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ORIGINAL ARTICLE

Synthesis and in vitro characterization of a novel PAA–ATP conjugate

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Abstract

The objective of this study was to improve the multifunctional properties of poly(acrylic acid) (PAA) by covalent attachment of 4-aminothiophenol (ATP) to its backbone. The permeation enhancing effect of PAA–ATP together with glutathione was evaluated in Ussing-type chambers using fluorescein isothiocyanate dextran as model compound. The mucoadhesive properties were evaluated in vitro on freshly excised porcine intestinal mucosa through the rotating cylinder method. The resulting conjugates PAA–ATP1 and PAA–ATP2 displayed 168 ± 35 and 426 ± 55 μmol immobilized free thiol groups per gram polymer, respectively. In addition, 279 ± 28 and 139 ± 22 μmol disulfide bonds per gram polymer, respectively, were identified on PAA–ATP1 and PAA–ATP2. Within disintegration studies in aqueous buffer solution, the modified polymers showed improved cohesive properties. Because of the immobilization of ATP, the swelling of PAA–ATP1 and PAA–ATP2 improved 12.0- and 17.8-fold, respectively. The adhesion times of the conjugates PAA–ATP1 and PAA–ATP2 were more than 20- and 30-fold increased in comparison to unmodified PAA. Furthermore, conjugates PAA–ATP1 and PAA–ATP2 exhibited a 1.86- and 2.07-fold higher permeation enhancing effect, respectively, over unmodified PAA. According to these results, PAA–ATP conjugates represent a very promising novel type of thiomer for the development of various mucoadhesive drug delivery systems.

Key words: 4-Aminothiophenol, mucoadhesion, permeation enhancement, poly(acrylic acid), rheology, swelling behavior, thiolated polymer

Introduction

Mucoadhesive polymers represent a promising approach to oral protein delivery, which has been studied extensively over the last decade[1,2]. Using these polymers, intimate contact time with the mucus surface increases, which results in an increased drug retention at the site of absorption resulting in improved overall bioavailability. The widely studied mucoadhesive materials include chitosan, hydroxypropyl cellulose, poly(acrylic acid) (PAA), and their derivatives. Thiolated polymers in particular feature an interesting strategy. These polymers are divided into two different groups: cationic- and anionic- thiolated polymers, which are mainly based on chitosan and PAA, respectively[3,4]. Their improved mucoadhesive features can be explained by covalent bonding between their thiol-bearing ligands and cysteine-rich subdomains of glycoproteins in the mucus[5]. Further investigations showed that thiolation of polymers leads not only to improved mucoadhesiveness but also to additional improved controlled release, permeation enhancement[6], inhibitory effects on proteases as well as efflux pumps[7,8].

Recently, Grabovac et al.[9] compared the mucoadhesive potential of 19 different, frequently cited, mucoadhesive polymers and ascertained that thiomers based on PAA exhibit significantly lower mucoadhesive properties in comparison to thiomers based on chitosan. Therefore, the purpose of this study was to establish a new thiolated polymer based on PAA with significantly improved mucoadhesive features. As adhesiveness and cohesiveness of a polymer increase, when the water content

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decreases[10] a promising strategy seems to be the direct immobilization of a hydrophobic ligand on PAA, which would lead to a new thiomer offering comparatively higher mucoadhesive and cohesive properties. On the basis of these considerations, aromatic aminothiophenols (ATP) seem to be of high interest as they include the desired hydrophobicity as well as the needed thiol groups. Furthermore, the presence of an amino function as an electron donor increases the ability of an aromatic compound for the coupling with thiol-rich subdomains. In addition, Puri and Roskoski[11] ascertained in one of their studies that aromatic aminothiophenols show a much higher tendency to bind to cysteine-rich subdomains in yeast hexokinase through their thiol group in contrast to aliphatic aminothiols.

In this study, ATP was covalently attached to PAA through the formation of an amide bond between the amino group of ATP and a carboxylic acid group of the polymer. In addition, essential thiomer features such as cohesive properties, swelling behavior, mucoadhesion, and permeation enhancing features were evaluated.

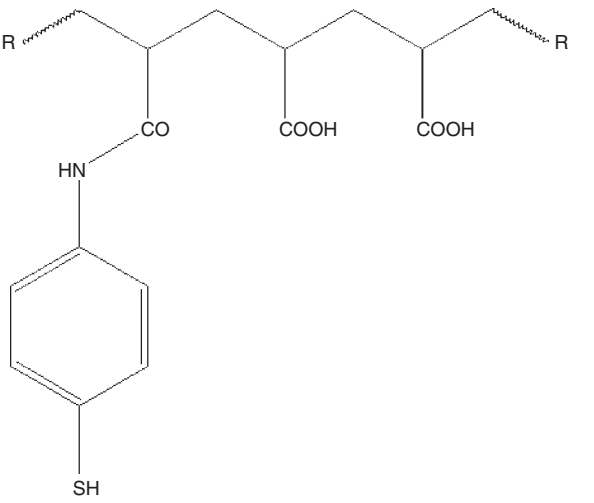

Figure 1. Chemical substructure of PAA–ATP conjugates. The presence of an amino function as an electron donor increases the ability of an aromatic compound for the coupling to thiol-rich subdomains. In addition, aromatic aminothiophenols show a much higher tendency to bind to cysteine-rich subdomains via their thiol group in contrast to aliphatic aminothiols.

