Published in final edited form as:

Eur J Pharm Biopharm. 2013 November ; 85(3 Pt B): 1266–1273. doi:10.1016/j.ejpb.2013.09.017.

Synthesis and in vitro characterization of entirely S-protected thiolated pectin for drug delivery

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Abstract

The study was aimed to synthesize a thiolated polymer (thiomer) that is resistant to oxidation in solutions above pH 5. In order to protect a pectin-cysteine conjugate against premature oxidation, the thiomer was S-protected by a disulfide connected leaving group. Therefore, 2-mercaptopyridine was first coupled to L-cysteine by a disulfide exchange reaction and the purified product was subsequently attached to pectin by a carbodiimide mediated amid bond formation. The obtained fully S-protected thiolated pectin was *in vitro* characterized with respect to co- and mucoadhesive properties and stability towards oxidation. The results indicated a 1.8-fold and 2.3-fold enhanced disintegration time at pH 6.8 of the S-protected thiolated pectin (Pec-Cys-MNA) compared to thiolated pectin (Pec-Cys) and unmodified pectin (Pec). Moreover, rheological measurements of polymer/mucus mixtures showed a 1.6-fold (compared to Pec-Cys) and 6.7-fold (compared to Pec) increased dynamic viscosity of Pec-Cys-MNA. On the other hand, in the presence of a strong oxidizing agent such as H₂O₂ (0.3% v/v), no increase in viscosity of Pec-Cys-MNA could be observed. A 6-month experiment also demonstrated the long-term stability of a liquid formulation based on Pec-Cys-MNA. Further investigations proved that the first time all thiol groups on a thiolated polymer could be protected owing to the novel synthesis. Accordingly, these features may help to develop thiomer based liquid or gel formulations targeting mucosal surfaces such as nasal, ocular or vaginal drug delivery systems.

Keywords

Thiomers; S-protected thiomers; Mucoadhesion; Oxidation; Disulfide exchange

1. Introduction

The concept of mucoadhesive excipients for drug delivery systems was invented to prolong the residence time of dosage forms at the site of action [1, 2]. The intimate contact combined with controlled drug release at delivery site may also lower drug concentrations and administration frequency for disease treatment. A promising approach to enhance the

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interactions between dosage form and mucosal membrane or mucus was the introduction of thiol bearing ligands to pharmaceutically used polymers, so-called thiomers [3]. Thiomers can form covalent disulfide bonds between cysteine-rich subdomains of mucus and the auxiliary agent, which are much stronger than van der Waal's forces, hydrogen bonds or dipole forces. However, in an aqueous environment above pH 5 thiomers are sensitive to oxidation unless they are sealed under inert conditions [4]. This deteriorating factor with respect to their pharmaceutical use limits the fields of application since they cannot be stored as aqueous solution (e.g. eye drops) and probably reduces the efficiency of the system, especially in body regions where pH is raised.

It was therefore the aim of the study to synthesize a thiomers that is stable towards oxidation and to investigate mucoadhesive properties. In order to reduce uncontrolled oxidation of thiomers, a protecting ligand was introduced. The idea is adapted from covalent chromatography, where thiol bearing resins can be preactivated by disulfide connected pyridyl substructures [5, 6]. These resins bind very efficiently peptides or proteins via disulfide exchange reactions. In analogy, preactivated thiomers may bind to high extent to cysteine-rich subdomains of mucus by disulfide exchange reactions and are protected against premature oxidation. However, a pyridyl substructure as leaving group is problematic from toxicological point of view and was therefore replaced by 2-mercaptosuccinic acid [7]. Previous studies attempted to protect thiomers by binding the leaving group to an already thiolated polymer backbone. However, the S-protection rate was only around 50% by using this method [8, 9]. In order to improve the S-protection rate, the thiol bearing ligand was bound to the leaving group before coupling to the polymer backbone and purified. Accordingly, the ligand L-cysteine was connected to 2-mercaptosuccinic acid by a disulfide exchange reaction and excess of L-cysteine was removed by ion exchange chromatography. The S-protected ligand was subsequently coupled to pectin as model polymer backbone. The final product was *in vitro* analyzed and compared with the conventional thiomers and unmodified pectin.

