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Mucoadhesive properties of low molecular weight chitosan- or glycol chitosan- and corresponding thiomer-coated poly(isobutylcyanoacrylate) core-shell nanoparticles

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List of abbreviations

PACA	Poly(alkylcyanoacrylate)
PIBCA	Poly(isobutyl cyanoacrylate)
CS	Chitosan
CS-GSH	Chitosan Glutathione conjugate
CS-NAC	Chitosan *N*-acetyl-cysteine conjugate
CSGSH-P-NPs	Chitosan glutathione coated poly(isobutyl cyanoacrylate) nanoparticles
CSNAC-P-NPs	Chitosan *N*-acetyl-cysteine coated poly(isobutyl cyanoacrylate) nanoparticles
CS-P-NPs	Chitosan coated poly(isobutyl cyanoacrylate) nanoparticles
CS-TBA	Chitosan-4-thiol-butylamidine
GCS	Glycol chitosan
GCS-GSH	Glutathione conjugate
GCS-NAC	N-acetyl-cysteine conjugate
GCS-GSH	Glycol Chitosan Glutathione conjugate
GCS-NAC	Glycol Chitosan *N*-acetyl-cysteine conjugate
GCS-P-NPs	Glycol chitosan coated poly(isobutyl cyanoacrylate) nanoparticles
GCSNAC-P-NPs	Glycol chitosan *N*-acetyl-cysteine coated poly(isobutyl cyanoacrylate) nanoparticles
GCSGSH-P-NPs	Glycol chitosan glutathione coated poly(isobutyl cyanoacrylate) nanoparticles
Isc	Short-circuit current
LMW	Low Molecular Weight
M%/cm^2	The percentage of NPs stuck on 1 cm2 of the mucosal surface
NPs	nanoparticles
PD	Transmucosal potential difference
PDI	Polydispersity index
QELS	Quasi-elastic light scattering
TEER	Transmucosal electrical resistance
TEM	Transmission electron microscopy

Abstract

The aim of the present work was to evaluate the mucoadhesive properties of poly(isobutyl cyanoacrylate) (PIBCA) nanoparticles (NPs) coated with Low Molecular Weight (LMW) chitosan (CS)- and glycol chitosan (GCS)-based thiomers as well as with the corresponding LMW unmodified polysaccharides. For this purpose, all the CS- and GCS-based thiomers were prepared under simple and mild conditions starting from the LMW unmodified polymers CS and GCS. The resulting NPs were of spherical shape with diameters ranging from 400 to 600 nm and 187 to 309 nm, for CS- and GCS-based NPs, respectively. The mucoadhesive characteristics of these core shell NPs were studied in Ussing chambers measuring the percentage of NPs stuck on the mucosal of fresh intestinal tissue after 2 h of incubation. Moreover, incubation of nanoparticle formulations with the intestinal tissue induced changes in transmucosal electrical resistance which were measured to gain information into the opening of tight junctions and to control the integrity of the mucosa. Thus, it was found that PIBCA NPs coated with the GCS–Glutathione conjugate (GCGPIBCA NPs) possessed the most favorable mucoadhesive performances. Moreover, both GCGPIBCA- and GCS-*N*-acetyl-cysteine (GCNPIBCA)-core-shell NPs might induced an enlargement of the epithelial cell tight junctions. In conclusion, coating of PIBCA NPs with GCS-based thiomers may be useful for improving the mucoadhesive and permeation properties of these nanocarriers.

Keywords: Mucoadhesion, Thiomers, Ussing chambers, Chitosan, Glycol Chitosan

Introduction

Mucoadhesive polymers have been extensively studied over the past of decades because they are able to interact with mucus and remain on mucosal tissue for extended period of time [1-4]. Therefore, associated drugs can efficiently accumulate in the mucus near absorption sites creating favorable conditions to enhance their absorption hence their bioavailability [5]. The physiological function of the mucus is to protect the underlying epithelia from the luminal content as well as to catch nutrients to be absorbed [6]. This viscous liquid is mainly constituted by glycoproteins ("mucins") composed of a protein backbone in which carbohydrate chains are grafted. At the pH of the gut, these side chains are highly negatively charged.

Most of polymers that interact with mucins found in the gut are hydrophilic and positively charged in the gut environment. Among these, chitosan (poly[β-(1-4)-2-amino-2-deoxy-D-glucopyranose]) (CS) is a cationic polysaccharide obtained by deacetylation of chitin, which is the most abundant polysaccharide in nature, after the cellulose. CS is a hydrophilic, biodegradable and biocompatible polymer that possesses hydroxyl and amine groups that can give rise to hydrogen bonding-mediated interactions with components of the mucus. These properties together with the polycationic nature, make the CS a good mucoadhesive polymer [7]. In fact, the positive charges on the chain of CS can interact with sialic and sulfonic acids of the mucus layer through strong electrostatic interactions. In general, the solubility of CS is rather poor in aqueous media including biological fluids. Various strategies were developed to increase the solubility of CS in these media. For instance, glycol chitosan (GCS) that can be obtained adding hydrophilic ethylene glycol groups on CS, is a typical water soluble CS derivative at neutral and acidic pH [8-10]. Reducing the molecular weight of CS based on depolymerization reaction is another strategy that was proposed to improve the solubility of CS at neutral pH [11].

Mucoadhesive properties of CS can also be strongly enhanced incorporating free thiol groups in the polysaccharide structure. This can be done by grafting free thiol group containing molecules such as thioglycolic acid [12], 2-iminothiolane[13], thioethylamidine [14], *N*-acetylcysteine (NAC) [15] or glutathione (GSH) [16] along the chitosan leading to a class of mucoadhesive polymers denoted as thiomers. For instance, the covalent attachment of thioglycolic acid and 2-iminothiolane improved the mucoadhesion of the corresponding thiomers by 10 and 140 times in comparison to the mucoadhesion shown by the parent chitosan. The mechanism that was proposed to explain the observed improvement in

mucoadhesion of the thiomers is based on the formation of disulfide bonds between thiol groups of the thiomer and thiol groups of cysteine residues found on mucus glycoproteins [17]. In addition to their mucoadhesive properties, chitosan derivatives including GCS-NAC (400 kDa) and GCS (400 kDa) were reported to inhibit the activity of efflux pump proteins like the P-gp occurring in most mucosal tissue including the gut [10, 18]. These efflux pumps were identified to participate to the poor bioavailability of numerous drugs from oral dosage [18]. In these cases, inhibition of the P-gp generally enhances drug absorption as reported considering important anticancer agents such as Paclitaxel [19].

In previous studies, it has been shown that poly(alkylcyanoacrylate) (PACA) nanoparticles (NPs) are able to encapsulate peptides, proteins [20-23] and anticancer agents [19]. However, due to the hydrophobic character of PACA as well as to the polyanionic nature of the corresponding NPs, their mucoadhesive potential is generally low and therefore they are of limited usefulness for transmucosal delivery. The mucoadhesion of these NPs, was enhanced coating them with CS and thiolated CS such as CS-4-thiol-butylamidine (CS-TBA) [24]. In addition to an improved mucoadhesion that was attributed to the formation of disulfide bonds between thiol groups of the thiomer and thiol groups found on mucin chains, a permeability of the gut epithelium through the paracellular route was also reported [25]. In this context, it appeared of interest to compare the fate and properties of different (thiomer-coated) poly(isobutyl cyanoacrylate) (PIBCA) core-shell NPs on native fully functioning intestinal mucosa. It with the aim to improve their drug delivery efficacy by the oral route. More precisely, the designed coatings were constituted by Low Molecular Weight (LMW) chitosan (CS)- and glycol chitosan (GCS)-based thiomers as well as by the corresponding LMW unmodified polysaccharides taking into account the favorable effect on the solubility of CS at neutral pH [11] due to the reduction of its molecular weight. In such a way not only GCS and derived polymers but also the corresponding CS based polymers should be endowed with adequate solubility at neutral and physiological conditions. Thus, our working hypothesis was that these polymers could confer to the nanoparticles interesting mucoadhesive properties with potential to increase mucoadhesion and permeability of the gut intestine in physiological conditions.

