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Thiolated nanocarriers for oral delivery of hydrophilic macromolecular drugs

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ABSTRACT

It was the aim of this study to investigate the effect of unmodified as well as thiolated anionic poly(acrylic acid) (PAA) and cationic chitosan (CS) utilized in free-soluble form and as nanoparticulate system on the absorption of the hydrophilic compound FD_4 across intestinal epithelial cell layer with and without a mucus layer. Modifications of these polymers were achieved by conjugation with cysteine to PAA (PAA-Cys) and thioglycolic acid to CS (CS-TGA). Particles were prepared via ionic gelation and characterized based on their amount of thiol groups, particle size and zeta potential. Effects on the cell layer concerning absorption enhancement, transepithelial electrical resistance (TEER) and cytotoxicity were investigated. Permeation enhancement was evaluated with respect to in vitro transport of FD_4 across Caco-2 cells, while mucoadhesion was indirectly examined in terms of adsorption behaviour when cells were covered with a mucus layer. Lyophilized particles displayed around 1000 μmol/g of free thiol groups, particle sizes of less than 300 nm and a zeta potential of 18 mV (CS-TGA) and −14 mV (PAA-Cys). Cytotoxicity studies confirmed that all polymer samples were used at nontoxic concentrations (0.5% m/v). Permeation studies revealed that all thiolated formulations had pronounced effects on the paracellular permeability of mucus-free Caco-2 layers and enhanced the permeation of FD_4 3.0- to 5.3-fold. Moreover, polymers administered as particles showed a higher permeation enhancement than their corresponding solutions. However, the absorption-enhancing effect of each thiolated formulation was significantly ($p < 0.05$) reduced when cells were covered with mucus layer. In addition, all formulations were able to decrease the TEER of the cell layer significantly ($p < 0.05$). Therefore, both thiolated polymers as nanoparticulate delivery systems represent a promising tool for the oral administration of hydrophilic macromolecules.

1. Introduction

Based on their hydrophilic nature and molecular mass, macromolecular drugs exhibit a poor permeability across mucosal membranes resulting in overall insufficient bioavailability. Accordingly, at present most of these drugs are primarily administered via the parenteral route as just a limited portion of the dose reaches the plasma to generate its pharmacological effect when administered orally (Aungst, 2000). The design of a suitable drug delivery system for oral administration of macromolecular drugs and their absorption to therapeutic levels is therefore a major aim. Promising systems may comprise of excipients providing the drug entire to the specific site of absorption, prolonging its residence time and increasing the permeability for an easier transport to the systemic circulation. Therefore, reversible modifications of epithelial barrier structure by permeation enhancers are required. Low molecular weight enhancers generally have physicochemical characteristics favouring their own absorption, whereas polymeric enhancers are not absorbed, whereby the risk of systemic toxicity is minimized. One example for a suitable polymeric enhancer are thiolated polymer or designed thiomers, which have been developed as a category of mucoadhesive polymers with reactive thiol groups

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immobilized on the polymeric structure (Bernkop-Schnürch, Schwarz, & Steininger, 1999). They can tightly adhere to the intestinal mucus layer for a prolonged time through covalent bonding with mucin glycoproteins via thiol-disulfide exchange reactions. Hence, they provide a steep drug concentration gradient at the absorption sites. In addition, thiomers demonstrated already a strong permeation enhancing effect for the uptake of poorly absorbed drugs from mucosal membranes (Clausen, Kast, & Bernkop-Schnürch, 2002; Clausen & Bernkop-Schnürch, 2001). The mechanism responsible for this permeation enhancing effect has been discovered within the last few years and shows a reversible opening of the tight junctions and the role of glutathione as permeation mediator (Clausen et al., 2002). Based on this, thiomers might show even improved features, when being formulated to nanoparticles (NP). Nanoparticulate delivery systems have been extensively investigated as oral delivery vehicles for macromolecular drugs due to their ability to protect these drugs from degradation, facilitate drug contact with the absorption sites, and promote drug absorption through the intestinal mucosa (Samstein, Perica, Balderrama, Look, & Fahmy, 2008). So far, however the full potential for thiolated nanoparticles as absorption enhancer for poorly absorbed drugs on the intestinal epithelium has not been tested in detail.

It was therefore the aim of the present study to examine and compare the capabilities of thiolated nanoparticles and solutions with varying characteristics on the absorption of a poorly absorbed model drug (FD_4) on the intestinal epithelial cell layer Caco-2. This confluent monolayer of polarized epithelial cells has previously been used as a model for studying effects of various absorption enhancers on intestinal epithelium (Anderberg, Nyström, & Artursson, 1992). The study focused on the application of the anionic poly(acrylic acid) and the cationic chitosan in different formulations to open epithelial tight junctions, thus allowing for paracellular transport. In addition, the influence of their mucoadhesive properties through addition of a mucus layer to the Caco-2 cells on permeation enhancing effects was evaluated.

2. Materials and methods

2.1. Materials

Chitosan with an average molecular weight (M_w) of 150 kDa and a deacetylation degree of 85% as well as PAA with an average M_w of 100 kDa were obtained from Sigma Aldrich, Austria. Sodium tripolyphosphate pentabasic (TPP), 5,5′-dithiobis(2-nitrobenzoic acid) (Elman's reagent), 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide (MTT), 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDAC), Hank's balanced salts for Hank's balanced salts solution (HBSS), thioglycolic acid (TGA), sodium borohydride ($NaBH_4$) and porcine gastric mucin were purchased from Sigma Aldrich, Austria. LDH based CytoTox 96 kit was obtained from Roche. Fluoresceinisothio-cyanate-dextran (FD_4, 4400 Da, purity >95%) was supplied from TdB Consultancy AB (Uppsala, Sweden). Caco-2 cells were kindly donated by Prof. Pfaller, Institute of Physiology, Medical University of Innsbruck. Cell culture medium, penicillin/streptomycin solution and fetal calf serum (FCS) were purchased from PAA, Austria. ThinCert 12-well transwells with polycarbonate membrane and 0.4 μm pore size as well as toxicity plates (24-well) were obtained from Greiner, Austria.

