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Research Paper

Development of a Novel Method for the Preparation of Thiolated Polyacrylic Acid Nanoparticles

Melanie Greindl[1] and Andreas Bernkop-Schnürch[1,2]

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Purpose. To develop a novel method for the preparation of thiolated polyacrylic acid nanoparticles via ionic gelation.
Materials and Methods. In a first step nanoparticles were generated by ionotropic gelation of polyacrylic acid (PAA) of three different molecular weights (100, 240 and 450 kDa) and various cations including Ca^{2+}, Mg^{2+}, Zn^{2+}, Al^{3+} and Fe^{3+}. Via *in vitro* characterization of the particles (particle size, size distribution and zeta potential) the optimal preparation conditions were established. Taking into consideration, that thiolated polyacrylic acid (PAA-Cys) displays higher mucoadhesive and permeation enhancing properties than unmodified PAA, PAA-Cys nanoparticles were produced in the same manner with Ca^{2+}, as the most promising results concerning particle size and stability of particles could be achieved with this ionic crosslinker. The nanoparticles were stabilized via the formation of inter- and intrachain disulfide bonds within these particles due to oxidation with H_2O_2. Ca^{2+} was removed proximately by the addition of EDTA and exhaustive dialysis.
Results. Using the preparation method described above PAA-Cys nanoparticles of a mean diameter of about 220 nm (PAA_{100}-Cys), 250 nm (PAA_{240}-Cys) and 295 nm (PAA_{450}-Cys) can be generated. In comparison to PAA nanoparticles ionically crosslinked with Ca^{2+}, the removal of the crosslinker Ca^{2+} from PAA-Cys particles led to a nearly three-fold decrease in the zeta potential, from about -7 up to -20 mV. Apart from this advantage, covalently crosslinked PAA-Cys nanoparticles were more firm as they remained stable when incubated in hydrochloride solution, whereas ionically crosslinked particles dissolved at pH lower than 5.
Conclusions. This novel nanoparticulate delivery system seems to be a promising vehicle for the administration of therapeutic proteins, genes and antigens via mucosal membranes.

KEY WORDS: calcium; crosslinking; nanoparticles; polyacrylic acid; thiomer.

INTRODUCTION

In recent years polymer nanoparticles have been widely investigated as a carrier for drug delivery. For non-invasive drug administration particulate delivery systems offer the advantage of providing a prolonged residence time on mucosal membranes (1) and the possibility to reach greater mucosal surface areas leading to a comparatively higher drug uptake (2). Although the efficacy of such systems has already been demonstrated in various clinical trials (3), it is believed that the full potential of non-invasive nanoparticulate delivery systems has by far not been reached. Nanoparticulate delivery systems for non-invasive administration are based on various polymers such as polyacrylates (4), PLGA (5) or chitosans (6). Among these polymers polyacrylic acid offers the advantage of high mucoadhesive properties because of the formation of non-covalent bonds such as hydrogen bonds, ionic interactions and van der Waals forces or physical interpenetration effects of polymer chains and mucus (7,8). These mucoadhesive properties of PAA were even significantly further improved by the immobilization of thiol groups on the polymer. Grabovac *et al.* (9), for instance, could show that the mucoadhesive properties are even 20-fold improved due to the immobilization of thiol groups on the polymer (10). These strongly improved mucoadhesive properties are based on the formation of disulfide bonds between the thiolated polymer and cysteine-rich subdomains of the mucus gel layer (11). Accordingly, thiolated PAA nanoparticles should display comparatively higher mucoadhesive properties than unmodified PAA nanoparticles being advantageous for the mucosal administration of various drugs.

In order to benefit from the high mucoadhesive properties of thiolated PAA on the one hand and the advantages of nanoparticulate delivery systems such as an increase of the adhesive force or a prolongation of the GI transit time on the other hand, it was the aim of this study to develop a novel method for the preparation of thiolated polyacrylic acid nanoparticles. The strategy pursued to achieve that goal is outlined in Fig. 1. In a first step nanoparticles were produced

[1] Department of Pharmaceutical Technology, Institute of Pharmacy, Leopold-Franzens-University Innsbruck, Innrain 52, Josef-Möller-Haus, 6020 Innsbruck, Austria.
[2] To whom correspondence should be addressed. (e-mail: andreas.bernkop@uibk.ac.at)