2. Materials and methods

2.1 Materials

Chitosan (CS) (low molecular mass: 20 kDa; degree of deacetylation: 92%) was purchased from Amicogen.inc (Jinju, South Korea). Glycol chitosan (GCS), *N*-acetyl-L-cysteine (NAC), L-Glutathione reduced form (GSH), Rhodamine B, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), N-Hydroxysuccinimide (NHS) were all purchased by Sigma-Aldrich (St. Louis, MO, USA).

2.2 Methods

2.2.1 Purification of chitosan

One gram of CS was purified by dissolution in MilliQ® water (50 mL) and the mixture was stirred at room temperature for 15 min. Then, the polymer solution was purified by dialysis (molecular weight cutoff 3500 g/mol), twice (2 h, overnight) against 1L of 1 mM hydrochloric acid solution .

2.2.2 Depolymerization of glycol chitosan

The depolymerization of GCS was developed following the procedure proposed by Knigh et al. [26]. Briefly, 500 mg of GCS were dissolved in 12.5 mL of acetic acid solution (6% v/v) and stirred for 2 h at room temperature in the presence of 2.5 mL of sodium nitrite solution (11 g/L). The polymer solution was purified by dialysis in tubing (molecular weight cutoff 3500 g/mol), twice during 1 h and once overnight against 1 L of distilled water. After purification, the purified reaction mixture was lyophilized using an Alpha 1-2 LD plus lyophilizator (Bioblock Scientific instrument).

2.3 Synthesis of chitosan and glycol chitosan conjugates

The CS-conjugates with *N*-acetyl-L-cysteine (CSNAC) and L-Glutathione (CSGSH) were obtained adapting the methods described by Bernkop-Schnürch *et al.* with CS at high molecular weight (HMW) [15,16,18]. Briefly, to prepare CSNAC, 150 mg of CS were dissolved in 10 mL of MilliQ® water. In another flask, 150 mg of NAC were dissolved in 10 mL of MilliQ® water. The carboxylic group of NAC was activated by addition of 1.37 mmol of EDAC followed by 20 min reaction. Then, the two preparations were combined and stirred at room temperature for 7 h.

The preparation of CSGSH was carried out following the above described procedure for CSNAC with a little modification. The carboxylic group of GSH was activated by addition of

both EDAC (2.13 mmol) and NHS (1.78 mmol). The reaction was allowed to proceed for 20 min. Both CSNAC or CSGSH were purified by dialysis in tubing (molecular weight cutoff 3500 g/mol) at room temperature. The dialysis was performed with the following sequence of counter dialyzing media: MilliQ® water for 24 h, EDTA (0.2 µM) solution for 24 h, and MilliQ® water for 24 h. Purified CSGSH was recovered after freeze-drying of the dialysate using an Alpha 1-2 LD plus lyophilizator (Bioblock Scientific instrument).

LMW-GCSNAC and -GCSGSH were prepared adapting the methods described by Trapani *et al.* [10] for GCS at high molecular weight.

2. 4 Preparation of PIBCA nanoparticles

NPs were prepared according to the redox radical emulsion polymerization method developed by Chauvierre *et al.* [27-29]. Briefly, the appropriate polysaccharide (130 mg) was dissolved in HNO_3 0.2 M (8 mL) at 42°C under vigorous stirring and argon atmosphere. Once the dissolution was completed, ammonium cerium (IV) nitrate (8×10^{-2} M) in HNO_3 0.2 M (2 mL) and IBCA (0.5 mL) were added, the argon flux was stopped and the reaction was allowed to continue at 42°C for another 50 min. Then, the nanoparticle suspension was purified by dialysis (cutoff 100.000 g/mol, Spectra/Por membranes) three times against HCl at pH 2.54 for 1 h and once overnight. The suspensions of PIBCA NPs coated with CS (CS-P-NPs), GCS (GCS-P-NPs), CSNAC (CSNAC-P-NPs), CSGSH (CSGSH-P-NPs), GCSNAC (GCSNAC-P-NPs) and GCSGSH (GCSGSH-P-NPs) (Figure 1) were stored under argon atmosphere until further use.

[Insert Figure 1]

The NPs were labeled with Rhodamine B as follows. Rhodamine B was dissolved in CH_3CN to give a solution of 4 mg/mL and, together with IBCA, added to the mixture containing the polysaccharide in HNO_3 above mentioned. The polymerization reaction in the presence of Rhodamine B was carried out in the dark and working up as above mentioned, the required labeled NPs were obtained.

All the NPs prepared were stored in HCl at pH 2.5, in absence of air and in dark vials in order to avoid aggregation and oxidation of thiol groups of the modified chitosan.

Hydrodynamic mean diameter, d, and size distribution, PDI, of the NPs were determined by quasi-elastic light scattering (QELS) using a Zetasizer Malvern SZ90 (Malvern Instrument, Orsay, France) and fixing the scattered angle at 90°. Samples were diluted in MilliQ® water

until a concentration of 0.35 mg/mL. Results were expressed as the mean hydrodynamic diameter, and polydispersity index (PDI) of the size distribution.

The ζ potential was deduced from the electrophoretic mobility of the particles measured by Laser Doppler Electrophoresis (Zetasizer Malvern SZ90, Malvern Instrument, Orsay, France) in a NaCl solution (1 mM) after suitable dilutions (1/100 (v/v)) of the different nanoparticle suspensions. The ζ potentials were evaluated at two pH values *i.e.*, 2.5 and 7.0.

2. 5. Morphology of the nanoparticles

The morphology of the NPs was investigated by transmission electron microscopy (TEM). The nanoparticle aqueous dispersion (50 µL) was dropped on the grid and left to dry. Then nanoparticles were stained with a solution of phosphotungstic acid at 1% (pH 7.3) for 5 minutes. Electron micrographs were acquired using an electron microscope JEOL 1400 MET operating at 80 kV (Electron Microscopy Facility of I2BC, CNRS, Gif sur Yvette, France, http://www.cgm.cnrs-gif.fr/spip.php?article282&lang=fr) equipped with a high-resolution digital CCD Gatan digital camera (11 megapixels). The nanoparticles were characterized by measuring their long and short axis.

2. 6 . Quantification of thiol groups content by iodometric titration

The total amount of SH-groups of CS- and GCS-P-NPs was measured by iodometric titration [24,30]. Briefly, an aliquot of NPs dispersion (2.5 mL) was incubated with 250 µL of acetate buffer (pH 2.7), 300 µl of a solution of iodine (1 mmol/L) and 500 µL of a solution of starch (4 %). The iodine solution was prepared at 0.1 M concentration by dissolving 0.63 g of I_2 and 1.93 g of KI in 25 mL of MilliQ® water, stored protected from light and diluted before the assay. The mixture was left at room temperature for 24 h protected from light. Then, the samples were ultra-centrifuged (15 min, 30,000 rpm) to allow the sedimentation of NPs and the absorbance of the supernatant was measured at a wavelength of 570 nm. The amount of thiol groups was calculated from the calibration curve obtained using solutions of cysteine-HCl at known concentrations (20-70 mmol/L). Iodine promotes the oxidation of thiol groups, while the excess of iodine reacts with starch to form a blue complex, the intensity of which depends on the amount of iodide remained in solution.

2. 7. Mucoadhesion ex vivo assays

Ex vivo experiments implied the collection of tissues from animals. All experiments that involved the use of animals were performed following a protocol that was approved by the ethic comity of the Ministry of Research and Superior Education in France (CEEA 26).

The mucoadhesive properties of the thiolated NPs were determined in Ussing-chambers employing the surface of fresh intestinal tissue. Electrical parameters were also recorded to determine the tissue viability and the opening of tight junctions during the assay.

