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Research paper

Specific permeability modulation of intestinal paracellular pathway by chitosan-poly(isobutylcyanoacrylate) core-shell nanoparticles

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Abstract

This work is focused on the evaluation of the *in vitro* permeation modulation of chitosan and thiolated chitosan (chitosan-TBA) coated poly(isobutylcyanoacrylate) (PIBCA) nanoparticles as drug carriers for mucosal administration. Core-corona nanoparticles were obtained by radical emulsion polymerisation of isobutylcyanoacrylate (IBCA) with chitosan of different molecular weights and different proportions of chitosan/chitosan-TBA. In this work, the effect of these nanoparticles on the paracellular permeability of intestinal epithelium was investigated using the Ussing chamber technique, by adding nanoparticle suspensions in the mucosal side of rat intestinal mucosa. Results showed that permeation of the tracer [^{14}C]mannitol and the reduction of transepithelial electrical resistance (TEER) in presence of nanoparticles were more pronounced in those formulations prepared with intermediate amounts of thiolated polymer. This effect was explained thanks to the high diffusion capacity of those nanoparticles through the mucus layer that allowed them to reach the tight junctions in higher extent.

It was concluded that, although a first contact between nanoparticles and mucus was a mandatory condition for the development of a permeation enhancement effect, the optimal effect depended on the chitosan/chitosan-TBA balance and the conformational structure of the particles shell.

Keywords: Chitosan; Thiolated chitosan; Poly(isobutylcyanoacrylate); Core-shell/core-corona nanoparticles; Permeation enhancement; TEER; Ussing-type chambers

1. Introduction

Significant advances in biotechnology and biochemistry have led to the discovery of a large number of bioactive molecules and vaccines based on peptides, proteins and oligonucleotides [1]. However, the two major obstacles for those drugs reaching the adequate biological compartments include poor stability and limited transport across epithelia, representing the limiting steps for their application in clinics [2–4]. The development of suitable carrier systems able to enhance their oral bioavailability remains a major challenge for the pharmaceutical scientist [1,4,5].

Those bio-engineered compounds are mostly large pharmaceutical molecules, highly hydrophilic in nature that do not partition in a large extent into the cell membrane. Their absorption is generally limited to the paracellular pathway, which occupies only a very small surface area in the gut (around 1%) compared to the total surface available for the transcellular route [6]. This paracellular pathway is controlled by the permeability of multiprotein complexes forming the tight junctions [7,8]. Although the adaptive mechanisms and specific regulation of these tight junctions are areas of active investigation and remain incompletely understood [4,8–10], it is known that some polymers can promote their widening. While anionic polymers such as carbomer can bind extracellular calcium, involved in the

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maintenance of tight junctions integrity [11], cationic polymers (e.g. chitosan, poly-lysine, etc.) can interact with the tight junction proteins, promoting their re-organization and subsequent opening [12,13]. Strategies were developed considering those polymers to design colloidal carrier systems able to enhance the paracellular absorption of active molecules. In this sense, chitosan has raised great attention in pharmaceutical and biomedical fields [5,14–16], not only inducing permeation enhancing properties [1,17] but also for its bioadhesiveness [18,19]. Additionally, this polymer can be chemically modified in order to include new functionalities. For instance, thiolated chitosan derivatives, formulated in gels and tablets, showed higher mucoadhesive properties and mucosal permeation enhancement than the unmodified polymer [20–23].

In a recent work, we showed that chitosan and thiolated chitosan can be associated with nanoparticle technologies obtaining poly(isobutylcyanoacrylate) (PIBCA) nanoparticles consisting of a polymeric core coated with these polysaccharides [24,25]. Such nanoparticles can be prepared either by anionic or by radical emulsion polymerisation of the corresponding monomers and showed improved bioadhesion when they were applied under static conditions on rat intestinal mucosal surfaces. It was observed that up to a defined threshold, chitosan and thiolated chitosan improved the bioadhesion of the nanoparticles. Above this threshold, formation of intra- and interchains disulphide bonds in thiolated chitosan created a cross-linked shell, reducing interactions with mucins and bioadhesiveness of the nanoparticles. Only those nanoparticles with high amounts of free (reduced) thiol groups at the surface could develop high bioadhesive properties by formation of covalent bonds with cysteine residues of glycoproteins in the mucus [26].

