured before the assay in culture medium and after a preequilibration of 20 min in the transport media (Time 0). Then measurements were made between sample withdrawals, after a stabilization period of 30 min. Untreated cell monolayers had TEER values of 544.5 ± 58.9 $\Omega.cm^2$.

Lucifer Yellow Analysis

LY is a fluorescent dye exhibiting a maximum wavelength of excitation at 430 nm and a maximum of emission at 540 nm. Samples (100 μL) were placed in 96-well black microtiter plates. Quantification was performed with a Cytofluor® 4000 microtiter plate reader (Perkin Elmer, Courtaboeuf, France) using an excitation wavelength of 450/50 nm and emission wavelength of 530/25 nm.

Cytotoxicity Studies

Cytotoxicity studies were performed to evaluate the viability of cells in the presence of chitosan solutions and microspheres. Cells were seeded on 96-well microtiter plates (Corning® Costar, Acton, MA, USA) at a density of 40,000 cells/well. The cells were allowed to grow and differentiate during 21 days in complete medium supplemented with penicillin (110 IU/mL) and streptomycin (110 μg/mL). Two different tests were used: lactate dehydrogenase (LDH) release from damaged cells and incorporation of neutral red (NR) in viable cells.

Monolayers were incubated with 200 μL of the samples described for the permeability studies in a controlled atmosphere for 3 h. The negative control was fresh HBSS/MES and the positive control was

Triton® X100 (0.1%) in HBSS/MES. The supernatant was withdrawn for investigation of LDH release using a cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer specifications. Cells were rinsed with phosphate-buffered saline (PBS) and incubated with 100 μL of NR at 50 μg/mL in HBSS/MES, for 3 h in a controlled atmosphere. After incubation, cells were washed once with PBS. The NR that was incorporated in viable cells was extracted using 200 μL of a solution of 50% (v/v) ethanol and 1% (v/v) acetic acid for 10 min. Optical density at 550 nm was read using an enzyme-linked immunosorbent assay (ELISA) plate reader.

Statistical Analysis

Each value was expressed as the mean ± SD. The one-way analysis of variance (ANOVA) was used followed by the Bonferroni post test for multiple comparisons. A value of p less than 0.05 was considered to be significant.

RESULTS AND DISCUSSION

Morphology of the Microspheres

Alginate microspheres appeared monodisperse and spherical as illustrated in Fig. 1A. Chitosan coating caused agglomeration of microspheres (Fig. 1B), which resulted in the incorporation of some oil among the microspheres, as seen by the presence of dark spots. Granulometric distribution based on volume distribution of microspheres showed a unimodal population with mean diameters of 20 μm and 350 μm for uncoated and coated alginate microspheres, respectively (Fig. 2). This increase on mean diameter size occurred due to agglomeration of alginate microspheres during the coating process and can be explained by strong electrostatic interaction between alginate and chitosan, two polyelectrolytes of opposite charge.

In a previous work from our group, the formation of CS-ALG complex on chitosan-coated alginate microspheres using a continuous procedure was confirmed by differential scanning calorimetry and Fourier-transform infrared spectroscopy. Also, the presence of a membrane around microspheres was confirmed after calcium alginate dissolution by citrate treatment (Ribeiro et al., 2005).

Permeability of Caco-2 Monolayer

TEER values were evaluated since they are representative of the tight junction complexes functionality between cells. A decrease in TEER is caused by increased ion permeability through the tight junction between adjacent cells. Caco-2 cells monolayers were incubated for 3 h with chitosan solutions, at a pH of 6.1, having concentrations ranging from 0.1% to 0.5% (w/v). A statistically significant decrease ($P < 0.05$) in TEER, in comparison to control, was observed only with the highest concentration (Fig. 3), but the behavior of the solutions at 0.25% and 0.5% was not statistically different ($P > 0.05$). The reduction in TEER observed with chitosan solution at 0.5% occurred 90 min after the start of the experiment and reached near 72% of the initial value.