2. Materials and methods

2.1. Materials

2-Mercaptosuccinic acid (MNA) was purchased from abcr GmbH & Co. KG, Germany. Pectin (classic, from citrus fruits, degree of esterification: 35-40 %) was supplied by Herbstreith & Fox, Germany. Resazurin sodium salt powder was purchased from Acros Organic, Austria. Fetal bovine serum and MEM with phenol red was purchased from PAA laboratories, Austria. All other chemicals were purchased from Sigma-Aldrich, Austria.

2.2. Synthesis of Cys-MNA

The ligand 2-((2-amino-2-carboxyethyl)disulfanyl)nicotinic acid (Cys-MNA) for conjugation with pectin was synthesized in a two-step reaction. 2-Mercaptosuccinic acid (MNA) was first oxidized to the corresponding MNA-dimer (2,2'-disulfanediyldinicotinic acid) with hydrogen peroxide according to a method described previously [8, 9]. The obtained MNA-dimer was subsequently modified by a disulfide exchange reaction with L-cysteine to Cys-MNA as shown in Figure 1.

Briefly, 2 g of MNA was dispersed in 80 mL of demineralized water by ultrasonication for 10 min. Afterwards the pH was adjusted to pH 8 with NaOH (5 M) obtaining a yellow clear solution. In order to oxidize MNA, 2.5 mL of hydrogen peroxide (30% v/v) was added and stirred for 10 min until the solution became colorless. The pH was maintained during the reaction at pH 8-9 with NaOH (5 M). Following that, a solution containing 1 g of L-cysteine in 40 mL of demineralized water was adjusted to pH 8 and added drop-wise to the reaction mixture within 60 min. The pH was maintained during the reaction at pH 8.5-9.5 and a slightly yellow clear solution was obtained.

Thin layer chromatography (TLC) was used to verify that Cys-MNA was successfully generated. The stationary phase was silica gel with a fluorescent indicator coated on aluminum foils and a mixture of butanol:acetic acid:water (4:1:1) was used as mobile phase. The plates were analyzed under blacklight (UV₂₅₄) and subsequently sprayed with a ninhydrin solution (0.3 g in 100 mL ethanol) in order to visualize primary amino groups after 10 min drying at 110°C.

2.3. Purification of Cys-MNA

In order to eliminate unbound L-cysteine and L-cystine from the reaction mixture, two purification steps were performed. The pH of the slightly yellow solution was decreased to pH 7.5 to precipitate L-cystine, since it is poorly soluble in an aqueous solution in a pH range of 2-8 [10]. The precipitation was removed by filtration and the filtrate was further purified with an ion exchange column. At pH 7.5 L-cysteine is uncharged, whereas MNA, MNA-dimer and Cys-MNA are mainly negatively charged (calculated by ChemAxon, www.chemicalize.org). Therefore, the column was packed with 30 g anionic exchange resin (Dowex® 1X4 chloride form, 100-200 mesh), loaded with 250 mL of a NaCl-solution (2 M) and washed with 250 mL of demineralized water. Afterwards, the filtrate was filled onto the column and washed two times with 250 mL of demineralized water. The bound compounds were released by adding three times 250 mL of a NaCl-solution (2 M) and the collected eluent was reduced to 200 mL with a rotary evaporator. The solution was taken for synthesis of S-protected thiolated pectin conjugate, since only compounds displaying primary amino groups can couple to the preactivated pectin.

