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Distribution of thiolated mucoadhesive nanoparticles on intestinal mucosa

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ABSTRACT

It was the aim of the present study to evaluate and compare the distribution of thiolated mucoadhesive anionic poly(acrylic acid) (PAA) and cationic chitosan (CS) nanoparticles on intestinal mucosa. Modifications of these polymers were achieved by conjugation with cysteine (PAA–Cys) and 2-iminothiolane (CS–TBA). Nanoparticles (NP) were prepared by ionic gelation and labelled with the strong hydrophilic fluorescent dye Alexa Fluor 488 (AF 488) and hydrophobic fluorescein diacetate (FDA). Unmodified and modified CS and PAA NP were examined in vitro in terms of their mucoadhesive and mucus penetrating properties on the mucosa of rat small intestine. To investigate the transport of NP across the mucus layer, their diffusion behaviour through natural porcine intestinal mucus was studied through a new diffusion method developed by our group.

Lyophilised particles displayed 526 μmol/g (CS) and 513 μmol/g (PAA) of free thiol groups and a zeta potential of 20 mV (CS) and −14 mV for PAA NP. Nanoparticle distribution on rat intestine suggested that mucoadhesion of thiolated NP is higher than the diffusion into the intestinal mucosa. Modified particles displayed more than a 6-fold increase in mucoadhesion compared to unmodified ones. The rank order with regard to mucoadhesion of all particles was: CS–TBA > PAA–Cys > CS > PAA, whereas CS–TBA showed 2-fold higher mucoadhesive properties compared to PAA–Cys NP. Diffusion through intestinal mucus was much higher for unmodified than for thiolated as well as for anionic compared to cationic particles. Overall, it was shown that thiolated particles of both anionic and cationic polymers have improved mucoadhesive properties and could be promising carriers for mucosal drug delivery.

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1. Introduction

The oral route, most accepted route by patients, exhibits poor bioavailability of therapeutic agents, which depends on the rapid degradation in contact with the gastrointestinal fluids and is a consequence of poor absorption through the GI epithelium. Related to these problems, mucoadhesive nanoparticulate delivery systems have been investigated as oral drug delivery vehicles for poorly absorbed therapeutic agents (Kawashima et al., 2000). They promise several advantages that arise from protecting drugs from degradation, localization at a given target site, prolonged residence time at the site of drug absorption and an intensified contact with the mucosa increasing the drug concentration gradient (Florence et al., 1995; Lehr, 1996; Takeuchi et al., 2001). The uptake and consequently bioavailability of the drug may be increased and frequency of dosing reduced with the result that patient compliance is improved. In vitro and in vivo behaviour of nanoparticles tends to be greatly dominated by their physicochemical properties such as size, zeta potential, surface properties and other variables (Sakuma et al., 2001). Nanoparticulate delivery systems for non-invasive administration are based on various polymeric enhancers and mucoadhesive polymers like polyacrylates and chitosans (Agnihotri et al., 2004; Kriwet et al., 1998). Poly(acrylic acid) as anionic polymer offers mucoadhesive properties via the formation of non-covalent bonds as hydrogen bonds, van der Waals forces or physical interpenetration effects of polymer chains and mucus (Peppas et al., 2000). The cationic polymer chitosan can adhere to the mucus layer by establishment of electrostatic interactions with anionic sialic groups of mucin (Hassan and Gallo, 1990). Attempts have been made to enhance mucoadhesion by means of chemical modification of these polymers by thiol groups on their structure. Thiolated polymers (thiomers) can tightly adhere to intestinal mucus layer for a prolonged time through covalent bonding with mucin glycoproteins via thiol-disulfide exchange reactions. Therefore they can provide a steep drug concentration gradient at the absorption sites, which might be advantageous for the mucosal administration of various drugs (Bernkop-Schnürch et al., 2004a,b). Their properties make them also highly suitable excipients for controlled drug release dosage forms (Bernkop-Schnürch et al., 2003; Kast et al., 2002). Therefore the objectives of the present study

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were to evaluate and compare the penetration and mucoadhesive properties of anionic and cationic unmodified and thiolated nanoparticles. Particles were prepared by ionic gelation and characterized by particle size, zeta potential, amount of thiol groups and disulfide bonds. To visualise and quantify the distribution of these NP on intestinal rat mucosa and in natural mucus, fluorescent dyes Alexa Fluors 488 (AF 488) and FDA were incorporated in the particles. Mucoadhesion/penetrating properties were evaluated with respect to ex vivo transport in rat intestine and diffusion behaviour in natural porcine mucus. We expect that thiolated compared to unmodified as well as cationic compared to anionic particles have higher mucoadhesive properties which might be a reason for delaying the diffusion of particles into the epithelium.

2. Materials and methods

2.1. Materials

Medium viscous chitosan (average MW 400 kDa), poly(acrylic acid) (average MW 450 kDa), dimethyl sulfoxide (DMSO), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's Reagent), sodium borohydride ($NaBH_4$), sodium nitrite, hydrogen peroxide (H_2O_2), dialysis tubes (MW cutoff 2 and 12 kDa), 2-iminothiolane HCl (Traut's reagent), L-cysteine hydrochloride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and fluorescein diacetate were obtained from Sigma–Aldrich, Austria. Alexa Fluor 488 succinimidylester and cadaverine sodium salt were purchased from Invitrogen, Austria. All other chemicals were of analytical grade.

2.2. Depolymerisation of chitosan

Chitosan with an average molecular mass of 20 kDa (degree of deacetylation 87%) was depolymerised from chitosan medium molecular weight utilising sodium nitrite as described previously (Huang et al., 2004; Schmitz et al., 2007). First, 2 g of chitosan (400 kDa) were dissolved in 100 ml of acetic acid 6% and 80 mg of sodium nitrite, dissolved in 10 ml of distilled water were added. After incubation for 1 h under continuous stirring, chitosan was precipitated by adding 4 M NaOH until a pH of 9 was reached. The precipitate was filtered and washed three times with cold acetone. The residue was resolubilised in 15 ml of 0.1 M acetic acid again and dialysed against distilled water for 24 h (dialysis tubing, MW cutoff 2 kDa). The depolymerised product was lyophilised and stored at 4 °C until further use.