2.2. Synthesis of polymer conjugates

2.2.1. Modification of chitosan with thioglycolic acid (CS-TGA)

Thiolated chitosan can be synthesized by derivatization of its primary amino groups with coupling reagents bearing thiol functions. Modification in this study was achieved via the covalent attachment of thioglycolic acid (TGA) to chitosan (CS) as described previously (Kast & Bernkop-Schnürch, 2001). Briefly, 1.0 g of CS was dissolved in acetic acid 0.05% (v/v). TGA was chemically treated with EDAC in a final concentration of 100 mM in order to activate the carboxylic acid moieties. Thereafter, 500 mg of activated TGA were added drop wise to CS solution and the reaction mixture was incubated for 3 h at room temperature under vigorous stirring. Unbound compounds were isolated by exhaustive dialysis for five times at acid pH conditions. The thiomer (CS-TGA) was lyophilized and kept at 4 °C for further use.

2.2.2. Modification of poly(acrylic acid) with cysteine (PAA-Cys)

The poly(acrylic acid)-cysteine (PAA-Cys) conjugate was synthesized according to a method described previously by our group (Greindl & Bernkop-Schnürch, 2006). First, 500 mg of PAA were hydrated in distilled water and the pH was adjusted to 4.5. Thereafter, EDAC in a final concentration of 100 mM was added drop wise in order to activate the carboxylic acid moieties of the hydrated polymer. After 20 min incubation at room temperature, 500 mg of cysteine were added and pH was readjusted to 4.5. Reaction mixture was incubated again for 24 h at room temperature under constantly stirring. The resulting conjugate was isolated by dialysis according to the method described previously (Greindl & Bernkop-Schnürch, 2006). Afterwards the frozen aqueous polymer solution was dried by lyophilization and stored at 4 °C until further use. Samples prepared in exactly the same way but omitting EDAC during the coupling reaction served as controls.

2.2.3. Determination of modification degree

The degree of modification as amount of thiol groups immobilized on the polymer was quantified by Ellman's method using a spectrophotometer (Wilson, Bayer, & Hupe, 1977). Briefly, 0.5 mg of modified polymers were dissolved in phosphate buffer, into which 500 μl of Ellman's reagent (pH 8) were added. Samples were incubated at 37 °C in a water bath and protected from light for 2 h. Subsequently, 100 μl of each sample solution were transferred to a microplate reader (FluoStar Galaxy, BMG, Offenburg, Germany) to determine the content of thiol groups at a wavelength of 450 nm. Moreover, disulfide contents were evaluated after reduction with $NaBH_4$ and as well determined by Ellman's reagent. The total amount of these moieties is represented by the summation of free and oxidized thiol groups in form of disulfide bonds (Werle & Hoffer, 2006). Cysteine and TGA served as standards to calculate the quantity of thiol groups immobilized on the polymer.

2.3. Particle preparation and characterization

Cationic CS is able to form nanoparticles (NP) with negatively charged TPP and anionic PAA with positively charged Ca^{2+} by ionic gelation (Calvo, Vila-Jato, & Alonso, 1997; Greindl & Bernkop-Schnürch, 2006). Therefore, 100 mg of unmodified and modified CS were dissolved in 20 ml of acetic acid 0.05% (v/v) pH 5.5. A 0.5% (m/v) TPP solution (pH 7) was drop wisely added to the chitosan solutions until turbidity occurred.

For unmodified and modified PAA, 100 mg of each polymer were dissolved in distilled water to obtain a 0.5% (m/v) solution and pH was adjusted to 8. $CaCl_2$ was dissolved in distilled water at 10 mg/ml and added to PAA or PAA-Cys solution under continuous stirring until turbidity occurred. Each particle suspension was stirred with 300 rpm for 1 h at room temperature. To remove the anionic and cationic cross linkers (TPP and Ca^{2+}) each nanoparticle suspension was centrifuged 3 times (4300 rpm, 30 min) and resuspended in water (PAA) or 0.05% of acetic acid (CS). To avoid any particle aggregation, trehalose in a concentration of 3% was

added to each suspension. Finally, the pH of all suspensions was readjusted before lyophilizing.

The amount of thiol groups on all particles was determined photometrically with Ellman's reagent as described earlier. Size and zeta potential of particle suspensions were determined by photon correlation spectroscopy using a Nicomp particle sizer (Nicomp 380 ZLS, PSS Nicomp) with laser wavelength of 650 nm and an E-fields strength of 10 V/cm. For particle size measuring their intensity distribution was fit to a Gaussian size distribution curve.

2.4. Assessment of cytotoxic effects

2.4.1. Cytotoxicity assay

In order to evaluate possible cytotoxic effects of polymer solutions or nanoparticles, cytotoxicity studies were determined by the lactate dehydrogenase assay (LDH) (Decker & Lohmann-Matthes, 1988). Caco-2 cells of passage 14–17 were cultured into 24 well plates (1×10^5 cells/ml) for 21 days at 37 °C under 5% CO_2 and 90% relative humidity in minimum essential medium (MEM). During this time the cells were fed with 500 μl of MEM every 48 h. After 21 days, medium was removed, cells were washed with PBS and 500 μl HBSS pH 6.4 containing 0.5% (m/v) of each formulation was added. A 10% solution of Triton-X was used as positive control, whereby untreated cells with MEM served as negative control. Supernatants were withdrawn at time point 0, 3 and 24 h, samples centrifuged and stored at 4 °C before analysis utilizing the CytoTox 96 kit. After 15 min incubation in the dark, absorbance was measured with a microplate reader at 492 nm. Cytotoxicity was calculated by the following equation:

$$\text{cytotoxicity}\,[\%] = \frac{(\text{average absorbance of samples} - \text{negative control})}{\text{positive control} - \text{negative control}} \times 100 \quad (1)$$