The aim of the present work was to analyse the potential permeation enhancement of the paracellular route induced by chitosan and thiolated chitosan coated PIBCA nanoparticles. Studies were performed *ex vivo* on rat intestinal tissue using Ussing chambers. The method was adapted to investigate simultaneously the influence of the bioadhesive properties of the nanoparticles on the intestinal permeation enhancement effect.

2. Materials and methods

2.1. Materials

Isobutylcyanoacrylate (IBCA) was kindly provided as a gift by Henkel Biomedical (Dublin, Ireland). Chitosan Mw 400,000 g/mol, forskolin and [^{14}C]mannitol were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). 2-Iminothiolane HCl (Traut's reagent) was synthesised in the Department of Organic Chemistry (Biocis, UMR CNRS 8076), Faculty of Pharmacy, University Paris-XI (Châtenay-Malabry) France. All other chemicals were of reagent grade and used as received.

Chitosan of different molecular weights was obtained by depolymerisation reaction with sodium nitrite at different concentrations (7.0; 2.7 and 1.6 g/l) [25,27]. The molecular weight of the resulting chitosans was, respectively, 20,000 g/mol (Chito20), 50,000 g/mol (Chito50) and 100,000 g/mol (Chito100) as evaluated from capillary viscosimetry (viscometer AVS400, Schott Gerate) [28]. ^{1}H NMR analysis (Bruker MSL-400 spectrometer, Bruker Instrument Inc. Wissembourg, France) showed no changes in the percentage of deacetylation after depolymerisation, with values around 86–88% in all cases [25].

Thiolated chitosans were prepared by the inclusion of thiol groups in the different hydrolysed chitosans by reaction with 2-iminothiolane [22]. The resulting polymers were chitosan-4-thiol-butylamidine, named Chito20-TBA, Chito50-TBA and Chito100-TBA, according to the original molecular weight of the corresponding unmodified polymers. Elemental analysis (Analyzer LECO SC144, Service central d'analyse du CNRS, Vernaison, France) was used to determine the total sulphur content of the polymer (6.6% for Chito20-TBA, 6.0% for Chito50-TBA and 5.1% for Chito100-TBA). Subsequently, the iodine titration method was used to quantify the percentage of total sulphur present in its reduced form (thiol groups), being 43%, 45% and 36% of the total sulphur, for Chito20-TBA, Chito50-TBA and Chito100-TBA, respectively [29].

2.2. Methods

2.2.1. Preparation and characterization of nanoparticles

Nanoparticles were elaborated following the radical polymerisation method developed by Chauvierre and coworkers [30–32] and adapted to chitosan [24,25]. Briefly, 0.069 g of mixtures of modified and unmodified hydrolysed chitosan (chitosan%/chitosan-TBA%: 0/100; 25/75; 50/50; 75/25; 100/0) was dissolved in 4 ml of 0.2 mol/l nitric acid in MilliQ® water in a glass tube at 40 °C, under gentle stirring and argon bubbling. After 10 min, 1 ml of a solution of 8×10^{-2} M ammonium cerium (IV) nitrate in 0.2 mol/l nitric acid, and 0.25 ml of IBCA were added under vigorous magnetic stirring. Argon bubbling was kept for additional 10 min and stopped. The reaction was allowed to continue at 40 °C under gentle stirring for 40 min. After cooling to room temperature, NaOH (1 mol/l) was added to raise the pH to 4.5. The nanoparticle suspensions obtained were purified by dialysis (Spectra/Por membranes, 100,000 g/mol molecular weight cut-off (MWCO), Biovalley, Marne la Vallée, France) against 1 l of acetic acid solution (16 μmol/l) in MilliQ® water twice for 90 min and once overnight. Under this condition, the total purification of nanoparticles can be ensured.

Control PIBCA cores were prepared by anionic polymerisation and stabilized with 1% Poloxamer 188 (Pluronic® F68) [33].

The *hydrodynamic mean diameter and the size distribution* of the nanoparticles were determined at 20 °C by quasi-elastic light scattering using a Nanosizer® N4 PLUS (Beckman-Coulter, Villepinte, France). The scattered angle

was fixed at 90°. Samples were diluted in acetic acid solution (16 μmol/l) in MilliQ® water to achieve a signal level ranging from 5×10^4 to 5×10^6 counts per second. The results were expressed as the mean hydrodynamic diameter, the standard deviation and the polydispersity index of the size distribution. Results corresponded to the average of three determinations.