2.3. Synthesis of polymer conjugates

2.3.1. Modification of chitosan with 2-iminothiolane

The covalent attachment of 2-iminothiolane to depolymerised chitosan (CS–TBA) was carried out according to a method described previously by our group (Bernkop-Schnürch et al., 2003). Initially, 500 mg of depolymerised chitosan were dissolved in 50 ml of 1% acetic acid to obtain a 1% (w/v) polymer solution. After adjusting the pH to 6.5 with 5 M NaOH, 400 mg of 2-iminothiolane HCl were added. The coupling reaction was allowed to proceed for 24 h at room temperature under continuous stirring. The resulting conjugate was purified by extensive dialysis for five times to eliminate unbound reagent. Finally the polymer solution was lyophilised and stored at 4 °C until further use.

2.3.2. Modification of poly(acrylic acid) with cysteine

The poly(acrylic acid)–cysteine (PAA–Cys) conjugate was synthesised according to a method described previously (Marschütz and Bernkop-Schnürch, 2002). First, 500 mg of PAA were hydrated in distilled water and the pH was adjusted to 6.0 by addition of 5 M NaOH. Then, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) in a final concentration of 200 mM was added in order to activate the carboxylic acid moieties of the hydrated polymers. After 20 min incubation under stirring at room temperature, 500 mg of cysteine were added and the pH was readjusted to 6.0. Reaction mixture was incubated for 3 h at room temperature under continuous stirring. The resulting conjugate was isolated by dialysis according to the method described previously by our group (Greindl and Bernkop-Schnürch, 2006). Afterwards the frozen polymer solution was dried by lyophilisation and stored at 4 °C until further use.

2.4. Characterisation of polymer conjugates

The degree of modification was determined by quantifying the amount of thiol groups on the thiolated conjugates. The total amount of thiol groups immobilised on thiomers is a composition of free thiol groups and oxidised thiol groups in form of disulfide bonds. The amount of free thiol groups fixed on CS and PAA was determined spectrophotometrically with Ellman's reagent as described previously by our research group (Hornof et al., 2003). The absorbance of free thiol groups was measured at a wavelength of 450 nm with a microplate reader (FluoStar Galaxy, BMG, Offenburg, Germany). A calibration curve was used to calculate the amount of free thiol groups immobilised on the polymer. To determine the degree of disulfide bond formation during synthesis, the reaction with Ellman's reagent was performed after reducing disulfides with $NaBH_4$. Measurement of the absorbance and quantification of the total amount of thiol groups was performed as described above.

2.5. Preparation of nanoparticles

Nanoparticles of all polymers were prepared by ionotropic gelation, CS with the anion TPP and PAA with the cation Ca^{2+}. Unmodified and modified CS was dissolved in 0.05% acetic acid (pH 5.5) to obtain a 0.5% solution. TPP was dissolved in distilled water at 2 mg/ml. Particles were formed spontaneously by adding the TPP solution to each polymer solution under continuous stirring (300 rpm) until turbidity occurred. Particle suspension was stirred with 300 rpm for one more hour.

PAA or PAA–Cys was dissolved in distilled water to obtain a 0.5% solution. The pH was adjusted to 8.0 by adding 1 M NaOH solution. $CaCl_2$ was dissolved in distilled water at 10 mg/ml and added to PAA or PAA–Cys solution under continuous stirring (300 rpm) until turbidity occurred. Particle suspension was stirred with 300 rpm for one more hour.

Stabilisation of all thiolated particle suspensions was carried out via formation of inter- and intramolecular disulfide bonds by oxidation with 10 μmol (v/v) of H_2O_2 solution.

To visualise the particles, the hydrophilic fluorescent model compound AF 488 was incorporated in the particles using the same procedure. AF 488 succinimidyl ester, dissolved in DMSO at 1 mg/ml was added to the TPP solution and AF 488 cadaverine, dissolved in water at 1 mg/ml was added to the $CaCl_2$ solution obtaining the final concentration of 8.7 μg/ml in the polymer crosslinker mixtures. Mixtures were incubated for 2 h, protected from light under permanent shaking. To remove the anionic and cationic crosslinkers as well as H_2O_2 and free Alexa, each nanoparticle suspension was centrifuged three times at 4000 rpm for 20 min with 1% trehalose to avoid any particle aggregation, resuspended in water (PAA) or in 0.05% of acetic acid (CS) and subsequently dialysed. After this centrifugation/washing procedure a transparent solution and white pellet were obtained.

FDA-labelled particles were prepared according to a method described previously by our research group (Bernkop-Schnürch

et al., 2006). In brief, 10 ml of each particle suspension prepared as described above was transferred to 10 ml of a 0.1% (w/v) FDA in acetonitrile solution. The mixtures were then incubated for 2 h at room temperature under permanent shaking following centrifugation at 4000 rpm for 20 min (with 1% trehalose), resuspension in water (PAA) or 0.05% acetic acid (CS) and dialysis. Finally, the pH of all suspensions was readjusted to 6.0 before lyophilising (with 1% trehalose).

2.6. Particle characterisation

The amount of thiol groups on all particles was determined spectrophotometrically with Ellman's reagent as described earlier. For determining a certain oxidation of thiol groups, CS–TBA and PAA–Cys particles were separately dissolved in water at 5 mg/ml and incubated with 10 μmol of H_2O_2 at room temperature under permanent shaking. At predetermined time intervals, aliquots of 200 μl were withdrawn and 50 μl of 5 M HCl were added to quench further reactions. The amount of disulfide bonds and remaining thiol groups was determined using Ellman's reagent (Marschütz and Bernkop-Schnürch, 2002). Tests were performed in triplicate for each sample.

Size and zeta potential of unmodified and modified particle suspensions were determined by photon correlation spectroscopy using PSS Nicomp 380 ZLS particles sizer with laser wavelength of 650 nm and an E-fields strength of 10 V/cm. For size measuring particles were suspended in water (0.5%, w/v) and the intensity distribution was fit to a Gaussian size distribution curve.