Materials and methods

Materials

PAA with a molecular mass of 450 kDa, glutathione reduced form (GSH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), 5,5′-dithiobis(2-nitrobenzoic acid), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), and fluorescein isothiocyanate dextran (FD_4) were purchased from Sigma (St. Louis, MO, USA). All chemicals were of analytical grade.

Synthesis of PAA–ATP conjugate

Synthesis of PAA–ATP1 conjugate

ATP was covalently attached to PAA through the formation of amide bonds between the amino group of ATP and a carboxylic acid group of the polymer as illustrated in Figure 1. First, 1 g of PAA was hydrated in 80 mL demineralized water and the pH value of the obtained polymer solution was adjusted to 5.5 by adding 5 M NaOH. Then, EDAC, dissolved in 5 mL demineralized water, was added in a final concentration of 50 mM to activate the carboxylic acid moieties of PAA. After 15 minutes of incubation at room temperature, 5 mL of dioxane was added dropwise over 10 minutes under continuous stirring and subsequently 0.2 g of ATP, dissolved in 2 mL of dioxane, was added dropwise. The reaction mixture was stirred further at room temperature for 3 hours.

The thiomer was then purified by solvent extraction in a separation funnel with ethyl acetate as extraction solvent and subsequent precipitation with acetone to remove excess unbound low-molecular-mass thiol compound and/or the coupling reagent. Controls were prepared and isolated in the same way as the polymer conjugate but the carbodiimide (EDAC) was omitted during the coupling reaction. Finally, the resulting polymers and corresponding control were frozen and lyophilized at −70°C and 0.01 mbar (Lyolab B; Inula, Vienna, Austria). Samples were stored at 4°C until further use.

Synthesis of PAA–ATP2 conjugate

ATP was covalently attached to PAA through the formation of amide bonds between the amino group of ATP and a carboxylic acid group of the polymer as illustrated in Figure 1. First, 1 g of PAA was hydrated in 80-mL demineralized water and the pH value of the obtained polymer solution was adjusted to 5.5 by adding 5 M NaOH. Then, EDAC, dissolved in 5-mL demineralized water, was added in a final concentration of 50 mM to activate the carboxylic acid moieties of PAA. After 15 minutes of incubation at room temperature, 5 mL of dioxane was added dropwise over 10 minutes under continuous stirring and subsequently 0.2 g of ATP, dissolved in 2 mL of dioxane, was added dropwise. The reaction mixture was stirred at room temperature for 3 more hours.

Thereafter, the resulting polymer was reduced with dithiothreitol (DTT). Immediately after the pH has been adjusted to 8, DTT was added in a final concentration of 100 mM. After 30 minutes of incubation at room temperature, the pH was adjusted to 6 with 1 M HCl. The thiomer was then purified by solvent extraction in a separation funnel with ethyl acetate as extraction solvent and subsequent precipitation with acetone to remove excess unbound low-molecular-mass thiol compound and/or the coupling reagent. Controls were prepared and isolated in the same way as the polymer conjugate but the carbodiimide (EDAC) was omitted during the coupling reaction. Finally, the resulting polymers and corresponding control were frozen and lyophilized at

–70°C and 0.01 mbar (Lyolab B). Samples were stored at 4°C until further use.

Determination of the thiol group and disulfide bond content

The amount of thiol groups immobilized on conjugates PAA–ATP1 and PAA–ATP2 was determined spectrophotometrically using Ellman's reagent as described previously[6]. Disulfide content was determined after reduction with $NaBH_4$ and addition of 5,5′-dithiobis(2-nitrobenzoic acid) as described by Habeeb[12].

Tablets manufacture

Lyophilized conjugates PAA–ATP1 and PAA–ATP2 and controls were compressed into 20 mg, 5.0 mm diameter flat-faced tablets (single punch eccentric press-Korsch EK, Berlin, Germany). The compaction pressure (force of 11 kN) was kept constant during the preparation of tablets. Tablets were checked for resistance to crushing (Schleuniger-type apparatus) according to the European Pharmacopoeia. The tensile strength (σ_x) of the tablets was then calculated using the following equation[13]:

$$\sigma_x = \frac{2P}{\pi dt},$$

where P is crushing strength (N), d is the diameter of the tablet (mm), and t is the thickness of the tablet (mm). The mean tensile strength of at least five tablets was calculated.

Evaluation of the swelling behavior

The water-absorbing capacity was determined by a gravimetric method. Briefly, 20 mg of each lyophilized conjugate or corresponding control was compressed (single punch eccentric press-Korsch EK) to 5.0 mm diameter flat-faced tablets. Tablets were fixed to a needle and immersed in a beaker containing 100 mM phosphate buffer, pH 6.8, at 37°C. At predetermined time points the swollen tablets were taken out of the incubation medium, excess water was removed, and the amount of water uptake was determined gravimetrically[14]. The swelling ratio was then calculated according to the following equation:

$$\text{Swelling ratio} = \frac{W_u}{W_o},$$

where W_u is the weight of uptaken water and W_o the initial weight of the dry tablet.

Disintegration studies

The stability of the polymer tablets and the corresponding control tablets was analyzed in 100 mM phosphate buffer, pH 6.8, at 37 ± 0.5°C with the disintegration test apparatus according to the European Pharmacopoeia. The oscillating frequency was adjusted to 0.5 s^{-1}.