The mucoadhesion experiments were carried out according to protocols previously described [25]. Briefly, a jejunum portion isolated from fresh small intestine of sacrificed male Wistar rats (200–250 g) (Charles River, Paris) was excised, rinsed with Ringer buffer and cut into segments of 2–3 cm length. After visual examination of the tissue, sections of such jejunum portion were mounted in Ussing chambers (the intestinal surface tested was 1 cm^2), bathed with Ringer solution at 7.4, 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 (2 mL) of preheated (37 °C) Ringer solution containing the fluorescence nanoparticle dispersion (5 mg/mL). At scheduled time intervals, four aliquots of 200 µL were withdrawn from the donor and from the acceptor chamber (mucosal and serosal side, respectively) and replaced with the same volume of fresh medium pre-equilibrated at 37 °C. The tests were carried out for 120 min. At the end of the experiment (after 120 min), the tissue was collected and degraded with 1 mL of a lytic solution (Sodium dodecyl sulfate, SDS 2 % v/v and NaOH 1 % v/v in MilliQ® water) for 24 h at 37 ° C. The samples were analyzed measuring the fluorescence of Rhodamine B with a spectrofluorimeter (L550B spectrometer, Perkin Elmer , Norwalk, USA) , emitting at 575 nm and excitation at 555 nm and using CS- and GCS-P-NPs as controls. Four jejunum portions from four different rats were used to evaluate each formulation and the experiments were replicated on different days. The percentage of NPs stuck on 1 cm^2 of the mucosal surface ($M\%/cm^2$) was calculated using Eq. (1)

$$M\%/cm^2 = [F(m) - F(b)] / [F(t_0)] \times 100 \qquad \text{Eq. (1)}$$

where F (m) was the fluorescence of the tissue treated with the dispersion of NPs, F (b) was the fluorescence of the untreated intestinal tissue and F (t_0) was the fluorescence of the nanoparticle dispersion before it was placed in contact with the intestinal tissue (at time 0).

The values of M % were calculated between 0 and 120 min after addition of the fluorescent NPs in all experiments.

2.7.1 Measurement of electrical parameters.

In the course of the mucoadhesion experiments, the transmucosal electrical resistance (TEER) was measured using a four electrode system according to the protocol previously described [25]. Briefly, transmucosal potential difference (PD) was continuously monitored 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). Isc values were corrected for fluid resistance and recorded at scheduled times. The TEER was calculated from the Ohm's law (Eq. (2)):

$$TEER = PD/Isc \qquad \text{Eq (2)}$$

Only tissues showing PD > 2 x 10^3V and Isc > 40 x 10^{-6} A/cm^2 after 30 min incubation were included in the study. At the end of the experiment, a further control was performed by adding 90 µL of a 10^{-3} M histamine Ringer buffer solution in the serosal compartment. Histamine increases Cl^- secretion by the cells with consequent enhancement of Isc. Whenever no increase in Isc was observed, damages in the tissue were suspected and all samples collected from the corresponding chambers were discarded.

2. 7.2. Quantification of attached nanoparticles to intestinal mucosa

The number of attached NPs to intestinal mucosa (N) was calculated from Eq (3):

$$N = M_T\, 6/\rho\pi d^3 \qquad \text{Eq (3)}$$

where M_T is the mass of both attached and non-attached NPs (g) incubated with intestinal mucosa, ρ is the NPs volumetric mass (1.2 g/cm^3 (24)), d is the NP hydrodynamic diameter (cm). M_T, in turn, was calculated taking into account the volume of the donor compartment of the Ussing-chamber (2 mL) and the nanoparticle dispersion concentration (5 mg/mL). By the percentage of NPs stuck on 1 cm^2 of the mucosal surface (*i.e.,* M%/cm^2, Eq. (1)) it was possible to calculate the number of attached NPs to the intestinal mucosa.

On the other hand, the number of theoretical layers of nanoparticles covering an apparent surface area of intestinal mucosa of 1 cm^2 may be calculate as suggested by Bravo-Osuna *et al* [24] using the following Eq (4):

Number of theoretical layers = Nd^2 Eq (4)

2. 8 Statistical analysis

Statistical comparisons were performed utilizing analysis of variance (ANOVA) followed by the Tukey post hoc tests (GraphPad Prism v. 4 for Windows, Graph-Pad Software, San Diego, CS). Differences were considered statistically significant at $p < 0.05$.

3. **Results and Discussion**

The aim of the present study was to compare the mucoadhesive properties of PIBCA NPs coated with LMW-CS and -CS thiomers with those of the corresponding PIBCA NPs coated with LMW-GCS and -GCS thiomers. Among the thiolate polymers currently known, our attention was focused on those modified with *N*-acetyl cysteine (NAC) and reduced glutathione (GSH). In addition to their interesting mucoadhesive properties, these thiomers are endowed with interesting permeation-enhancing and P-gp inhibitory characteristics [10,18]. For the mentioned purpose, the required NPs were prepared following the radical polymerization method described by Chauvierre *et al* [27-29]. Table 1 summarizes the hydrodynamic mean diameters of all the nanoparticles synthesized for this study. The mean hydrodynamic diameter of CS- and thiolated CS- coated NPs was about twice that observed for GCS-based NPs.

[Insert Table 1]

This result can be accounted for the average molecular weight of the starting unmodified CS. Depolymerization of GCS with HNO_2, indeed, leads to a significant reduction of molecular weight up to 7 kDa at pH ≤ 3 (26) which is quite lower than that of the CS used in this study (*i.e.*, 20 kDa). Consistently with data of the literature, the size of PIBCA NPs generated by radical emulsion polymerization synthesized in this work depended on the molecular weight of the polysaccharide [24, 28]. Moreover, the size of thiolated CS- and thiolated GCS-coated NPs (*i.e.,* CSGSH-P-NP, GCSGSH-P-NP) was increased by half of the size of the corresponding non thiolated nanoparticles (CS-P-NP, GCS-P-NP, respectively). Rhodamine B loaded NPs showed almost the same size of unloaded and the differences were not

significant (data not shown). Consistently with the polycationic nature of the material used to coat the nanoparticles, zeta potential of all nanoparticles were positive. The value depended on the type of polysaccharide and on the pH value of the nanoparticle dispersion. The highest values of zeta potential found in acidic pH agreed with a protonation of the polysaccharide at this pH. The PDI values observed for CS-P-NPs and GCS-P-NPs (*i.e.*, 0.05 and 0.04, respectively), are indicative of a quite homogeneous dispersion in size of these NPs, while a much more large size dispersion was observed for the other NPs since their PDI values were in the range 0.26-0.58. Thus, the PDI values clearly showed that NPs coated with thiomers were more hetero-dispersed than the corresponding unmodified parent polymer-coated NPs and this may be explained by a higher average molecular weight of the thiomers used.

The morphology of the NPs was studied by TEM. Electron micrographs indicated that the NPs are generally spherical in shape (Figure 2).

[Insert Figure 2]

The amount of free thiol groups on the surface of thiomer-coated PIBCA NPs was determined by iodometric titration using a calibration curve ($y = -0.0311x + 0.1004$ $R^2 = 0.981$) obtained with different concentrations of cysteine-HCl, and the results are also showed in Table 1. As can be seen, it was found that the amount of free thiol groups on the surface of GCS thiomer-coated PIBCA NPs was lower than the corresponding NPs coated with CS thiomers.

The mucoadhesive performances of the studied NPs were evaluated on mucosa of fresh intestinal tissue after 2 h of contact in Ussing-chambers. The *in vitro* mucoadhesive studies employing the Ussing-chamber technique are significant enough for evaluating the mucoadhesive potential of NPs and offer many advantages, including more bio-mimetic conditions, over other *in vitro* mucoadhesive tests. A distinct advantage of the Ussing chambers is that they allow the monitoring of the trans-epithelial electrical potential (TEER) of the intestinal tissues during the experiment [31]. Variation of TEER overtime indicates the opening of tight junctions while the viability and integrity of the mucosa was controlled. In general, control of the integrity of the tissue consists on addition of pump inhibitors at the end of the experiments inducing so a voluntary variation of TEER due to the increased permeability of the tissue by paracellular routes. Having controlled the integrity of the tissue at the end of the experiment, a variation of TEER observed in the present work could indicate that the formulation increased the permeability of the mucosa. Results obtained from mucoadhesive studies are shown in Table 2. In general, thiomer-coated NPs adhered in a

higher extend on the mucosal surface than the corresponding NPs coated with the non-thiolated parent polysaccharide. The GCSNAC-P-NPs seemed to display a different mucoadhesion compared with that monitored with GCS-P-NPs, the ANOVA demonstrated that there was no significant difference of interaction with the mucus between these GCS-based nanocarriers.