For encapsulation efficiency (EE%), 5 mg of fluorescent (AF 488) lyophilised particles were suspended in 1 ml of water and determined spectrophotometrically with a microplate reader. Both Alexa dyes (succinimidyl ester and cadaverine) were determined at an excitation wavelength of 495 nm and an emission wavelength of 519 nm. EE was calculated as the ratio between the fluorescent dyes loaded into the nanoparticulate system with respect to the total amount of the dye employed for nanoparticle preparation as follows:

$$\mathrm{EE\%} = \frac{\mathrm{AF\ \ 488\ \ partices}}{\mathrm{AF\ \ 488\ \ total\ amount}} \times 100$$

Whereas the amount of the fluorescent dye loaded into the nanoparticulate system was calculated according to a calibration curve of fluorescent particles.

FDA loaded particles were dissolved in 5 M NaOH at 5 mg/ml and incubated for 30 min at 37 °C while shaking in order to hydrolyze FDA to the fluorescent sodium fluorescein, which was quantified spectrophotometrically at an excitation wavelength of 485 nm (Albrecht et al., 2006). Encapsulation efficiency was calculated as described above.

2.7. In vitro mucoadhesion studies on freshly excised rat intestine

2.7.1. Mucoadhesion studies

Unmodified and modified CS and PAA NP were examined in an ex vivo rat jejunum model in terms of their mucoadhesive and penetration behaviour. For these studies Sprague–Dawley rats weighing between 300 and 400 g were used. After sacrificing the rats the fresh intestine (jejunum) was removed and rinsed carefully with physiological saline (NaCl 0.9%) to remove all food residues. The tissue was cut into segments of 2 cm length. Each segment was filled with 0.1 ml of fluorescent labelled nanoparticle suspension (5 mg/ml in water pH 6.5) by using a syringe and incubated for 2 h at 37 °C. Thereafter, the tissues were rinsed extensively with saline to eliminate all unabsorbed particles before opening them through the mid-line incision. Afterwards tissue sections were conserved in formalin and dehydrating in ascending concentrations of isopropanol (70–100%). In following steps, tissues were embedded in different infiltration solutions (methylbenzoate, chloroform) and in the end two days in parablast. A motorized microtome (Schlittenmikrotom 1400, Leitz, Germany) was used to make 10 μm thick slices of each preparation, which were transferred to microscope slides. A Nikon fluorescence microscope (Nikon Eclipse 80i, Germany) was used to qualitatively detect the fluorescent particles in the tissue sections. The light was adjusted in the green fluorescence mode, which yielded an excitation wavelength at 495 nm. Image recognitions for detected particles in the tissue sections were performed on a ProgResCFscan Color/Monochrome CCD camera (Jenoptik) and analysed by PicEd Cora to determine the distribution and number of particles on the tissue sections. All settings for image processing were kept constant. Alexa solution and blank chitosan particles were used as references.

2.7.2. Nanoparticle quantification

To determine the amount of particles either attached to the mucus layer or taken up into the tissue, mucus was removed from the mucosal tissue by scraping off with a scalpel.

The segments were filled with 0.1 ml of fluorescent labelled nanoparticle suspension (5 mg/ml) and incubated for 2 h at 37 °C as described above. Thereafter the tissues were rinsed three times with 1 ml of saline to remove all unabsorbed particles. Subsequently, the mucus layer was scraped off the membrane and dispersed in 1 ml of 5 M NaOH solution. The tissue samples were immersed in water for 10 min to wash off the residual mucin layer (Ebel, 1990). Mucus and tissue samples were incubated for 30 min in a water bath at 37 °C while shaking in order to hydrolyse FDA to the fluorescent sodium fluorescein. Afterwards samples were treated by ultrasonication and left overnight at room temperature until mucus/tissue and NP were completely dissolved. The fluorescent signal of absorbed NP was quantified spectrophotometrically at an excitation wavelength of 485 nm. Data were compared with the corresponding calibration curves elaborated in the same conditions. Results were expressed as the amount of attached NP in mucus layer or epithelium. Each sample was tested in triplicate.

2.8. Diffusion studies

To investigate the transport of nanoparticles across the mucus barrier, we studied the quantitative transport rates of unmodified and modified anionic as well as cationic NP in natural porcine intestinal mucus secretions. Fresh porcine intestine was rinsed carefully with physiological saline (NaCl 0.9%) to remove all food residues. The mucus collection was done by scrapping off the mucus layer from membrane after washing process. Afterwards 300 μl of fresh mucus were filled in small silicon tubes with a diameter of 6 and a length of 40 mm and closed on one end with small silicon caps. Each fluorescein diacetate labelled particle suspension of 0.5% (w/v) was applied to the open end of a mucin-filled silicon tube and closed with another silicon cap.

For each sample, standard curves were prepared by mixing 10 standards from 0.5 to 9 mg with 300 μl of fresh mucus in small tubes. As blank value 300 μl of fresh mucus was used. All tubes were kept at 37 °C under continuous rotation for 24 h. After this incubation time the tubes were frozen at −80 °C for 1 h and cut into 20 slices of 2 mm length, starting with the end, where particles were added. Each slice was incubated in 400 μl of 5 M NaOH for 30 min in a water bath at 37 °C while shaking in order to hydrolyze FDA to the fluorescent sodium fluorescein. Afterwards slices were treated by ultrasonication and left overnight at room temperature. Fluorescence was determined in order to assess the depth of diffusion into the mucus. Data were compared with the corresponding calibration curves elaborated in the same conditions.