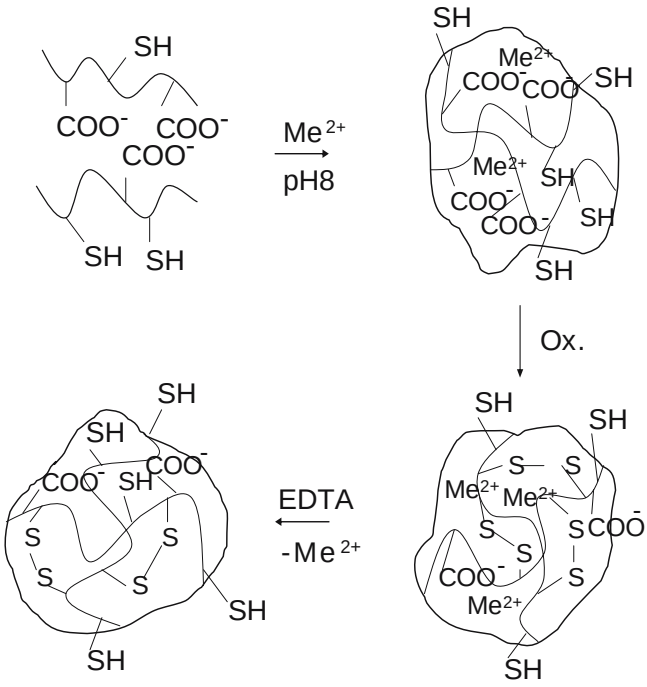

Fig. 1. Schematic presentation of the preparation method of covalently crosslinked nanoparticles. In a first step nanoparticles were produced via ionic gelation with thiolated PAA and Ca^{2+}. Afterwards the particles were oxidised in a second step. In a third step Ca^{2+} was removed by the addition of EDTA and dialysis in the following. The nanoparticles remained stable when incubated in hydrochloride solution.

via ionic gelation with unmodified and thiolated PAA of three different molecular weights (100, 240 and 450 kDa) and various cations including Ca^{2+}, Mg^{2+}, Zn^{2+}, Al^{3+} and Fe^{3+}. In case of thiolated PAA thiol groups within the particles were oxidised forming stabilizing intra- and intermolecular disulfide bonds in a second step. In a third step the cations were removed by the addition of EDTA and dialysis. In addition, certain parameters such as the type of cation or the molecular weight of the PAA influencing the properties of resulting particles were evaluated within this study.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich, Steinheim, Germany.

Synthesis of Polyacrylic Acid-Cysteine Conjugates

Polyacrylic acid-cysteine conjugates of 100-, 240-, 450 kDa (PAA_{100}-Cys, PAA_{240}-Cys, PAA_{450}-Cys) were synthesized according to a method described previously by our research group (12). In brief, PAA was first hydrated in demineralised water (Table I) and the pH value of the PAA solution was adjusted to 6 by the addition of 5 M NaOH. Then, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was added in order to activate the carboxylic acid moieties of the hydrated polymers. After 15 min incubation under stirring at room temperature, L-cysteine was added and the pH was readjusted to 6. Reaction mixtures were incubated for 3 h at room temperature under stirring. The resulting conjugates were dialysed two times against 0.2 mM HCI, then two times against 0.2 mM HCI containing 1% NaCI and finally again two times against 0.2 mM HCI. After dialysis, the pH of all samples was readjusted to 6. Thereafter, the polymer was freeze-dried at −70°C and 0.01 mbar (Benchtop 2K, VirTis, NY, USA) and stored at 4°C until further use.

Determination of Thiol/Disulfide Groups

The amount of thiol groups on the PAA-Cys conjugates was determined via Ellman's reagent [DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)] as described previously (13). Disulfide content was measured after reduction with $NaBH_4$ and addition of DTNB (14).

Table I. Reaction Conditions for the Formation of Polymer–cysteine Conjugates and Resulting Amount of Thiol Groups and Disulfide Bonds Immobilized on PAA100, PAA240 and PAA450