.

[Insert Table 2]

Despite the low surface thiol group content and the moderate positive zeta potential (*i.e.,*+31 ± 1 mV), GCSGSH-P-NPs exhibited the highest mucoadhesivity as demonstrated by the high percentage of NPs attached on the rat intestinal surface (*i.e.,* 76.3 %/cm^2). The results that are generally consistent with those of the literature were obtained in very different experimental conditions [25,30]. Indeed, here, mucoadhesion studies were performed in Ussing chambers in which the mucosa was placed in vertical position while it was surrounded by significant volumes of survival medium on both side of the mucosae. The nanoparticles added in the donor compartment were rather diluted and maintained under agitation while the design of the experimental conditions were designed to maintain the viability and functionality of the tissue. In contrast, in previous works evaluating mucoadhesion of chitosan/thiolated chitosan-coated PIBCA NPs, a small volume of the dispersion of nanoparticles was placed directly on the luminal side of the intestinal tissue mounted on the horizontal position in a device that only allowed to expose a well define surface area of the tissue to samples to be tested. In this model, no condition was provided to maintain the viability and functionality of the tissue. The mucoadhesion of the nanoparticles considered in the present work was evaluated from the calculation of the number of NPs attached to mucosa. Results were reported in Table 2. Besides, calculations were performed to estimate the number of theoretical NPs layers formed onto the 1 cm^2 mucosal apparent surface area. These calculations suggested that the nanoparticles adhered on the mucosa forming several layers. However, it is noteworthy that the apparent surface area that was taken in the calculation can underestimated the actual surface area available on the mucosa surface. Indeed, in the apparent surface area calculated from the geometry of the aperture separating the two compartments of the Ussing chamber the roughness of the mucosal surface created by villi were not taken into account. Nevertheless, this result indicated that a huge amount of nanoparticles adhered on the mucosa eventually covering the mucosa surface by the accumulation of several layers of nanoparticles. The nanoparticles that adhered the most were GCSGSH-P-NPs. Consistently

with data of the literature, the hydrodynamic diameter of the nanoparticles seemed to play an important role influencing the transport by diffusion phenomena through the mucus layer and hence the mucoadhesion properties of CS coated PIBCA NPs [24,32]. In Figure 3a, the amount of attached NPs has been plotted against the hydrodynamic mean diameter observed by QELS. Besides the superior mucoadhesive properties of GCSGSH-P-NPs, in the remaining cases, a negative correlation between the number of attached NPs and their hydrodynamic mean diameter was noted consistently with results already reported on thiolated chitosan-coated PIBCA nanoparticles [24,32]. In Figure 3b, the number of attached NPs was plotted against the content free thiol groups at the NPs surface. As shown, a negative exponential correlation between the number of attached NPs and their free thiol content was observed. Again, the highest number of NPs attached was noted for GCSGSH-P-NPs despite their low surface thiol group amount. These findings are also in agreement with data obtained by Bravo-Osuna *et al* [24] in the same range of the surface thiol group amount and at the same NPs concentration. As observed by these authors, in fact, a decrease in mucoadhesion occurred when the surface thiol group content was lower than 0.02 μmol SH/cm^2. Finally, since it was also suggested that enhanced electrostatic interactions take place between CS-coated PIBCA NPs and glycoproteins of mucus [24], it was of interest to examine the effect of ζ potential as function of the free thiol content at the NPs surface. However, as shown in Figure 3c, no clear correlation could be observed between the number of attached NPs and their surface charge, again according to that previously reported with CS-coated PIBCA NPs and evaluated by another method [24].

[Insert Figure 3]

Taken together, all these results indicated that, in the series herein examined, the highest mucoadhesion properties was observed with a hydrodynamic diameter of about 300 nm corresponding to the size of the GCSGSH-P-NPs. This finding agreed well with the results of previous studies suggesting that hydrodynamic diameters in the range 200-300 nm may be considered optimal for a satisfactory interaction with mucus chains [24,32]. Consequently, it may account for the lower mucoadhesive properties of CS-P-, CSNAC-P- and CSGSH-P-NPs characterized by larger hydrodynamic diameters (*i.e.*, 400-600 nm). In this context, however, it was surprising that the mucoadhesion of GCSNAC-P-NPs appeared much lower than that of GCSGSH-P-NPs despite the former nanocarriers were endowed with a size of 296 nm, a high zeta potential (+49 ± 1mV) and a higher concentration of thiol groups on the surface. Even though this aspect remains to be fully clarified, to explain the marked difference in

mucoadhesive properties between GCSNAC-P-NPs and GCSGSH-P-NPs, additional parameters should be considered including flexibility of the macromolecules, electrostatic interactions, molecular weight of the polymer chains as well as swelling, ionic strength and pH of the medium. In fact, according to the accepted mechanism for the mucoadhesion process [33], two sequential steps should be considered and namely, a first contact stage where a close contact between mucoadhesive polymer and mucus occurs. In the second one, *i.e.,* the so-called "consolidation stage", several physical (noncovalent) and chemical (covalent) interactions are established between the mucoadhesive polymer and mucus glycoproteins. In this consolidation stage, a chain interpenetration and entanglement takes place leading to strong enough adhesion. The chain flexibility, which is directly related to chemical structure and molecular weight of the polymer, is thought to be important in the consolidation stage since it allows the approach of binding groups and consequent interpenetration and entanglement of macromolecules. Thus, it can be hypothesized that introduction of GSH moieties on the GCS polymeric backbone constituting the shell of the corresponding PIBCA NPs leads to more flexible chains than those resulting from covalently linking NAC to GCS [16]. A further aspect to be taken into account refers to the fact that, introduction of GSH moieties, but not the NAC ones, on the GCS polymeric backbone can positively influence the water solubility of the coating polymer GCSGSH leading to its swelling with consequent improved flexibility and, hence, enhanced mucoadhesive properties.

Moreover, it should be also considered that, unlike GCSNAC, GSH moieties linked to GCS polymer contain ionizable carboxyl groups. Considering the pH of the buffer employed in the Ussing chamber experiments (*i.e.*, 7.4), these groups should be in dissociate form. Thus, they may be involved in electrostatic and non-covalent interactions with mucus components hence enhancing the mucoadhesion of the GCSGSH-P-NPs.

Besides the percentage of NPs stuck on the mucosal surface after 2 h of contact, it was also measured by fluorescence intensity both in the donor and in the acceptor compartment of the Ussing chamber in the presence of NPs. As shown in Figure 4, the fluorescence intensity was markedly reduced after 120 min in the donor compartment, while, in the acceptor compartment, the fluorescence was negligible (data not shown). This suggests that these NPs were stuck on the mucosal surface remaining firmly adherent to the surface of the intestinal lumen without crossing of the intestinal tissue

[Insert Figure 4]

The incubation of the different nanoparticle formulations with the intestinal tissue induced changes in TEER. In contrast to that observed with the CS- and thiolated CSs, when thiolated GCS-based coatings were used, the TEER was lower than that measured with the unmodified polymer after 120 min of incubation time. Figure 5 shows the TEER % changes induced by the different NPs formulations between 30 min and 120 min incubation time. The notable decrease of TEER values with GCSNAC-P-NPs and CSGSH-P-NPs between 60-120 min of incubation time, suggests an enhancement of the paracellular transport when these colloidal GCS-based carriers were employed. Hence, they can bring about a permeation effect since they induce an enlargement of epithelial cell tight junctions but the nanoparticles remained unable to go across the epithelium. Nevertheless, the increase of permeability of the tight junctions may be interesting to promote the absorption of poorly absorbed drug molecules that could then be absorbed through this route. It is in agreement with results reported by other authors who observed by Ussing-type techniques a permeation of thiolated CS higher than that observed for the unmodified polymer [34].