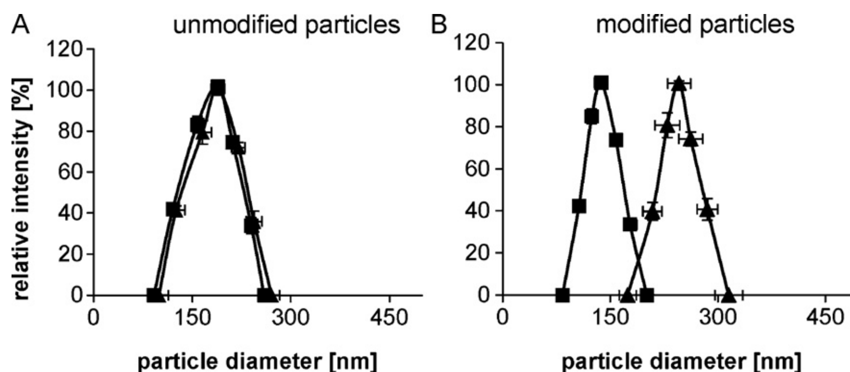


Fig. 1. Particle size distribution of (A) unmodified and (B) modified chitosan (■) and poly(acrylic) acid (▲) nanoparticles prepared by ionic gelation with TPP and $CaCl_2$. Indicated values are means ± SD (n = 3).

2.9. Statistical data analyses

Statistical data analyses were performed by SPSS version 17 (SPSS Inc, Chicago, IL, USA) to calculate the mean values of each sample. Unpaired student's t-test was performed to test the significance of the difference between two mean values. Level of $p \leq +0.05$ was set for significance and $p \leq 0.01$ for high significance.

3. Results and discussion

3.1. Synthesis and characterisation of polymer conjugates

Depolymerised chitosan (20 kDa) was hydrolysed from chitosan medium molecular weight utilising sodium nitrite. On this depolymerised chitosan, thiol groups in form of 4-thiobutylamidine substructures were immobilised by formation of amidine bonds. Cysteine was covalently linked to poly(acrylic acid) via formation of amide bonds between the primary amino group of cysteine and a carboxylic acid group of the polymer. Both thiomers appeared as white, odourless powders of fibrous structure after lyophilisation, which were soluble in aqueous solutions. About 1218 μmol (CS–TBA) and 1131 μmol (PAA–Cys) of thiol groups per gram polymer were immobilised on the thiomers with approximately 84% remaining free thiol groups while 16% being oxidised to disulfide bonds during the conjugation as listed in Table 1.

3.2. Preparation and characterisation of NP

Unmodified and thiolated NP were prepared via ionic gelation with TPP (CS) and Ca^{2+} (PAA) followed by formation of intra- and intermolecular disulfide bonds within the thiolated particles and the removal of the ionic crosslinkers. The ratio of polymers to crosslinkers, pH and stirring rate had a great impact on the formation of small nanoparticles. Several settings were tested for the preparation of nanoparticles in a size range of approximately 200 nm. The best polymer:crosslinker ratio tested was 7:1 with no aggregation and a satisfying yield (60%) of particles (data not shown). Especially for unmodified and modified PAA particles the pH of the solutions was quite important. In previous studies pH values from 6 to 9 were tested, whereas pH 8 seemed to be the best (Greindl and Bernkop-Schnürch, 2006). Values above pH 8 resulted in aggregates, values below pH 8 in clear solutions. A stirring rate of 300 rpm for 1 h was identified to form nanoparticles with a narrow distribution in a size range of 150–250 nm. As shown in Fig. 1 and Table 2, particles possessed sizes of 139–246 nm and positive (CS) and negative (PAA) zeta potentials, respectively. Due to the carboxylic groups in unmodified and modified PAA, particles were negatively charged. It was found that cross-linking with cysteine resulted in a 1.12-fold decrease in the zeta potential of the PAA–Cys particles compared to unmodified ones. 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 negatively charged mucus layer. Therefore particles of a more positive zeta potential should display comparatively more pronounced mucoadhesive properties. Moreover it was shown that thiolation of chitosan led to a 1.21-fold increase in the zeta potential and to a 1.36-fold decrease in particle size as listed in Table 2. The lyophilised NP appeared as white powder and were resuspendable in aqueous solutions. After suspending in water, particles displayed a mean particle size in the range of 230–317 nm as shown in Fig. 2. The oxidation process by addition of H_2O_2 demonstrated that the formation of disulfide bonds could be well controlled in this way. The total amount of thiol groups was 1210 μmol/g for CS–TBA NP of which 56% thiol groups were oxidised to disulfide bonds and 1117 μmol/g for PAA–Cys NP with 54% oxidised thiol groups. As shown in Table 2, due to the removal of the anionic and cationic crosslinkers the zeta potential for thiolated CS NP was raised from 4 up to 20 mV and reduced from −2 down to −14 mV for thiolated PAA NP without losing stability. Within this work mucoadhesion studies required the labelling of particles in order to localise them in vitro. The aim was to obtain fluorescent NP by inclusion of fluorescent dyes (AF 488 and FDA) into the particles. Their incorporation led to an encapsulation efficiency of approximately 10%. For diffusion studies and nanoparticle quantification it was really necessary to have approximately the same encapsulation efficiency for all tested samples.

Table 1
Used reagents, amount of thiol groups and disulfide bonds immobilised on the basic thiomers, on ionically cross-linked as well as on covalently cross-linked nanoparticles. Indicated values are means ± SD (n = 3).

Polymer	Used reagents	–SH [μmol/g]	–S–S– [μmol/g]	$\sum$–SH [μmol/g]
Chitosan-TBA (CS-TBA)	2-Iminothiolane	1022	98	1218 ± 8
CS–TBA particles	TPP Alexa Fluor 488 succinimidyl ester	986	116	1219 ± 21
CS–TBA particles (ox)	H_2O_2	526	342	1210 ± 17
Poly(acrylic acid-Cys) (PAA-Cys)	Cysteine	955	88	1131 ± 9
PAA–Cys particles	$CaCl_2$ Alexa Fluor 488 cadaverine sodium salt	943	91	1125 ± 10
PAA–Cys particles (ox)	H_2O_2	513	302	1117 ± 20

Table 2
Mean particle size and zeta potential and encapsulation efficiency (EE) of unmodified and modified nanoparticles after preparation (pH 8.0 for PAA and 5.5 for CS) as well as after lyophilisation, dissolved in water (pH 6.5). Indicated values are means ± SD (n = 3).