The behavior of chitosan solutions was compared with that of microspheres suspended in apical transport

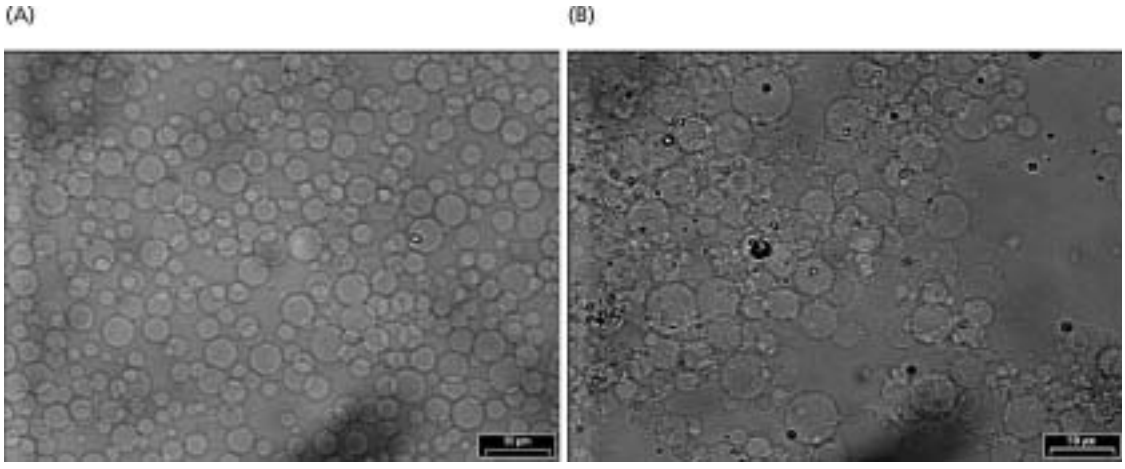

FIGURE 1 Optical Microscope Photograph of Uncoated (A) and Chitosan-Coated (B) Alginate Microspheres (Magnification × 200).

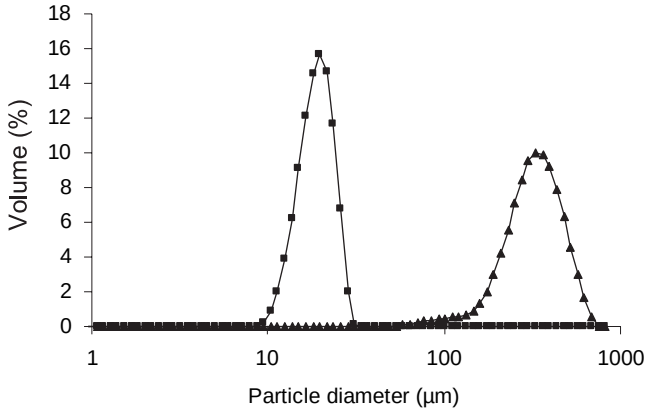

FIGURE 2 **Size Distribution of Uncoated (■) and Chitosan-Coated (▲) Alginate Microspheres.**

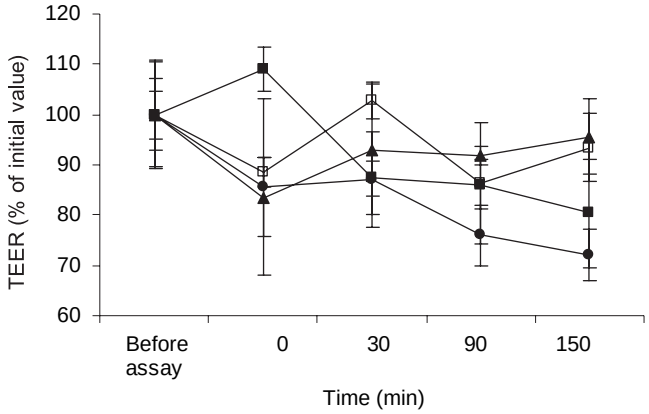

FIGURE 3 **Effect of Control (□) and Chitosan Solutions at 0.1% (▲), 0.25% (■), and 0.5% (●) on the TEER Values of Caco-2 Cell Monolayers. Error Bars Represent Standard Deviations About the Mean of 3 Replicates.**

