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Research paper

# Surface-functionalized polymethacrylic acid based hydrogel microparticles for oral drug delivery

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ABSTRACT

Aim of the present work was to develop novel thiol-functionalized hydrogel microparticles based on poly(methacrylic acid)–chitosan–poly(ethylene glycol) (PCP) for oral drug delivery applications.

PCP microparticles were prepared by a modified ionic gelation process in aqueous medium. Thiol modification of surface carboxylic acid groups of PCP micro particles was carried out by coupling L-cysteine with a water-soluble carbodiimide. Ellman's method was adopted to quantify the sulfhydryl groups, and dynamic light-scattering technique was used to measure the average particle size. Cytotoxicity of the modified particles was evaluated on Caco 2 cells by MTT assay. Effect of thiol modification on permeability of paracellular marker fluorescence dextran (FD4) was evaluated on Caco 2 cell monolayers and freshly excised rat intestinal tissue with an Ussing chamber set-up. Mucoadhesion experiments were carried out by an ex vivo bioadhesion method with excised rat intestinal tissue.

The average size of the PCP microparticles was increased after thiol modification. Thiolated microparticles significantly improved the paracellular permeability of FD4 across Caco 2 cell monolayers, with no sign of toxicity. However, the efficacy of thiolated system remained low when permeation experiments were carried out across excised intestinal membrane. This was attributed to the high adhesion of the thiolated particles on the gut mucosa. Nevertheless, it can be concluded that surface thiolation is an interesting strategy to improve paracellular permeability of hydrophilic macromolecules.



## 1. Introduction

Advanced delivery systems capable of improving drug permeability across biological barriers are of prime significance in modern pharmaceutical research [1]. Intestinal epithelium represents the major barrier for the absorption of orally administered therapeutic macromolecules into the systemic circulation. It is formed by a high-resistance epithelial cell barrier which restricts the diffusion of various hydrophilic compounds across the small intestine. Between cells, tight junctions form a paracellular barrier which limits the passage of molecules through the intercellular spaces of the intestinal epithelium [2]. The paracellular permeability depends mainly on the regulation of the intercellular tight junctions, which in turn is governed mostly by a group of proteins often referred as tight junction proteins [3,4]. The utility of the paracellular route for oral drug delivery has remained unexplored mainly due to the lack of substances capable of modulating reversible opening of the tight junctions, hence enhancing drug transport without irreversibly compromising the integrity of the gut epithelium [5].

Polymeric absorption enhancer systems based on poly(acrylic acid) (PAA) or poly(methacrylic acid) (PMAA) and chitosan (CS) seem to be a promising approach in this aspect [6,7]. They received good attention in recent years as transmucosal penetration enhancers improving absorption of hydrophilic drugs [8,9]. Unlike small molecular weight permeation enhancers, polymeric systems can improve the intestinal permeability by imparting less or no toxicity on the biological membrane [10]. Most of these systems enhance drug transport by a completely reversible mechanism, which exerts minimal damage on the integrity of tight junctions [10]. The mechanism by which permeation enhancement occurs seems to be slightly different for each polymer. PAA-based polymers are believed to be responsible for permeation-enhancing effects due to its capacity to bind calcium ions. Indeed, calcium chelators can disturb cell–cell adhesion phenomena by depleting the concentration in the extracellular calcium ions, which play a major role in maintaining the integrity of the epithelial tight junctions. Chelation of calcium further activates protein tyrosine kinases (PTK), which subsequently leads to the phosphorylation of the tyrosine moieties in the transmembrane protein – occludin [11,12]. Although phosphorylation of tyrosine groups lead to the opening of the tight junctions, protein tyrosine phosphatases (PTP) have the role of closing the tight junctions by dephosphorylating

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the tyrosine groups. The second polymer, chitosan, is polycationic in nature. Its protonated form interacts with the epithelial tight junctions inducing a redistribution of actin filaments and of the tight junction protein ZO-1 [13,14]. This ultimately leads to the opening of tight junctions across the intestinal epithelium.

A novel class of polymers called "thiomers" was introduced to improve the bioadhesion of polymeric drug delivery systems [15]. Thiomers are thiolated polymers which display thiol groups. They are expected to form disulfide bonds, thanks to thiol/disulfide exchange reactions and/or simple oxidation processes between thiomers and cysteine-rich subdomains of the mucus glycoproteins [16]. Polymers such as poly(acrylic acid), alginate and chitosan were modified with thiol-containing molecules to yield thiomers. Drug delivery systems formulated with such polymers showed higher mucoadhesion capacity compared with the corresponding non-thiolated systems. Interestingly, the introduction of thiol groups has also improved the permeation-enhancing capability of these polymers. However, the exact mechanism at the origin of the permeation-enhancing effect still remains unclear. It was postulated that thiolated systems have the ability to inhibit PTP and regulate the opening of the tight junctions in a reversible manner. PTP has a cysteine residue in its active site, which is largely involved in the activity of this protein. Thus, it is believed that thiomers can form disulfide linkage with these cysteine residues and hence modifies the activity of the PTP. This could be the major mechanism involved for the permeation-enhancing effect of thiol-containing system [17]. However, the leading factor to the activation of PTK in such a case still remains unclear.

Surface modification of polymeric nano- and microparticles seems to be a promising approach in advanced drug delivery. A novel core–shell type nanoparticulate delivery system, including poly(iso-butyl cyanoacrylate) (PIBCA) core with chitosan/thiolated chitosan brushes at the surface were developed in our laboratory [18,19]. Presence of polysaccharide brushes on the particle surface significantly enhanced the adhesion behavior of the PIBCA nanoparticles to the mucosa and also improved the permeation of hydrophilic markers across the intestinal membrane through the intercellular pathway [20,21]. Surface thiolation may be an interesting approach to be applied to hydrogel systems. It may be expected that thiol groups may increase permeability of the gut epithelium to hydrophilic macromolecules and help in anchoring the delivery system onto the mucus layer, whereas the hydrogel system with optimum chain flexibility and mobility may help in the diffusion of the delivery system across the mucus layer to access the underlying epithelium.

The objective of the present work was to study the effect of surface thiolation of hydrogel microparticles on the mucoadhesion and paracellular permeability through the gut epithelium. We suggest that the specific surface modification of the particles may increase the amount of thiol groups available on the surface of the microparticles. The microparticles used in this work were obtained by a modified ionic gelation method, and surface thiolation was achieved by the activation of surface carboxylic acid groups followed by the coupling with amino groups of cysteine. The characteristics of these novel surface-modified particles were evaluated in terms of their amount of thiol groups, size and ability to enhance the paracellular permeability of hydrophilic macromolecular marker with Caco 2 cell-culture model and Ussing chamber facility.

## 2. Materials and methods

Methacrylic acid (MAA), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), ethylene glycol dimethacrylate (EDMA), 5,5′-dithiobis (nitrobenzoic acid) (Ellman's reagent), sodium borohydride, L-cysteine, 2-[N-morpholno]ethane sulfonic acid (MES), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), N-acetyl cysteine, fluorescein isothiocyanate–dextran, MW 4400 (FD4), polyethylene glycol (PEG) MW 20,000, potassium persulfate and sodium borohydride were from Sigma–Aldrich (St. Louis, MO). Chitosan (CS) with approximate molecular weight of 270 kDa and 85% deacetylated was obtained from Indian Sea Food (India).