Oscillatory rheology

The viscoelastic properties of the gels of conjugates PAA–ATP1 and PAA–ATP2 were determined with a HAAKE MARS (Haake GmbH, Karlsruhe, Germany), a thermostatically controlled plate–plate rheometer with a plate diameter of 35 mm. All rheological measurements were shear stress controlled whereas the shear stress increased from 0 to 500 Pa. Initially, 5 mL of 1% (w/v) polymer solution was incubated at 37°C. After determination of the linear viscoelasticity range, dynamic oscillatory tests were performed for aliquots of about 750 µL at a frequency of 1 Hz over a time period of 18 hours. Throughout the experimental period, the plate temperature was maintained at 37.0 ± 0.1°C and the gap was 0.5 mm. The parameters obtained are the complex modulus G^* and the phase angle δ. The elastic modulus G', the viscous modulus G'', and the dynamic viscosity η' are calculated by the following equations:

$$G' = G^* \cos(\delta),$$

$$G'' = G^* \sin(\delta),$$

$$\eta' = \frac{G''}{\omega},$$

where ω is the angular frequency, which is related to the oscillatory frequency ν by the relationship $\omega = 2\pi\nu$. Loss tangent (tan δ), a parameter that represents the ratio between the viscous and elastic properties of the polymer, was also calculated ($\tan\delta = G''/G'$).

Permeation studies

According to previous studies, Ussing-type chambers with a volume of 1 mL within both donor and acceptor chamber and a permeation area of 0.64 cm^2 were used to perform permeation studies with FD_4 as model compound. The pH of the prepared incubation medium containing 250 mM NaC1, 2.6 mM $MgSO_4$, 10 mM KCl, 40 mM glucose, and 50 mM $NaHCO_3$ buffered with 40 mM HEPES was adjusted to 6.0.

Right after killing the rat, the first 15 cm of the small intestine was excised and mounted in Ussing-type chambers. Thereafter, the media of the donor compartment was substituted by either 0.5% (w/v) PAA–ATP1 or PAA–ATP2 or 0.5% (w/v) PAA. All three test solutions included 0.5% (w/v) GSH and 0.005% (w/v) FD_4. Control studies were carried out with FD_4 in incubation medium. Over a period of 3 hours, aliquots of 100 µL were taken

out from the acceptor compartment every hour and the volume was substituted by 100 µL incubation medium preequilibrated at 37°C. The amount of permeated FD_4 was determined using a microplate reader (Infinite® M200, Tecan, Maennerdorf Switzerland). Cumulative corrections were made for the previously removed samples. The cumulative mass Q (µg) of FD_4 transported from donor to acceptor chamber was assessed over the time course of the experiment (dQ/dt). Using linear regression analysis, the following equation was fit to the data:

$$\frac{dQ}{dt} = P_{app} \times A \times C^{\circ},$$

where P_{app} is the permeability coefficient (cm/s), A is the diffusion area of the Ussing chamber (cm^2), and C° is the initial concentration of the marker in the donor compartment ($\mu g/cm^3$).

Transport enhancement ratios (R) were calculated from P_{app} values by the following equation:

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

In vitro mucoadhesion studies with the rotating cylinder method

In vitro mucoadhesion was tested using 'Paddle Apparatus' according to the European Pharmacopoeia. An Erweka DT 700 (Erweka GmbH, Heusenstamm, Germany) dissolution tester was used. Stirrers in cylinder form were provided as part of the mucoadhesion tester. The vessel was filled with 900 mL of 0.1 M phosphate buffer, pH 6.8, at 37°C ± 0.5°C. The rotational speed of the cylinders was 125 min^{-1} in various phases of product testing. Polymer and control tablets were attached by hand to a freshly excised intestinal porcine mucosa, which has been attached to a stainless steel cylinder (diameter 4.4 cm, height 5.1 cm). Detachment of test tablets was determined visually during an observation time of 3 hours[15].

Assessment of cytotoxic effects

To investigate potential cytotoxic effects of PAA–ATP1 conjugate, the extracellular activity of lactate dehydrogenase (LDH) was determined. LDH is released by cells that have lost their membrane integrity. Therefore, extracellular LDH activity can be regarded as a measure for membrane toxicity of test compounds. Cytotoxicity Detection Kit (LDH) was purchased from Roche, Berlin, Germany, to determine the LDH activity.

The assay was performed on Caco-2 cells which were cultured on 12-well plates at a density of 1×10^4 cells/mL MEM medium for 24 hours. After attachment of Caco-2 cells to the plate, they were incubated with PAA–ATP1 conjugate and unmodified PAA dissolved in MEM medium at a concentration of 0.5%. MEM served as negative control whereas a 2% solution of Triton X-100 in MEM was used as positive control. The pH value of all solutions was kept constant at 7.4. Caco-2 cell were then incubated for 3 hours at 37°C and 5% CO_2. Samples of 100 µL were taken from the supernatant of each well and transferred to a 96-well microplate. A 100 µL of the provided reaction mixture was added. The LDH activity was quantified by measuring the absorbance at a wavelength of 490 nm in a microplate reader (Infinite® M200, Tecan). Cytotoxicity was calculated according to the following equation:

$$\text{Cytotoxicity}(\%) = \frac{A(\text{sample}) - A(\text{negative})}{A(\text{positive}) - A(\text{negative})}.$$

Statistical data analysis

Statistical data analyses were performed using the Student's t-test with $P < 0.05$ as the minimal level of significance. Calculations were done utilizing the software Xlstat version 5.0 (b8.3). Concerning permeation studies, results are given as means ± SD for $n = 4$ experiments. Statistical comparisons (P-values) were made using Student's unpaired t-test, two-sided.