[Insert Figure 5]

A model can be proposed to explain the mucoadhesion of the nanoparticles developed in the present work and their permeation enhancing properties (Figure 6). In a first step, the nanoparticles penetrate in the hydrogel mucus thanks to convection and Brownian motions. Then, they are retained in the mucus due to the occurrence of electrostatic interactions and formation of di-sulfur bonds between the polymer chains of the nanoparticle shell and complemental groups found on mucin glycoproteins (Figure 6a). The particle size notably influence the penetration and diffusion step and we believe that the differences observed between the GCS/GCS thiomer-based NPs and the corresponding CS/CS thiomer ones were mainly due to differences in their respective hydrodynamic diameters. Finally, nanoparticles interacting the most with the mucosa, the GCSGSH-P- and GCSNAC-P-core-shell NPs, were able to increase the permeability of the paracellular route that involved the opening of epithelial cell tight junctions (figure 6b). This was possible assuming that the nanoparticles could deeply penetrate in the mucus layer thanks to their smaller size and could interact with proteins of the tight junctions of the underlying epithelium thanks to their thiol groups. The last type of interactions can induce conformational changes of the tight junction proteins leading to leakage of the tight junctions increasing the permeability of the epithelium through the paracellular route [16,17,34].

[Insert Figure 6]

Conclusions

Novel PIBCA NPs coated with thiol derivatives of LMW-CS, -GCS and corresponding unmodified parent polymers were successfully prepared and characterized. All these new colloidal systems showed mucoadhesive properties as evaluated by the Ussing-chamber technique. In this way, it was found that GCSGSH-P-NPs possessed the most favorable mucoadhesive performances. Data also showed that the mucoadhesive properties of all the polymers examined can be arranged in the following rank order: GCSGSH-P- >> GCS-P- ~ GCSNAC-P- > CS-P- > CSNAC-P-- ~ CSGSH-P-NPs. These results were interpreted on the basis of literature suggestions concerning both the mucoadhesion process of CS- and thiolated CS-PIBCA core shell NPs and the basic principles underlying the mechanism of mucoadhesion. Moreover, both GCSGSH-P- and GCSNAC-P-core-shell NPs showed an enhancement of the paracellular transport without crossing of the intestinal tissue. This effect may be an advantage from a toxicological point of view and it is probably due to the capability of the latter NPs to only enlarge the epithelial cell tight junctions as proved by TEER measurements. Therefore, LMW- GCSGSH-P- and GCSNAC-P-NPs may be considered new and interesting delivery systems for enhanced absorption of different kind of drugs including peptides, proteins and anticancer drugs by transmucosal administration, thanks to their mucoadhesion and permeation promoter effect on tight junctions.

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Captions to Figures

Figure 1. Schematic representation of the low molecular weight chitosan- or glycolchitosan- and corresponding thiomer-coated poly(isobutylcyanoacrylate) core-shell nanoparticles prepared in this study.

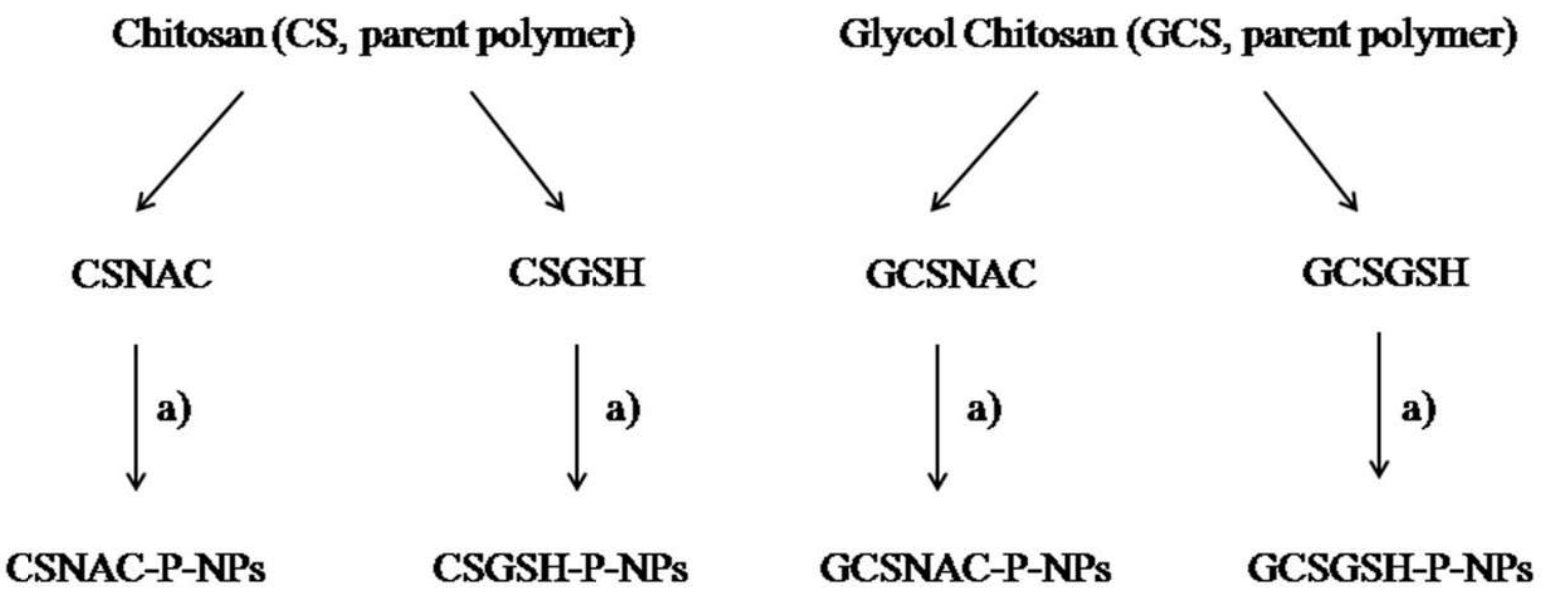
Figure 2. Transmission electron microscopy images of CS-P-NPs a), CSNAC-P-NPs b), CSGSH-P-NPs c), GCS-P-NPs d), GCSNAC-P-NPs e) GCSGSH-P-NPs f).

Figure 3. Effect of various parameters on the number of attached nanoparticles (5 mg/mL concentration) on intestinal mucosa in Ussing chambers. See Figure 2 for legend. a: nanoparticle hydrodynamic diameter b: free thiol content, c: nanoparticle ζ potential.

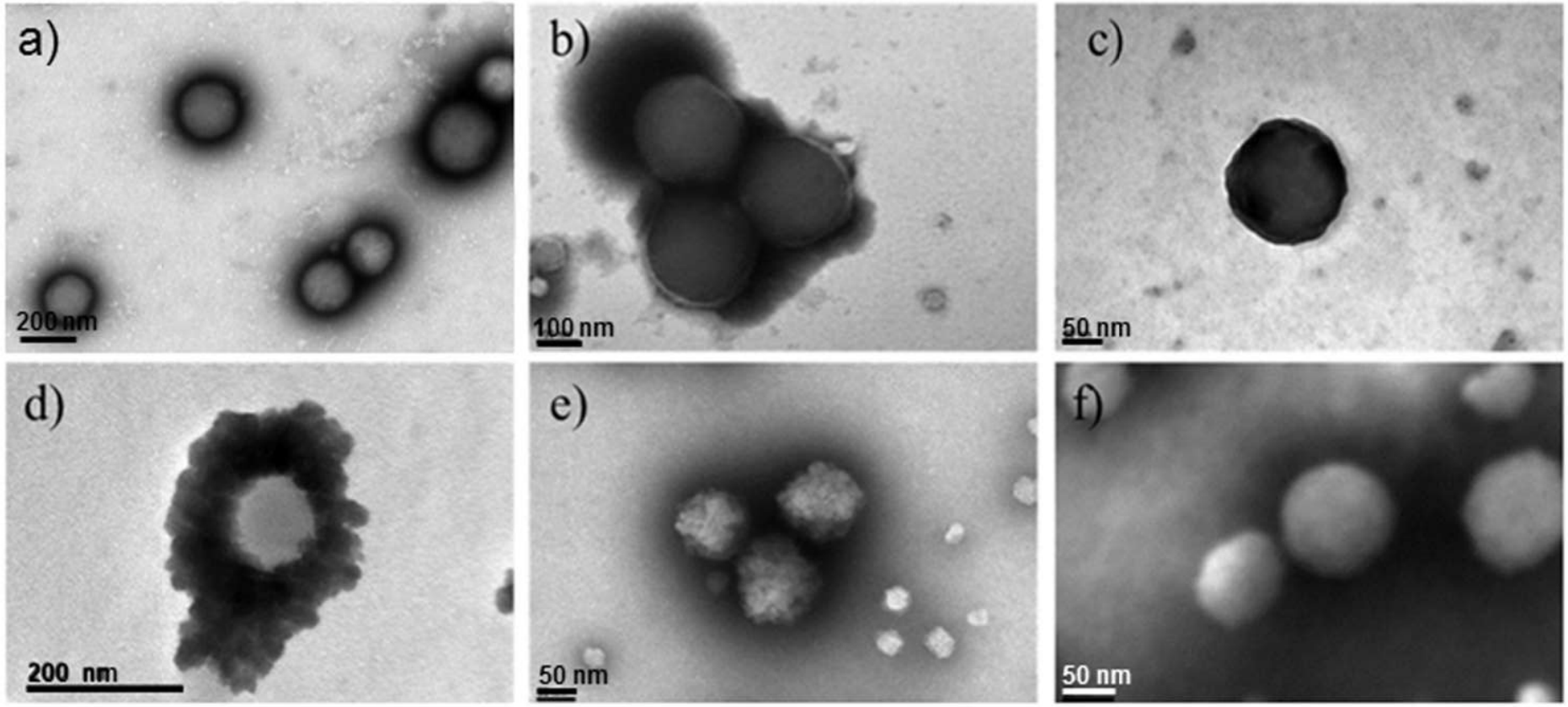
Figure 4. Variation in fluorescence in Ussing donor chamber in the presence of CSNAC-P-NPs, CSGSH-P-NPs, CS-P-NPs, GCSNAC-P-NPs, GCSGSH-P-NPs and GCS-P-NPs.