### 2.1. Preparation and characterization of microparticles

#### 2.1.1. Preparation of PCP microparticles

Methacrylic acid was purified with the help of an inhibitor removal column (HEMQ specific, Aldrich), and purified monomer was stored at −20 °C. PMAA–PEG–CS (PCP) microparticles were obtained by a modified ionic gelation process as described in our previous reports [22,23]. Briefly, MAA was copolymerized with EDMA, in the presence of CS and PEG in aqueous medium. In a typical experiment, MAA (3 g), EDMA (0.2 g), PEG (0.15 g) and chitosan (0.01 g) were mixed in a round-bottom flask at ambient temperature. Double-distilled water (300 ml) was added to this reaction medium, and potassium persulfate was added to initiate the polymerization. Polymerization was allowed to proceed for 6 h at a temperature ranging from 60 to 70 °C under magnetic stirring. The obtained suspension was centrifuged at 10,000 rpm for 10 min. The microparticles were collected and redispersed in distilled water. PCP microparticles were further purified by dialysis (Spectrapor dialyzing membranes, MWCO 15,000) against double-distilled water for 48 h to remove the unreacted monomers. Finally, particles obtained from the reaction were dried under vacuum and stored at ambient temperature.

#### 2.1.2. Surface modification of PCP microparticles

The surface carboxylic groups on PCP microparticles were activated with EDC followed by the addition of cysteine to introduce thiol functionalities on the particle surface [24]. PCP particles (100 mg) dispersed in 10 ml MES buffer (pH 6.0) were mixed with EDC solution in water to obtain a final concentration of 50 mM. The reaction was allowed to proceed for 3 h at room temperature under magnetic stirring. Thereafter, unreacted EDC was removed by repeated centrifugation, and the activated microparticles collected were re-suspended in 20 ml of phosphate buffer (pH 7.0, 100 mM). L-cysteine at different concentrations (Table 1) was added, and the reaction was allowed to proceed for another 3 h

**Table 1**
Thiol content of surface-modified PCP microparticles.

| Sample code | PCP microparticles (mg) | Concentration of *L* cysteine (mg) | Final concentration of EDC (mM) | Free thiol groups[a] (μmol/100 mg of particles) | Total thiol content[b] (μmol/100 mg of particles) |
|---|---|---|---|---|---|
| Cys PCP 1 | 100 | 50 | 50 | 237 | 756 |
| Cys PCP 2 | 100 | 100 | 50 | 405 | 1129 |
| Cys PCP 3 | 100 | 200 | 50 | 506 | 1214 |
| Cys PCP 4 | 100 | 300 | 50 | 510 | 1217 |

[a] Free thiol groups were determined by the Ellman's test.
[b] Total amount of thiol groups on the particles was determined by Ellman's test with pre-treated microparticles with sodium borohydride to cleave disulphide bonds.

at room temperature under magnetic stirring with occasional purging of nitrogen. Finally, cysteine-conjugated PCP microparticles (Cys-PCP) were centrifuged several times at 5000 rpm until all remaining free cysteine was removed from the supernatant as determined by the Ellman's method. Particles were dried under vacuum and stored at 2–4 °C under inert atmosphere to prevent the oxidation of thiol groups on storage.

*2.1.3. Quantification of thiol concentration by Ellman's method*

Ellman's method was adopted for the quantification of thiol groups over the Cys-PCP microparticles. Microparticles (PCP and Cys-PCP) (2.5 mg) were dispersed in 1 ml of phosphate buffer pH 7.0, and 200 μl of Ellman's reagent (0.3 mg/ml in phosphate buffer pH 8.0) was added. The mixture was incubated for 15 min at room temperature in the dark, and thereafter the suspension was centrifuged at 10,000 rpm for 10 min to separate the supernatant containing 2-nitro-5-thiobenzoic acid (NTB). The supernatant from each sample (200 μl) was transferred into a microplate and the absorbance was measured at a wavelength of 450 nm using a microplate reader (Labsystems, Multiskans MS). The amount of thiol groups was calculated from a standard curve of L-cysteine in a concentration range of 50–500 μmol/ml prepared in exactly the same way as the samples.

After reduction of disulfide bonds with sodium borohydride, Ellman's test was performed to determine the total amount of thiol groups present in the system [25]. Particles (2.5 mg) were suspended in 1 ml of phosphate buffer pH 7.0, and 1.0 ml of a freshly prepared sodium borohydride solution 1% (w/v) was added. The samples were incubated for 1 h at 37 °C, and 200 μl of 1 M HCl was added in order to inactivate the remaining sodium borohydride. The pH of the reaction mixture was adjusted to 8.0 with phosphate buffer (pH 8.0), and the thiol concentration was determined with Ellman's assay.

*2.1.4. Measurement of particle size*

The hydrodynamic mean diameter of the particles was determined by dynamic light scattering using Zetasizer (Nano-ZS, Malvern, UK). All dynamic light scattering measurements were performed at a wavelength of 633 nm at 25 °C with an angle detection of 90°. The particles were dispersed in phosphate buffer (pH 7.0, 0.2 M), and three measurements were made for each sample.

*2.1.5. Determination of zeta potential*

The zeta potential measurements were performed by Laser Doppler Electrophoresis using Zetasizer (Nano-ZS, Malvern, UK). Dispersed suspension of particles in phosphate buffer was diluted (1:50 v/v) in KCl 1 mM (pH 6.5), and three measurements were made on each sample.

*2.2. Cell-culture experiments*

The colonic adenocarcinoma cell line, Caco 2, was obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco's modified Eagle medium with high glucose (DMEM) (Lonza, Belgium) containing 20% v/v fetal bovine serum (FBS, Gibco), 1% v/v non-essential amino acids (Lonza), 160 U/ml benzylpenicillin and 100 U/ml streptomycin (Lonza). The cells were maintained at 37 °C in an atmosphere of 95% air and 5% $CO_2$ at 90% relative humidity. Cells were trypsinized once per week with 0.05% trypsin–EDTA solution (Sigma), and Caco 2 cells of passage numbers 80–90 were used in these experiments.

*2.2.1. Cytotoxicity assay*

Caco 2 cells used for MTT assay were seeded on 24-well culture plates at seeding density of $8 \times 10^4$ cells per well in DMEM culture medium. The cells were grown in an atmosphere of 95% air, 5% $CO_2$ at 37 °C and 90% humidity for 6–7 days. Subsequently, culture medium was replaced with DMEM (without FBS) in which various amounts of either thiolated or non-thiolated-dried microparticles (0.25, 0.5, 1, 2 mg) were added. Cells were then incubated with particles (PCP, Cys-PCP 1–3) at 37 °C for 6 h. Thereafter, the polymer microparticles were removed by carefully washing with PBS buffer. Then, 200 μl MTT solution (0.5 mg/ml in PBS) was added to each well before the cells were incubated for another 3 h at 37 °C. The reaction product was then solubilized in 200 μl of DMSO before quantifying the color of reaction product using a microplate reader at 570 nm. DMEM culture medium and sodium dodecylsulfate (SDS) 2% w/v in PBS were used as negative and positive controls, respectively. Experiments were carried out in triplicate.