Results and discussion

Synthesis of PAA–ATP conjugate

As thiolated polymers were introduced into the pharmaceutical arena, their outstanding qualities have been demonstrated in various studies regarding mucoadhesive[6], permeation enhancing[16] as well as enzyme inhibitory[17] properties. Moreover, thiolated polymers became an integral part of the development of new formulations and showed their potential in various in vitro and in vivo studies[18,19]. In many cases, thiomers display significantly different features according to the utilized ligands. This offers the chance to generate appropriate thiomers for a diverse range of purposes using different ligands.

Thiomers based on PAA represent a good example as they offer good permeation enhancing and enzyme inhibitory capacities on the one hand but exhibit much lower mucoadhesive features in comparison to thiomers based on chitosan on the other hand. Therefore, the target-orientated development and evaluation of new thiolated polymers for specific applications seems to be feasible.

Grabovac et al.[9] could ascertain in an earlier study that Carbopol 980, displaying comparatively the highest molecular mass and a high degree of cross-linking, was found to adhere to the mucosa for the longest time period among the polyacrylates. Furthermore, the results of this study support the findings that increasing the

molecular mass of polymer leads to the higher internal cohesion of the molecule that consequently increases mucoadhesion. However, the relatively high residence time on the mucosa is to be attributed not only to the high-molecular mass of polyacrylates but also to the degree of cross-linking. It is known that cross-linkage diminishes the dissolution rate of hydrophilic polymer chains in aqueous environment, providing comparatively greater cohesion of polymers[9]. Nevertheless poly(acrylic) acid with a molecular weight of 450 kDa was chosen by reason of its better solubility behavior in the reaction solution. For the development of a thiomer based on PAA with improved mucoadhesive and cohesive properties, ATP was chosen. The sulfhydryl group of ATP plays a pivotal role. Because of its high nucleophilicity and its reasonably low pK_a, it shows high chemical reactivity in nucleophilic additions and substitutions[20]. Thiol–disulfide interchange involves the nucleophilic attack of thiolate anion along the S–S bond axis of the disulfide. The apparent rate of thiol–disulfide reaction reaches its maximum when the thiol pK_a is close to the pH of the surrounding medium[21]. Considering this effect, we chose the ligand ATP, which exhibits pK_a value of 5.25. The pK_a values of all thiol-bearing ligands that have been coupled to polymers — cysteine, cysteamine, homocysteine, and glutathione — are in the range of 8.3–9.5. For these thiols, only a small fraction (1–0.1%) is presented as thiolate anion at pH 6.8. Therefore, the oxidation potential is limited at a low pH at which there is no significant thiol ionization. Nevertheless, a higher amount of negatively thiolated anions is highly needed as the anion represents the active form for oxidation which leads to cross-linking of the polymer[22].

Generally, the fabrication of thiomers is performed in aqueous solution and consists of three major steps. Initially, a low-molecular-mass thiol group bearing compound is coupled to a polymeric backbone using a coupling reagent such as *N*-(3-dimethylaminopropyl)*N*-ethylcarbodiimide hydrochloride (EDAC). The carboxylic acid moieties of PAA are activated by EDAC forming an *O*-acylurea derivative as intermediate product, which further reacts with the amino groups of the ligand. The main features affecting the coupling rate are the pH, coupling agent, reaction time, and thiol-bearing compound that are used for the synthesis of the thiolated polymers. Therefore, a preparation method was used that has been evaluated in a preliminary study by Kafedjiiski et al.[23] and was modified for this novel thiomer. A major obstacle was the dissolution of hydrophilic PAA on the one hand and hydrophobic ATP on the other hand in the same medium. As the activation of PAA through lipophilic carbodiimide in various organic solvents was not feasible because of occurring precipitation of PAA after the addition of the lipophilic carbodiimide (data not shown), PAA was activated with a hydrophilic carbodiimide in demineralized water. To avoid precipitation of ATP in aqueous solution, dioxane was added dropwise before ATP (2 g/5 mL dioxane) was added likewise. After 3 hours the thiomer was purified by solvent extraction with ethyl acetate and subsequent precipitation with acetone to remove excess unbound low-molecular-mass thiol compound and/or the coupling reagent. Prolonging the reaction time up to 8 hours had no significant effect on the coupling rate (data not shown). Finally, the polymer was dried through lyophilization.