	After preparation		After lyophilisation, dissolved in water			
	Size [nm]	Zeta potential [mV]	Size [nm]	Zeta potential [mV]	EE [%] AF 488	EE [%] FDA
CS particles						
Labelled	189 ± 8	3 ± 2	290 ± 17	16 ± 4	10.7 ± 2	12.9 ± 3
Blank	181 ± 7	3 ± 2	230 ± 9	17 ± 3		
CS–TBA particles						
Labelled	137 ± 9	4 ± 2	287 ± 16	20 ± 3	10.1 ± 4	10.9 ± 3
Blank	135 ± 10	5 ± 1	254 ± 12	20 ± 4		
PAA particles						
Labelled	189 ± 13	−3 ± 1	310 ± 12	−13 ± 3	11 ± 3	11.2 ± 4
Blank	189 ± 8	−3 ± 1	300 ± 10	−12 ± 4		
PAA–Cys particles						
Labelled	246 ± 16	−2 ± 2	317 ± 15	−14 ± 3	9.2 ± 3	9.9 ± 4
Blank	232 ± 12	−3 ± 2	305 ± 10	−14 ± 4		

Several particles were prepared and their encapsulation efficiency analysed. Among these particles, the ones with approximate the same amount of incorporated fluorescent dye were used for the studies. The direct observation of tissue sections with fluorescent particles by fluorescent microscopy confirmed that particles were labelled with AF 488. The labelling process of NP resulted in no increase of the hydrodynamic diameter of NP. As illustrated in Figs. 1 and 2, the effect of an increase in particle size was just shown after lyophilisation and suspending in water and might be related to an aggregation of particles due to the oxidation process with H_2O_2. Another reason could be a too low amount of trehalose for the centrifugation and drying process, which can avoid this aggregation. Also the insolubility of chitosan in aqueous solutions might be responsible for a swollen particle suspension with increasing diameter. Tables 1 and 2 present the characteristics of all particles such as diameter, zeta potential, amount of thiol groups and disulfide bonds as well as encapsulation efficiency of the fluorescent dyes.

3.3. *In vitro mucoadhesion studies on freshly excised rat intestine*

The mucoadhesiveness of a particle suspension depends mainly on the ability of the particles to interact with mucins glycoproteins or other mucus components being immobilised within the mucus layer (Durrer et al., 1994; Ponchel and Irache, 1998). The complex structure of the mucus offers many opportunities for the development of adhesive interactions with small polymeric particles either through non-specific (van der Waals) or specific (ionic) interactions between complementary structures. In vitro studies with the particle suspensions in terms of their mucoadhesive and penetration behaviour on the mucosa of rat small intestine suggested that all thiolated NP display significantly ($p < 0.0001$) higher mucoadhesive properties than the corresponding unmodified ones. When NP suspensions are administered, they rapidly encounter the mucosal surface. Because of their high mucoadhesive properties, the thiolated particles were arranged much more in the mucus layer than in the epithelia (Figs. 3 and 4). This considerable improvement in mucoadhesive properties of thiolated NP is based on the formation of disulfide bonds between thiol-bearing side chains of the polymer and cysteine-rich subdomains of mucus glycoproteins (Bernkop-Schnürch, 2005). The rank order with regard to mucoadhesion of all particles established within this study showed that CS–TBA > PAA–Cys > CS > PAA, which was in good agreement with that found by Grabovac et al. (2005). As shown in Figs. 3a and 4a after 2 h of contact time with rat intestine, more than 50% of the thiolated green spots (which can be identified as AF 488 loaded NP) were located in the mucus layer compared to particles which were in contact with the epithelium. Among all particles tested, the CS–TBA particles displayed the most favourable properties concerning mucoadhesion (Figs. 5 and 6). In Fig. 6 it is shown that more than 90% of CS–TBA NP were detected in the mucus layer, whereas just 10% of these particles were in contact with the mucosal tissue. Hence, the thiolated CS particles showed more than 10-fold mucoadhesive in comparison to penetration properties.

Fig. 6 suggested that the thiolation process enhanced the intestinal mucoadhesion of CS by 3.3-fold compared to unmodified particles. In comparison to all other in vitro test systems, the improvement in mucoadhesion due to the immobilisation of thiol groups on chitosan was less pronounced. The same thiomer showed in tensile studies and on the rotating cylinder 117-fold and 140-fold higher mucoadhesive properties than the unmodified polymer (Roldo et al., 2004). One reason for this observation might be seen

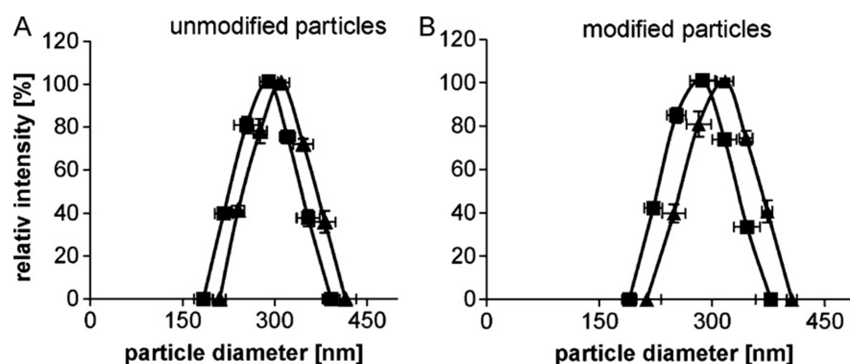


Fig. 2. Particle size distribution of the used (A) unmodified and (B) modified chitosan (■) and poly(acrylic acid) (▲) nanoparticles after lyophilisation, suspended in water (pH 6.5). Indicated values are means ± SD (n = 3).