2.4. Synthesis and purification of S-protected thiolated pectin

The S-protected thiolated pectin conjugate (Pec-Cys-MNA) was synthesized by the covalent attachment of primary amino groups of Cys-MNA to the carboxylic acid group of pectin according to a modified method, as described previously by our research group [11]. Briefly, 2 g of pectin (Pec) was dissolved in 200 mL of demineralized water and pH was adjusted to 5.5. The carboxylic acid moieties were activated for conjugation by the addition of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC) in a final concentration of 150 mM and *N*-hydroxysuccinimide (NHS) in a final concentration of 40 mM. After stirring for 15 min at pH 5.5 the pH was adjusted to pH 7 and the solution obtained in above described purification process was slowly added within 30 min. The reaction mixture was stirred for 3 hours at room temperature. In order to purify the Pec-Cys-MNA conjugate, the solution was filled in a dialysis tube (MWCO 10,000-12,000 Da) and dialyzed ten times

against 5 L of demineralized water for 12 hours. Thereafter, the dialyzed product was freeze dried and stored under dry conditions until use.

2.5. Synthesis and purification of thiolated pectin

The pectin-cysteine conjugate (Pec-Cys) was synthesized according to a method described previously [11, 12]. In brief, 2 g of pectin was dissolved in 200 mL of demineralized water, the pH was adjusted to pH 4.5 and EDAC was added in a final concentration of 150 mM. After 15 min of activation, 1 g of L-cysteine dissolved in 10 mL of demineralized water was added and the solution was stirred for 3 hours at room temperature at pH 5. For purification, the reaction mixture was filled in a dialysis tube (MWCO 10,000-12,000 Da) and dialyzed ten times against 5 L of HCl (200 μ M) in the dark at 10°C to prevent oxidation of free thiol groups. The product was lyophilized by drying frozen solutions at -30°C and 0.01 mbar. The polymer was stored in the dark at 4°C under dry conditions until further use.

2.6. Characterization of thiolated and S-protected thiolated pectin

The thiolated (Pec-Cys) and S-protected thiolated pectin (Pec-Cys-MNA) were analyzed for functional groups to characterize the polymers. Moreover, the S-protected thiolated pectin was analyzed with respect to the extent of preactivation with MNA.

The free thiol group content was determined spectrophotometrically using Ellman's reagent according to a method described previously [3]. The disulfide bond test was performed to quantify disulfide bonds formed due to oxidation during the thiolation process of Pec-Cys and secondly to quantify disulfide bonds of Pec-Cys-MNA. The test was performed in a similar way as described for thiol groups after reduction with NaBH₄[3]. To verify that the isolation by dialysis was successful, the amount of primary amino groups was determined by 2,4,6-trinitrobenzenesulfonic acid (TNBS) detecting unbound L-cysteine and L-cystine [13, 14]. In order to underline the results of disulfide bond tests, the amount of conjugated MNA at Pec-Cys-MNA was analyzed with reduced glutathione [8, 9]. Therefore, 0.5 mg of the polymer was dissolved in 500 μ L of phosphate buffer pH 8 (0.5 M) and mixed with 500 μ L of reduced glutathione (2% m/v). After 90 min of incubation absorbance was measured at 354 nm. MNA was employed to establish the calibration curve. All experiments were performed in triplicate.

2.7. Cytotoxicity screening – resazurin assay

Evaluating the cytotoxic potential of Pec-Cys-MNA, Pec-Cys and unmodified pectin (Pec) the resazurin assay on Caco-2 cells was performed. In the environment of metabolic active cells the blue, non-fluorescent resazurin is reduced to red and fluorescent resofurin [15, 16]. Briefly, Caco-2 cells ($d = 1 \times 10^5$ cells/well) were cultured on 24-well plates in a final volume of 500 μ L MEM supplemented with 20% fetal bovine serum (FBS) at 37°C in 5% CO₂ environment. The medium was exchanged every other day for 2 weeks. Before incubation with test compounds, the cells were washed twice with 500 μ L of prewarmed phosphate buffer saline (PBS). Following that, 500 μ L of 0.5% (m/v) test solutions (Pec, Pec-Cys, Pec-Cys-MNA), negative control (MEM without phenol red) and positive control (4% m/v Triton X-100) were filled into wells in triplicate. The cells were incubated for 3 h and 24 h, respectively, and washed again two times with PBS. The resazurin solution (2.2 μ M in

MEM without phenol red) was added in 250 μ L quantity and incubated for another 3 hours. The supernatant was removed and analyzed at 540 nm with background subtraction at 590 nm using a fluorimeter (Tecan infinite M200).