The *ζ potential* of nanoparticles was deduced from their electrophoretic mobility measured by Laser Doppler Electrophoresis (Zetasizer Nano serie. Malvern Instruments Ltd., Worcestershire, UK) at room temperature in a NaCl 1 mmol/l solution after suitable dilutions (1/200 (v/v)) of the different nanoparticle suspensions. The pH of the final solution was around 6.8.

The *total amount of sulphur in nanoparticles* was determined by elemental analysis using an Analyzer LECO SC144 (Service central d'analyse du CNRS, Vernaison, France). Samples of 10 mg were burned at 1350 °C over oxygen flux and the detection of SO_2 was performed by infrared measurements.

The *quantification of reduced thiol groups* on the nanoparticle surface was determined using the iodine titration method [29].

2.2.2. Permeation assays

Ussing chambers were used to determine the permeability of fresh intestinal tissue to a paracellular marker ([^{14}C]mannitol) in presence of nanoparticles. Electrical parameters were also recorded to determine the tissue viability and the opening of tight junctions during the assay. The methodology used was the following.

Preparation of the intestinal tissue. Jejunum from fresh small intestine of sacrificed male Wistar rats (200–250 g) (Charles River, Paris) was excised, rinsed with chilled physiological saline solution (NaCl 0.9%) and cut into segments of 2–3 cm length. After visual examination of the tissue, sections containing Peyer's Patches were discarded from the studies [34].

Permeation experiments. Jejunum portions were mounted in Ussing chambers (the intestinal surface tested was 1 cm^2) bathed with Ringer solution at pH 6 containing mannitol 1 mmol/l. The system was maintained at 37 °C and continuously oxygenated with O_2/CO_2 95%/5%. After 30 min of incubation, the liquid of the donor chamber was replaced by the same volume of preheated (37 °C) Ringer solution containing [^{14}C]mannitol and in which 5 mg of the different formulations of nanoparticles was added (Fig. 1A). At pre-set time intervals, three aliquots of 200 μl were recovered from the acceptor chamber (serosal side) and replaced with the same volume of fresh medium pre-equilibrated at 37 °C. Assays were carried out for 2 h. Sample analysis was performed by measuring the radioactivity of [^{14}C] by liquid scintillation (Scintillation liquid: Ultima gold from Perkin-Elmer, Apparatus LS 6000 TA, Beckman). Control test was carried out with PIBCA cores and with chitosan and thiolated chitosan solutions (0.2%). Four tissue portions from four different rats were used to evaluate each formulation. The experiments were repeated on different days.

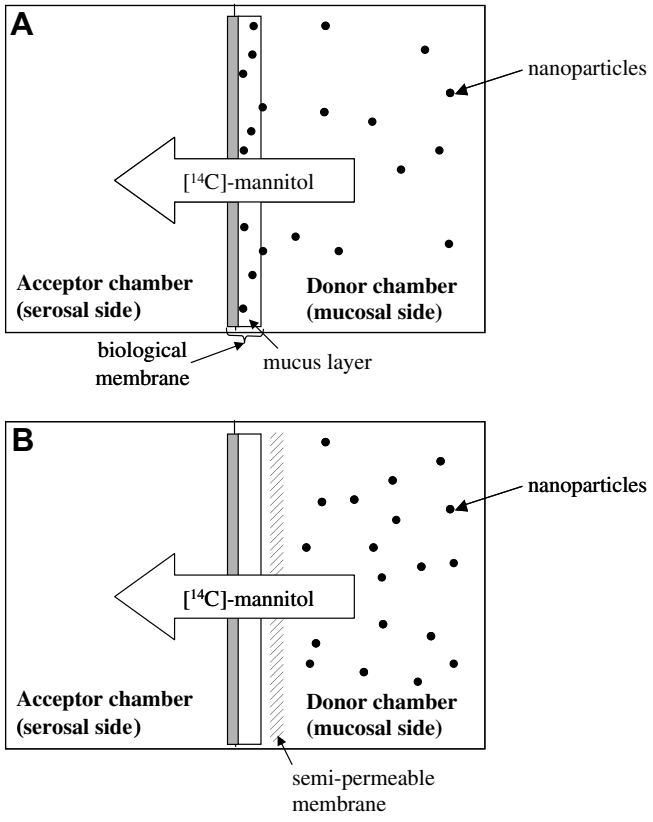

Fig. 1. Experimental setup of Ussing chambers used for determining mannitol permeability (A) and simultaneously avoiding nanoparticles bioadhesion (B).