Conjugate	Aqueous Polymer Solutions (g/100 ml)	EDAC (final conc., mM)	Cysteine (g/100 ml)	–SH [μmol/g]	–S–S– [μmol/g]	Σ –SH [μmol/g] mean ± S.D.; $n = 3$
PAA_{100}-Cys I	1.0	50	1.0	88	18	124 ± 11
PAA_{100}-Cys II	1.0	75	1.0	152	23	198 ± 19
PAA_{100}-Cys III	1.0	100	1.0	260	42	344 ± 10
PAA_{100}-Cys IV	1.0	150	1.0	464	60	584 ± 35
PAA_{240}-Cys I	1.0	50	1.0	95	12	119 ± 7
PAA_{240}-Cys II	1.0	75	1.0	172	25	222 ± 13
PAA_{240}-Cys III	1.0	100	1.0	306	29	364 ± 28
PAA_{240}-Cys IV	1.0	150	1.0	490	31	552 ± 18
PAA_{450}-Cys I	1.0	50	1.0	98	21	140 ± 15
PAA_{450}-Cys II	1.0	75	1.0	152	31	214 ± 9
PAA_{450}-Cys III	1.0	100	1.0	252	37	326 ± 33
PAA_{450}-Cys IV	1.0	150	1.0	486	56	598 ± 22
Control	1.0	0	1.0	0	0	0

The degree of modification was determined using Ellman's reagent.

Investigation of the Conditions for the Formation of PAA/PAA-Cys Nanoparticles

The PAA or PAA-Cys nanoparticles were obtained by inducing the gelation of unmodified and thiolated PAA_{100}, PAA_{240}, and a PAA_{450} solution with various cations. Preliminary experiments were done in order to determine the optimal conditions for the formation of the nanoparticles. For this purpose PAA or PAA-Cys was dissolved in distilled water at various concentrations [0.025, 0.05, 0.1 and 0.25% (w/v)] and the pH of the solutions was adjusted to different values [6, 7, 8 and 9] with 1 M NaOH. Afterwards, the cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, Al^{3+} and Fe^{3+}), used as chlorides, were dissolved in distilled water at the following concentrations: 2.5, 5, 10 and 25 mg/ml. Finally, increasing volumes of the Ca^{2+}, Mg^{2+}, Zn^{2+}, Al^{3+} and Fe^{3+} solutions (0.2, 0.4, 0.6, 0.8, 1.0, 1.25, 1.5, 2.0, 3.0, 5.0 ml) were added to 10 ml of the PAA or PAA-Cys solution under magnetic stirring at room temperature. As shown in Table II, samples were visually analyzed and divided into three categories: clear solution, opalescent suspension and aggregates. The opalescent suspension, corresponding to a suspension of very small particles, was then further examined with a particle sizer in order to further improve the preparation conditions.

Preparation of PAA Nanoparticles

Nanoparticles were formed spontaneously due to the addition of 0.6 ml $CaCl_2$ (10 mg/ml), 1.0 ml $MgCl_2$ (10 mg/ml), 0.4 ml $ZnCl_2$ (10 mg/ml), 0.6 ml $AlCl_3$ (10 mg/ml) or 0.4 ml $FeCl_3$ (10 mg/ml) solution to 10 ml of the PAA_{100}, PAA_{240}, or PAA_{450} solution (0.5 mg/ml) at pH 8 under magnetic stirring, respectively.

Preparation of PAA-Cys Nanoparticles

Nanoparticles were obtained spontaneously upon incorporation of 1.0 ml $CaCl_2$ solution (10 mg/ml) to 10 ml of the PAA_{100}-, PAA_{240}-, or PAA_{450}-Cys solution (0.5 mg/ml), respectively, at pH 8 under magnetic stirring. In the following step 10 ml of the particle suspension were oxidised due to the addition of 0.05 ml hydrogen peroxide (30%) and the solution was stirred for 80 min. Thereafter Ca^{2+} was removed by the addition of an equimolar amount of EDTA and exhaustive dialysis.

Particle Characterisation

Size distribution and zeta potential of particles were determined with a particle sizer (Zeta Potential/Particle Sizer, NicompTM 380 ZLS, Tokyo, Japan). The amount of thiol groups on covalently crosslinked particles was determined via iodometic titration (1 mM iodine solution; indicator: starch) at pH 1–2 as described previously (15). Disulfide content was measured after reduction with $NaBH_4$ and iodometic titration as described above. Stability of the particle suspension concerning the particles size was examined at pH 2. Remaining traces of Ca^{2+} were determined via complexometric titration (0.01 M EDTA; indicator: calconcarboxylic acid) according to (16). Shape of particles was monitored with an in-column energy filter transmission electronic microscope (ZEISS 902; Zeiss AG; Oberkochen; Germany). Particles dispersed in distilled water (pH 8.0) were transferred onto a copper grid coated with pioloform and then examined under TEM without the use of a contrasting agent.