2.4.2. Cell viability assay

Viability of cells was measured using the MTT staining method (Mosmann, 1983). Therefore, MTT was dissolved in MEM at 0.5 mg/ml and filtered to be sterilized. Caco-2 cells were grown and fed as described before. After 21 days, cells were incubated with 0.5% of each polymer sample for 3 and 24 h. Untreated cells were used as positive control, whereas a 10% solution of Triton-X was used as negative control. After incubation periods, medium was replaced by 500 μl of MTT solution per well. The cells were incubated for 3 h along with the reagent. Cell supernatant was aspirated, 500 μl of DMSO added to every well and mixed thoroughly to dissolve the blue-violet crystals. Aliquots of 100 μl were transferred to a microtiter plate and absorbance was measured at wavelength of 570 nm with a microplate reader. Percentage of cell viability was calculated by Eq. (2) compared to 0% viability (negative control) and 100% viability (positive control):

$$\text{cell viability}\,[\%] = \frac{\text{average absorbance of samples}}{\text{average absorbance of positive control}} \times 100 \quad (2)$$

2.5. In vitro permeation studies across Caco-2 monolayer

2.5.1. Permeation studies without a mucus layer

For cell culture experiments cells of passage 14–17 were used. They were placed in a density of 1×10^5 cells/ml onto the polycarbonate membrane inserts of 12-well plates directly after splitting and were allowed to grow and differentiate over 21 days. During this time, cells were fed with 1 ml of the medium mentioned above in the apical and 2 ml of the same medium in the donor chamber every 48 h. Permeation studies were performed in the transwell monolayer system with a permeation area of 1.13 cm^2 and 1 ml of HBSS buffered with 40 mM Hepes pH 6.4 in the donor and the same medium pH 7.4 in the acceptor chamber. The paracellular transport was investigated through the hydrophilic compound FD_4 for the apical-to-basolateral (A to B) direction. Hence, the absorptive transport of FD_4 in a final concentration of 1.0 mg/ml (0.23 nM) was investigated in the absence and presence of 0.5% (m/v) of each test compound: unmodified CS and PAA (solution and NP) as well as thiolated CS and PAA (solution and NP). Within 3 h incubation, samples of 100 μl were taken out every 30 min of the apical chamber and substituted by incubation medium preheated at 37 °C. The amount of permeated FD_4 was determined by fluorescence measurements (λ_{ex} = 485 nm and λ_{em} = 535 nm) with a microplate reader. Cumulative corrections were made for previously removed samples. Apparent permeability coefficients (P_{app}) for FD_4 across the Caco-2 monolayer were calculated from the following equation:

$$P_{app} = \frac{Q}{A \times c \times t} \quad (3)$$

where Q is the total amount permeated within 3 h (μg), A is the diffusion area of the transwells (1.13 cm^2), c is the initial concentration in the donor chamber (1000 μg/cm^3) and t is the time of the experiment (10,800 s). Improvement ratios were calculated from P_{app} values for each test compound by the following equation:

$$IR = \frac{P_{app}\,(FD_4 + \text{compound})}{P_{app}\,(FD_4\,\text{control})} \quad (4)$$

2.5.2. Measurements of transepithelial electrical resistance

The integrity and permeability of the monolayer was studied by determining the transepithelial electrical resistance (TEER) with an epithelial voltohmmeter (EVOM®, World Precision Instruments, Germany). TEER was measured prior to each experiment to ensure the confluence of the monolayer and during transport studies (every 30 min for 3 h) to observe the effect of transport enhancer at each sampling time. At the end of each experiment, cells were washed thoroughly with phosphate buffered saline (PBS) to ensure complete removal of all samples. Thereafter, both apical and basolateral chambers were replenished with fresh complete medium and incubated at 37 °C. TEER values were measured again after 24 and 48 h to observe TEER increase indicating cell recovery. The measured TEER previous to each experiment ranged between 680 and 830 Ω cm^2 and set as 100%. All other values were calculated according to this.

2.5.3. Influence of mucus on permeation enhancement

A mucus layer is of limited importance as barrier to drug diffusion as it restricts the access of higher molecular weight compounds to the epithelial surface. As it is known that cultured Caco-2 cells do not produce a mucus layer, which reduces the number of factors playing a role in transepithelial penetration, permeation studies were also performed by mucus covered Caco-2 cells. The monolayers were therefore grown onto the transwell inserts of 12-well plates and allowed differentiate over 21 days as described above. For permeation studies, Caco-2 cells were completely covered with 28 μl sterilized artificial mucus to achieve a thickness of 250 μm, which was determined to be the mean mucus thickness of the whole intestine (Atuma, Strugala, Allen, & Holm, 2001). The absorptive transport of FD_4 in a final concentration of 1.0 mg/ml was examined again in absence and presence of 0.5% (m/v) anionic and cationic unmodified as well as thiolated polymers in solution and formulated as NP. In addition, permeation experiments with thiolated solutions and nanoparticles were repeated including plate shaking on a Vibromax 100 (Heidolph Instruments) with 300 rpm to investigate, whether movement of particles had an influence for the A to B transport. For all studies, the amount of permeated FD_4 was determined and calculated as described previously for permeation studies without a mucus layer.

In order to provide the same properties of natural and commercial available artificial mucus, their viscoelastic features were

Table 1
Comparison of the total amount of thiol groups, disulfide bonds and remaining free thiol groups on thiolated conjugates as well as mean particle size and zeta potential of unmodified and modified nanoparticles. Indicated values are means ± SD ($n = 3$).