The apparent permeability coefficient (P_{app}) was calculated using Eq. (1):

$$P_{app} = dQ/dt \times 1/AC_0 \quad (1)$$

where dQ/dt is the flux of [^{14}C]mannitol from the mucosal to the serosal side of the mucosa, C_0 is the initial concentration of [^{14}C]mannitol in the donor compartment and A is the area of the membrane. The values of P_{app} were calculated between 15 and 90 min after addition of the marker molecule in all experiments, in order to standardize the calculations [35]. Absorption enhancement ratio (R) was calculated from P_{app} values (Eq. (2)) [6].

$$R = P_{app}(\text{sample})/P_{app}(\text{control}) \quad (2)$$

Measurement of electrical parameters. During the experiments, a four electrode system was used to perform electrical measurements. Transmucosal potential difference (PD) was continuously recorded between two KCl saturated agar bridges connected to an MDVC-2C voltage clamp (Titis Business Corporation, Paris, France) via calomel electrodes filled with saturated KCl solution. Potential difference was short-circuited throughout the experiment by a short-circuit current (Isc) via agar bridges placed in each half-cell, and adapted to platinum electrodes connected to an automatic

MDVC-2C voltage clamp (Titis Business Corporation, Paris, France). Delivered Isc (short-circuit current) was corrected for fluid resistance and recorded at pre-set times. The transmucosal electrical resistance (TEER) was calculated from Ohm's law (Eq. (3)):

$$\mathrm{TEER} = \mathrm{PD}/\mathrm{Isc} \quad (3)$$

Only tissues showing $\mathrm{PD} > 2 \times 10^{-3}$ V and $\mathrm{Isc} > 40 \times 10^{-6}$ A/cm^2 after 30-min incubation were retained for the study. An additional control was performed at the end of the experiment by adding 25 μl of a 2 mM forskoline ethanol solution in the serosal compartment. Forskolin increases Cl^- secretion by the cells and therefore the Isc [36]. If there was no increase in Isc, damages in the tissue were suspected and all samples collected from the corresponding chambers were discarded.

Permeation experiments were repeated *avoiding bioadhesion* of the nanoparticles on the intestinal tissue. In these experiments, a semi-permeable membrane (cut-off: 100,000 g/mol) was placed in the donor chamber at the surface of the luminal side of the intestinal mucosa, to avoid direct contact between nanoparticles and intestinal mucus. The membrane was placed in such a manner that it was not in contact with the living tissue (Fig. 1B).

2.2.3. Statistical analysis

The results obtained were statistically analysed by using Mann–Whitney's *t*-test with a 95% confidence level ($p < 0.05$).

3. Results and discussion

3.1. Preparation and characterization of the nanoparticles

To obtain core-shell particles in the nano-size range by radical emulsion polymerisation of IBCA, low molecular weights chitosan were necessary [24,25]. Although it was possible to elaborate nanoparticles with different proportions of Chito20/Chito20-TBA in the range 100%/0%–0%/100%, the limited solubility of Chito50-TBA and Chito100-TBA in the polymerisation medium made impossible the preparation of nanoparticles with percentage of thiolated chitosans higher than 75% and 25%, respectively. The quite high percentage of oxidized sulphur groups found in Chito20-TBA (57%); Chito50-TBA (55%) and Chito100-TBA (64%) suggested the formation of a cross-linked structure in the modified chitosans which might influence their solubility in the polymerisation medium [25].

The characteristics of nanoparticles prepared for this study are given in Table 1. Results are in good agreement with those corresponding to nanoparticles obtained in previous studies [25,26]: (i) monodispersed nanoparticle populations were obtained in all cases; (ii) the hydrodynamic mean diameter increased with the percentage of chitosan-TBA and with the molecular weight of chitosan, which was observed to be consequence of the increment of the chitosan gel layer around the cyanoacrylic core [25]; (iii) the total amount of sulphur in nanoparticles increased with the percentage of chitosan-TBA used in their preparation. However, as already explained in a previous work, the proportion of total sulphur presented in the "reactive" (reduced) form decreased with the amount of total sulphur in the formulation, because of formation of disulphide bonds, creating a higher cross-linked structure on the nanoparticle surface; (iv) the positive ζ potential values measured for all chitosan and thiolated chitosan coated nanoparticles were due to the cationic nature of chitosan. The absolute value, which decreased by increasing the content of chitosan-TBA, can be related to the increased viscosity in the nanoparticle shell as consequence of cross-linkage.