Figure 5. TEER % changes induced by the different NPs formulations between 30 min and 120 min incubation time.

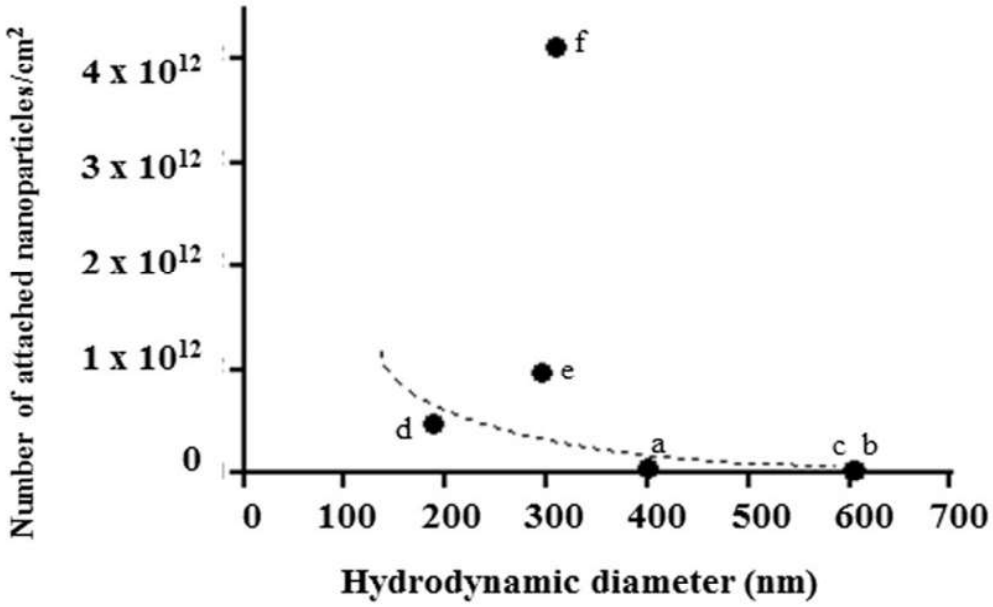
Figure 6. Schematic representation of the interaction of nanoparticles with the mucin glycoproteins in the *attachment* step of the mucoadhesion process (a) and interactions with the underlying epithelium (b).


Chitosan (CS, parent polymer)
Glycol Chitosan (GCS, parent polymer)
CSNAC
CSGSH
GCSNAC
GCSGSH
a)
a)
a)
a)
CSNAC-P-NPs
CSGSH-P-NPs
GCSNAC-P-NPs
GCSGSH-P-NPs

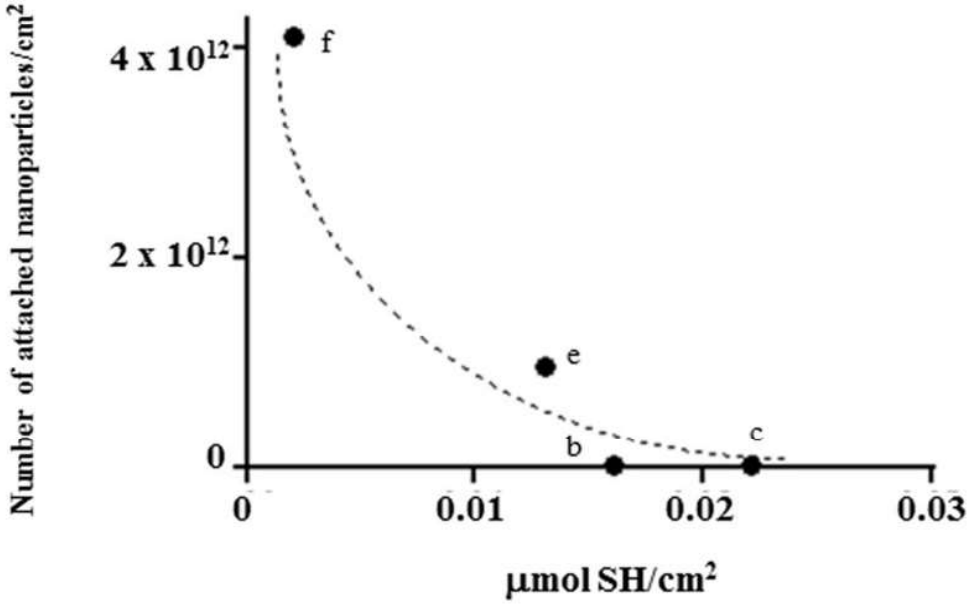
a) PIBCA (P)-NPs


a)
200 nm
b)
100 nm
c)
50 nm
d)
200 nm
e)
50 nm
f)
50 nm

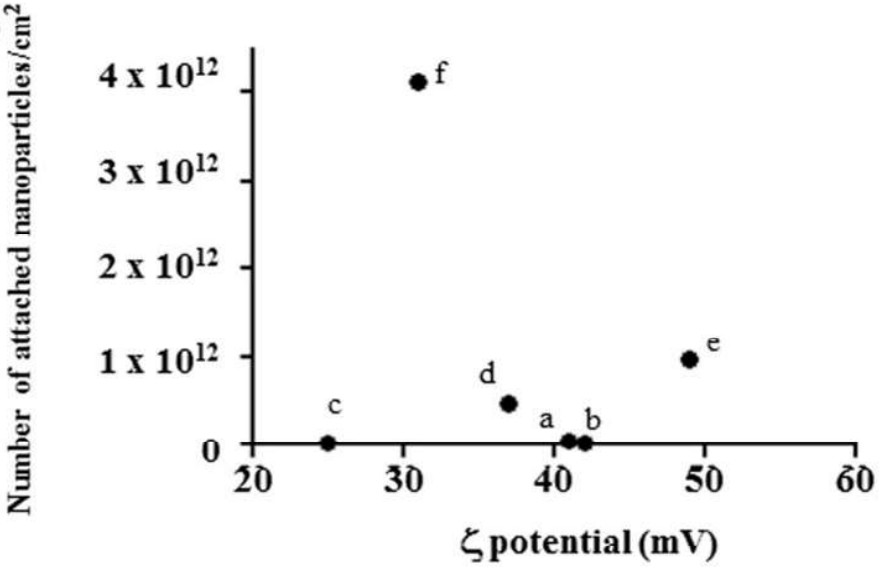
a)