Determination of the thiol group and disulfide bond content

Thiol residues within close proximity can be oxidized to disulfides by either an intra- or an intermolecular reaction. Disulfide bond formation is controlled by the presence of oxygen and by the pH value of aqueous media during test procedures. Kafedjiiski et al.[24] demonstrated in a previous study that 25–40% of thiol groups were oxidized to disulfide bonds during the reaction process. This raised the question of reducing the disulfide bonds to achieve a higher amount of thiol groups, which will inevitably influence the properties of the polymer. The addition of DTT is a common method to reduce disulfide bonds through two sequential thiol–disulfide exchange reactions[25]. The reducing capacities of DTT are limited to pH values above 7 because only the negatively charged thiolate form is reactive in contrast to the protonated form (pK_a of thiol groups is typically ~8.3). Consequently, pH had to be adjusted to 8. The new conjugate exhibited 168 ± 35 μmol immobilized free thiol groups and 279 ± 28 μmol disulfide bonds per gram polymer for the oxidized form (PAA–ATP1) and 426 ± 55 μmol immobilized free thiol groups and 139 ± 22 μmol disulfide bonds per gram polymer for the reduced form (PAA–ATP2). The conjugate appeared as a white, odorless powder of fibrous structure and it was hardly hydratable in aqueous solution. The efficacy of the purification method used here could be verified by controls which were prepared exactly in the same way as the polymer conjugate but omitting EDAC during the reaction, resulting in a negligible amount of thiol groups.

Swelling behavior

The general properties of the conjugates were also strongly influenced by its swelling behavior. The rapid and continued swelling of thiolated polymer favors the interdiffusion process between the polymer and the mucus layer. Because of a relatively more pronounced interdiffusion and chain interpenetration, more interactions between the conjugate and the mucus occur. Thus, the mucoadhesive properties could be improved compared to unmodified PAA. Slightly cross-linked polymers with a high number of charged groups, which can be dissociated and are firmly fixed on the polymer, are good water absorbers. Consequently, they can absorb water in quantities which are multiples of their own weight and can fixate them firmly within their polymer networks.

The degree of swelling depends on the number of fixed charges on the polymer, the density of cross-linking, and the salt concentration in the solvent. The ability to take up water increases with the number of charges on the polymer and decreases if the density of cross-linking increases[26]. Results within this study could confirm this theory as in the beginning tablets of unmodified PAA swelled more rapidly than thiolated ones but then a gradual decrease in the tablet weight of unmodified PAA was observed followed by complete dissolution erosion within 30 minutes.

The swelling behavior can be influenced as well by the type of the cross-linking agent. Gels cross-linked by a higher molecular cross-linking agent can swell more pronounced in the same solution than gels with junction points which are formed by a lower molecular cross-linking agent. The size of the cross-linking point influences the distances of the linked polymer chains from each other, and therefore the swelling is disturbed depending on the cross-linking agent. The weight of tablets comprising PAA–ATP1 and PAA–ATP2 conjugates increased rapidly and continuously over the 100 minutes of the experiment. Tablets comprising PAA–ATP1 and PAA–ATP2 reach a maximum weight and a swelling ratio of 12.0 and 17.8 after 120 minutes, respectively. The swelling behavior of PAA–ATP1, PAA–ATP2, and unmodified PAA is shown in Figure 2.

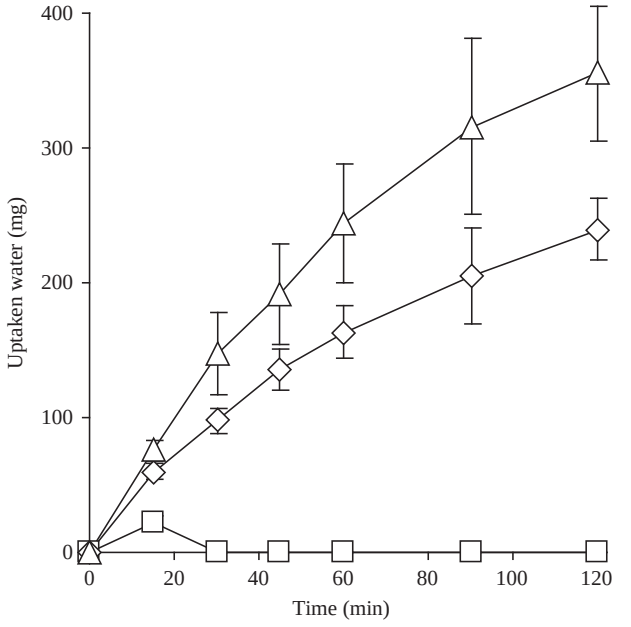

Figure 2. Swelling behavior of 20 mg tablets based on PAA–ATP1 (◇) and PAA–ATP2 (△) conjugate and unmodified PAA (□) in 100 mM phosphate buffer pH 6.8 at 37°C; indicated values are means (±SD) of at least three experiments. Tablets of unmodified PAA display a gradual decrease in the tablet weight of unmodified PAA followed by complete dissolution erosion within 30 minutes. In comparison weight of tablets comprising PAA–ATP1 and PAA–ATP2 conjugates increased rapidly and continuously over the 100 minutes of the experiment. *differs from control <0.05.

Cohesive properties

Disintegration time of PAA–ATP is sufficient to guarantee the mucoadhesive properties of a thiomer-based drug delivery system. Disintegration studies were carried out with tablets comprising PAA–ATP1, PAA–ATP2, and unmodified PAA (Figure 3). The matrix tablets of PAA–ATP1 and PAA–ATP2 were stable for 63 and 75 hours, respectively, and no erosion was observed over this period of time. In contrast, the tablets of unmodified PAA disintegrated very quickly within 2 hours. The observed higher cohesiveness can be explained with the formation of intra- as well as intermolecular disulfide bonds within the polymeric network. This cross-linking process takes place immediately in the aqueous medium and supports the integrity and stability of the polymer matrix tablet[24]. Therefore, disulfide bonds are an integral part of the structure of the thiolated polymers and contribute to the cohesiveness of the tablets. To check the cohesion of the dry tablets, the crushing strength was determined and the tensile strength was calculated. The tensile strength of the PAA tablets was calculated to be 0.484 ± 0.020 mPa. In addition, the tensile strength of the conjugate tablets was also in the same range, that is, 0.452 ± 0.019 mPa.