*2.2.2. FD4 permeation experiments with Caco 2 cells*

For transport studies, the Caco 2 cells were grown on porous polycarbonate filter membranes with a pore size of 0.4 μm and 12 mm diameter (Costar Transwell®). Following trypsinization, cells were seeded at a density of $2 \times 10^5$ cells into each insert. These cells were maintained at 37 °C in an atmosphere as described earlier, medium was replaced every 48 h, for 21–23 days. On the day of the transport experiments, the culture medium was replaced with an equal volume of Hank's balanced salt solution (HBSS, Gibco) without phenol red (pH 7.4), and cells were incubated with the transport medium for an hour prior to the beginning of the experiment.

HBSS with three different concentrations of calcium chloride were used for the experiments.

HBSS 1 – with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$.
HBSS 5 – with 5 mM $CaCl_2$ and 0.5 mM $MgCl_2$.
HBSS 10 – with 10 mM $CaCl_2$ and 0.5 mM $MgCl_2$.

An incubation medium without calcium chloride, HBSS 0, was also used in these experiments.

After removing the transport medium, 2.5 mg of microparticles of thiolated and non-thiolated particles were added to the apical side of the insert. FD4 dissolved in transport medium was added to the apical compartment of the insert. Transport medium without FD4 was added to the basolateral side, and an aliquot of samples (100 μl) were withdrawn at pre-determined times of incubation, 0, 30, 60, 90, 120 min. Control experiments were carried out in exact manner, without the addition of microparticles to the inserts. Concentration of FD4 was assessed spectrophotometrically using fluorescence spectrophotometer (HITACHI F 2000).

*2.2.3. TEER measurement*

The transepithelial electrical resistance (TEER) was measured using a WPI EVOM resistance meter with Endohm chambers to ensure the integrity of the monolayers formed on the filters and inserts with resistance of 450 Ω and above were only selected for the permeation experiments. TEER measurements were also performed during the experiment in order to check the effect of polymers on opening of the tight junctions at time intervals of 0, 30, 60, 90, 120 min.

*2.2.4. Trypan blue test*

Cell survival after transport experiments was evaluated by Trypan blue exclusion test. After completion of transport experiments, the thiolated and non-thiolated microparticles were removed carefully by washing with corresponding HBSS. Cells were stripped off with 0.25% trypsin–EDTA solution and 20 μl of 0.1% trypan blue solution (in PBS) was added to 20 μl of the cell suspension. The cells were examined under a light microscope. The intact cells

showed no inclusion of dye, whereas the damaged cells showed dye inclusion.

### 2.3. Ussing chamber experiments

#### 2.3.1. Preparation of intestinal tissues

Male Wistar rats (250–300 g) (Charles River, Paris) were used for the in vitro permeation experiments, and animals were sacrificed with overdose of anesthesia pentobarbital. The jejunum portion of sacrificed rats (250–300 g) was excised, rinsed with chilled physiological saline solution (NaCl 0.9%) and cut into segments of 2–3 cm in length. After visual examination of the tissue, sections containing Peyer's patches were discarded from the studies because it was not in our purpose to investigate the retention of the particles by the immune system of the gut. Animal experiments were carried out according to the recommendations from the Declaration of Helsinki. The agreement for animal experiments is A92-019-01 from 30 March 2005.

#### 2.3.2. FD4 permeation experiments with Ussing chamber

Intestinal portions were mounted in Ussing chambers, and the system was maintained at 37 °C, continuously oxygenated with $O_2/CO_2$ 95%/5% and incubated for 20 min with the Krebs–Ringer buffer containing the appropriate amount of calcium chloride. After removing the transport buffer, microparticles (5 mg) were applied to the mucosal surface and transport buffer (5 ml) containing FD4 and L-glutamine (1 mM) was added to the apical compartment. Transport buffer (5 ml) with glutamine and without FD4 was added to the acceptor compartment. At pre-set time intervals, aliquots of 200 μl were recovered from the acceptor chamber and replaced with the same volume of fresh medium. Assays were carried out for 2 h, and four tissue portions were used to evaluate each formulation. The experiments were repeated on different animals to check for relevant reproducibility. Concentration of FD4 was assessed spectrophotometrically using fluorescence spectrophotometer.

Permeation experiments were also performed by avoiding the direct interaction of the particles with the intestinal tissue, in order to evaluate the effect of the adhesion process on the paracellular permeability [20]. In these experiments, a dialysis membrane (Spectrapor MWCO 100,000) was placed in the donor chamber at the surface of the luminal side of the intestinal mucosa. The permeation experiments were then carried out in an exact manner as described above.

### 2.4. Analysis of permeation experiments

The apparent permeability coefficient ($P_{app}$) for Caco 2 cells and Ussing chamber experiments was calculated using the following equation

$$P_{app} = (dQ/dt)(1/A \cdot Co)$$

where $dQ/dt$ is the flux of FD4 from the mucosal to the serosal side of the mucosa, $Co$ is the initial concentration of FD4 in the donor compartment, and $A$ is the area of the membrane (1 $cm^2$ for Ussing chamber and 1.12 $cm^2$ for Caco 2 cells).

Absorption enhancement ratio ($R$) was calculated as follows

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

### 2.5. Evaluation of the mucoadhesion behavior of the microparticles

Mucoadhesion studies were performed on freshly excised rat intestinal mucosa according to a method described previously with slight modification [26]. Excised jejunum portion of the rat intestine was flushed with normal saline to remove luminal contents. Around 10 cm of intestinal tissue was cut opened and placed in a polyethylene support with help of cyanoacrylate adhesive. A defined amount of PCP and Cys-PCP 3 microparticles (10 mg) were uniformly spread on the mucosal surface and were allowed to interact with mucus gel layer for 10 min. Tissue was then mounted on a platform at an angle 45° and washed under a constant flow rate (10 ml/min) of phosphate buffer (20 mM, pH 7.0). Particles washed from the mucosal surface, and particles retained over the mucosal surface after the experiment were collected separately and placed in a mucolytic solution composed of N-acetyl cysteine (5%) in 100 mM phosphate buffer. Suspensions were sonicated for 15 min and left overnight. Desorbed mucus was removed, and particles were dried under vacuum. The weight of the dried particles was compared with the weight of the particles applied to the mucosal surface.

### 2.6. Statistical analysis

Each value was expressed as the mean ± SD. Significant differences in the mean values were evaluated by the Student's unpaired $t$-test. A $p$ value of less than 0.05 was considered significant.