Table II. Results from the Formulation Studies: Stated Concentrations of PAA or PAA-Cys and Ionic Crosslinkers Resulted in the Formation of No Nanoparticles (△), Nanoparticles (●) and Aggregates (□)

		PAA (100, 240, 450 kD)		PAA-Cys (100, 240, 450 kD)	
		0.5 mg/ml	2.5 mg/ml	0.5 mg/ml	2.5 mg/ml
Ca^{2+}	0.4 ml (2.5 mg/ml)	△	△	△	△
	1.0 ml (2.5 mg/ml)	△	□	△	□
	3.0 ml (2.5 mg/ml)	●	□	●	□
	0.4 ml (10 mg/ml)	●	□	△	□
	1.0 ml (10 mg/ml)	●	□	●	□
	3.0 ml (10 mg/ml)	□	□	□	□
Mg^{2+}	0.4 ml (2.5 mg/ml)	△	△	△	△
	1.0 ml (2.5 mg/ml)	△	□	△	□
	3.0 ml (2.5 mg/ml)	●	□	□	□
	0.4 ml (10 mg/ml)	△	△	△	□
	1.0 ml (10 mg/ml)	●	□	□	□
	3.0 ml (10 mg/ml)	□	□	□	□
$Zn^{2+}/Fe^{3+}/Al^{3+}$	0.4 ml (2.5 mg/ml)	△	□	△	□
	1.0 ml (2.5 mg/ml)	●	□	□	□
	3.0 ml (2.5 mg/ml)	□	□	□	□
	0.4 ml (10 mg/ml)	●	□	□	□
	1.0 ml (10 mg/ml)	□	□	□	□
	3.0 ml (10 mg/ml)	□	□	□	□

Statistical Data Analysis

Statistical data analyses were performed using the Student *t*-test with $p < 0.05$ as the minimal level of significance. Calculations were done using the software OriginPro 7G version 7.0552 (B552).

RESULTS AND DISCUSSION

Preparation and Characterisation of Ionically Crosslinked Nanoparticles

The main goal of the present work was to develop a novel method for the preparation of thiolated PAA nanoparticles showing essential advantages in comparison to established polymerization techniques for generating PAA nano- (17) and microparticles (18), as these production processes require the use of organic solvents and surfactants, high temperatures and free radicals for the initiation of the polymerization. Nanoparticles were obtained under exceptionally mild conditions via an ionic gelation method previously described for chitosan and tripolyphosphate as crosslinker (19), alginate and calcium as crosslinker (20,21) or block ionomer complexes formed between poly(ethylene oxid)-*b*-polymethacrylate and divalent metal cations (22). Reaction conditions were appropriately modified for PAA by our research group.

For the development of this new nanoparticulate system a preliminary study focusing on the production of unmodified PAA nanoparticles was conducted. In order to evaluate the influence of various cations on the particle size and size distribution, five different ionic crosslinkers namely Ca^{2+}, Mg^{2+}, Zn^{2+}, Al^{3+} and Fe^{3+} were utilized. Furthermore, the influence of the molecular weight of PAA on the particle size was investigated. For this purpose PAA of three different molecular weights, 100, 240 and 450 kDa, were chosen. Results of these studies demonstrated a great influence of the cations and a significant impact of the molecular weight of PAA on the particle size and size distribution. As shown in Fig. 2 the smallest particles with the least size distribution could be achieved with PAA_{100} and with Ca^{2+} as crosslinker, displaying a mean particle size in the range of 60 nm, whereas the use of PAA_{450} and trivalent cations (Al^{3+}, Fe^{3+}) as crosslinkers resulted in the formation of the biggest particles, showing a mean particle size of 490 and 610 nm, respectively. Nanoparticles produced with PAA_{100} showed minimum size, followed by PAA_{240} nanoparticles and at last, nanoparticles made of PAA_{450}. Moreover, the particle size and size distribution increased in the following way concerning the use of afore listed cations: $Ca^{2+} < Mg^{2+} < Zn^{2+} < Al^{3+} < Fe^{3+}$.

Preparation and Characterisation of Thiolated Polymer

Three types of PAA, namely PAA_{100}, PAA_{240} and PAA_{450}, were chosen for the preparation of thiolated PAA nanoparticles and different amounts of thiol groups, as listed in Table I, were immobilized on the polymers to explore the influence of the molecular weight of PAA and the amount of thiol groups on various parameters such as the mean particle size, size distribution or zeta potential of the particles. The lyophilized thiolated polymers were of fibrous structure, white and odourless.