In situ gelling properties

Rapid clearance from the site of drug absorption is an important factor that limits the efficacy of drug

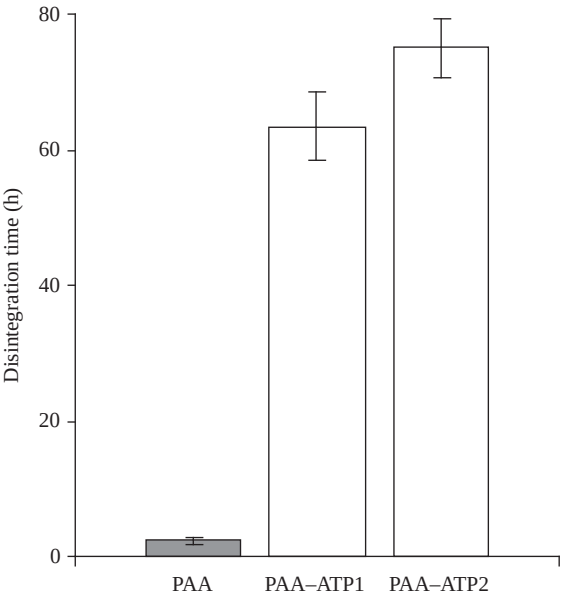

Figure 3. Disintegration behavior of matrix tablets based on the conjugates PAA–ATP1 and PAA–ATP2 and unmodified PAA. Studies were carried out with a disintegration test apparatus in 100 mM phosphate buffer pH 6.8 at 37°C. Indicated values are means (±SD) of three experiments. The matrix tablets of PAA–ATP1 and PAA–ATP2 were stable for 63 and 75 hours, respectively, and no erosion was observed over this period of time. In contrast, the tablets of unmodified PAA disintegrated very quickly within 2 hours. *differs from control <0.05.

administration to the ocular, nasal, and vaginal mucosa. It is widely accepted that limiting the clearance by increasing the viscosity of a drug formulation will result in an increased bioavailability of these drugs. A very promising strategy to obtain drug formulations of an appropriate viscosity is based on in situ gelling. The formation of a gel at the site of drug delivery combines the advantage of a solution, which can be administered easily, with the favorable viscoelastic properties of a gel, providing a prolonged residence time of the formulation. The sol–gel transition occurs in the physiological environment as a result of physicochemical changes, such as changes in the pH[26], in temperature[27], or in electrolyte concentration[28]. Thiolated polymers display in situ gelling properties because of the oxidation of thiol groups at physiological pH values, which results in the formation of inter- and intramolecular disulfide bonds. Further investigations of thiolated polymers demonstrated a clear correlation between the total amount of polymer-linked thiol groups and the increase in elasticity of the formed gel. The more thiol groups were immobilized on polymers, the higher was the increase in the elastic modulus G' in solutions of thiolated polymers[29]. The sol–gel transition of thiolated polymers was completed when highly cross-linked gels were formed. In parallel, a significant decrease in the thiol group content of the polymers was observed, indicating the formation of disulfide bonds[29]. Results within this study could confirm this theory because PAA–ATP2 displayed a significant higher increase in elastic modulus G' than PAA–ATP1 due to a much higher amount of free thiol groups (Figure 4).

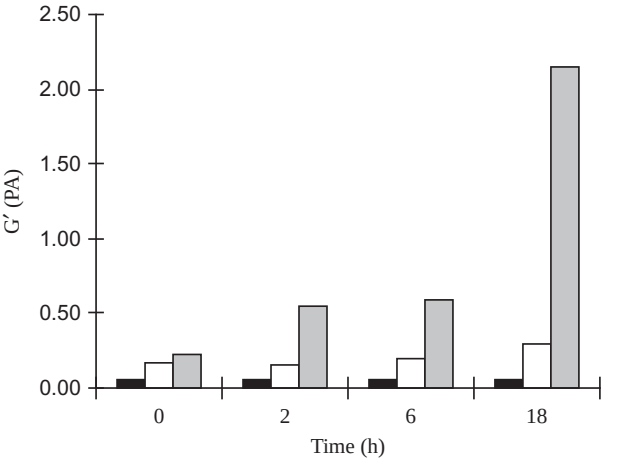

Figure 4. Increase in the elastic properties (G') of a 4% (m/v) PAA gel (black bars) in contrast to a PAA–ATP1 conjugate gel (white bars) and a PAA–ATP2 conjugate gel (gray bars) at pH 6.8 and 37°C as a function of time. Indicate values are means (±SD) of at least three experiments. The more thiol groups were immobilized on polymers, the higher was the increase in the elastic modulus G' in solutions of thiolated polymers. PAA–ATP2 displayed a significant higher increase in elastic modulus G' than PAA–ATP1 because of a much higher amount of free thiol groups. *differs from control <0.03.