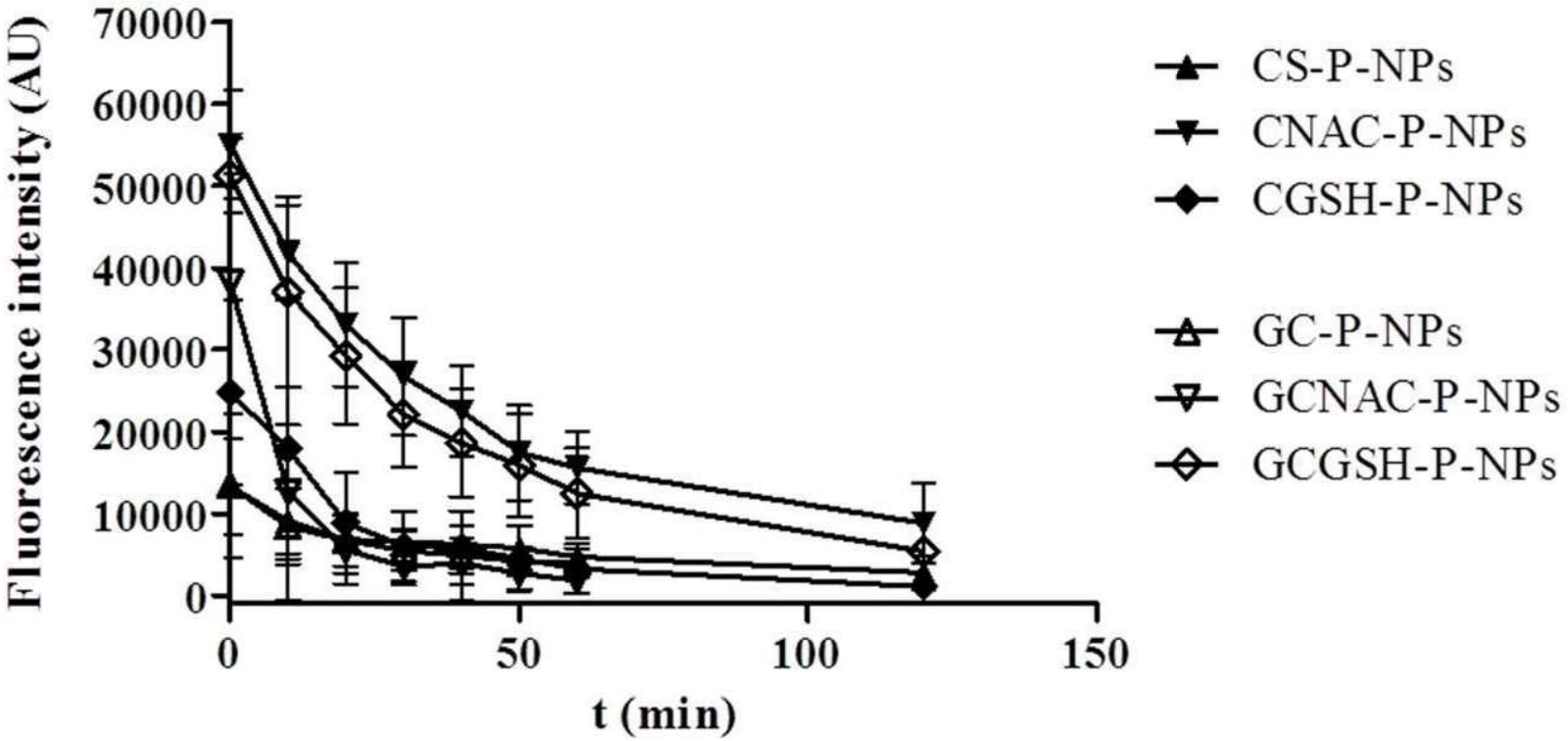

Number of attached nanoparticles/cm²
4 x 10¹²
3 x 10¹²
2 x 10¹²
1 x 10¹²
0
f
e
d
a
c b
0 100 200 300 400 500 600 700
Hydrodynamic diameter (nm)

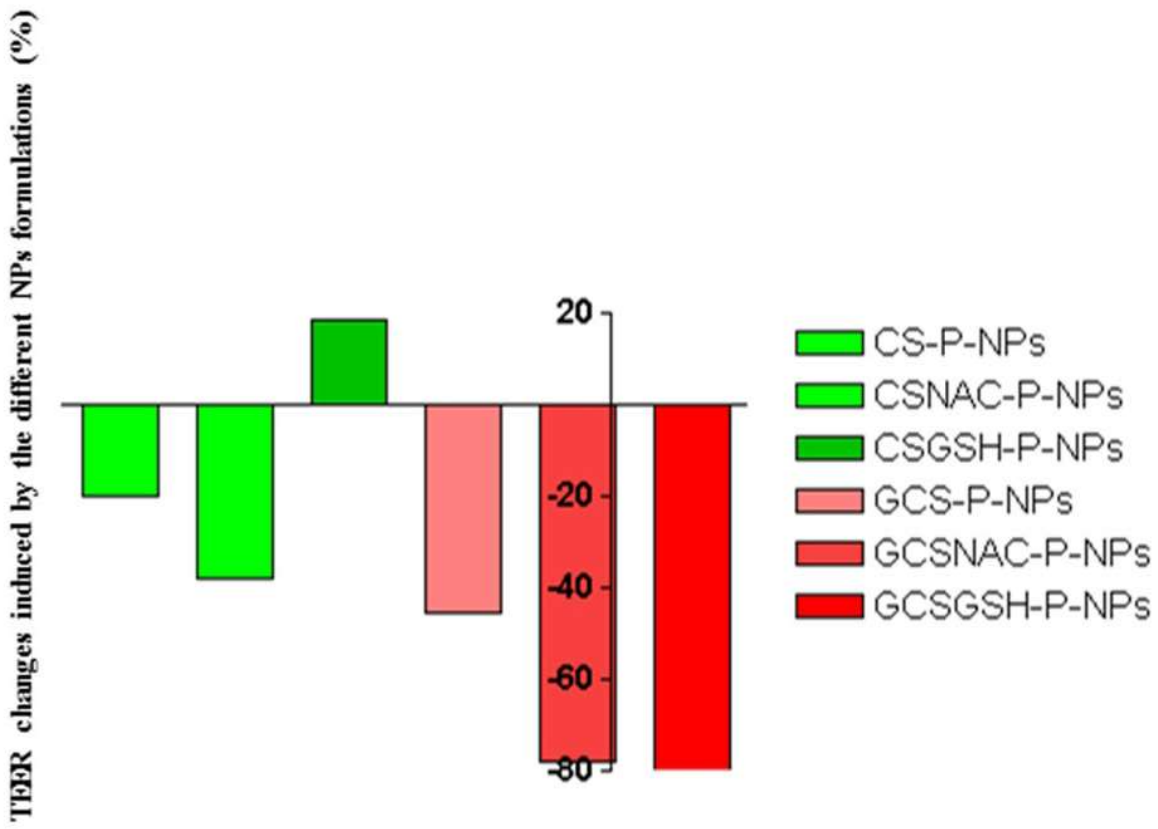
b)