2.8. Preparation of tablets

The lyophilized polymers (Pec, Pec-Cys, Pec-Cys-MNA) were compressed into 30 mg flat-faced tablets (5.0 mm diameter) using a single punch excentric press (Paul Weber, Remshalden-Grünbach, Germany). The compaction pressure of 12 kN was kept constant during the manufacturing process and all tablets were stored under dry conditions until further use.

2.9. Evaluation of the disintegration behavior

The disintegration behavior of polymer tablets was analyzed according to the European Pharmacopoeia (Ph. Eur.). The stability of each polymer tablet was tested in 0.1 M phosphate buffer pH 6.8 and 0.1 M HCl pH 1 at 37°C using a disintegration test apparatus with discs (Ph. Eur. Apparatus A). The oscillating frequency was 0.5 s⁻¹ and the time was taken when tablets were completely disintegrated. The experiments were performed with 3 tablets of each polymer.

2.10. Evaluation of the water-uptake capacity

The water-uptake capacity of polymer tablets in 0.1 M phosphate buffer pH 6.8 was measured gravimetrically as described previously [17]. In brief, the tablets were fixed on a needle, accurately weighed and immersed in phosphate buffer at 37°C. At several time points tablets were taken out, excess water was removed by gentle soaking with a tissue paper and weighed again. The amount of water uptake was calculated by following equation:

$$\text{Wateruptake} \left[\% \right] = \frac{(w_t - w_0)}{w_0} * 100$$

Where, W_t is the weight of swollen tablet at given time point and W_0 is the initial weight of the tablet. The results were expressed as a function of time and all tests were performed in triplicate.

2.11. In vitro evaluation of mucoadhesive properties

2.11.1. Rotating cylinder method—In order to investigate mucoadhesive properties of produced polymers, samples were analyzed by the rotating cylinder method as reported [11]. Native small intestinal porcine mucosa was fixed on stainless steel cylinders and tablets were attached to the mucosa by little force. The cylinder was used as stirrer in a dissolution tester (Erweka GmbH, Heusenstamm, Germany) according to the European Pharmacopoeia (Paddle Apparatus). The dissolution tester was preheated at 37°C and the vessels were filled with 900 mL of 0.1 M phosphate buffer pH 6.8. Afterwards, the cylinders were immersed with a rotational speed of 100 rpm and the detachment of tablets was observed visually.

2.11.2. Rheological investigations of polymer/H₂O₂ and polymer/mucus mixtures

Confirming that all thiol groups immobilized on Pec-Cys-MNA were S-protected by MNA, polymer solutions were oxidized with hydrogen peroxide and rheologically investigated. Therefore, 1000 µL of polymer solutions (1% m/v Pec, Pec-Cys and Pec-Cys-MNA in 0.1 M phosphate buffer pH 6.8) were oxidized with 20 µL of hydrogen peroxide (0.3% v/v) and incubated at 37 °C until the dynamic viscosity was measured after 30 min and 120 min. Controls without oxidant were directly placed on the rheometer.

In analogy, to evaluate the adhesion properties of Pec, Pec-Cys and Pec-Cys-MNA 500 µL of polymer solutions (1% m/v Pec, Pec-Cys and Pec-Cys-MNA in 0.1 M phosphate buffer pH 6.8) were mixed with 500 mg of native small intestinal porcine mucus and incubated for 30 min and 120 min at 37°C. Controls without mucus were mixed with 500 µL of 0.1 M phosphate buffer pH 6.8.

Afterwards, 750 µL of the polymer/H₂O₂ mixtures, polymer/mucus mixtures and control solutions were transferred to a cone-plate rheometer (Haake Mars Rheometer, 379-0200, Thermo Electron GmbH, Karlsruhe, Germany) to measure the dynamic viscosity (η^*). The oscillating measurements were performed with a shear stress in the range of 0.5-10 Pa and the temperature was maintained at 37 ± 0.1 °C.