Permeation studies

The permeation enhancing effect of thiomers has been described in numerous publications[16,17]. Permeation studies carried out with various model compounds across intestinal mucosa demonstrated that the combination of thiolated polymers with reduced glutathione as low-molecular-mass permeation mediator led to significantly improved permeation enhancing effect of thiomers. The underlying mechanism of this system seems to be based on the inhibition of the protein tyrosine phosphatase (PTP) being involved in the opening process of the tight junctions. The reduced form of free GSH reacts with PTP through thiol–disulfide exchange reaction causing an inactivation of PTP[30]. The inhibitory effect of glutathione is limited as it is rapidly oxidized on the mucosal surface. Recent studies supported the assumption that thiomers expressing reactive thiol groups might be able to reduce oxidized glutathione. Thus, the amount of reduced glutathione on the absorption membrane would be increased. Therefore, the permeation enhancing effect of the thiomer–GSH system depends on the reducing properties of thiomers. In addition, thiomers display some advantages in the permeation studies of model compounds such as comparatively more prolonged effects on the mucosa on the one hand and low toxicity on the other hand[30]. The results of this study are presented in Figure 5. Unmodified PAA [0.5% (w/v)] showed an apparent permeability coefficient of $5.00 \pm 0.13 \times 10^{-5}$ cm/s, not significantly different from the coefficient of the control $4.77 \pm 0.11 \times 10^{-5}$ cm/s. The P_{app} value of the 0.5% (w/v) PAA–ATP1 conjugate was determined to be $8.90 \pm 0.19 \times 10^{-5}$ and $9.87 \pm 0.19 \times 10^{-5}$ cm/s for the 0.5% (w/v) PAA–ATP2 conjugate. Accordingly, the transport enhancement ratio (R) was calculated to be 1.86 for 0.5% (w/v) PAA–ATP1 conjugate and 2.07 for the 0.5% (w/v) PAA–ATP2 conjugate, respectively. These data demonstrate that the immobilization of ATP on the polymer backbone leads to significantly improved permeation of the model drug FD_4. ATP has a potent electron-donating capacity, linked to its thiol group (pK_a value 5.25).

Mucoadhesion studies

Mucoadhesion is a complex phenomenon that can be influenced by numerous physicochemical circumstances. Clearly one of these is the electrostatic forces. The chemical nature of the cell surface and mucin causes negative charges at physiological pH, and so positively charged molecules interact with them[31]. On the contrary, the anionic PAA polymers are negatively charged but their mucoadhesive properties are based on other physicochemical processes, such as hydrogen bonding and van der Waal's interactions. However, all these polymers are based on the formation of noncovalent bonds and therefore they provide only relative weak mucoadhesion in contrast to thiolated polymers. Their improved mucoadhesive properties are explained by the formation of

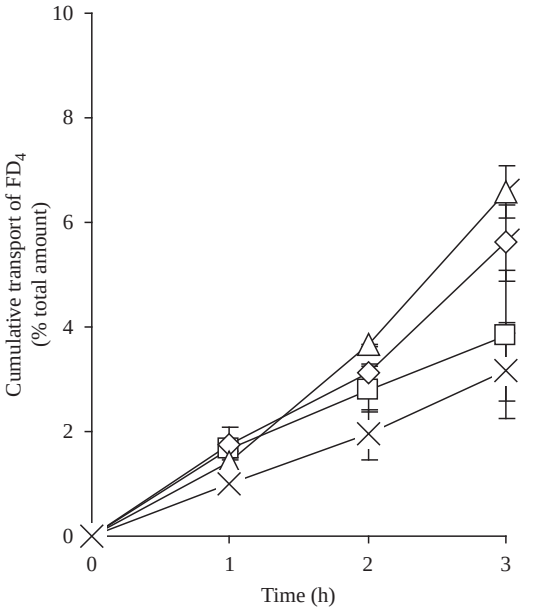

Figure 5. Permeation enhancing effect of 0.5% (w/v) PAA–ATP1 conjugate (◇) and 0.5% (w/v) PAA–ATP2 (△) conjugate with 0.5% reduced glutathione in comparison to 0.5% (w/v) unmodified PAA (□) with 0.5% reduced glutathione and FD_4 (X) on the permeation of FD_4 across freshly excised small intestinal mucosa. Indicated values are means (±SD) of at least four experiments. Unmodified PAA showed no significant difference to the control. In contrast, the utilization of PAA–ATP1 and PAA–ATP2 conjugates led to an enhancement ratio of 1.86 and 2.07, respectively. These data demonstrate that the immobilization of ATP on the polymer backbone leads to significantly improved permeation of the model drug FD_4. *differs from control <0.05.