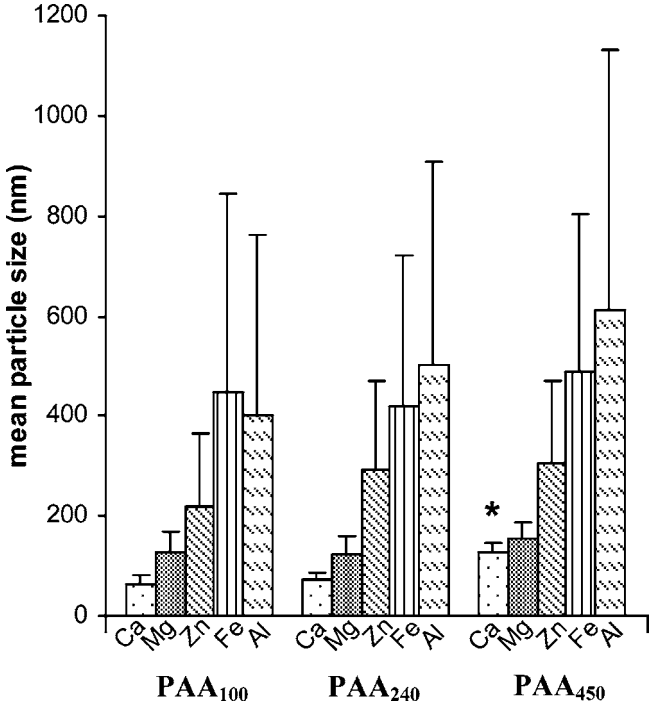

Fig. 2. Influence of cationic crosslinkers and molecular weight of polyacrylic acid on the mean diameter of resulting particles prepared by ionic gelation. Indicated values are the means of at least three experiments (± S.D.). *, differs from PAA_{100}/Ca^{2+} nanoparticles, $p < 0.05$.

Preparation of Covalently Crosslinked Nanoparticles

Within this study thiolated PAA was chosen for the preparation of nanoparticles as the permeation enhancing effect (23) and the mucoadhesive properties (9) of PAA can be significantly improved due to the immobilization of thiol groups on the polymer. According to this, PAA-Cys nanoparticles should exhibit strong mucoadhesive and permeation enhancing properties. Furthermore, the inhibitory properties of PAA against various enzymes such as aminopeptidase N or chymotrypsin are also improved due to the attachment of thiol groups on the polymer (24,25).

PAA-Cys nanoparticles were prepared via gelation with Ca^{2+}, Mg^{2+}, Zn^{2+}, Al^{3+} or Fe^{3+} followed by the formation of intra- and intermolecular disulfide bonds within the particles and the removal of the ionic crosslinker. Regarding the 5 different cations, it can be pointed out that it was only possible to prepare nanoparticles with calcium as crosslinker. Using Zn^{2+}, Al^{3+} and Fe^{3+} as crosslinkers, formation of nanoparticles could not be obtained since the addition of little amount (200–400 µl) of $ZnCl_2$ (10 mg/ml) or $AlCl_3$ (10 mg/ml) or $FeCl_3$ (10 mg/ml) solution to 10 ml of the PAA_{100}-Cys, PAA_{240}-Cys, or PAA_{450}-Cys solution (0.5 mg/ml) already induced the creation of agglomerates which showed a particle size in the range of 1 to 1,000 µm. Variation of diverse parameters such as the decrease of the cation or PAA-Cys concentration did not lead to more assumable results. Concerning the use of Mg^{2+} as crosslinker, although nanoparticles were formed during the first preparation step showing a particle size in the range of 200 to 300 µm, the size of these particles increased rapidly within 5 to 10 min and aggregates were