## 3. Results

### 3.1. Preparation and characterization of the PCP and Cys-PCP microparticles

Poly(methacrylic acid) (PMAA)-based hydrogel microparticles were prepared by ionic gelation method, and thiol groups were introduced by grafting cysteine on the microparticle by a surface modification strategy. Thiol content of the microparticles was determined by Ellman's assay, and Ellman's test did not show any reactivity towards non-thiolated-PCP microparticles. Table 1 gives the results of the thiol concentration found in microparticles obtained by using different initial concentrations of cysteine during the grafting of the thiol group. Total sulfhydryl content (including –S–H and –S–S–) was determined after treatment of the microparticles with sodium borohydride. It was more than twice the amount of free thiol groups originally present in the particles. Cys-PCP 1–3 showed an increase in the thiol concentration with respect to the initial concentration of cysteine used during the modification process. In contrast, Cys-PCP 4 obtained with the higher concentration of cysteine demonstrated almost the same amount of thiol as Cys-PCP 3. This could be due to the saturation of the accessible carboxylic acid groups on the microparticle surface. Thus, further investigations were pursued on the three formulations, Cys-PCP 1–3.

Particle size analysis of modified and unmodified particles was carried out by dynamic light scattering. Results are summarized in Table 2. Surface thiolation increased the average size of PCP microparticles. Indeed, unmodified microparticles showed an average diameter around 1 μm, whereas thiol-modified microparticles showed a mean diameter in the range of 1.5–2 μm depending on thiol content. In contrast, zeta potential of the microparticles was not affected by the thiolation process. This could be possibly due to the addition of a carboxylic acid-containing amino acid on the particle surface.

### 3.2. Cell-culture experiments

#### 3.2.1. Cytotoxicity assay

The effect of PCP and Cys-PCP microparticles (Cys-PCP 1–3) on mitochondrial dehydrogenase activity of the cells as evaluated by the MTT test is given in Fig. 1. Overall, the particles exhibited no

**Table 2**
Microparticle characterization: hydrodynamic diameter (DH); polydispersity index (PI) and surface charge ($\zeta$ potential).

| Sample code | DH (nm) | PI | $\zeta$ Potential (mV) |
|---|---|---|---|
| PCP | 1030 | 0.346 | −33 |
| Cys-PCP 1 | 1660 | 0.384 | −33.5 |
| Cys-PCP 2 | 1860 | 0.294 | −34 |
| Cys-PCP 3 | 1900 | 0.264 | −32.5 |

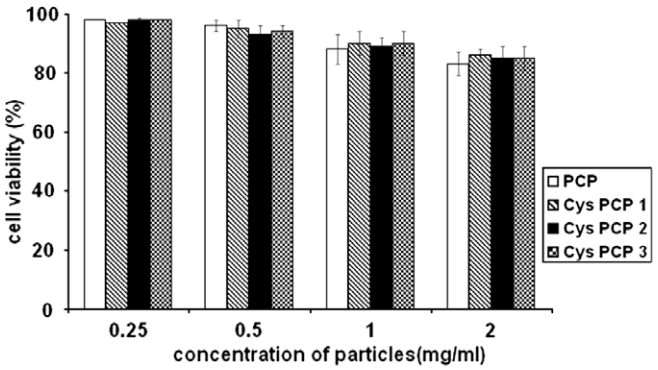



**Fig. 1.** Effect of thiolated and non-thiolated microparticles on mitochondrial dehydrogenase activity (MTT assay). White bars: PCP microparticles, hash bars: Cys-PCP 1, black bars: Cys-PCP 2, dotted bars: Cys-PCP 3 ($n = 3$).

cytotoxicity within the range of concentrations studied here. Cells remained viable (more than 90%) in the presence of a low amount of thiolated and non-thiolated microparticles (0.25 and 0.5 mg). When the amount of microparticles increased (1 mg and 2.5 mg), the cell viability tended to decrease slightly (down to 85%). No difference in cytotoxicity could be observed between thiolated and non-thiolated microparticles.

### 3.2.2. Transport of FD4 across Caco 2 cell monolayers

Permeation experiments were conducted with Caco 2 cell monolayers. Results of the determination of $P_{app}$ and of the absorption-enhancing ratio, $R$, for a macromolecular marker, FD4, are summarized in Table 3. Since calcium plays a key role in maintaining the Caco 2 cell monolayer integrity, transport media containing magnesium chloride and different concentrations of calcium chloride were used in all experiments except in the case of HBSS 0. At first, an experiment was conducted on PCP and on Cys-PCP 1–3 with HBSS 1 as the transport medium. Thiolated microparticles enhanced the permeability of FD4 across the cell monolayers. The enhancement was a function of the thiol content in the microparticles. The cumulative amount of FD4 transported across the monolayers (Fig. 2a) was also significantly improved with thiol-modified microparticles when compared to the unmodified microparticles.

In the next step, transport buffers with higher calcium content (5 mM and 10 mM) were used. The permeation-enhancing effect of PCP and the most effective thiolated system from the initial investigations (Cys-PCP 3) were evaluated in this experiment. Table 3 summarizes these results. PCP microparticles were not effective in improving transport of FD4 under high calcium transport medium (HBSS 10), whereas thiolated systems displayed higher permeation-enhancing capability in the presence of high calcium concentration. Total amount of FD4 permeation from the apical to basolateral side of the Caco 2 cell monolayers was also higher with thiolated microparticles (Fig. 2b and c) when compared with the unmodified microparticles.

The $P_{app}$ value of FD4 with the control microparticles (PCP) in the transport medium without calcium chloride (HBSS 0) was $2.0 \times 10^{-6}$ when compared with the $P_{app}$ value $0.5 \times 10^{-6}$ obtained in the calcium supplemented medium.

### 3.2.3. TEER measurement

TEER of the cell monolayers was reduced by almost 50% with the apical application of 2.5 mg of thiolated and non-thiolated microparticles (Fig. 3a). This drastic drop in the resistance of the epithelium was observed within 10 min after the application of the microparticles. Interestingly, around 40% decrease was observed in TEER values for PCP and Cys-PCP 3 microparticles in HBSS 5 and HBSS 10 (Fig. 3b and c). However, there was no improvement in FD4 permeation (Fig. 2b and c) corresponding to the decrease in TEER value when PCP microparticles were evaluated in HBSS containing high concentrations of calcium chloride. No reduction in TEER values was observed for the control system (i.e. without any particles) except in case of HBSS 0, where a 20% reduction in TEER value was observed even with the control groups (Fig. 3d).

### 3.2.4. Trypan blue test

Trypan blue staining was used to check the viability of the cells at the end of the permeability experiments. After completion of transport experiments, the Caco 2 cell monolayer did not show any intracellular uptake of the dye, indicating that the cells remained viable during the duration of the transport experiments.