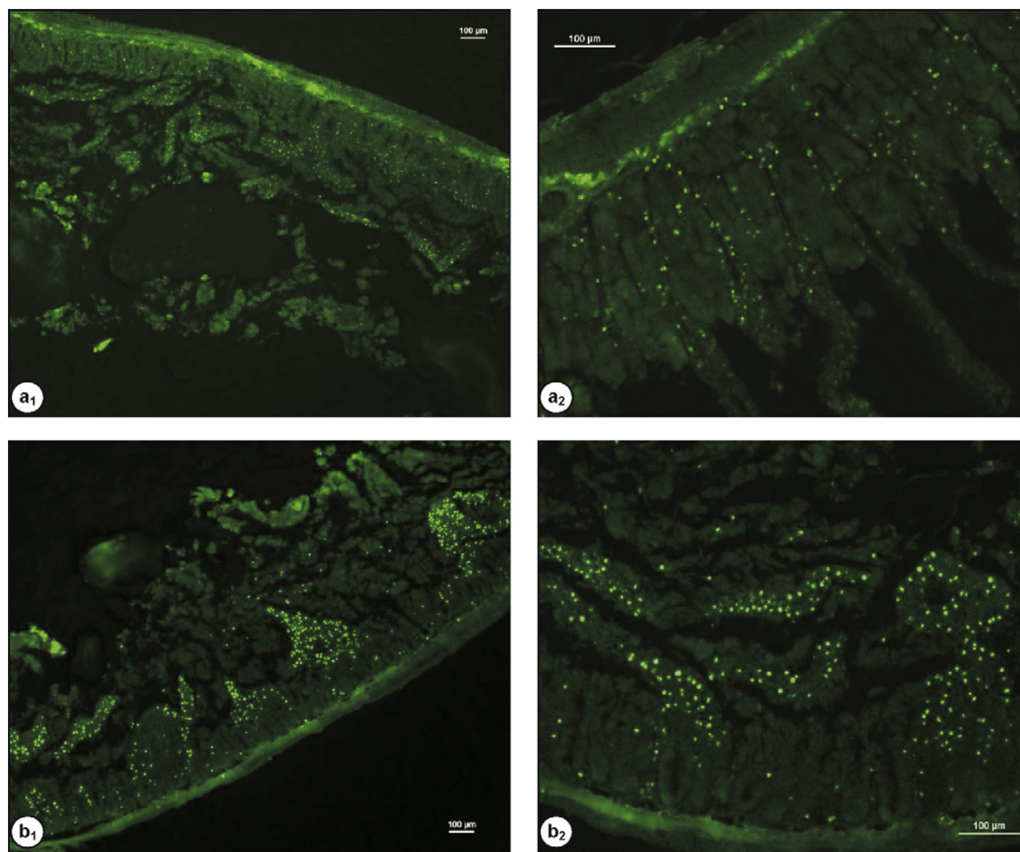


Fig. 3. Microscopic viewings of tissue segments through the intestine taken after 2 h incubation with 100 μl (0.5%, w/v) of unmodified (a) and modified (b) chitosan particles labelled with the fluorescent hydrophilic dye (AF 488) (a_1 and b_1, 40×; a_2 and b_2, 100×).

in the comparatively much lower molecular mass used here. In previous studies CS with a molecular mass of 400 kDa was used, whereas in this study CS of an average molecular mass of 20 kDa was utilised. Tobyn and Bravo-Osuna et al. showed that the smaller the chain length of a mucoadhesive polymer is the lower are its mucoadhesive properties, but using chitosan of comparatively higher molecular mass led to much greater particles in the range of above 1000 nm (data not shown) (Bravo-Osuna et al., 2007; Tobyn et al., 1995). Another reason might be seen in the hydration of particles. In previous studies with more than 100-fold improvement in mucoadhesion dry tablets were used, which show more hygroscopic properties than hydrated ones (Roldo et al., 2004). Mortazavi et al. compared dry mucoadhesive materials (Carbopol 934P) with their hydrated ones (4% gels) on a mucus gel layer (Mortazavi and Smart, 1993). It was shown that the dry materials were rapidly hydrating/swelling and extracting the water from the mucus layer (dehydration process), whereas the hydrated materials lost water. This dehydration process of the mucus layer alters its physicochemical properties, making it locally a more adhesive layer, which in turn generate a strong mucoadhesive effect. This theory might be another reason for the lower improvement in mucoadhesion by using hydrated particles within this study compared to dry tablets used in previous studies. As well as the thiolated particles, the unmodified CS particles showed also more than doubled mucoadhesive properties in comparison to their penetration behaviour into the mucosal tissue. Approximately 70% of unmodified CS particles were determined in mucus layer and just 30% were in contact with the epithelium as illustrated in Figs. 5 and 6. Both cationic (CS and CS–TBA) particles showed no significant difference ($p = 0.5231$) between their penetration behaviour through mucosal tissue. In comparison to anionic PAA particles it could be demonstrated that positively charged particles were absorbed by mucus gel layer to a significant higher degree ($p = 0.0008$) than carboxylated particles. Cationic CS–TBA particles showed doubled mucoadhesive features than anionic PAA–Cys particles. Also unmodified CS NP displayed more than a 3.6-fold increase in mucoadhesion compared to unmodified PAA particles, which might be based on ionic interactions of the positively charged polymer with negatively charged moieties within the mucus, such as sulfonic and sialic acid substructures. More positively charged particles will consequently be relatively more mucoadhesive (Hassan and Gallo, 1990). Thus the penetration rate to the epithelia was much higher for the anionic particles of PAA than the cationic CS particles. Approximately 73% of unmodified PAA particles were in contact with the tissue, whereas just 17% of particles showed mucoadhesive properties (Fig. 6). Due to the immobilisation of thiol groups on PAA, the mucoadhesive properties could be significantly improved ($p < 0.0001$), whereby much more particles were located in the mucus layer compared to particles in contact with mucosal tissue. As illustrated in Figs. 5 and 6, more than 60% of PAA–Cys particles were determined in the mucus layer compared to 40% of particles in contact with mucosal tissue. However, unmodified and modified PAA particles showed no significant difference ($p = 0.8652$) in their penetration behaviour through mucosal tissue. As described previously, the considerable improvement in mucoadhesion due to the thiolation is based on the formation of strong disulfide bonds between thiomers and cysteine-rich subdomains of the mucus layer. According to this, a further improvement in mucoadhesion of NP might be feasible by a further increase in the content of thiol groups on the particles. The more free thiol groups are available for the formation of disulfide bonds with mucus glycoproteins, the higher are their mucoadhesive properties (Bernkop-Schnürch et al.,