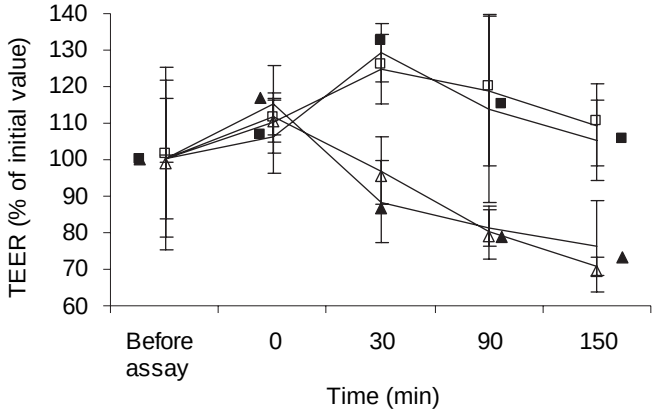

FIGURE 4 **Effect of Uncoated (UM) and Chitosan-Coated (CM) Microspheres on the TEER Values of Caco-2 Cell Monolayers. UM 4 mg/mL, (■), UM 8 mg/mL (□), CM 4 mg/mL (▲) and CM 8 mg/mL (Δ). Error Bars Represent Standard Deviations About the Mean of 3 Replicates.**

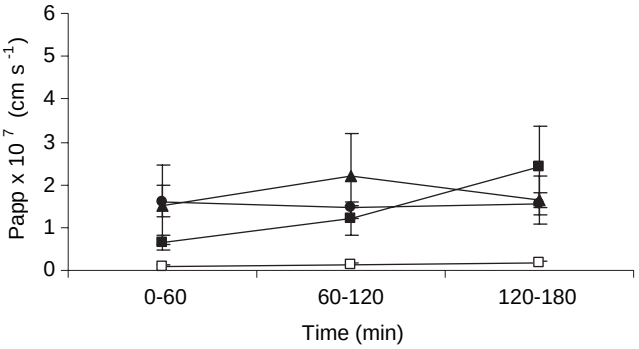

FIGURE 5 **Effect of Control (□) and Chitosan Solutions at 0.1% (▲), 0.25% (■), and 0.5% (●) on the Permeability of LY. Error Bars Represent Standard Deviations About the Mean of 3 Replicates.**

medium. After the addition of the suspension to the cells monolayer, sedimentation occurred. Uncoated microspheres were evaluated as control, and they slightly increased TEER values after 30-min incubation, but a linear decrease was observed afterward reaching the initial values at the end of the assay (Fig. 4). On the other hand, chitosan-coated microspheres were able to significantly decrease TEER measurements after 30 min of incubation. At 3-h incubation, values similar to those obtained with chitosan solution at 0.5% were reached ($P = 0.353$). Increasing the concentration of microspheres from 4 to 8 mg/mL did not result in significant differences in TEER values ($P = 0.139$).

The effect of chitosan solutions at different concentrations on the permeability of LY across Caco-2 monolayer along the assay is shown in Fig. 5, and Table 1 shows the mean Papp values calculated for the all 3-h period. The control, resulting from the incubation with LY in the apical transport medium, gave a mean Papp of 0.13×10^{-7} cm.sec^{-1}. In the presence of chitosan a 10- to 13-fold increase in permeability was observed, and the effect was not dose-dependent. At a concentration of 0.25% the effect became more pronounced during the 3-h incubation, but for the other chitosan concentrations there was no increase in LY permeability throughout the experiment. Nevertheless, the mean Papp value observed with chitosan at 0.25% (1.4×10^{-7} cm.sec^{-1}) was not statistically significantly different from the values found for the other concentrations ($P > 0.05$).