Polymer	—SH [μmol/g]	—S—S— [μmol/g]	Σ—SH [μmol/g]	Particle size [nm]	Zeta potential [mV]
CS particles	–	–	–	250 ± 35-	20 ± 5
CS-TGA polymer solution	1060	20	1100 ± 80	–	–
CS-TGA particles	940	55	1050 ± 70	230 ± 30	18 ± 4
PAA particles	–	–	–	280 ± 15	−15 ± 3
PAA-Cys polymer solution	1110	45	1200 ± 75	–	–
PAA-Cys particles	1040	70	1180 ± 70	220 ± 14	−14 ± 5

determined by a plate-plate combination rheometer (RotoVisco RT20, Haake GmbH, Germany). In brief, different amounts of porcine gastric mucin were hydrated in PBS (pH 7.4) to obtain a 10, 20, 30 and 40% (m/v) mucin solution. Immediately after hydration and an incubation period of 3 h at 37 °C, aliquots of 500 μl of each mucin solution were transferred to the plate-plate viscometer and the apparent viscosity (η) measured as described previously by our group (Kast & Bernkop-Schnürch, 2001). The shear stress was set at a range of 0.5–500 Pa, the gap between two plates was 0.5 mm and the temperature maintained at 37 °C.

2.6. Statistical data analysis

Unpaired Student's *t*-test was performed by GraphPad Prism 5 to test the significance of the difference between the mean value of the control and the respective sample. Level of $p \leq 0.05$ was set for significant, $p \leq 0.01$ for very significant and $p \leq 0.001$ for highly significant.

3. Results and discussion

3.1. Characterization of thiomers

Chitosan was modified by covalent attachment of thioglycolic acid due to the formation of amide bonds between the primary amino groups of the polymer and carboxylic acid groups of TGA. Cysteine was covalently linked to PAA via formation of amide bonds between the primary amino group of cysteine and a carboxylic acid group of the polymer. The efficacy of the purification method could be verified by controls which were prepared in exactly the same way as the polymer conjugates but omitting EDAC during the coupling reaction, exhibiting a negligible amount of thiol groups. Both thiomers appeared as white, odourless powders of fibrous structure after lyophilization, which were soluble in aqueous solutions. As listed in Table 1, about 1100 μmol (CS-TGA) and 1200 μmol (PAA-Cys) of thiol groups per gram polymer were immobilized on the thiomers with approximately 94% remaining free thiol groups while 6% being oxidized to disulfide bonds during the conjugation. Substructures of both thiomers are illustrated in Fig. 1.

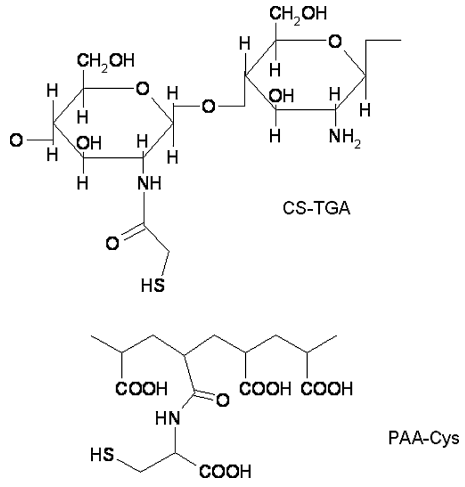

Fig. 1. Presumptive chemical substructure of thiolated poly(acrylic acid) (PAA-Cys) as well as modified chitosan (CS-TGA) conjugates.

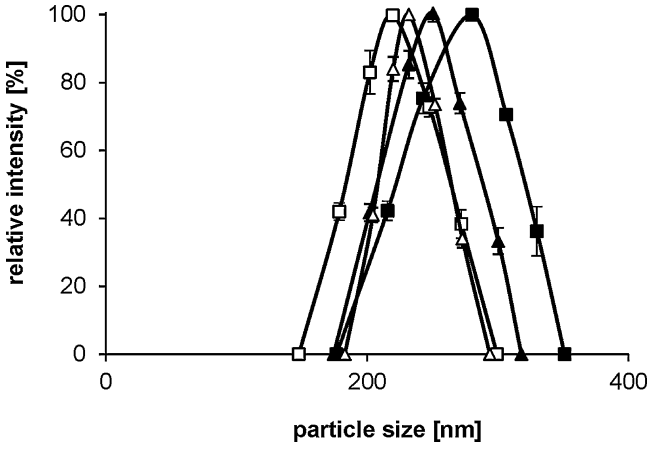

Fig. 2. Particle size distribution of unmodified PAA (■) and CS (▲) as well as modified PAA-Cys (□) and CS-TGA (Δ) particles. Indicated values are means ± SD of at least three experiments.

3.2. Characterization of nanoparticles

Unmodified and thiolated NP were prepared via ionic gelation with TPP (CS) and Ca^{2+} (PAA) followed by the removal of the ionic cross linkers. As shown in Fig. 2 and Table 1, particles possessed sizes of 220 to 280 nm and positive (CS) or negative (PAA) zeta potentials, respectively. Due to the carboxylic groups in unmodified and modified PAA, particles were negatively charged. Concerning to the free amino groups in chitosan, modified and unmodified particles display a positive charge. Concerning to the free amino groups in chitosan, modified and unmodified particles display a positive charge. Positive charges of these particles could give rise to a strong electrostatic interaction with tight junctions and negatively charged mucus layer. Hence, particles of a more positive zeta potential should display comparatively more pronounced permeation and mucoadhesive properties. Moreover, it was shown that thiolation of both polymers led to minor changes in the zeta potential and to a slight decrease in particle size as listed in Table 1. The lyophilized NP appeared as white powder and were resuspendable in aqueous solutions.

3.3. Cytotoxicity and cell viability studies

The toxicity and viability of NP and solutions on Caco-2 cells was evaluated via membrane integrity LDH assay and cellular viability MTT assay. In the LDH test, enzyme release after cell damage was measured, whereas in the MTT test the decrease in mitochondrial activity in cells was determined.