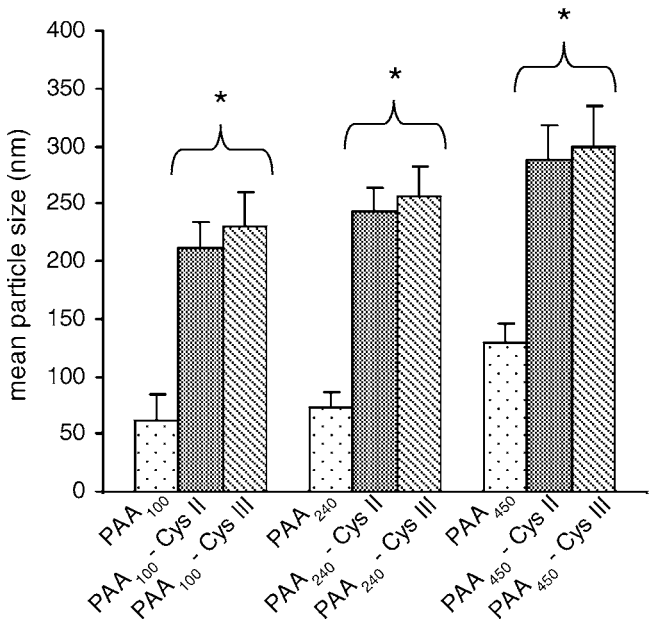

Fig. 3. Comparison of the mean particle size of ionically crosslinked PAA/Ca^{2+} nanoparticles and covalently crosslinked PAA-Cys nanoparticles. Indicated values are the means of at least three experiments (± S.D.). *, differs from nanoparticles prepared with the correspondent unthiolated PAA, $p < 0.05$.

built during the oxidation process. However, in comparison with Mg^{2+}, Zn^{2+}, Al^{3+} and Fe^{3+}, the addition of Ca^{2+} to a PAA-Cys solution resulted in the preparation of nanoparticles which remained stable during the entire preparation process and further studies were consequently performed using just Ca^{2+} as crosslinker.

Three different types of thiolated PAA, namely PAA_{100}-Cys, PAA_{240}-Cys and PAA_{450}-Cys, were chosen in order to determine the effect of the molecular weight on the physical features of the nanoparticles. As shown in Fig. 3 the smallest particles could be achieved with PAA_{100}-Cys, displaying a mean particle size in the range of 220 nm, whereas the use of PAA_{450}-Cys resulted in the formation of the biggest particles, showing a mean particle size in the range of 290 nm. Furthermore, every type of PAA-Cys was utilized several times, each time containing a different amount of thiol groups, as due to different amounts of thiol groups variable numbers of disulfide bonds are formed during the oxidation process and the nanoparticles should therefore feature different mean particle diameters. Results of these studies demonstrated a significant impact of the amount of thiol groups on the particle size. Whereas thiolated PAA containing thiol groups in the range of 200 to 400 μmol/g polymer provided nanoparticles with a mean particle size of about 200 to 300 nm dependent on the molecular weight of the PAA, particles composed of PAA-Cys with an amount of thiol groups under 150 μmol/g polymer dissolved immediately after the addition of EDTA since the number of disulfide bonds formed during the oxidation were not sufficient for a stabilization of the nanoparticles. Moreover, with PAA-Cys comprising more than 500 μmol thiol groups per gram polymer no nanoparticles but aggregates were formed in the course of the addition of Ca^{2+} to the PAA-Cys solution.

Characterisation of Covalently Crosslinked Nanoparticles

Thiol/Disulfide Content

The degree of oxidation as listed in Table III has been achieved via oxidation of the thiol groups due to the addition of H_2O_2. In case of covalently crosslinked particles having been generated by utilizing Ca^{2+} during the preparation, 20 to 30% of all thiol groups remained unoxidized. Bernkop-Schnürch *et al.* (26), for instance, could show that the mucoadhesive properties of chitosan nanoparticles could be doubled due to the immobilization of thiol groups on the particles in the range of 30 to 120 μmol/g polymer. Furthermore, mucoadhesion studies with PCP-Cys particles were performed by our research group, showing three-fold improved mucoadhesion properties in comparison to unmodified PCP *in vitro* (27). According to these results, the remaining unoxidized thiol groups on PAA-Cys nanoparticles should thereby contribute to increased mucoadhesive properties of the particles.