### 3.3. FD4 transport studies across excised rat intestinal tissue

FD4 transport across the intestinal tissue was evaluated with an Ussing chamber set-up. The results are summarized in Table 4. Two formulations of thiolated particles (Cys-PCP 1 and 3), one with low and one with high thiol content, respectively, were used for these experiments. The results were compared with those obtained in the presence of non-thiolated microparticles. PCP microparticles were able to improve FD4 transport across intestinal membrane as

**Table 3**
FD4 transport experiment on Caco 2 cells – apparent permeability coefficient ($P_{app}$) and absorption enhancement ratio ($R$) for FD 4 (MW 4400) across Caco 2 cell monolayers ($n = 3$).

| Sample code | HBSS 1 | | HBSS 5 | | HBSS 10 | | HBSS 0 | |
|---|---|---|---|---|---|---|---|---|
| | $P_{app}$* | $R$ | $P_{app}$* | $R$ | $P_{app}$* | $R$ | $P_{app}$* | $R$ |
| Control (no particles) | 0.5 ± 0.1 | – | 0.30 ± 0.05 | – | 0.20 ± 0.05 | – | 2.9 ± 0.2 | – |
| PCP | 2 ± 0.3 | 4 | 1.1 ± 0.1 | 3.6 | 0.6 ± 0.1 | 3 | 7.9 ± 0.5 | 2.7 |
| Cys-PCP 1 | 2.9 ± 0.2 | 5.8 | nd | – | nd | – | nd | – |
| Cys-PCP 2 | 5.4 ± 0.2 | 10.8 | nd | – | nd | – | nd | – |
| Cys-PCP 3 | 6.1 ± 0.2** | 12.2 | 3 ± 0.1** | 10 | 2.3 ± 0.3** | 11.5 | 10.4 ± 0.4** | 3.6 |

nd – Not determined.
'–' Represents no value of $R$ because it was the control experiment [25,27] or there were no data.
* $P_{app}$ is given in $P \times 10^{-6}$ cm/s.
** Statistically significant difference from corresponding PCP group, $p < 0.05$.

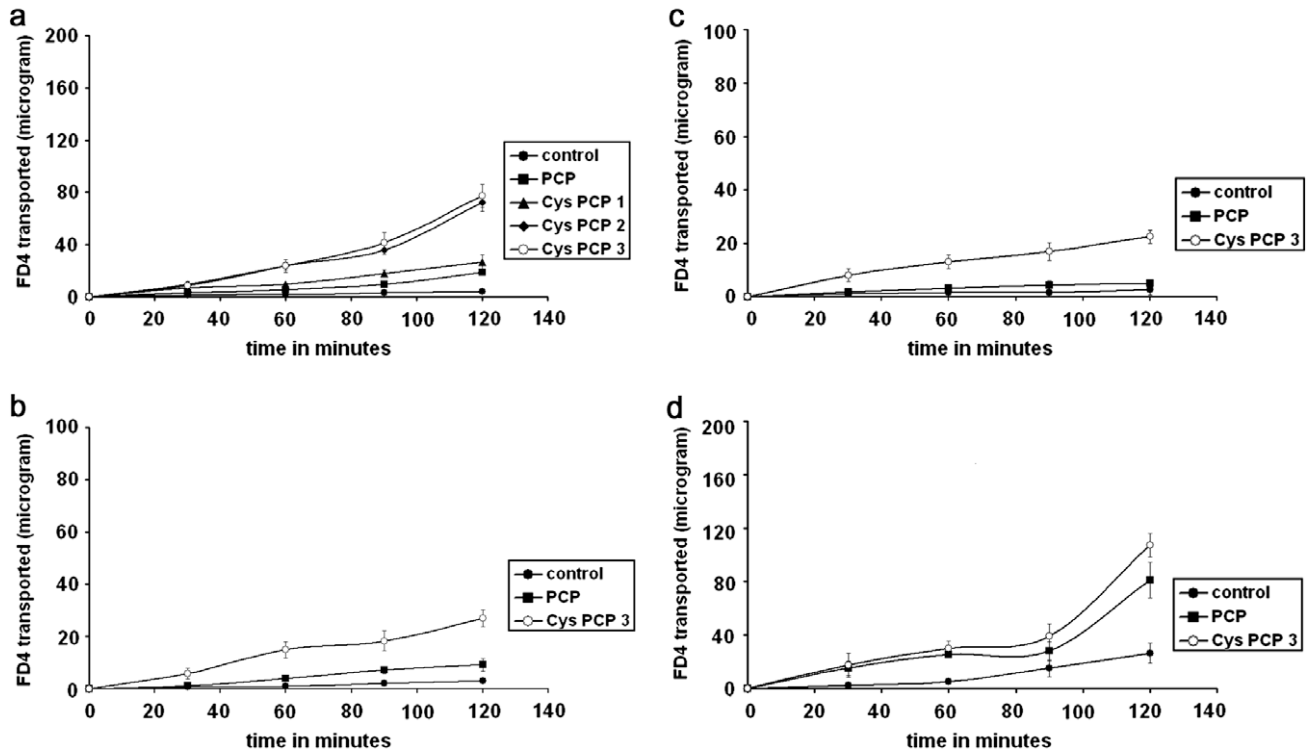



**Fig. 2.** Cumulative amount of FD4 (in μg) transported across Caco 2 cell monolayers incubated in different medium. (a) HBSS 1, (b) HBSS 5, (c) HBSS 10 and (d) HBSS 0. ● Control (no particles), ■ PCP, ▲ Cys-PCP 1, ♦ Cys-PCP 2 and ○ Cys-PCP 3 ($n = 3$).