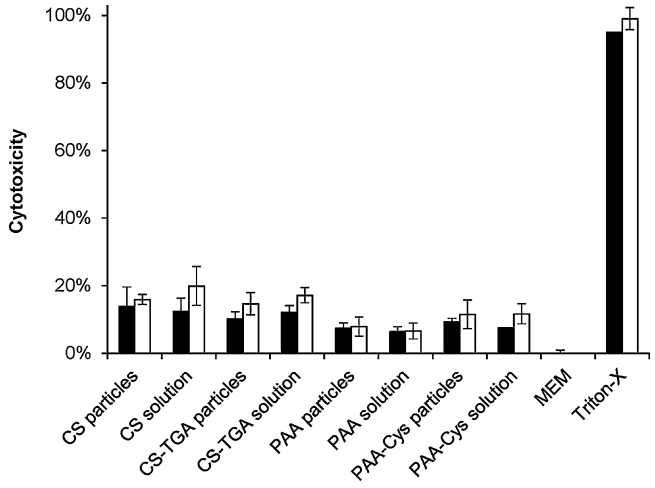

Fig. 3. Cytotxicity via LDH test of all samples after 3 h (black bars) and 24 h (white bars). Indicated values are means ± SD of at least three experiments.

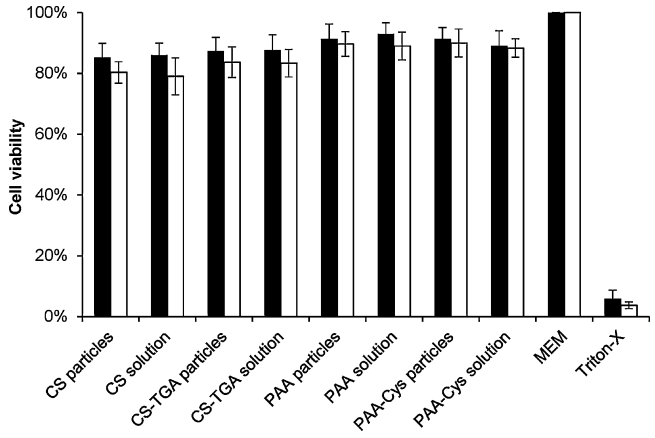

Fig. 4. Cell viability via MTT assay of all samples after 3 (black bars) and 24 h (white bars). Indicated values are means ± SD of at least three experiments.

Generally, neither the polymeric NP nor the solutions induced severe toxicity. More than 80% viable cells were determined by both tests after 24 h incubation with all samples as shown in Figs. 3 and 4. LDH test revealed that cells remained intact, and less than 20% LDH release was observed after overnight incubation with polymer formulations. In case of unmodified CS solution, LDH release increased from 12% after 3 h to 20% after 24 h incubation, which might be related to its strong positive charge. Cytotoxicity of cationic polymers is directly related to their surface charge density (Chang, Westcott, Henson, & Voelkel, 1987). Quantitative assessment of viable cells by MTT assay after 3 and 24 h of incubation with unmodified or modified particles and solutions showed at least 85 and 80% mitochondrial activity, respectively. Hence, anionic as well as cationic NP and polymer solutions can be regarded relatively non-toxic for Caco-2 cells during the exposure time. In addition, results of both assays showed that the decrease in the viability of cells after 3 and 24 h was not significant ($p > 0.05$). Results of the LDH cytotoxicity and MTT viability test are shown in Figs. 3 and 4, respectively.

3.4. Permeation studies

3.4.1. In vitro evaluation of permeation enhancement

The two multifunctional polymers of different ionic charge were studied with respect to their probable use in a suitable formulation for oral delivery of hydrophilic drugs. The influence of both polymer solutions as well as NP on the transport of the hydrophilic macromolecule FD_4 was evaluated across Caco-2 monolayers. FD_4 is well known as hydrophilic marker for studying cell processes such as cell permeability, phagocytosis or endocytosis and as well as mechanisms of biomolecular delivery (Makhlof, Werle, Tozuka, & Takeuchi, 2011). Advantages of these fluorescent dextrans include water solubility, low toxicity and resistance against acid and alkaline hydrolysis due to its amount of alpha-1.6-polyglucose linkages (Andrieux, Lesieur, Lesieur, Ollivon, & Grabielle-Madelmont, 2002). Regarding FD_4 analyses, the marker was dissolved in decreasing concentrations ranging from 1000 μg/ml to 0.976 μg/ml in 40 mM HEPES buffer pH 6.4, whereby the limit of detection was determined to be 1.95 μg/ml. Calibration curve of FD_4 presented as Supporting Material in Fig. S2 showed a good correlation with a linear regression coefficient (R^2) of 0.9921.

Caco-2 cells, when grown on transwell filters, spontaneously differentiate in culture to form confluent monolayers which both structurally and functionally resemble the small intestinal epithelium. Owing to this property they show promise as a simple, in vitro model for the study of drug absorption in the intestinal mucosa (Hilgers, Conradi, & Burton, 1990). In order to overcome biological tissue barriers, molecules have to pass the epithelium which can be achieved by several pathways. Among all routes the paracellular route is the main absorption way for hydrophilic compounds and controlled by the tight junctions. Regarding this, both polymers were supposed to influence the structure of epithelial tight junctions and therefore assigned for the paracellular route of drug uptake.