2.12. Long-term stability of a liquid formulation

In order to evaluate the stability of a liquid formulation based on fully S-protected thiolated pectin a long-term experiment was conducted. Pec-Cys-MNA was dissolved in a concentration of 0.5% in phosphate buffer pH 6.8 (0.1 M) and benzalkonium chloride was added in a final concentration of 0.01 % (m/v) in order to improve the microbial stability. The liquid formulations were stored in Eppendorf tubes protected from light at room temperature for 1 day and 6 month, respectively. After storing time the liquid formulation was investigated with respect to their thiol, disulfide and MNA content as described for lyophilized Pec-Cys-MNA in section 2.6.. Moreover, the solution was rheologically investigated as described in section 2.11.2. to determine the viscosity after the long-term experiment.

2.13. Statistical analysis

Statistical data analysis were performed using one-way ANOVA with 95 % confident interval (p value < 0.05) as the minimal level of significance followed by Dunns multiple-comparison post test (p < 0.05). Calculations were done using the software GraphPad Prism 5 version 5.01. The results are expressed as the mean of at least 3 experiments $\pm$ SD.

3. Results and discussion

3.1. Synthesis and purification of Cys-MNA

The first step to synthesize entirely S-protected thiolated pectin was the generation and purification of the ligand Cys-MNA as shown schematically in Figure 1. The successful formation of Cys-MNA by a disulfide exchange reaction between MNA dimer and L-

cysteine was confirmed by thin layer chromatography (Figure 2). The left hand plate compared the reaction mixture (I), MNA (II), MNA dimer (III) and L-cysteine (IV) under UV-light and the right hand plate was subsequently sprayed with ninhydrin. The reaction mixture showed three spots on the left hand plate: MNA, MNA dimer and probably Cys-MNA. The ligand Cys-MNA is the only compound in the reaction mixture that can be detected under UV-light and visualized by ninhydrin since it displays a primary amino group. MNA and MNA-dimer, however, could not be detected with ninhydrin, as they have no amino groups. On the other hand, L-cysteine has an amino group, but it cannot be seen under UV-light. The ninhydrin sprayed TLC plate verified these considerations. Accordingly, the only compound that could be seen under UV-light and after ninhydrin detection was identified as Cys-MNA.

In order to prepare the reaction mixture for the covalent attachment to pectin mediated by EDAC (Figure 1), all compounds that display primary amino groups, except Cys-MNA, should be removed as they might be also coupled to pectin. Since L-cysteine can be oxidized to L-cystine by oxygen in air or by residual hydrogen peroxide, these two molecules are of interest. L-cystine was eliminated by filtration at pH 7.5 due to the very low solubility at a pH between pH 2 and 8. Moreover, at this pH L-cysteine is uncharged, whereas MNA, MNA dimer and Cys-MNA are mainly negatively charged (calculated by ChemAxon, www.chemicalize.org). Therefore, unbound L-cysteine was efficiently removed by anionic exchange chromatography. MNA and MNA dimer, however, did not interfere with the carbodiimide mediated coupling reaction to pectin and were therefore later eliminated by dialysis.

3.2. Synthesis and characterization of thiolated and S-protected thiolated pectin

The lyophilized pectin (I), thiolated pectin (II) and S-protected thiolated pectin (III) were white and of fibrous structure. The thiolated pectin (Pec-Cys) showed 223.5 ± 13.4 $\mu\text{mol/g}$ free thiol groups determined by Ellman's reagent, whereas no free thiol groups could be detected at the S-protected thiolated pectin (Pec-Cys-MNA). After reduction with sodium borohydride, however, Pec-Cys-MNA showed 407.8 ± 22.8 $\mu\text{mol/g}$ thiol groups; accordingly 50% of that was immobilized as L-cysteine on the polymer backbone and the other half belonged to the leaving group MNA. Using the assay with reduced glutathione 210.6 ± 11.1 $\mu\text{mol/g}$ MNA could be detected, which confirmed the previous results. In both products no unbound L-cysteine and L-cystine could be detected with TNBS confirming quantitative isolation. The characterization tests proved that the thiolation rate of designed polymers was comparable and that the S-protection of Pec-Cys-MNA was complete.