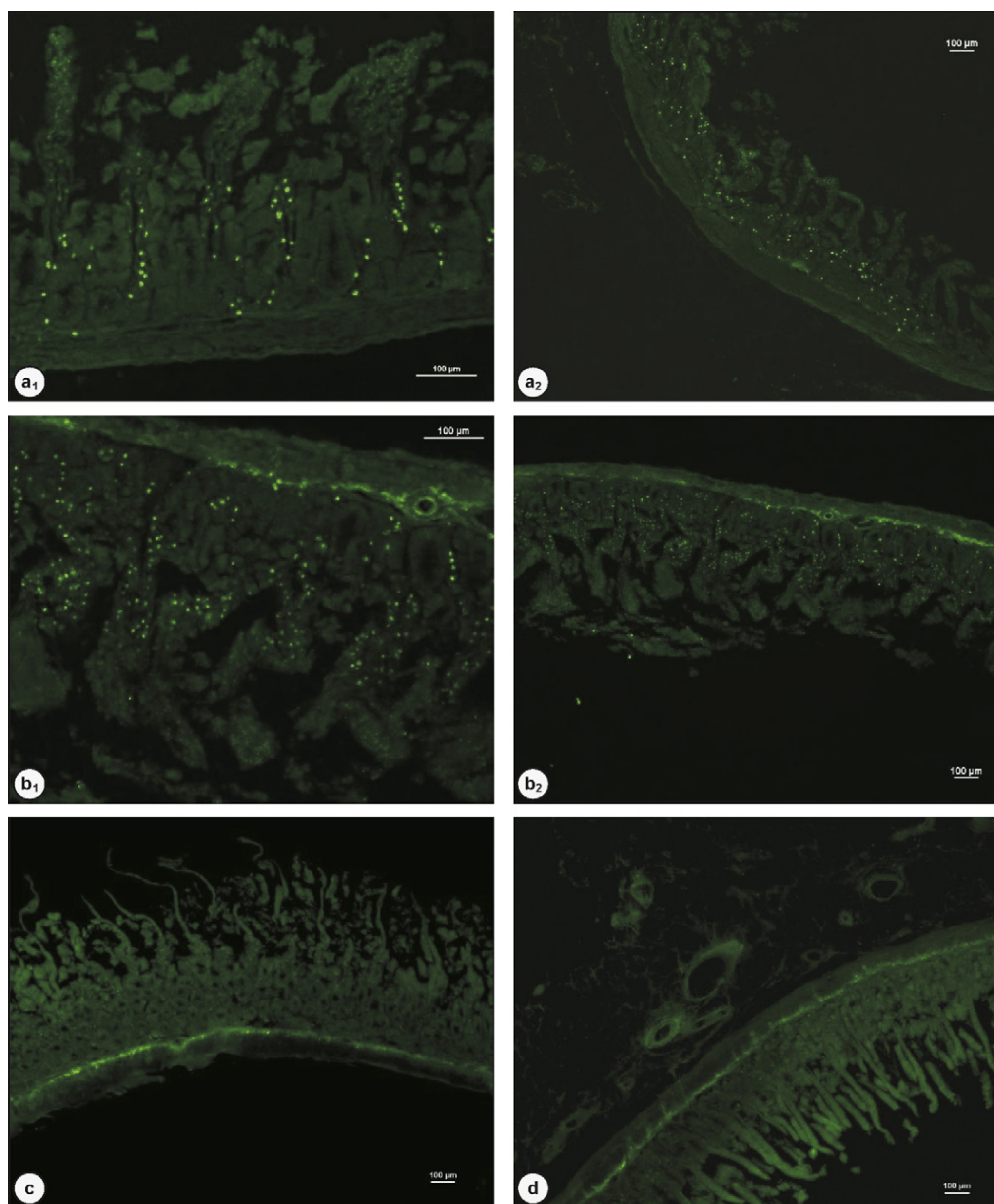


Fig. 4. Fluorescent micrographs of tissue segments through intestinal mucosa incubated for 2 h with 100 µl of unmodified (a) and thiolated (b) PAA particles (0.5%) labelled with AF 488. Photomicrographs of tissue segments through intestinal mucosa with blank chitosan particles (c) and AF 488 solution (d) were used as references (a_1 and b_1, 100×; a_2, b_2–d, 40×)

2006). In addition, based on these strong disulfide bonds thiolated particles remain very stable on the mucus, whereas unmodified particles could disintegrate faster (data not shown).

3.4. *Diffusion studies through fresh intestinal mucus*

Nanoparticles in the range of 200 nm in mucus have been thought to be much too large to undergo diffusion transport through mucus barriers. However, large nanoparticles are preferred for higher drug encapsulation efficiency and the ability to provide sustained delivery of drugs.

A new diffusion method developed by our group was used for quantifying transport rates of different charged polymeric particles with a size of nearly 230 nm in samples of fresh porcine intestinal mucus.

To emanate from previous mucoadhesion studies on fresh intestine, we expected that unmodified negatively charged particles will show the highest diffusion rate. Diffusion of positively charged particles in mucus may be reduced due to ionic interactions with the negatively charged mucin molecules. As illustrated in Fig. 7, results of the diffusion studies confirmed our expectation that unmodified PAA particles showed the highest diffusion rate. They diffused through the silicon tube with natural mucus reaching slice 16, which is equivalent to 32 mm. In slice 17–20 no particles could be detected. In comparison, because of their thiol groups and formation of disulfide bonds with cysteine-rich subdomains in the mucus, thiolated PAA particles stopped their diffusion in slice 14 and no particles could be detected in the following slices. Contrary to unmodified PAA particles, which had nearly the same amount of remaining particles in slices 1–7, the thiolated ones had their highest amount in slice 4 as shown in Fig. 7. In comparison to anionic PAA particles, the cationic CS particles were much more immobile and their diffusion stopped between slice 9 and 11, which correspond to 18–20 mm. In the following slices no particles