Within this study, it could be demonstrated that due to the addition of 0.5% (m/v) PAA solution to the buffer, transport of FD_4 was 1.4-fold (P_{app}: 2.18×10^{-6} cm/s) improved in comparison to FD_4 (P_{app}: 1.58×10^{-6} cm/s) applied in buffer only. Moreover, due the addition of 0.5% (m/v) unmodified PAA particles to the buffer, transport of FD_4 was 1.7-fold (P_{app}: 2.67×10^{-6} cm/s) enhanced. Furthermore, nanoparticle formulation and polymer solution comprising 0.5% (m/v) unmodified CS improved the transport of FD_4 3.1- and 2.8-fold (P_{app}: 4.94 and 4.39×10^{-6} cm/s), respectively. However, this permeation enhancing effect of CS and PAA was much more pronounced through the immobilization of thiol groups on the polymeric backbones. In presence of all thiolated PAAs, corresponding to their administration as solution or nanoparticulate system, FD_4 transport was 3.0- and 3.7-fold improved compared to the buffer control, respectively. P_{app} values of PAA-Cys in solution or as NP were determined to be 4.77 and 5.83×10^{-6} cm/s, respectively. As opposed to this, P_{app} values of the cationic CS-TGA utilizing as polymer solution or as NP were determined to be 6.42 and 8.37×10^{-6} cm/s, and therefore significantly ($p < 0.001$) increased compared to control value of 1.58×10^{-6} cm/s. Permeation enhancement ratios of 4.1 and 5.3 for CS-TGA in solution and utilized as NP were thereby reached, respectively. P_{app} values indicate that cationic chitosan and its derivatives were slight more effective permeability enhancers for FD_4 than the respective anionic PAA derivates. The rank order of effectiveness in enhancing FD_4 permeability across the Caco-2 monolayer was determined to be CS-TGA particles > CS-TGA solution > PAA-Cys particles > CS particles > PAA-Cys solution > CS solution > PAA particles and PAA solution. These outcomes demonstrated that anionic PAA showed only a slight enhancement in the permeation of the hydrophilic compound. This observation might be related to the low molecular weight (100 kDa) of PAA used within this study. As it was shown by Kast and Bernkop-Schnürch (2002), PAA with a M_w of 450 kDa showed significantly higher permeation enhancing effects than PAA with M_w of 100 kDa. However, using a comparatively higher molecular mass led to much greater particles (Greindl & Bernkop-Schnürch, 2006). In contrast, use of cationic chitosan was attributed to significantly ($p < 0.05$) improved permeation enhancing features. It was already demonstrated in previous studies that

Table 2
Comparison of the apparent permeability coefficients (P_{app}) and their improvement ratio (IR) related to the control of the hydrophilic marker FD_4 in presence of indicated test compounds on Caco-2 monolayers with or without a mucus layer. Indicated values are means ± SD ($n = 5$), (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to Control FD_4).

Test compound 0.5% (m/v)	Caco-2 cells without mucus		Caco-2 cells with mucus		Ratio P_{app} without mucus/P_{app} with mucus
	P_{app} ($\times 10^{-6}$ cm/s)	IR	P_{app} ($\times 10^{-6}$ cm/s)	IR	
Control FD_4	1.58 ± 0.24	–	1.53 ± 0.26	–	1.0
CS solution*	4.39 ± 1.57	2.8	2.92 ± 0.23	1.9	1.5
CS particles**	4.94 ± 0.66	3.1	3.11 ± 0.25	2.0	1.6
CS-TGA solution***	6.42 ± 0.33	4.1	2.03 ± 0.11	1.3	3.2
CS-TGA particles***	8.37 ± 1.79	5.3	2.58 ± 0.50	1.7	3.2
PAA solution	2.18 ± 0.24	1.4	2.05 ± 0.16	1.3	1.1
PAA particles	2.67 ± 0.20	1.7	2.39 ± 0.29	1.6	1.1
PAA-Cys solution**	4.77 ± 0.39	3.0	2.59 ± 0.22	1.7	1.8
PAA-Cys particles**	5.83 ± 1.74	3.7	3.01 ± 0.20	1.9	1.9

CS possessed promising abilities to increase the paracellular drug transport (Kotzé, Lueßen, de Boer, Verhoef, & Junginger, 1999; Lueßen et al., 1996). The mechanism underlying this enhancement of CS involves electrostatic interactions between its positive charge and negatively charged sites in the tight junctions, which led to drug transport via transiently opening of tight junctions (Merwe, Verhoef, Verheijden, Kotzé, & Junginger, 2004). Hence, CS bound to the epithelial cell membrane through a charge-dependent mechanism, resulting in enhanced permeability to the apical membrane. The anionic PAA affected also by opening tight junctions, which might, however, be partly due to their calcium-binding activity (Kast & Bernkop-Schnürch, 2002). PAA inhibited proteolytic enzymes and increased therefore the oral absorption of FD_4 through this mechanism, but to an essential lower effect than the cationic CS. The proposed mechanism for the permeation enhancement of thiomers seems to be based on inhibition of the enzyme protein tyrosine phosphatase (PTP), which is responsible for the dephosphorylation of tyrosine subunits of occludin, an important protein building up the tight junctions (Clausen et al., 2002). The inhibition of PTP can be achieved via formation of disulfide bonds among its cysteine residues and intestinal GSH resulting in an increased number of phosphorylated tyrosine groups and therefore opening of tight junctions (Barrett et al., 1999). Glutathione is known to be present in its reduced (GSH) and oxidized (GSSG) form at the apical side of mucosa. Immobilized thiol groups present on the surface of thiomers reduce GSSG thereby raising the amount of GSH at the absorption site for PTP inhibition. Accordingly, significantly improved paracellular permeability through tight junctions could be observed.

Overall, it could be demonstrated that unmodified or modified polymers formulated as nanoparticles had a greater effect as permeation enhancers than their corresponding polymer solutions. The use of thiolated particulate absorption enhancers, which improve the mucosal permeation of macromolecules without causing serious tissue damage seemed to be very promising approaches for their efficient oral delivery.

Results of the in vitro permeation studies are shown in Table 2. The cumulative transport of FD_4 across the cells is shown as a function of time in Fig. 5.