Particle Size

Comparing unthiolated PAA nanoparticles with nanoparticles produced with thiolated PAA, it is shown in Fig. 3, that replacing the ionic crosslinking with Ca^{2+} with disulfide crosslinking leads to 3.5-fold greater particles in the case of PAA_{100} and PAA_{240} and 2.3-fold bigger particles in the case of PAA_{450}, which might be explained by the lower number of disulfide crosslinkages compared to ionic crosslinkages. Based on the assumption that each Ca^{2+} provides just one ionic crosslinking 10.8 mmol Ca^{2+} crosslinkages per gram PAA are feasible, whereas in maximum only 172 μmol (PAA_{100}-Cys), 182 μmol (PAA_{240}-Cys) and 162 μmol (PAA_{450}-Cys) disulfide crosslinkages per gram polymer can be formed. Moreover, the difference in the swelling properties of unmodified and thiolated PAA might also influence the size of the nanoparticles. Guggi *et al.*, for instance, showed that the swelling behaviour of PAA-Cys tablets differed totally from that of unmodified PAA tablets (28). Whereas the weight of PAA-Cys tablets was ten-fold increased when the tablets were incubated in phosphate buffer pH 6.8 for 60 min, unthiolated PAA tablets progressively lost their weight after an initial augmentation until they were completely dissolved and/or eroded. Furthermore, different rheological properties of covalently crosslinked and ionically crosslinked nanoparticles might also contribute to different mean particle sizes, as the viscosity of PAA-Cys increases significantly due to the formation of disulfide bonds (12).

Table III. Amount of Thiol Groups and Disulfide Bonds Immobilized on the Nanoparticles after Ionic Gelation with Ca^{2+} and Oxidation with H_2O_2

	–SH [μmol/g]	–S–S–[μmol/g]	Σ –SH [μmol/g]
PAA_{100}-Cys II	40	76	192 ± 14
PAA_{100}-Cys III	83	124	331 ± 16
PAA_{240}-Cys II	44	93	229 ± 9
PAA_{240}-Cys III	98	129	355 ± 21
PAA_{450}-Cys II	47	86	218 ± 11
PAA_{450}-Cys III	93	126	344 ± 27

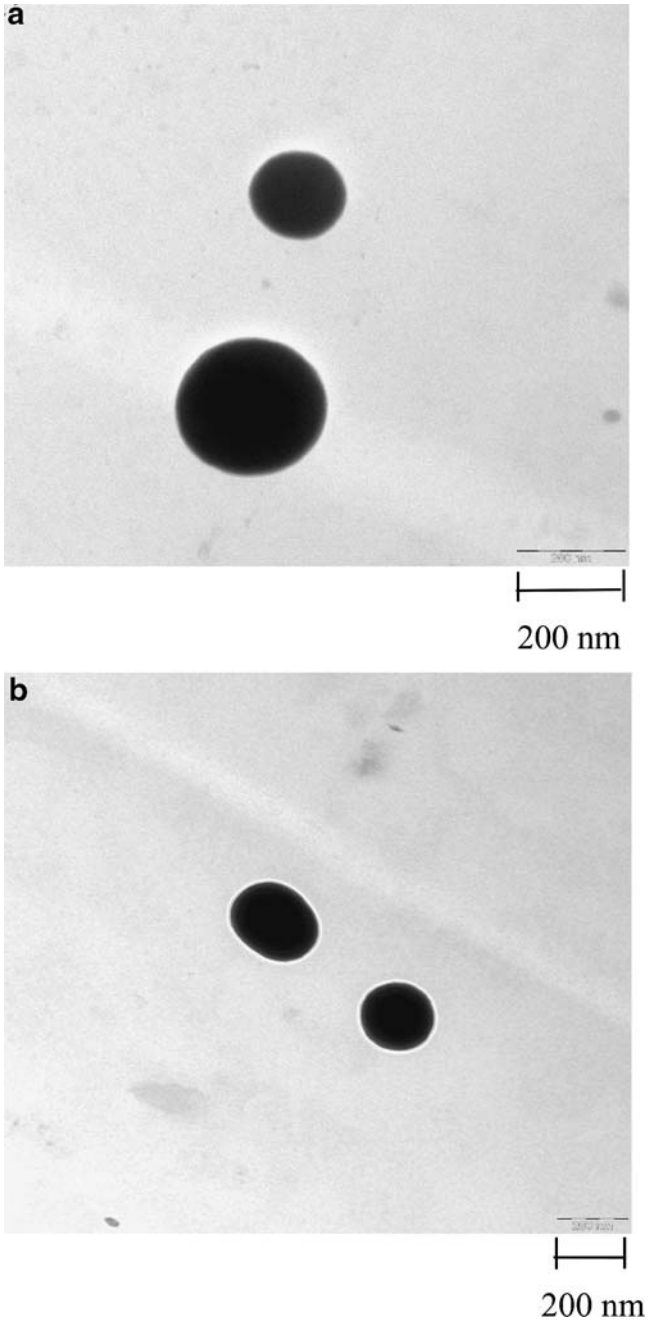

Fig. 4. TEM micrographs of covalently crosslinked nanoparticles based on PAA-Cys. Particles were obtained by ionic gelation with Ca^{2+} and subsequent oxidation by the addition H_2O_2 (a) PAA_{100}-Cys nanoparticles; (b) PAA_{450}-Cys nanoparticles.