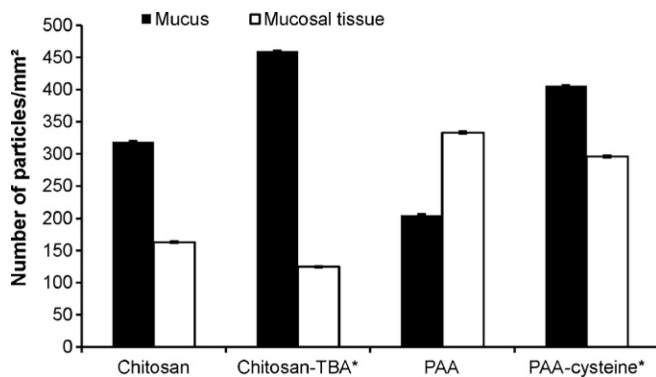


Fig. 5. Number of unmodified/modified chitosan and poly(acrylic acid) particles detected on the tissue sections and analysed with PicEd Cora software. Indicated values are the means of at least three pictures (±SD) (*differs from CS $p = 0.0039$ and PAA $p = 0.0006$).

could be determined. Reason for this lower diffusion rate might be their positive charge and ionic interactions with the negatively charged mucin molecules. Unmodified CS particles had their highest amount with 3 mg/ml of remaining FDA between slices 1 and 3 as shown in Fig. 7. In comparison CS–TBA particles, showed more than doubled amount of remaining particles in the same slices. This higher detected amount is based on the formation of disulfide bonds between thiol groups of the particles and mucus in the first slices, whereby their further diffusion rate was delayed. Transport of these thiolated CS particles through natural mucus stopped between 16 and 18 mm, whereas thiolated PAA particles reached 28 mm. This higher diffusion rate of negatively charged unmodified and modified PAA particles is believed to be a result of repulsion with the negatively charged mucin molecules. However, it could be shown that nanoparticles in the range of 230 nm are not too large for undergoing diffusion transport through mucus barriers. Opposed to this, cationic as well as anionic thiolated particles indicated that their interaction with mucus, due to the thiol groups, had a great impact on their diffusion rate. Mucoadhesive properties of these particles were more than 2-fold improved compared to unmodified ones. Therefore it can be said that mucoadhesion can slow down the particle transit time through the GI tract, which results in prolonged residence time, localisation of the delivery system at a specific target site and an increase in drug concentration gradient.

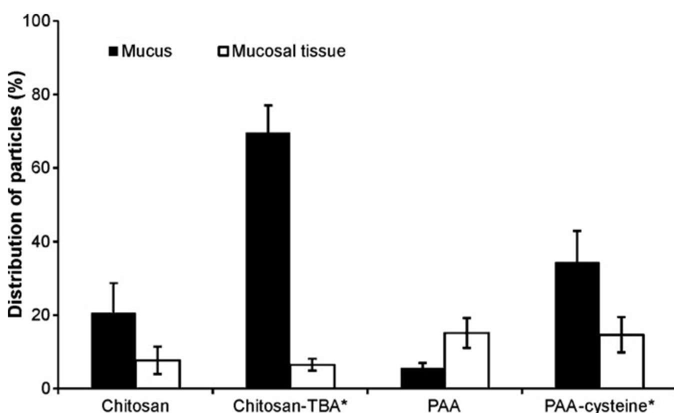
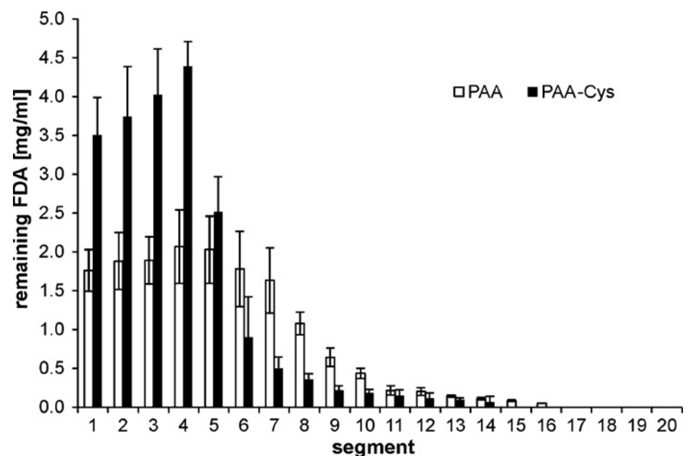


Fig. 6. Distribution of unmodified and modified chitosan and poly(acrylic acid) particles labelled with FDA on mucus layer and on mucosal tissue. Indicated values are the means of at least five experiments (±SD) (*differs from control CS and PAA $p < 0.0001$).



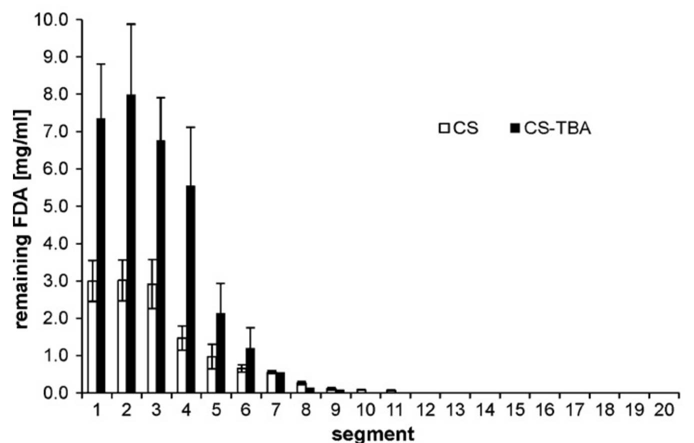


Fig. 7. Diffusion studies of FDA labelled unmodified and modified chitosan and poly(acrylic acid) particles through natural porcine intestinal mucus pH 6.5. Indicated values are the means of at least three experiments (±SD).

4. Conclusion

In this study we compared mucoadhesive and diffusion behaviour of particles with different characteristics on intestinal mucus layer and tissue. As expected before it could be demonstrated that thiolated NP of PAA and CS have promising mucoadhesive properties, whereby these particles were arranged much more in the mucus layer than in the mucosal tissue. The therapeutic potential of such drug 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 mucus membrane. Therefore both thiolated polymers as nanoparticulate delivery systems seem to be promising carriers for mucosal drug delivery.

Acknowledgments

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