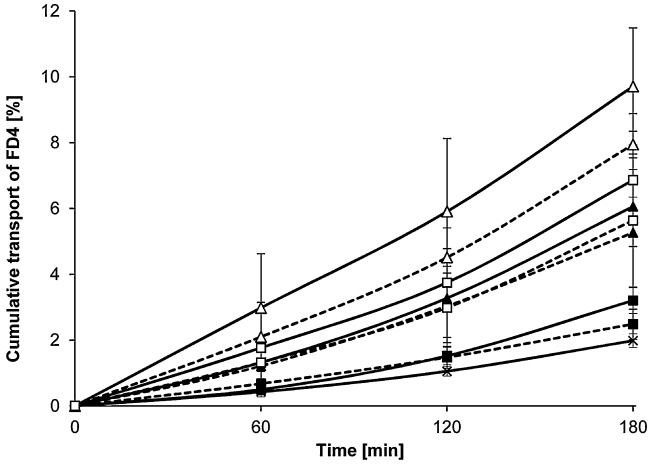

Fig. 5. Cumulative transport of FD_4 across Caco-2 cells based on 0.5% (m/v) unmodified polymers [(–▲–) CS* and (–■–) PAA], unmodified particles [(▲) CS** and (■) PAA], modified polymers [(–△–) CS-TGA*** and (–□–) PAA-Cys**] and modified particles [(△) CS-TGA*** and (□) PAA-Cys**] in comparison to the FD_4 control (×). Indicated values are means ± SD ($n = 5$), (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to FD_4 solution).

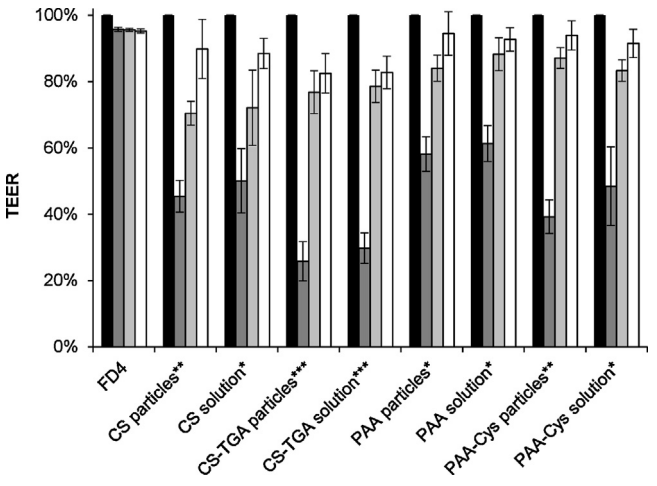

Fig. 6. TEER measurements on Caco-2 cells before (black bars), during permeation studies (dark grey bars) and after 24 h (grey bars) as well as after 48 h (white bars). Indicated values are means ± SD ($n = 5$), (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to FD_4 solution).

3.4.2. Effects on TEER measurements

TEER measurements are a good indication of the tightness of the junctions between cells. Thus, a decrease in TEER during a permeation experiment can be explained by the regulation and opening of the tight junctions and is therefore an informative value for the paracellular transport.

Results of TEER measurements are shown in Fig. 6 and demonstrated that all samples were able to decrease significantly ($p < 0.05$) the TEER of the monolayer. This reduction during the transport studies was in accordance to the enhancement effects of all polymer samples on FD_4 permeability (Fig. 5). In case of the cationic CS samples, the TEER decreased more rapidly with enhanced loosening/opening of the intercellular tight junctions than in case of the anionic PAA samples. The relevant data presented in Fig. 6 indicate that this effect was even significantly ($p < 0.05$) stronger through the immobilization of thiol groups on the polymer

Table 3
Viscosity values of natural and artificial mucus. Measurements were carried out immediately after suspending (0 h) and after an equilibration period of 3 h. Indicated values are means ± SD ($n = 3$).

Mucus	Concentration (%)	Volume (μl)	Dynamic viscosity η (mPa s)	
			0 h	3 h
Natural	–	500	4.89 ± 0.22	5.00 ± 0.29
Artificial	10	500	0.04 ± 0.01	0.05 ± 0.01
	20	500	0.87 ± 0.01	0.88 ± 0.01
	30	500	4.38 ± 0.29	4.42 ± 0.36
	40	500	29.87 ± 0.87	31.97 ± 0.65

backbones. CS-TGA particles, for instance, showed the strongest effect in reducing the TEER and were therefore the most effective ones in enhancing the permeability of the hydrophilic macromolecule FD_4. FD_4 is supposed to penetrate via the paracellular route, the hypothesis that the polymers act by reversibly loosening/opening the epithelial tight junctions could thereby be proven (Schipper et al., 1997). This improved paracellular uptake through Caco-2 cells by unmodified and modified chitosans or polyacrylates could be already demonstrated in previous studies utilizing different low permeable transport markers. Bejugam et al. for instance evaluated with low permeable compounds the permeation effects of chitosans and polyacylates and could demonstrated that chitosan improved significantly the paracellular permeation of FITC-dextran as well as Rhodamine-123 and was able to decrease the transepithelial electrical resistance indicating the paracellular transport mechanism (Bejugam, Sou, Uddin, Gayakwad, & D'Souza, 2008). In addition, also thiolated formulations could guarantee a permeation enhancement of the low-molecular paracellular marker Rhodamine-123 (Hombach & Bernkop-Schnürch, 2009).

Moreover, TEER values do not completely recover at the end of the experiments, which might be related to the incomplete removal of polymer samples from cell surface caused by bioadhesion and viscosity of the polymers. Certainly, there was no significant suggestion for cell damage. TEER of cells treated with CS or PAA samples recovers to at least 70 or 78% of the initial value, respectively. Nevertheless, a reversibility of the achieved effects could be observed after 2 days incubation. A recovery of TEER after 48 h implied that cells were undamaged and functionally intact. Hence, it can be concluded that all substances permeated the epithelial barrier via the paracellular route, and was not due to cell damage. The effect over time of each compound on the TEER of Caco-2 cells appears in Fig. 6.