3.2. Evaluation of the permeation of [^{14}C]mannitol in presence of nanoparticles

In vitro permeability studies are relevant approaches to evaluate the absorption enhancing effect of a colloidal drug carrier systems of the intestinal tissue [2,37]. They offer

Table 1
Nanoparticles characterization: mean hydrodynamic diameter (D_H); polydispersity index (PI); surface charge (ζ potential); total sulphur content measured by elemental analysis and reduced thiol groups content to the nanoparticles surfaces obtained by iodine titration

Chitosan (g/mol)	Chitosan/chitosan-TBA	D_H (nm)	PI	ζ Potential (mV)	Total sulphur (%w/w)	Thiol groups (μmol SH/cm^2)	% of total sulphur
20,000	100/0	181	0.110	+41.2 ± 0.9	nd	nd	nd
	75/25	210	0.161	+37.0 ± 0.7	0.19	114×10^{-6}	50
	50/50	360	0.139	+36.3 ± 0.7	0.52	127×10^{-6}	11
	25/75	463	0.162	+36.0 ± 0.4	0.77	170×10^{-6}	8
	0/100	491	0.200	+26.9 ± 0.8	0.99	209×10^{-6}	7
50,000	100/0	240	0.029	+40.9 ± 0.7	nd	nd	nd
	75/25	249	0.181	+31.5 ± 0.5	0.24	53×10^{-6}	14
	50/50	465	0.125	+37.1 ± 0.7	0.54	107×10^{-6}	7
	25/75	548	0.086	+35.2 ± 0.8	0.75	206×10^{-6}	7
100,000	100/0	509	0.133	+51.2 ± 0.9	nd	nd	nd
	75/25	512	0.091	+53.0 ± 0.3	0.18	104×10^{-6}	27
PIBCA cores	0/0	260	0.088	−17.7 ± 0.3	nd	nd	nd

nd, non-detected.

many advantages over *in vivo* studies: they allow more rapid determinations, need less number of animals, and generally involve simpler analytical procedures easier to standardize [35]. However, there is one restriction that must be considered, concerning the viability and integrity of the tissue [35]. The feature of Ussing-like chambers is that they allow the monitoring of the spontaneous transepithelial electrical potential difference and the short-circuit current across the membrane, assessing viability of tissues during the experiment [2,7]. In addition, the transepithelial electrical resistance (TEER), deduced from those parameters [35], decreased when tight junctions are opened, so TEER determination can be used as indirect measurement of the paracellular absorption enhancement induced by pharmaceutical formulations [38–41].

Similarly, low molecular weight marker such as mannitol is routinely employed for the evaluation of paracellular permeability [35,36,40]. Metabolically inert and highly hydrophilic in nature, it is an ideal substance to evaluate the permeability of the intestinal tissue through the alternative aqueous paracellular pathway [6,39,41].

Figs. 2 and 3 present changes in TEER % induced by the incubation of intestinal tissue with the different nanoparticle formulations and chitosan solutions. The apparent permeability coefficient of [^{14}C]mannitol and the absorption enhancement ratio are listed in Table 2.

As control experiments, chitosan and thiolated chitosan solutions were tested at the same concentration than those found in the nanoparticle suspension. Compared to the blank experiment (Table 2), the permeation enhancement ratio (R) values, $R = 3.3$ and $R = 2.0$, obtained with chitosan and thiolated chitosan, respectively, indicated that the paracellular transport was improved in the presence of these polysaccharides in the soluble form. The data were confirmed by the electrical parameters, showing a significant decrease of TEER values with both products, especially in the first hour of incubation (Fig. 2). Other authors have observed higher permeation for thiolated chitosan than for the unmodified polymer by Ussing-type techniques. The discrepancy observed with the results obtained for Chito50 and Chito50-TBA solutions in this work could be explained by the different experimental conditions used. For example, the use of higher polymer molecular weight, the co-administration of permeation mediators (glutathione) with thiolated polymers, the use of thiolated polymers with different disulphide bond proportions, etc. [22,42].