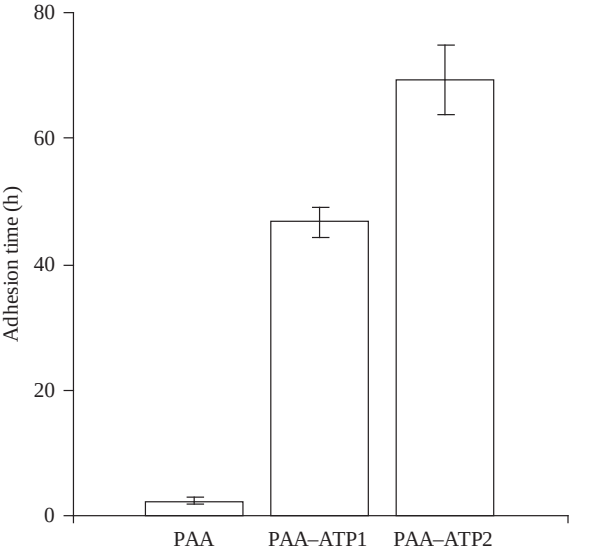

Figure 6. Comparison of the adhesion time of PAA–ATP1 and PAA–ATP2 conjugates and unmodified PAA on the rotating cylinder. The indicated time of adhesion represents the mean (±SD) of at least three experiments. The adhesion time of the conjugates PAA–ATP1 and PAA–ATP2 was more than 20- and 30-fold increased, respectively, in comparison to unmodified PAA. The new conjugates displayed a prolonged adhesion time on the mucosa as a consequence of sufficiently balanced cohesive and mucoadhesive properties. *differs from control <0.03.

covalent bonds between thiol groups of the polymer and cysteine-rich subdomains of glycoproteins in the mucus layer[3]. Another likely mechanism being responsible for the improved mucoadhesive properties is based on their in situ cross-linking properties. During and after the interpenetration process[32], disulfide bonds are formed within the thiolated polymer itself leading to additional anchors through chaining up with the mucus gel layer. In addition, disulfide bonds are also formed within the polymer itself leading to cohesive properties[33]. Altogether these properties result in a prolonged residence time at the site of application and demonstrated that the covalent attachment of ATP increased the mucoadhesive properties of the thiolated polymer in comparison to unmodified PAA. The adhesion time of the conjugates PAA–ATP1 and PAA–ATP2 was more than 20- and 30-fold increased, respectively, in comparison to unmodified PAA. The new conjugates displayed a prolonged adhesion time on the mucosa as a consequence of sufficiently balanced cohesive and mucoadhesive properties (Figure 6). To evaluate the influence of different ligands of PAA conjugates on their permeation enhancing effect, the experimental data were compared with previous studies[34,35] (Table 1).

Assessment of cytotoxic effects

Polyacrylates are widely used and investigated excipients in pharmaceutical sciences. It was demonstrated that orally administered polyacrylates do not reach systemic circulation because they cannot pass through intestinal epithelia due to their macromolecular structure[36]. Hence, systemic toxicity can be excluded. Nevertheless, local toxic effects need to be taken into consideration. In previous studies, it could be demonstrated that thiolation of

Table 1. Comparison of the mucoadhesive properties of various anionic thiomers. Mucoadhesive studies were performed by the rotating cylinder method.

Thiomer	Thiol groups per gram polymer (μmol/g ± SD, $n = 3$)	Adhesion time (h)	Improvement ratio	Ref.
PAA–GSH	354 ± 42	21	14*	(34)
PAA–Cys	404 ± 66	22	13*	(35)
Polycarbophil–Cys	404 ± 66	9	1.7*	(35)
PAA–ATP1	168 ± 35	47	20*	—
PAA–ATP2	426 ± 55	69	30*	—

*Differs from control $P < 0.05$.

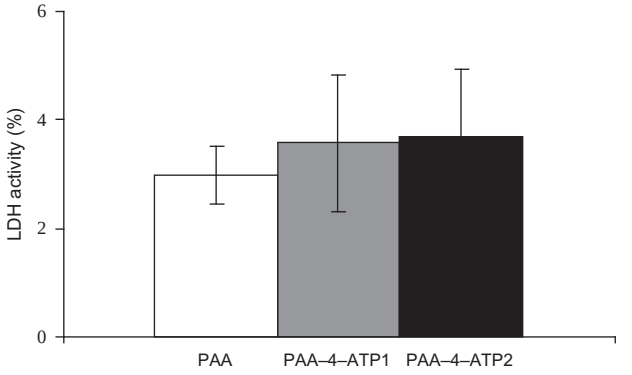

Figure 7. LDH activities of cultured Caco-2 cells after incubation with test solutions for 3 hours. Results are expressed as means (±SD) of at least three experiments. As the result for PAA–ATP does not significantly differ from unmodified PAA, it can be concluded that attachment of ATP to PAA is not accompanied by an increase in membrane toxicity. *differs from control <0.05.

several polymers does not result in significant toxification. Because a ligand of considerable hydrophobicity, compared to formerly used ligands, was covalently attached to PAA within this study, investigations of cytotoxic effects were carried out. The extracellular LDH activity after incubation with test solutions of unmodified PAA and PAA–ATP1 was measured. LDH activities of these test solutions were related to those of a Triton X-100 solution (positive control) and those of MEM (negative control); results of these correlations are depicted in Figure 7. From the fact that the result for PAA–ATP does not significantly differ from unmodified PAA, it can be concluded that attachment of ATP to PAA is not accompanied by an increase in membrane toxicity.

Conclusion

Within this study, PAA–ATP conjugate has been synthesized and characterized for the first time. The covalent attachment of ATP to the anionic polymer PAA leads to conjugates displaying improved mucoadhesive properties in comparison to unmodified PAA. Because of the chemical modification, the swelling behavior and cohesive properties of this novel conjugate were strongly improved. Within this study PAA–ATP conjugate was identified as the most mucoadhesive polyacrylate that has been generated so far to our knowledge. Therefore, this novel conjugate represents a very promising tool for the development of various mucoadhesive drug delivery systems.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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