3.4.3. *Influence of mucus on permeation enhancement*

Rheological measurements of natural intestinal porcine and artificial mucus pointed out that commercially available mucin in a concentration of 30% exhibited approximately the same viscoelastic features as the natural mucus, which are listed in Table 3. Based on these outcomes, a 30% sterilized mucilage was used for covering the cells and to examine the influence of this barrier for drug absorption.

Results of the permeation studies of mucus covered Caco-2 cells demonstrated that mucus has a significant influence on the permeability of the two mucoadhesive polymers. Unmodified CS in solution or formulated as NP, for instance, showed a 1.5- and 1.6-fold lower P_{app} value, respectively in comparison to P_{app} values achieved by mucus free permeation studies. This reduction in drug absorption was even more pronounced when thiolated formulations were used. The transport enhancement ratio for CS-TGA solution or NP in contrast to the buffer was determined to be only 1.3- and 1.7-fold, respectively. P_{app} values were therefore 3.2-fold lower than the corresponding values omitting the mucus layer. Results of PAA-Cys administered as solution or NP showed a 1.8- and 1.9-fold decreased permeation enhancing effect of the system compared to the previous achieved results, respectively. In contrast, when PAA solution or particles were administered to the monolayer, P_{app} values were only 1.1-fold lower than P_{app} values obtained without a mucus layer. Results and comparisons of permeation studies across mucus free and mucus covered Caco-2 cells are shown in Table 2. In addition, permeation experiments performed under permanent shaking as shown in Table S1 (seen in Supporting material) were in good accordance with previous results without shaking as no significant difference ($p > 0.05$) could be determined The only difference was seen in the uniformity of the results as plate shaking resulted in smaller standard deviations. The very thin mucus layer used within these studies might be the reason that plate shaking was herein not necessary. Furthermore, the use of usually lab standing devices seemed to be unsuitable for a cell lab as cells need really sterile and harmonized conditions.

Based on their different structure and ionic charge, the mechanisms of both polymers exhibited different characters, which emphasize the variation of permeability. In brief, permeation of FD_4 utilizing unmodified PAA was not essential altered when cells were covered with a mucus layer. A reason for these outcomes can be seen in the anionic charge of PAA and thus its weak absorption or even repulsion behaviour with the negatively charged mucin molecules. In contrast, incubation with CS formulations revealed that mucus largely inhibited the interaction between the cationic polymer and the epithelial cell membrane. CS decreased the uptake of FD_4 by forming ionic interactions between its positively charged groups and negatively charged sub structures of the mucus (Schipper et al., 1997). The mucoadhesiveness of a particle suspension depends mainly on the ability of particles to interact with mucins glycoproteins or other mucus components being immobilized within the mucus layer (Durrer, Irache, Puisieux, Duchäne, & Ponchel, 1994; Ponchel & Irache, 1998). However, due to just weak electrostatic interactions, the absorption of FD_4 using unmodified CS and PAA on mucus covered cells was less reduced than in case of all thiolated formulations. As shown in Table 2, their P_{app} values for permeation studies omitting the mucus layer were at least 1.8-fold higher than P_{app} of experiments containing mucus. Hence, it could be demonstrated that owing to their mucoadhesive properties their permeation enhancing features were significantly ($p < 0.05$) reduced when cells were covered with mucus. Both thiolated polymers interact via formation of disulfide bonds with cysteine-rich subdomains of mucus glycoproteins demonstrating the potential of these conjugates for being effective as absorption enhancers at mucosal surfaces (Bernkop-Schnürch, 2005). These mucoadhesive features prevented all thiolated formulations from reaching and/or adhering to the epithelial cell membrane and inhibited their absorption-enhancing effects. In addition, comparisons between anionic and cationic derivates demonstrated that positively charged CS formulations were absorbed by mucus gel layer to a significant higher degree than carboxylated particles. Furthermore, both polymers administered as NP reached the epithelial cell membrane to a slight higher degree than their corresponding solutions and exhibited therefore a preferable mucus diffusion behaviour. Owing to their small size, nanoparticles are able to penetrate into comparatively more loose regions of the mucus. By contrast, their polymer solutions with high molecular weights and chain length do not have the possibility to enter the mucus layer as deep as their corresponding particulate systems (Coupe, Davis, & Wilding, 1991). If nanoparticles are able to move through the mucus barrier and reach the epithelium, they might remain there and release their payload in a concentrated manner to the absorption membrane. Hence, owing to their prolonged residence time, a comparatively longer time is available for drug uptake by the cells resulting in an improved bioavailability.

Overall, potential reactive groups such as thiol groups make polymers a varied polymer with unique properties for utilization

in drug delivery technology. The combination of thiol groups and nanoparticulate systems offer promising permeation enhancing effects in relation to mucoadhesive and mucus diffusion features. These polymeric particulate systems are attractive drug delivery carriers able to improve the oral delivery of poorly absorbed drugs.

4. Conclusion

Within this present study, permeation enhancement properties of unmodified and thiolated anionic or cationic polymers utilized as nanoparticles or in solution were evaluated on the Caco-2 monolayer. It could be demonstrated that both polymers when administered as NP exhibited higher transport rates than their polymer solutions. In addition, the covalent attachment of a sulfhydryl ligand on the polymer backbones led to enhanced permeation properties of the hydrophilic macromolecule FD_4 along the paracellular route. Owing to the immobilized free thiol groups on the thiomer surface and their ability to form covalent bonds with the mucus layer, absorption enhancing effects were significantly reduced when cells were covered with a mucus layer. However, the therapeutic potential of such drug particulate carriers is probably not to deliver the drug directly in the blood flow, but rather to increase bioavailability by protecting from degradation or by increasing drug concentration for a prolonged time at the mucosal membrane. Therefore, both thiolated polymers as nanoparticulate delivery systems seem to be promising carriers for oral delivery of hydrophilic macromolecular drugs.

Supporting material

This article contains additional Supporting material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.09.078.

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