Concerning the nanoparticles behaviour, a significant increase in paracellular permeability was also observed for all thiolated chitosan coated formulations evaluated, according to TEER (Fig. 3) and R values (Table 2), which is in agreement with the behaviour observed for chitosan-TBA solutions (Fig. 2 and Table 2). On the contrary, the non-modified chitosan coated nanoparticles did not show

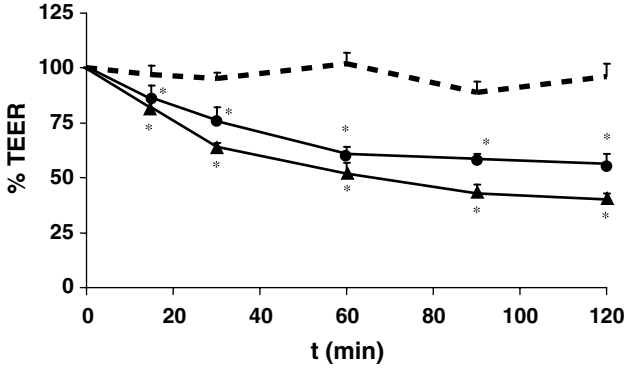

Fig. 2. Effect of chitosan solutions (0.2%) on the TEER of jejunum mucosa of Wistar rats mounted in Ussing chambers at pH 6.0. Each point represents mean ± SD of four experiments. Keys: reference blank solution (dotted line); Chito50 (▲); Chito50-TBA (●). *Significant differences ($P < 0.05$) with the control blank solution.

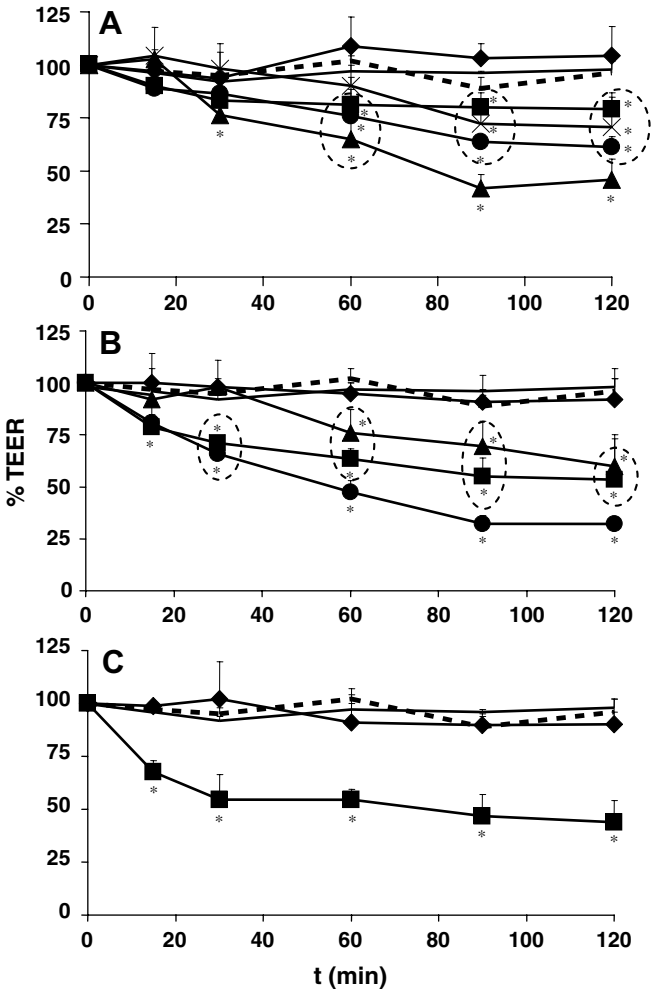

Fig. 3. Effect of different nanoparticle formulations on the TEER of jejunum mucosa of Wistar rats mounted in Ussing chambers at pH 6.0. Each point represents mean ± SD of four experiments. (A) Nanoparticles elaborated with Chito20; (B) Nanoparticles elaborated with Chito50; (C) nanoparticles elaborated with Chito100. Keys: control blank solution (dotted line); non-coated PIBCA nanoparticles (black line); chitosan/chitosan-TBA = 100/0 (♦); 75/25 (■); 50/50 (●); 25/75 (▲); 0/100×. *Significant differences ($P < 0.05$) with the control blank solution. Dotted circles represent groups of values statistically similar.