The cytotoxicity screening results of pectin (I), thiolated pectin (II) and S-protected thiolated pectin (III) are shown in Figure 3. The findings indicate that none of the polymers was significantly ($p < 0.05$) cytotoxic on Caco-2 cells within 24 hours.

3.3. Evaluation of the disintegration behavior

The disintegration time of pectin (I), thiolated pectin (II) and S-protected thiolated pectin (III) discs in HCl 0.1 M (pH 1) and phosphate buffer 0.1 M (pH 6.8) are shown in Figure 4. Unmodified pectin disintegrated very quickly at pH 6.8, whereas at pH 1 the disintegration

was prolonged due to the mainly protonated and therefore uncharged carboxylic acid groups of the polymer. Hence, hydrophilicity was reduced and the polymer chains do not repulse each other, resulting in a tightly packed and relatively insoluble gel [12, 18]. These variations with respect to the solubility in different body regions can be reduced by the modification of carboxylic groups as described for thiomers since they are less susceptible to changes in ionic strength and/or pH [19]. The increased stability of Pec-Cys at pH 6.8 in comparison to unmodified pectin can be explained by improved crosslinking properties of the polymer after the thiolation process. The formation of covalent bonds (disulfide bonds) within the polymer leads to an enhanced stability and cohesion that is responsible for the disintegration time. In accordance, the introduction of MNA as S-protective leaving group further prolonged the disintegration of discs in both media. This might be caused by the lipophilic nature of MNA that increases hydrophobicity of the conjugate, in combination with an increased formation of inter- and/or intramolecular disulfide bonds since crosslinking influences chain mobility and resistance to dissolution [20].

3.4. Evaluation of the water-uptake capacity

The swelling behavior and water-uptake capacity, respectively, is a good indicator to predict mucoadhesive and cohesive properties of a polymer. Furthermore, the stability of the polymer and release of an embedded drug are related to this capacity. The amount of uptaken water in relation to the initial weight of compressed polymer tablets in phosphate buffer pH 6.8 is shown in Figure 5. The swelling behavior of thiolated and S-protected thiolated pectin were greatly improved compared to unmodified pectin. As unmodified pectin has many deprotonated COO^- groups at pH 6.8 that can repel each other, the polymer is hydrophilic and the tablet can swell very rapidly. On the other hand, stability is decreased and erosion of the tablet starts rapidly, which leads to a reduced amount of absorbed water. The thiolated pectin, however, can form a stable disulfide crosslinked network allowing it to retain its structure in the presence of water. Hence, more water can be incorporated owing to the increased cohesiveness. The introduction of the lipophilic MNA as S-protecting ligand further increases the ability to bind water, resulting in a 2256.3% water-uptake capacity. The considerably higher ability to swell might be caused by the decreased solubility in an aqueous environment and the increased behavior to form stabilizing disulfide bonds as reported for preactivated covalent chromatography resins or preactivated thiomers [5, 6, 9]. Moreover, the greatly improved swelling behavior may help to enhance the gastrointestinal transit time due to the increase in volume and viscosity. This may prolong the residence time at the site of action and the carrier can act as controlled release matrix [21, 22].

3.5. In vitro evaluation of mucoadhesive properties

3.5.1. Rotating cylinder method—In order to evaluate the mucoadhesion properties of novel synthesized polymers, freshly excised porcine intestinal mucosa was fixed on a rotating cylinder, loaded with polymers tablets and immersed in physiological buffer simulating *in vivo* conditions. The time until tablets were detached from the mucosa is illustrated in Figure 6. Although pectin is already mucoadhesive due to physical entanglement and secondary interactions (hydrogen bonds) that contribute to the formation of strengthened network [20], Pec-Cys and Pec-Cys-MNA showed more favorable adhesive properties. Thiolation and S-protection enhance the adhesion time by the formation of

covalent disulfide bonds between the polymer and mucus. However, it is noteworthy that cohesiveness of polymer discs also influences the residence time at the mucosa since the discs were mainly not detached, but gradually hydrated and eroded. Therefore, improved adhesive properties of Pec-Cys and Pec-Cys-MNA were caused by the decreased solubility and increased stability.