Chitosan effect on permeability seems to reach a saturation level, since no major differences were found in TEER and LY permeability between different

TABLE 1 **Mean Apparent Permeability (Papp) of LY Across Caco-2 Cell Monolayers, in the Presence of Control, Chitosan (CS) Solutions, and Uncoated and Coated Microspheres**

Sample	Concentration	Mean Papp $\times 10^7$ ($cm.sec^{-1}$)[b]	Ratio sample Papp/control Papp
Control[a]		0.134 ± 0.037	1.0
CS solutions	0.1%	1.790 ± 0.359	13.3
	0.25%	1.421 ± 0.898	10.6
	0.5%	1.552 ± 0.061	11.6
Uncoated microspheres	4 mg/mL	0.353 ± 0.165	2.6
	8 mg/mL	0.264 ± 0.016	2.0
Coated microspheres	4 mg/mL	3.587 ± 0.251	26.7
	8 mg/mL	4.509 ± 0.608	33.6

[a]HBSS/MES (10 mM) at pH 6.1 was used as control. [b]Mean Papp values were calculated based on a 3-h period of incubation with samples. Results are expressed as mean ± SD of 3 replicates.

concentrations. This result agrees with a previous study, where it was verified that when chitosan concentration was increased from 0.25% to 0.50%, the increase in mannitol permeability across Caco-2 cell monolayers was of the same extent (Artursson et al., 1994; Schipper et al., 1996). An apparent absorption plateau level was achieved, showing a shallow dose-response relationship.

The apical solutions were buffered at pH 6.1 to maintain chitosan in solution, which has a pK_a value of approximately 6.5. The pH of the various intestinal segments ranges from slightly acidic to slightly basic and at the immediate surface of epithelial cells a slightly acid "microclimate" is found (Daugherty & Mrsny, 1999). The pH value of the microclimate region was measured in vitro and in vivo using pH microelectrodes and estimated to be 5.8–6.3, which is apparently lower than that of the bulk solution in the intestinal tract (Yamashita et al., 2000).

Decreasing the pH of the chitosan solution from 6.0 to 4.0 has been shown to increase the transport of mannitol across Caco-2 cell monolayers, since at lower pH values chitosan has a higher charge density, assumes a more elongated shape and permits a more intimate contact with the epithelial membrane (Artursson et al., 1994; Schipper et al., 1996). However, these low pH values do not mimic the pH value of the microclimate region. In our study, chitosan solutions at pH 6.1 caused, at least, a 10-fold increase on LY permeability, suggesting that chitosan was still able to interact with the intestinal epithelia, although acting near its pK_a value. No precipitation of chitosan was detected at the end of the assay.

An increase on LY permeability was found following treatment with chitosan-coated microspheres, by comparison to the control (Table 1), which suggests an increase in paracellular permeability. The LY mean Papp value (4.51×10^{-7} $cm.sec^{-1}$ for 8 mg/mL) was approximately 3-fold higher when compared to the 0.5% chitosan solution. In agreement with the results of the TEER experiments, the mean Papp was not significantly different when microspheres concentration increased from 4 to 8 mg/mL. However, considering LY Papp behavior along the assay (Fig. 6), it could be observed that microspheres at 8 mg/mL gave higher Papp values for each time point. Furthermore, epithelial permeability significantly decreased with time, especially for the highest concentration ($P < 0.001$). Uncoated microspheres did not produce an increasing effect on LY permeability, so the absorption enhancing effect was not due to the presence of the particulate system by itself but rather to the chitosan coating.

LY recovery rates were above 95%, in all cases, showing no occurrence of adsorption to the cells or support, except for uncoated microspheres. In this