Table 2
Apparent permeability coefficient (P_{app}) and absorption enhancement ratio (R) obtained for tested formulations (mean ± SD, $n = 4$)

Additives in the donor chamber	Chitosan Mw (g/mol)	Chitosan/ chitosan-TBA	P_{app} (cm/s)	Absorption enhancement ratio (R)
Blank			$3.0 \pm 0.5 \times 10^{-6}$	1
PIBCA core nanoparticles			$3.4 \pm 0.8 \times 10^{-6}$	1.1
Chitosan solution	50,000	100/0	$10.0 \pm 0.4 \times 10^{-6}$	3.3
Chitosan-TBA solution	50,000	0/100	$6.0 \pm 0.5 \times 10^{-6}$	2.0
PIBCA coated nanoparticles	20,000	100/0	$3.8 \pm 0.6 \times 10^{-6}$	1.3
		75/25	$4.4 \pm 0.7 \times 10^{-6}$	1.5
		50/50	$5.4 \pm 0.8 \times 10^{-6}$	1.8
		25/75	$6.7 \pm 0.5 \times 10^{-6}$	2.2
		0/100	$5.2 \pm 0.8 \times 10^{-6}$	1.7
	50,000	100/0	$3.3 \pm 0.9 \times 10^{-6}$	1.1
		75/25	$6.2 \pm 0.8 \times 10^{-6}$	2.1
		50/50	$6.6 \pm 0.8 \times 10^{-6}$	2.2
		25/75	$5.7 \pm 0.8 \times 10^{-6}$	1.9
	100,000	100/0	$3.6 \pm 0.4 \times 10^{-6}$	1.2
		75/25	$7.8 \pm 0.9 \times 10^{-6}$	2.6

significant decrease in TEER values during the 2 h of the study (Fig. 3, diamonds), obtaining P_{app} values in the same range as for the control experiment and for the experiment performed with non-coated PIBCA nanoparticles (Table 2).

To explain the effect of polymeric nanoparticles on the paracellular permeability two hypotheses could be assumed: (*hypothesis I*) Mobile polysaccharide chains bearing in the nanoparticles surface could directly interact with proteins of the tight junction, changing the protein conformation and then widening the paracellular pathway and/or (*hypothesis II*) nanoparticles immobilized in the vicinity of the epithelium could deplete specific factors such as calcium, in this microenvironment, thus contributing to promote the opening of the tight junctions.

As can be seen in Fig. 3 and Table 2, the thiolated formulations that induced the highest permeation enhancements were those prepared with intermediate percentage of Chitosan-TBA: 75% and 50% of Chito20-TBA, 50% of Chito50-TBA and 25% of Chito100-TBA. Interestingly, these formulations were those that showed simultaneously lower bioadhesive properties, compared to non-modified chitosan PIBCA nanoparticles, after incubation with rat intestinal surface under static conditions [26]. Such a decrease has been shown elsewhere to be due to the formation of a cross-linked structure of the thiolated chitosan nanoparticle shell that might limit the occurrence of strong interactions (electrostatic and covalent) with the intestinal mucus [26]. In fact, the relative amount of TBA linked to the chitosan chains can be regarded as a key factor, which can modulate the structure and the conformation of chitosan chains constituting the shell formed at the surface of the particles, as a large amount of TBA will increase intra- and/or inter-chain bonding and finally restrain the polysaccharides chains mobility. As a consequence, the explanation of the high permeation enhancement observed for those formulations in the present study could be related with their mucoadhesion behaviour through *hypothesis I* as follows: once in contact with the mucus, only a fraction of the nanoparticles interacted strongly with mucins being immobilized in the gel layer. The other fraction of nanoparticles was not attached to the mucus, which was higher for the thiolated formulations for the reason mentioned above [26], and could then diffuse within the mucus layer [43]. Those nanoparticles could then reach the vicinity of the tight junction proteins, improving the paracellular permeability. This hypothesis would be also coherent with the fact that non-modified chitosan coated nanoparticles, which were the most bioadhesive of the series [26], do not increase the paracellular pathway (Fig. 3 and Table 2).

It is interesting to remark that, in the special case of non-modified chitosan, other authors have also reported an important discrepancy in the polymer permeation enhancement capacity between chitosan solutions and nanoparticles even in absence of mucus, using the Caco-2 cells monolayer model, which was explained by a restriction in the movement of the polysaccharide chains, hindering an easy contact of the chains with cell membranes and tight junctions [5].

It seems that a direct contact with intestinal membranes might play an important role in the development of permeation behaviour by the nanoparticles evaluated in this work, which would support *hypothesis I*. However, a moderated calcium binding capacity has been also observed for those chitosan and thiolated chitosan coated nanosystems [44], which could be in favour of *hypothesis II* for explaining the enhancement of paracellular transport. To evaluate the contribution of this second hypothesis and to try to confirm the first one, a second series of *permeation experiments* was performed in conditions *avoiding bioadhesion of the nanoparticles on the mucus*, by interposing a semi-permeable membrane between the tested formulation and the intestinal tissue (Fig. 1B).