3.5.2. Rheological investigations of polymer/H₂O₂ and polymer/mucus

mixtures—Rheological measurements of polymer solutions (1% m/v) in combination with hydrogen peroxide showed no increase in viscosity for Pec-Cys-MNA (Figure 7A). This confirmed that no free thiol groups were immobilized on the S-protected thiolated pectin. The thiolated pectin, however, showed an increase in viscosity after oxidation with hydrogen peroxide.

The increase in viscosity can be explained by the formation of inter- and/or intramolecular disulfide bonds that stabilize the polymeric network [23, 24]. On the other hand, oxidation of thiomers can also occur *in vivo* at pH > 5 that could reduce the ability to bind to the mucus and limits their use in pharmaceutical formulations. The viscosity of a 1% (m/v) pectin solution was so low, that it could not be seen at Figure 7A, but the addition of hydrogen peroxide did not affect the viscosity of this solution. Hence, it could be proven that Pec-Cys-MNA had constant characteristics without uncontrolled oxidation in solution due to the S-protective leaving group.

Figure 7B shows the results of rheological investigations of polymer solutions with native porcine mucus. The formation of hydrogen bonds with the mucus and the high initial viscosity of the native mucus likely cause the increase in viscosity of pectin. The comparatively higher increase in viscosity of thiolated pectin revealed once again of disulfide bonds between mucus and polymer. Furthermore, the viscosity of S-protected thiolated pectin overtopped the thiolated ones, indicating that S-protection by MNA improved the mucoadhesion characteristics of the polymer.

The degree of hydration is an important parameter influencing the mucoadhesive strength of auxiliary agents. The rotating cylinder method represents a method of relatively low hydrated conditions since the dry polymer come in contact with the wet mucosal surface. In that case, the adhesion might be a result of a combination of capillary attraction and osmotic forces that dehydrate and strengthen the mucus layer [20]. This process must be distinguished from swollen polymers that are attached to mucus such as in rheological investigations of polymer/mucus mixtures. Although hydration is important for mucoadhesion, excess of hydration can lead to the formation of a slippery mucilage. The highly crosslinked Pec-Cys-MNA may be also advantageous for a prolonged mucoadhesive effect since it only permit a certain degree of hydration. Moreover, the relatively hydrophobic nature of the conjugate may support this effect. The *in vitro* characterization of Pec-Cys-MNA showed that the introduction of a S-protected thiol bearing ligand to pectin further improved the mucoadhesion properties in “dry-on-wet” and “wet-on-wet” tests compared to the thiolated and unmodified pectin. Furthermore, the stability of the thiomers towards oxidation in an aqueous environment above pH 5 likely enable the application as eye drops or vaginal gel that are not designed as instant formulation. This allows the usage

in numerous pharmaceutical formulations, such as nasal, vaginal or ocular drug delivery systems [20].

3.6. Long-term stability of a liquid formulation

A long-term experiment was carried out to demonstrate the suitability of Pec-Cys-MNA in a liquid formulation. Table 1 shows the results of rheological and chemical characterization of the solution after 1 day and 6 months. It could be seen that the viscosity of the formulation after 6 months was not significantly different from the 1-day sample. It thus appears that Pec-Cys-MNA did not form inter- and/or intramolecular disulfide bond, which would lead to a viscosity-increasing network. Chemical characterization indicated a small decrease of disulfide bond and MNA content, but both were not significant. Moreover, after 6 months still no unbound thiol groups could be detected by Ellman's reagent. Consequently, the formulation was stable over the evaluated time period.