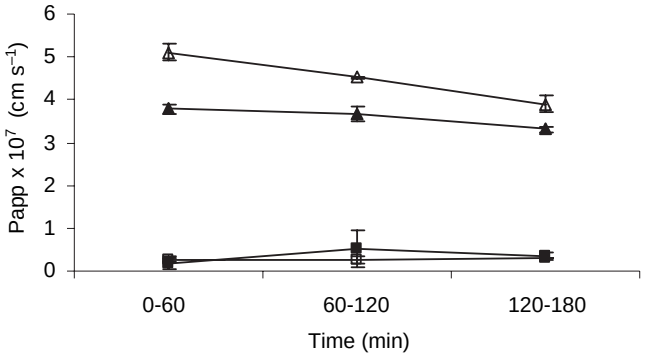

FIGURE 6 **Effect of Uncoated (UM) and Coated (CM) Microspheres on the Permeability of LY. UM 4 mg/mL, (■), UM 8 mg/mL (□), CM 4 mg/mL (▲), and CM 8 mg/mL (Δ). Error Bars Represent Standard Deviations About the Mean of 3 Replicates.**

case, a lower value near 90% was found, which could be attributed to adsorption of small amounts of LY to the alginate microspheres.

Coated microspheres were prepared by adding a 0.3% (w/v) chitosan solution to the recently prepared microspheres. Considering that all the chitosan could react with the alginate matrix, a maximal concentration of 0.13 mg of chitosan per milligram of microspheres could be obtained. Taking into account this value, when the dispersions of microspheres were prepared at concentrations of 4 and 8 mg/mL, the maximal concentration of 0.05% and 0.1% could be achieved for chitosan, respectively. Apparently, the particulate dosage form enhances the efficacy of chitosan for increasing paracellular permeability. The increased effect on permeability may be related with the achievement of highest local concentrations due to the sedimentation and possible adherence of microspheres.

Cytotoxicity

Neutral red is a cationic dye, which diffuses through the apical membrane of cells and is incorporated intracellularly in the lysosomes (Babich & Bohrenfreund, 1990; Fautz et al., 1991). Its uptake by the monolayer is proportional to the viability of cells. The percentage of viable cells was calculated in relation to the apical transport medium used as negative control (Fig. 7). A significant decrease ($P < 0.001$) in the percentage of living cells was observed with chitosan solution at 0.5% and uncoated microspheres, at a concentration of 8 mg/mL. However, these values were still above those found for Triton® X100, used as a positive control. Chitosan coated microspheres did not decrease cells viability. Nevertheless, this test requires the removal of the supernatant, which is difficult in the case of chitosan solutions due to its viscosity and adhesive character. In addition, it becomes difficult to remove all the microspheres over the monolayer without damaging the cells. Therefore, the LDH cytotoxicity test was performed using the supernatant.

LDH is a cytoplasmic enzyme (MW 140 kDa) released when apical membrane of cells is damaged. The amount of enzyme detected in supernatant correlates to the proportion of lysed cells (Decker & Lohmann-Matthes, 1988). It was verified that chitosan solutions significantly increased LDH leakage from damaged cells (Fig. 8) indicating an increased apical membrane permeability. A dose-dependent effect was observed, although a much higher effect was observed for the positive control. Values of 79.4 ± 26.4, 777.5 ± 159.7, and 1821.1 ± 184.9 mIU/mL were obtained for negative control, chitosan at 0.5%, and positive control, respectively.

Chitosan has recently received FDA approval and was shown to have little or no adverse effect on the rat (Illum et al., 1994) and human (Aspden et al., 1997) nasal membrane as evaluated by histological studies. These results showed that negligible amounts of cell destruction and membrane disruption occurred in the presence of chitosan solutions and were further confirmed by the release of low amounts of membrane-bound (5′-nucleotidase) and cytosolic (LDH) enzymes from the nasal mucosal membranes (Aspden et al., 1996). Also, it was found that no dramatic damage effect to Caco-2 cells monolayers