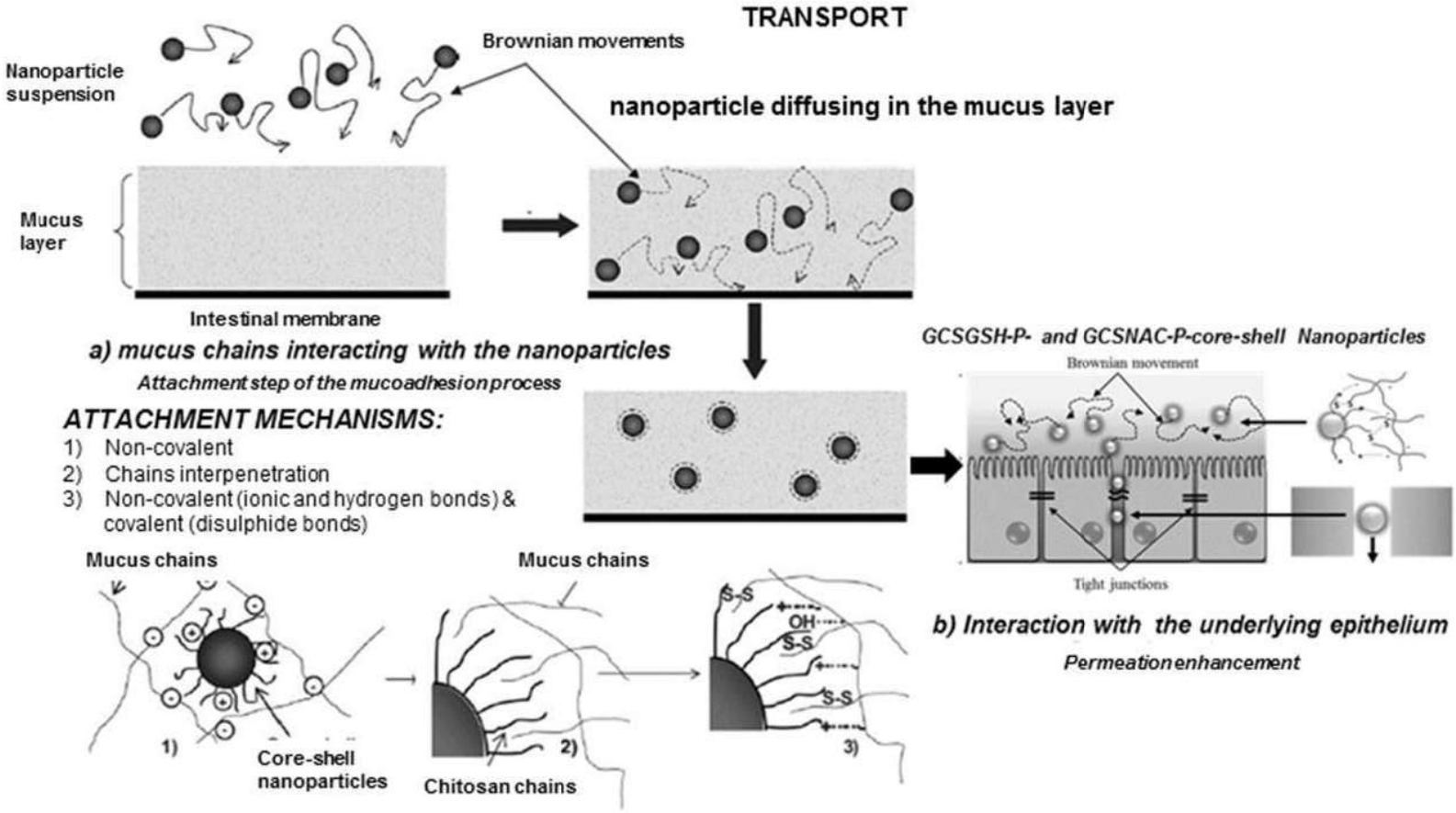

Number of attached nanoparticles/cm²
4 x 10¹²
2 x 10¹²
0
f
e
b
c
0 0.01 0.02 0.03
µmol SH/cm²

c)


Number of attached nanoparticles/cm²
4 x 10¹²
3 x 10¹²
2 x 10¹²
1 x 10¹²
0
f
e
d
c
a b
20 30 40 50 60
ζ potential (mV)


Fluorescence intensity (AU)
70000
60000
50000
40000
30000
20000
10000
0
0
50
100
150
t (min)
CS-P-NPs
CNAC-P-NPs
CGSH-P-NPs
GC-P-NPs
GCNAC-P-NPs
GCGSH-P-NPs


TEER changes induced by the different NPs formulations (%)
20
-20
-40
-60
-80
CS-P-NPs
CSNAC-P-NPs
CSGSH-P-NPs
GCS-P-NPs
GCSNAC-P-NPs
GCSGSH-P-NPs


TRANSPORT
Nanoparticle suspension
Brownian movements
nanoparticle diffusing in the mucus layer
Mucus layer
Intestinal membrane
a) mucus chains interacting with the nanoparticles
Attachment step of the mucoadhesion process
ATTACHMENT MECHANISMS:
1) Non-covalent
2) Chains interpenetration
3) Non-covalent (ionic and hydrogen bonds) & covalent (disulphide bonds)
GCSGSH-P- and GCSNAC-P-core-shell Nanoparticles
Brownian movement
Tight junctions
b) Interaction with the underlying epithelium
Permeation enhancement
Mucus chains
Mucus chains
S-S
OH
S-S
S-S
1)
2)
3)
Core-shell nanoparticles
Chitosan chains

Tab 1. Size, polydispersity index (PDI) and zeta potential (Z), and surface thiol content of LMW CS-, GCS- and corresponding thiomers-coated polyisobutylcyanoacrylate NPs.

	CS-P-NPs [a]	**CSNAC-P-NPs** [a]	**CSGSH-P-NPs** [a]	**GCS-P-NPs** [b]	**GCSNAC-P-NPs** [b]	**GCSGSH-P-NPs** [b]
Size (nm)	400 ± 9	605 ± 15	603 ± 98	187 ± 2	296 ± 19	309 ± 11
PDI	0.05 ± 0.00	0.26 ± 0.10	0.58 ± 0.20	0.04 ± 0.00	0.32 ± 0.10	0.33 ± 0.00
ζ at pH 2.5 (mV)	+ 41 ± 1	+ 42 ± 2	+ 25 ± 1	+ 37 ± 1	+ 49 ± 1	+ 31 ± 1
ζ at pH 7.0 (mV)	+ 23 ± 1	+ 9 ± 0	+ 2 ± 0	+ 16 ± 0	+ 2 ± 0	- 6 ± 1
µmol SH/cm²		160 x 10^{-4}	220 x 10^{-4}		130 x 10^{-4}	20 x 10^{-4}

[a] Nanoparticles prepared from CS 20 kDa. [b] Nanoparticles prepared from GCS 7 kDa.

Table 2. Percentage of nanoparticles stuck on the mucosal surface (M%/cm^2) after 2 h of contact, number of theoretical nanoparticles attached onto 1 cm^2 mucosal surface and number of theoretical nanoparticles layers formed onto 1 cm^2 mucosal surface

	CS-P-NPs	CSNAC-P-NPs	CSGSH-P-NPs	GCS-P-NPs	GCSNAC-P-NPs	GCSGSH-P-NPs
M%/cm^2	20.1 ± 9.9	44.9 ± 16.5	38.4 ± 20.3	19.4 ± 3.2	15.8 ± 9.8	76.3 ± 19.8
Number of NPs attached to mucosa(x 10^9)	50	32.3	28	472	970	4118
Number of NPs layers formed onto the mucosal surface (1 cm^2)	5	118	101	349	849	3931

Abstract

The aim of the present work was to evaluate the mucoadhesive properties of poly(isobutyl cyanoacrylate) (PIBCA) nanoparticles (NPs) coated with Low Molecular Weight (LMW) chitosan (CS)- and glycol chitosan (GCS)-based thiomers as well as with the corresponding LMW unmodified polysaccharides. For this purpose, all the CS- and GCS-based thiomers were prepared under simple and mild conditions starting from the LMW unmodified polymers CS and GCS. The resulting NPs were of spherical shape with diameters ranging from 400 to 600 nm and 187 to 309 nm, for CS- and GCS-based NPs, respectively. The mucoadhesive characteristics of these core shell NPs were studied in Ussing chambers measuring the percentage of NPs stuck on the mucosal of fresh intestinal tissue after 2 h of incubation. Moreover, incubation of nanoparticle formulations with the intestinal tissue induced changes in transmucosal electrical resistance which were measured to gain information into the opening of tight junctions and to control the integrity of the mucosa. Thus, it was found that PIBCA NPs coated with the GCS–Glutathione conjugate (GCGPIBCA NPs) possessed the most favorable mucoadhesive performances. Moreover, both GCGPIBCA- and GCS-*N*-acetyl-cysteine (GCNPIBCA)-core-shell NPs might induced an enlargement of the epithelial cell tight junctions. In conclusion, coating of PIBCA NPs with GCS-based thiomers may be useful for improving the mucoadhesive and permeation properties of these nanocarriers.

Keywords: Mucoadhesion, Thiomers, Ussing chambers, Chitosan, Glycol Chitosan