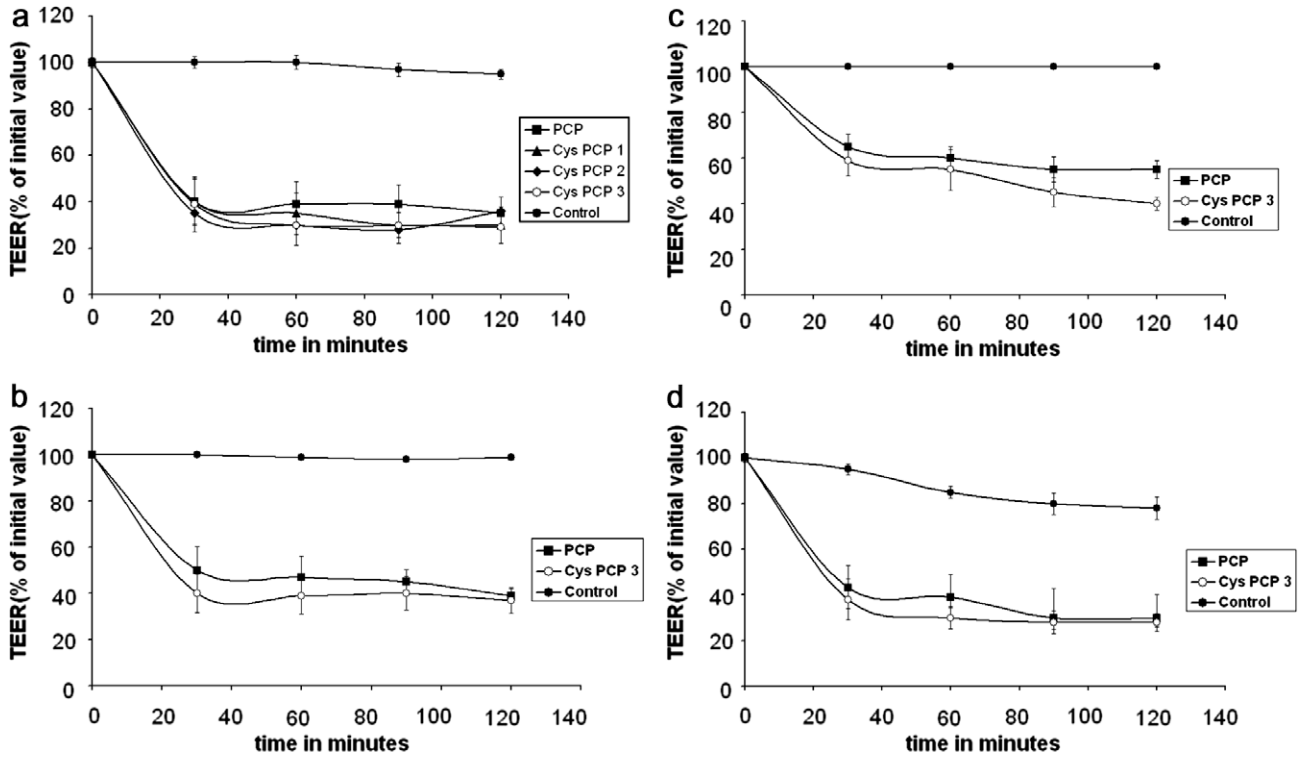



**Fig. 3.** Effects of the microparticles (2.5 mg) on TEER of Caco 2 cell monolayers incubated in different medium. (a) HBSS 1, (b) HBSS 5, (c) HBSS 10 and (d) HBSS 0. ● Control (no particles), ■ PCP, ▲ Cys-PCP 1, ♦ Cys-PCP 2 and ○ Cys-PCP 3 ($n = 3$).

demonstrated by a factor of 3.2 when compared with the control experiment performed in the absence of particles. *R* factor of 3.3 and 3.4 were obtained for microparticles Cys-PCP 1 and Cys-PCP 3. These values were slightly above the value of 3.2 found with the microparticles PCP. It can be concluded that only a marginal improvement in the amount of FD 4 permeation was observed with

**Table 4**
Apparent permeability coefficient and absorption enhancement ratio for FD4 across rat small intestinal tissue as evaluated with Ussing chamber set-up ($n = 3$).

| Sample code | $P_{app} \times 10^{-7}$ cm/s | $R$ |
|---|---|---|
| Control (no particles) | 2.3 ± 0.1 | – |
| PCP | 7.4 ± 0.2 | 3.2 |
| Cys-PCP 1 | 7.5 ± 0.2 | 3.3 |
| Cys-PCP 3 | 7.9 ± 0.3 | 3.4 |

'–' Represents no value of $R$ because it was the control experiment [25,27].

the thiolated microparticles when compared with the non-thiolated microparticles (Fig. 4).

Finally, studies carried out in conditions in which direct interactions between microparticles and intestinal tissue were inhibited by the use of a semi-permeable membrane layer on the intestinal tissue showed that the $P_{app}$ values of FD4 were comparable to the $P_{app}$ of the control experiments performed without microparticles (Fig. 5). This result indicated that the contact between the microparticles and the intestinal tissue is required to promote the permeability of FD4.

### 3.4. Evaluation of the mucoadhésion of the microspheres

Mucoadhesion experiments were carried on two formulations of thiolated microparticles (Cys-PCP 1 and 3) along with the non-thiolated microparticles. Thiol-functionalized microparticles displayed enhanced adhesion on isolated intestinal tissue (Table 5). Unmodified microparticles displayed 85% adhesion after 10 min and 74% after 20 min with a flow of 10 ml/min. In case of thiolated microparticles, the adhesion percentage was 95% and 90% after 10 and 20 min, respectively, for Cys-PCP 3. The percentage of adhesion was similar in the case of the microparticles containing the lower amount of thiol groups (Cys-PCP 1), as they displayed 93% and 90% adhesion after 10 and 20 min, respectively.

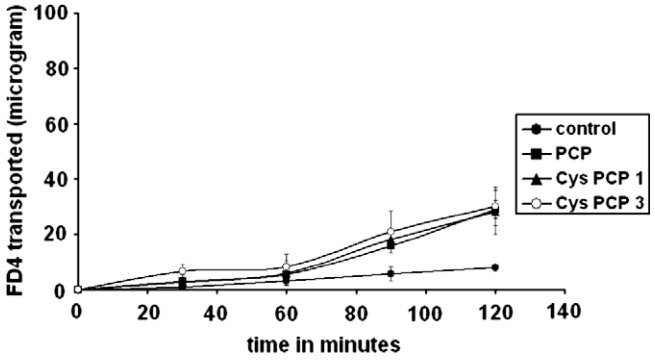



**Fig. 4.** Cumulative amount of FD4 transported (in μg) across intestinal membrane with Ussing chamber set-up ($n = 3$) in Krebs–Ringer buffer. ● Control (no particles), ■ PCP, ▲ Cys-PCP 1 and ○ Cys-PCP 3 ($n = 3$).

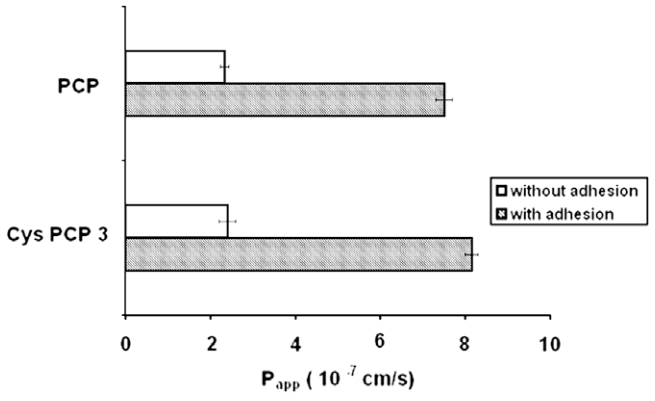



**Fig. 5.** Comparison of $P_{app}$ ($P \times 10^{-7}$ cm/s) obtained for particle formulations allowing (grey bars) and avoiding direct (white bars) contact with intestinal mucosa in Krebs–Ringer buffer ($n = 3$).

**Table 5**
Ex vivo bioadhesion experiments – percentage of bioadhesion of thiolated and non-thiolated particles ($n = 3$).

| Sample code | Percentage of bioadhesion | |
|---|---|---|
| | After 10 min | After 20 min |
| PCP | 85 ± 4 | 74 ± 3.5 |
| Cys-PCP 1 | 95 ± 2.5 | 90 ± 2 |
| Cys-PCP 3 | 90 ± 2 | 87 ± 2** |

Values obtained by comparing the amount of microparticles retained in the intestine with amount of particles washed out from the intestine segment during the adhesion experiments.
** Statistically significant difference from corresponding PCP group, $p < 0.05$.