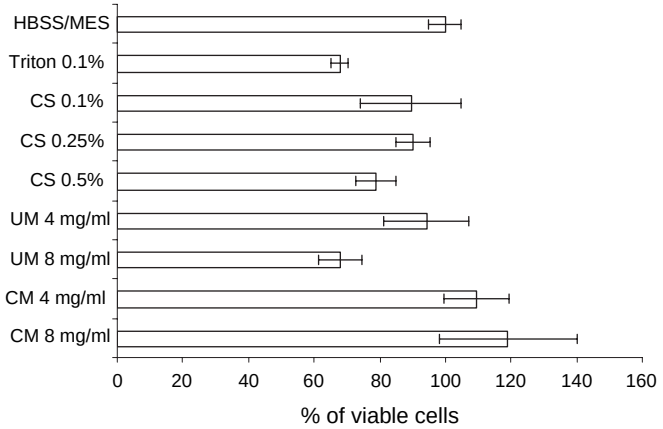

FIGURE 7 **Percentage of Viable Cells Relative to Negative Control Considering NR Uptake, After Incubation With Chitosan (CS) Solutions, Uncoated (UM), and Coated (CM) Microspheres. Fresh HBSS/MES (10 mM) at pH 6.1 and Triton X100 at 0.1% Were Used as Negative and Positive Control, Respectively. Results Are Expressed as Mean ± SD Values of 6 Replicates.**

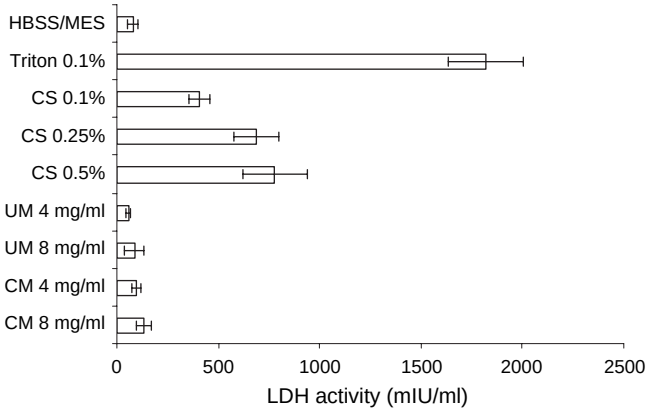

FIGURE 8 **Activity of LDH Released From Damaged Cells After Incubation With Chitosan (CS) Solutions, Uncoated (UM), and Coated (CM) Microspheres. Fresh HBSS/MES (10 mM) at pH 6.1 and Triton X100 at 0.1% Were Used as Negative and Positive Control, Respectively. Results Are Expressed as Mean ± SD Values of 6 Replicates.**

could be detected with the trypan blue exclusion technique (Kotze et al., 1997; Dodane et al., 1999). However, in other studies, it was observed that chitosan treatment appeared to slightly perturb the plasma membrane as assessed by a significantly increased release of LDH in the presence of a 0.5% chitosan solution (Dodane et al., 1999; Tengamnuay et al., 2000).

Schipper et al. (1996) verified that adverse effects of chitosan are dependent on the polymer structure and charge. A dose-dependent effect on intracellular dehydrogenase release from Caco-2 cells was found for chitosans having a low degree of acetylation (DA $<$ 35%) (Schipper et al., 1996). This observation agrees with our results, given that a chitosan with a degree of acetylation of 15% caused a dose-dependent LDH release from cells.

In contrast, uncoated and coated microspheres did not cause a statistically significant increase on LDH release, showing that incubation with microspheres caused no detectable damages on cells. It was recently described that transformation of chitosan into nanoparticles did not modify the cytotoxicity profile when a MTT reduction assay was performed on epithelial pulmonar cells (Huang et al., 2004). In our study, a remarkable decrease from 777.5 ± 159.7 to 96.9 ± 24.9 mIU/mL on LDH release was observed when chitosan was used as coating instead of a solution.

CONCLUSIONS

Chitosan solution increased the permeability of the hydrophilic marker LY and decreased TEER, which are generally considered to be indicators of increased paracellular permeability in experiments using cell lines. No relation to the chitosan solution concentration was found. However, a significant increase on LDH leakage was also verified. On the other hand, chitosan-coated microspheres increased LY permeability and decreased TEER to a higher extent than chitosan solutions. Nevertheless, a less deleterious effect was shown on the monolayers, as assessed by the cytotoxicity results. These results indicate that chitosan-coated alginate microspheres have potential to be used as safe carriers of poorly absorbable hydrophilic drugs to the intestinal epithelia and possibly increase their oral bioavailability.

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