The relevance of the method needed first to be evaluated to check that the artificial membrane did not modify the [^{14}C]mannitol permeability. In a preliminary experiment,

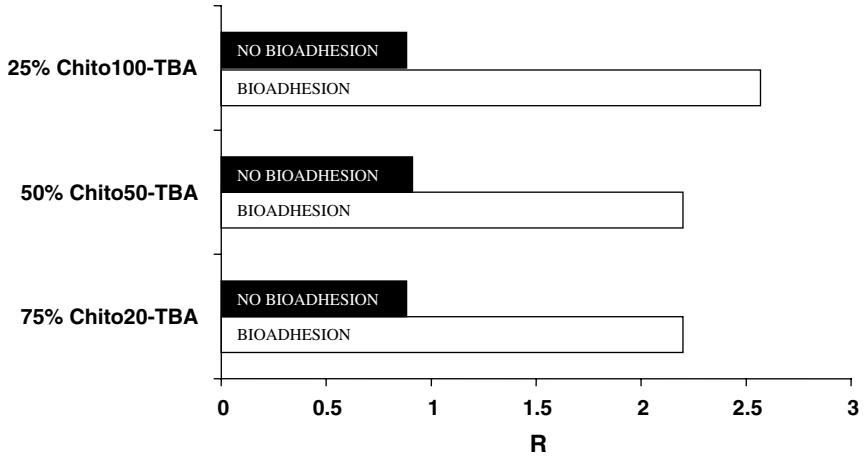

Fig. 4. Comparison of absorption enhancement ratio (R) obtained for nanoparticle formulations allowing (white bars) and avoiding (black bars) direct contact with intestinal mucosa.

permeability studies were carried out only with the artificial membrane alone and with both artificial and intestinal membrane. The apparent permeability coefficients were $(105.0 \pm 4.0) \times 10^{-6}$ cm/s and $(3.3 \pm 0.6) \times 10^{-6}$ cm/s, respectively. Considering Eq. (4), this result showed that there was no effect of the artificial membrane on the permeability of the marker across the intestinal mucosa, which corroborated the feasibility of these modified permeation assay experiments.

$$1/P_{app}(\text{artificial} + \text{natural}) = 1/P_{app}(\text{artificial}) + 1/P_{app}(\text{natural}) \quad (4)$$

The method was applied with the nanoparticles having showed the higher permeation enhancing capacity in the previous experiments expressed as high R values (Table 2). These were elaborated with 75% of Chito20-TBA; with 50% of Chito50-TBA and with 25% of Chito100-TBA. [^{14}C]Mannitol permeability dropped to values of $3.0 \pm 0.5 \times 10^{-6}$ cm/s, $3.1 \pm 0.5 \times 10^{-6}$ cm/s and $3.0 \pm 0.7 \times 10^{-6}$ cm/s, respectively. R values obtained for these formulations have been presented in Fig. 4.

So, according to these second set of experiments, even if thiol and amino groups remained on nanoparticles shell able to bind calcium (which could easily diffuse through the artificial membrane), this event did not lead to any permeation enhancement, undoubtedly showing that the absorption enhancing effect of the nanoparticles was intimately related to their capacity to adhere on the mucosa, and hence confirming *hypothesis I* as the main mechanism involved in the permeation enhancement effect observed for the thiolated chitosan coated nanoparticles evaluated in this work.

4. Conclusions

A moderated permeation enhancement effect was detected for thiolated chitosan coated PIBCA nanoparticles. From the whole results presented in this work, this behaviour could be explained by a direct contact of nanoparticles with tight junctions (*hypothesis I*). The following sequence of events can be proposed: (i) The presence of chitosan or thiolated chitosan on the surface of nanoparticles promoted their bioadhesion on the mucus layer covering the intestinal epithelium. (ii) The cross-linked structure of the thiolated chitosan nanoparticle shell might limit the occurrence of strong interactions with mucus, leaving particles still able to diffuse through the mucus gel and hence allowing them to better interact with epithelial cells. (iii) At this level, the combination of both, the remaining cationic nature of the particle surface and the presence of thiol groups, could promote the opening of the tight junctions.

Although many parameters (gastrointestinal environment from site to site, peristaltic movements, mucus turnover, etc.) may compromise extrapolations of data obtained *in vitro* to the *in vivo* conditions, the permeation enhancing properties observed for the thiolated nanoparticles evaluated in this work make them interesting candidates that could be effective to improve peptides bioavailability after oral administration.

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