Lamprecht *et al.* investigated the mucoadhesive properties of fluorescent particles with a size of 100 nm, 1 μm and 10 μm in the gastrointestinal tract of rats. Results of this study showed the highest mucoadhesive properties for 100 nm particles (29). According to these results, the size of covalently crosslinked nanoparticles made of PAA-Cys, which already provides significantly more pronounced mucoadhesive properties than unmodified PAA, should be advantageous in terms of a prolonged gastrointestinal residence time. Because of this covalent crosslinking particles became also stable at low pH levels. Whereas PAA/Ca^{2+} and thiolated PAA/Ca^{2+} particles disintegrated rapidly in aqueous solution at pH 2, oxidized thiolated PAA/Ca^{2+} particles remained stable concerning the particle size (data not shown). In contrast to ionically crosslinked PAA nanoparticles, covalently crosslinked particles will not disintegrate in the acidic milieu of the stomach because of their improved stability. Electronic microscopic investigations revealed that these particles were of spheric shape and had a smooth surface. Results are shown in Fig. 4.

Zeta Potential

The comparison of the zeta potential of ionically and covalently crosslinked particles is shown in Fig. 5. The zeta potential of particles altered significantly as a result of the elimination of the ionic crosslinkers. Removing Ca^{2+} from PAA-Cys particles led to an almost three-fold decrease in the zeta potential. Quantification of the remaining amount of Ca^{2+} after dialysis showed that less than 1% of the initial amount of the ionic crosslinker remained within the particles. Particles of a more negative zeta potential should display comparatively more pronounced mucoadhesive properties, as the negative charges of PAA are responsible for its mucoadhesive capacity (7,8). Moreover, as the negative charges of PAA seem to be essential for its permeation enhancing properties (30), a more negative zeta potential might contribute to a more pronounced permeation enhancing effect of such particles.

CONCLUSION

Within this study a novel method for the preparation of thiolated PAA nanoparticles being covalently crosslinked via

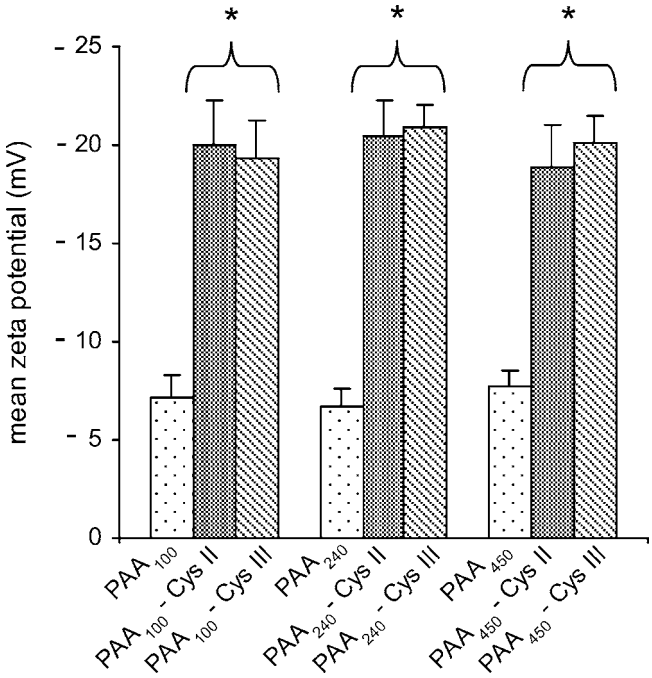

Fig. 5. Comparison of the zeta potential of ionically crosslinked PAA/Ca^{2+} nanoparticles and covalently crosslinked PAA-Cys nanoparticles. Indicated values are the means of at least three experiments (± S.D.). *, differs from nanoparticles prepared with the correspondent unthiolated PAA, $p < 0.05$.

disulfide bonds was developed. The major advantages of these nanoparticles are that they are made of a hydrophilic polymer and prepared under extremely mild conditions without the need of organic solvents and surfactants. Due to the covalent crosslinking particles were more stable and showed a more pronounced negative charge than the corresponding ionically crosslinked particles. Because of these interesting features such nanoparticles may be suggested as carrier systems for the transdermal delivery of therapeutic proteins, genes and antigens.

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