## 4. Discussion

The ionic gelation process was used to prepare hydrogel microparticles made of PMAA–PEG–Chitosan (PCP microparticles). The main advantage of the technique is that the microparticles are formed spontaneously during polymerization of methacrylic acid without the addition of any surfactants and/or steric stabilizers. The aim of the present work was to introduce new chemical groups, namely thiol groups, on the surface of the PCP microparticle to improve both microparticle mucoadhesion and to enhance permeability properties for macromolecular drugs across the intestinal mucosa. The thiol content of the microparticles increased with the initial cysteine concentration used in the grafting reaction. According to the method of grafting used, cysteine residues were grafted on the free carboxylic acid groups exposed on the microparticle surface. The increase in size of the microparticles after grafting of the thiol groups is due to the formation of intermolecular disulfide linkage created between microparticles.

The effects of thiol-functionalized microparticles on the permeability properties of Caco 2 cell monolayers were subjected to detailed investigation. Effects of PCP and thiol-modified PCP microparticles on the paracellular permeability of FD4 were evaluated, and the results clearly suggest that thiol groups have excellent permeation-enhancing capability in this simplified model of the intestinal epithelium. As could be expected, the increase in $P_{app}$ of FD4 in the presence of thiolated particles was consistent with the increased surface thiol concentration shown by the different microparticles. Mechanisms by which the microparticles enhanced permeability of Caco 2 cell monolayer for FD4 were investigated using incubation media containing concentration of calcium chloride ranging from 0 to 10 mM. In agreement with data of the literature, calcium ions were necessary to maintain the integrity of the functionality of the tight junction. Indeed, in incubation medium containing no calcium (HBSS 0), the permeability coefficient for FD4 was high. In contrast, in the presence of calcium the permeability of the Caco 2 cell monolayer for FD4 was much lower indicating the tight junctions were functional. It is noteworthy that the permeability of the tight junction decreased as calcium chloride concentration increased.

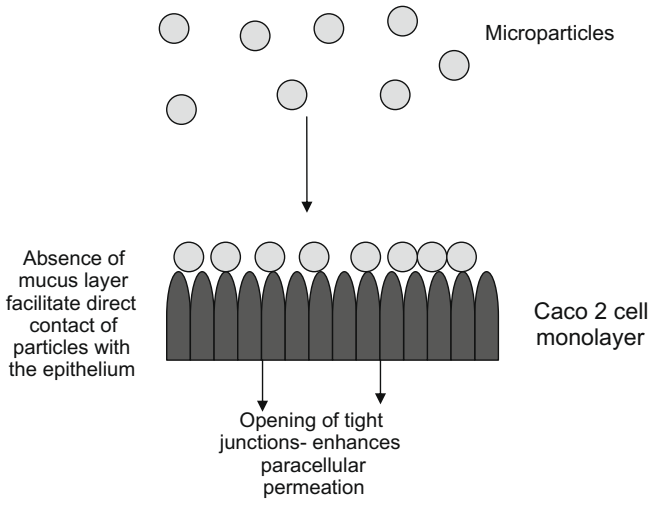
As expected from previous reports [7], the permeability of Caco 2 cell monolayer for FD4 increased after the addition of PCP microparticles. This is attributed to the capability of PMAA to bind the divalent cation, hence reducing the local concentration in calcium available to maintain the integrity of the functionality of the tight junctions. Results showing the absorption-enhancing

ratio decreased with the increase in calcium concentration in the incubation medium were in agreement with the calcium dependent enhancing permeability mechanism suggested previously for these particles. The addition of Cys-PCP microparticles in the incubation medium leads to a further increase in the permeability coefficient of the Caco 2 cell monolayer for FD4 in a manner which depended on the thiol content of the microparticles (Table 3). This result clearly indicated that thiol groups actively contributed to the increase in the permeability of the tight junction to FD4. In the case of the microparticles with the highest thiol content (Cys-PCP 3), the contribution of the thiol group was even predominant. MTT test and trypan blue tests performed at the end of each permeation experiment clearly demonstrated that there was no toxicity of the microparticles on the Caco 2 cell monolayer, and that the Caco 2 cell monolayer was still viable at the end of the experiments. Thus, the increase in permeability observed in the presence of the microparticles cannot be attributed to a lack of viability of the cells and/or to a toxicity of the microparticles. The fact that the contribution of the thiol group did not depend on the concentration in calcium (experiments performed with microparticles Cys-PCP 3 in different incubation media) suggests that the mechanism by which thiol groups increased permeability of tight junction was calcium concentration independent. Furthermore, the increase in the absorption enhancement ratio ($R$) value observed with the thiolated microparticles suggests that this mechanism was more efficient than the one based on simple calcium concentration depletion. Thus, it can be suggested that the mechanism of permeation enhancement of the tight junction induced by the thiol groups is highly specific. As suggested in the previous work, it can involve a specific interaction of the thiol groups with defined proteins of the tight junction protein complex leading to a reversible opening of the tight junction [17].

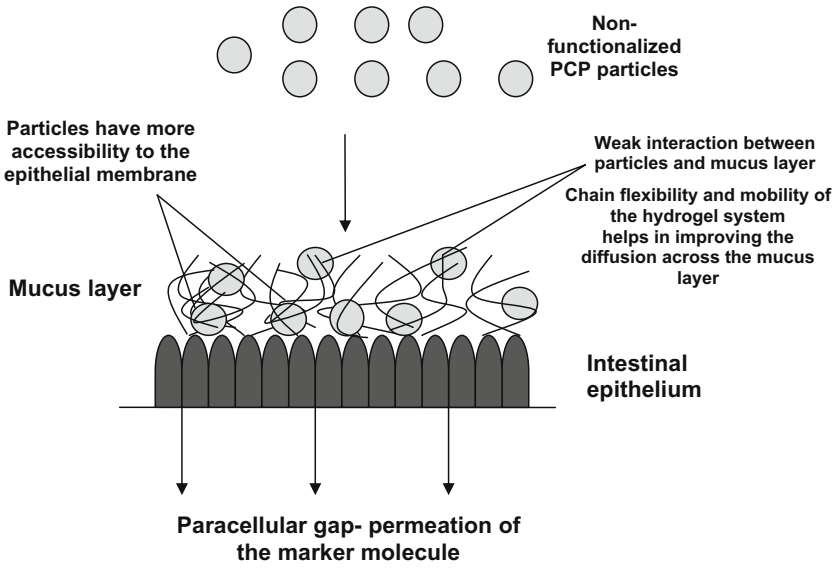
The ex vivo permeation experiments performed with an Ussing chamber set-up and using freshly excised rat intestinal tissue may provide a much closer approximation with the in vivo conditions. It was observed that the permeation-enhancing effect of thiolated microparticles was very low when compared with the effect observed previously on the Caco 2 cell model experiments. Indeed, $P_{app}$ value and the amount of FD4 permeating through the mucosa in the presence of thiolated and non-thiolated microparticles were almost comparable. Although no significant effect on thiolation of the particles could be highlighted, both thiolated and non-thiolated microparticles enhanced the permeability of FD4 compared to the control experiment performed without microparticles added in the donor compartment. As demonstrated by further experiments, a direct contact between the microparticles and the mucus were required to enhance the permeability of the intestinal tissue to FD4. Indeed, no improvement in permeability was observed when an artificial membrane was placed between the microparticles and the intestinal tissue to hinder the direct interactions between the microparticles and the intestinal tissue. This clearly demonstrated the role of the mucoadhesion process in improving the paracellular permeability of hydrophilic macromolecules. According to these results, particles must interact with the epithelial lining, in order to be able to disturb the tight junction architecture hence to widen the intercellular spaces.