4. Conclusion

The present work describes a novel synthesis to produce for the first time a fully S-protected thiomers. Interestingly, the S-protection not only improved the stability of the thiomers in solution at high pH, but also enhanced adhesive and cohesive properties of the thiomers. Moreover, the S-protecting ligand did not affect the cytotoxicity on Caco-2 cells. According to these results, the S-protection seems to be a promising modification tool to further improve the favorable characteristics of thiomers with respect to their use as excipient in mucoadhesive drug delivery systems and enables the development of liquid thiomers based formulations.

Acknowledgements

This work was supported by the FWF (Fonds zur Förderung der wissenschaftlichen Forschung) project no. ZFP 235150.

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Fig. 1. Schematic synthesis of S-protected thiolated pectin (Pec-Cys-MNA). First, 2-mercaptonicotinic acid was oxidized with hydrogen peroxide to the corresponding MNA-dimer (1). Subsequently, L-cysteine was added to form the Cys-MNA ligand via disulfide exchange reaction (2). The purified product was coupled to pectin by a carbodiimide mediated amid bond formation (3).

Fig. 2.

Thin layer chromatography (TLC) on silica gel to separate the reaction mixture (1) after addition of L-cysteine and to compare it with MNA (2), MNA-dimer (3) and L-cysteine (4). The mobile phase was butanol:water:acetic acid (4:1:1). The left hand plate was analyzed under UV₂₅₄-light, whereas the right hand plate was subsequently sprayed with ninhydrin.

Fig. 3.

Histogram shows the cytotoxic potential of S-protected thiolated pectin (Pec-Cys-MNA) compared to thiolated pectin (Pec-Cys) and unmodified pectin (Pec) after 3 hours (first bar) and 24 hours (second bar). Cytotoxicity assays were performed on Caco-2 cells using resazurin. As negative control MEM without phenol red was used and the positive control was Triton™ X-100 (4% m/v). Indicated values represent an average of at least three experiments ($\pm$ SD).

Fig. 4. Disintegration time of 30 mg tablets of Pec (□), Pec-Cys (■) and Pec-Cys-MNA (■) in 0.1 M phosphate buffer pH 6.8 and 0.1 M HCl pH 1. Indicates values are means $\pm$ SD of at least three experiments.

Fig. 5. Water-uptake capacity of Pec (O), Pec-Cys (▲) and Pec-Cys-MNA (■) in 0.1 M phosphate buffer pH 6.8 at 37°C. Indicates values are means $\pm$ SD of at least three experiments.

Fig. 6. Adhesion time of 30 mg tablets of Pec (□), Pec-Cys (■) and Pec-Cys-MNA (■) on intestinal porcine mucosa using rotating cylinder method. The cylinders were immersed in 0.1 M phosphate buffer pH 6.8 and rotated with 100 rpm. Indicates values are means $\pm$ SD of at least three experiments.

Fig. 7.

A+B: Comparison of the dynamic viscosity of polymer solutions (Pec (□), Pec-Cys (■) and Pec-Cys-MNA (■) which were oxidized with H₂O₂. 1 mL of polymer solutions (1% m/v in 0.1 M phosphate buffer pH 6.8) were oxidized with 20 μ L H₂O₂ (0.3% v/v). The polymer solutions were also mixed with native porcine mucus (A) in a weight ratio of 1:1 and rheologically investigated (B). Indicates values are means $\pm$ SD of at least three experiments.

Table 1

Rheological and chemical characterization of a liquid formulation (0.5% m/v) based on Pec-Cys-MNA in a long-term experiment. Indicates values are means $\pm$ SD of at least five experiments.

Parameter/Test	1 day	6 month
Viscosity (mPa s)	19.3 $\pm$ 4.7	19.7 $\pm$ 2.0
Disulfide bonds (μ mol/g)	391.8 $\pm$ 22.8	348.5 $\pm$ 7.7
MNA (μ mol/g)	204.6 $\pm$ 13.2	195.8 $\pm$ 7.5