Regarding mucoadhesion, it was shown in previous work that PCP microparticles can interact strongly with mucus glycoproteins through H-bonding interactions [28]. By virtue of their unique physical properties, hydrogel particles can also physically entangle through the mucus layer and may remain in the GI tract for an extended period of time [29]. Results from the mucoadhesion evaluation showed that the PCP particles displayed good mucoadhesion properties. The mucoadhesion of PCP microparticles was further increased with the addition of surface thiol groups. This can be explained by a different nature of the interactions between the two types of microparticles and the mucus layer overlapping the intestinal epithelium. Although the mucoadhesion of the PCP microparticles may only result from the occurrence of strong H-bonding interactions with the mucus glycoproteins, thiol groups included on the surface of the Cys-PCP microparticles can form additional disulfide bonds with the mucus glycoproteins [15]. Therefore, the formation of disulfide linkage may account for the improved mucoadhesion behavior.

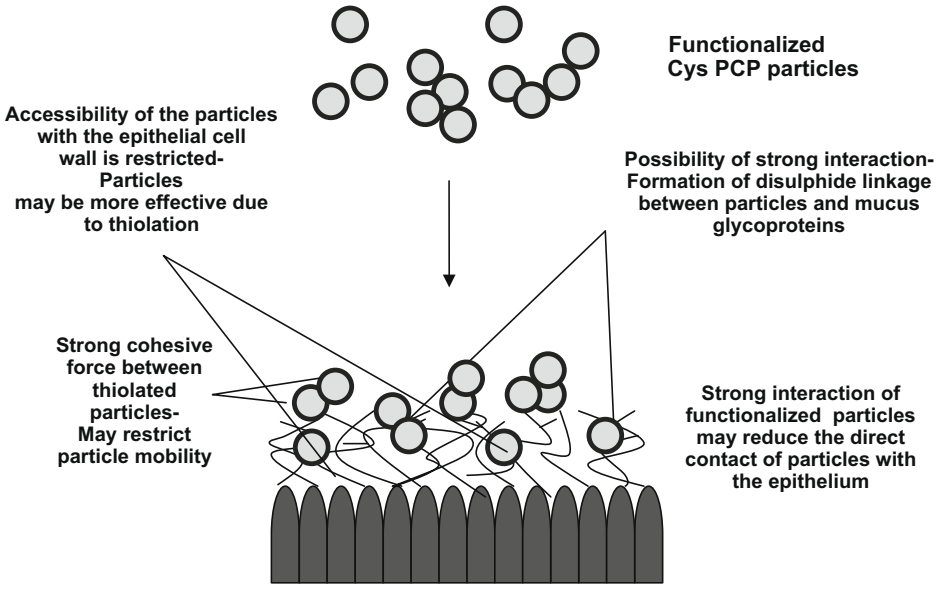
From this study, it appeared that surface thiolation is an effective strategy to improve permeation of hydrophilic macromolecules through the gut epithelium. However, several factors have to be taken into account with respect to this property. As shown by our results, the effect of thiolation was more predominant in Caco 2 cell-cultured monolayers when compared to the Ussing chamber experiments. Caco 2 cells are among the most accepted technique in evaluating the drug permeation, possibly due to their close resemblance with the intestinal epithelium [30]. They can provide with an exact mechanistic outlook on the direct interaction between the particles and epithelial cells, since there is no intervention of a mucus layer over the epithelial membrane (Scheme 1). This ensures a perfect contact between the particles and the epithelial cell monolayer membrane. However, the absence of mucus layer leaves the Caco 2 cell monolayer an incomplete model for the evaluation of drug permeability through the gut epithelium. The mucus layer plays a vital role in maintaining the integrity of the epithelial barrier and has been accepted as a strong strategic barrier for drug permeation across the biological membranes [31]. Caco 2 cell model may be inadequate in explaining the actual in vivo behavior and efficacy of a mucoadhesive-based delivery system. However, this model may serve as an excellent tool for the rapid screening and optimization of the advanced delivery systems. When PCP microparticles come in contact with an intestinal tissue, the surface carboxyl groups over the microparticles may establish H-bonds with the hydroxyl groups of the sialic acid residues in the mucus glycoproteins. The phenomenon of inter-diffusion between polymer-mucus layers eventually helps the particles to diffuse across and get access to the underlying epithelial membrane (Scheme 2). However, in case of the thiolated microparticles, the mechanism of mucoadhésion was slightly different. Thiolated microparticles by virtue of their ability to form covalent bonds with the mucus layer may strongly attach to the mucus gel layer. This was quite evident in the adhesion experiments, as thiolation significantly enhanced the interaction of the




**Scheme 1.** Interaction of functionalized/non-functionalized microparticles with Caco 2 cell monolayers. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)




**Scheme 2.** Interaction of non-functionalized PCP microparticles with intestinal epithelium. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)




**Scheme 3.** Interaction of surface-functionalized Cys-PCP microparticles with intestinal epithelium. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)

polymeric systems with the mucus layer. This is a critical factor to take into account. Indeed, covalent binding of the microparticles with mucus glycoproteins may reduce diffusion of microparticles towards the epithelial cells and ultimately affect the permeation-enhancing property of the system (Scheme 3), because the microparticles may remain entrapped in the upper mucus layer during the mucoadhesion process. The occurrence of such a phenomenon can explain the difference in the increase in permeability of the intestinal medium considering the two types of experimental models used in the present study.

## 5. Conclusions

Thiolation of PCP microparticles improved the intercellular permeability of a hydrophilic macromolecule through Caco 2 cell monolayers. The improvement of the paracellular permeability was due to the combination of two mechanisms. The depletion of calcium concentration in the vicinity of the cell epithelium can be due to the presence of PMAA in the composition of the microparticles. Indeed, this effect can be responsible for a loss of integrity in the tight junction functionality. An additional and stronger effect was attributed to the thiol groups grafted at the microparticle surface. They can act on the tight junction protein PTP inducing the opening of the intercellular pathway.

Experiments performed with gut mucosa demonstrated that a direct contact between microparticles and tissue was necessary to promote the permeability of the tissue to the hydrophilic macromolecule. However, in contrast to what was observed using the Caco 2 cell model, no further permeability-enhancing effect was observed with thiolated microparticles compared with the non-thiolated microparticles. This may be due to the enhancement of the mucoadhesion property of the thiolated particles. A strong adhesion phenomenon layer may restrict the diffusion of the microparticles through the mucus layer, and this effect may reduce

the potent permeation-enhancing property of the drug delivery system expected from experiments performed on intestinal epithelium models exempt of mucus.

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