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Synthesis and *in vitro* evaluation of thiolated hyaluronic acid for mucoadhesive drug delivery

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Abstract

It was the aim of this study to synthesize and characterize a novel hyaluronic acid-cysteine ethyl ester (HA-Cys) conjugate providing improved mucoadhesive properties and a significantly lowered biodegradation rate. Mediated by carbodiimide and *N*-hydroxysuccinimide, L-cysteine ethyl ester hydrochloride was covalently attached to hyaluronic acid (HA, hyaluronan) via the formation of an amide bond. The adhesive properties of HA-Cys conjugates were evaluated *in vitro* on a freshly excised porcine mucosa via the rotating cylinder method. The cohesive properties of the resulting conjugates were evaluated by oxidation experiments. Biodegradability studies were carried out by viscosity measurements and spectrophotometric assays. Release studies were performed with fluorescein isothiocyanate-dextrans (FD) as model compounds. The obtained conjugate displayed 201.3 ± 18.7 μmol immobilized free thiol groups and 85.7 ± 22.3 μmol disulfide bonds per gram polymer. Results from the rotating cylinder method showed more than 6.5-fold increase in the adhesion time of HA-Cys versus unmodified HA. In aqueous solutions, the obtained conjugate demonstrated improved cohesive properties. The hydrolysis degree of HA-Cys was lower compared with the corresponding unmodified HA in the framework of viscosity experiments. In addition, the cross-linking process via disulfide bonds additionally reduced the rate of degradation of the new derivative. Cumulative release studies out of matrix tablets comprising HA-Cys and the model compound FD demonstrated a sustained drug release for more than 12 h due to *in situ* formation of inter- and intramolecular disulfide bonds in the thiomer matrix. According to the results of the present study, this novel thiolated polymer seems to represent a promising multifunctional excipient for the development of various drug delivery systems.

Keywords: Hyaluronic acid-cysteine ethyl ester; L-Cysteine ethyl ester hydrochloride; Hyaluronic acid; Mucoadhesion; Enzymatic degradation

1. Introduction

Hyaluronic acid (HA) is a naturally occurring macromolecular polysaccharide found in synovial fluid, extracellular matrices, connective tissues and organs of all higher animals (Fraser et al., 1997). The structure of HA consists of repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine linked by β-1–3 and β-1–4 glycosides bonds (Laurent and Fraser, 1992). Purified native HA has many biomedical applications, including viscosurgery, viscosupplementation and wound healing (Balazs and Denlinger, 1989). More recently, HA has been investigated as a drug delivery agent for various routes of administration including ophthalmic, nasal, pulmonary, orally, parenteral and topical. DNA-HA matrix formulations and microspheres intended for use as gene delivery systems have been reported (Kim et al., 2003; Yun et al., 2004). However, pure HA does not remain in the joint or in tissues for prolonged periods because of its rapid degradation. HA is enzymatically degraded by hyaluronidase and is completely resorbable through multiple metabolic pathways (Fraser et al., 1997). Testes-type hyaluronidases are endo-β-*N*-acetylhexosaminidases that catalyse the random hydrolysis of the 1–4 bonds in HA, primarily to tetrasaccharide residues (Kreil, 1995). Therefore, chemically modified or cross-linked

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HA has been widely developed to prolong its degradation time and improve its mechanical stability *in vivo*. Chemical modifications of HA involve two most commonly used sites of its structure: carboxylic acid and hydroxyl groups. The carboxylic acid groups have been modified by esterification (Benedetti et al., 1993) and carbodiimide-mediated dihydrazide reactions (Luo et al., 2000). A variety of cross-linking strategies have been used to prepare insoluble or less soluble in water forms of HA. Thus, it has been cross-linked with different agents such as aldehydes, epoxides, divinylsulfone and disulfide (Shu et al., 2002). Carbodiimide-mediated coupling of HA to primary amines has been proven in a number of studies not to lead efficiently to the formation of amide bonds since the reactive intermediate rearranges rapidly to a stable *N*-acylurea adduct (Kuo et al., 1991). In this study we used *N*-hydroxysuccinimide, which provides non-rearrangable active ester of HA for conjugation (Luo and Prestwich, 2001). Herein, we describe the first chemical modification of HA with L-cysteine ethyl ester hydrochloride by means of the double catalytic system—carbodiimide/*N*-hydroxysuccinimide. The effect of the disulfide-cross-linking of the thiomer on its biodegradability was also evaluated.

Thiolated polymers or so-called thiomers have recently gained considerable attention as platforms for controlled drug delivery. Thiomers might be useful to overcome the oral bioavailability problems associated with various categories of therapeutic agents such as peptides, antisense oligonucleotides, heparins or cephalosporines. Mucoadhesive matrix tablets, patches or microparticles are useful for, intraoral, peroral and ocular, local or systemic delivery. Another purpose of that thiol modification was to combine the mucoadhesive features of the hyaluronic acid with the new thiomer technology for the improvement of mucoadhesion (Bernkop-Schnürch et al., 2004). Furthermore, the polymer's potential as a matrix tablet dosage form for oral macromolecule delivery was investigated.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salt from *Streptococcus equi* (medium molecular mass: 1.3 MDa) was donated by Croma Pharma GmbH. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), fluorescein isothiocyanate-dextrans (FD) with different molecular weights (FD-20, average MW 20,000; FD-40, average MW 40,000 and FD-70S, average MW 70,000), hyaluronidase from sheep testes (EC 3.2.1.35, 485 U/mg), albumin from bovine serum (Fraction V, min. 98%) and 5,5′-dithiobis(2-nitrobenzoic acid) were all purchased from Sigma (St. Louis, MO, USA). *N*-Hydroxysuccinimide (NHS) and L-cysteine ethyl ester hydrochloride were obtained from Acros (Geel, Belgium). All chemicals were of analytical grade.

2.2. Synthesis of hyaluronic acid-cysteine conjugate (HA-Cys)

The covalent attachment of L-cysteine ethyl ester hydrochloride to sodium hyaluronate was achieved via the formation of amide bonds between primary amino group of cysteine and carboxylic acid group of hyaluronate.

First, 0.200 g of sodium hyaluronate was hydrated in 50 mL of demineralized water to obtain a 0.4% (w/v) polymer solution. The pH of the reaction mixture was adjusted to 5.5 by the addition of 0.1 M HCl. Afterwards, EDAC and NHS were added in final concentrations of 50 mM. The pH was readjusted to 5.5 and the reaction mixture was stirred for 15 min at a room temperature. Then, 0.250 g of L-cysteine ethyl ester hydrochloride was added and the pH was readjusted to 6.0. The reaction mixture was incubated for 4 h at a room temperature under stirring. The resulting conjugate was dialyzed in tubings (molecular weight cutoff 12 kDa) three-times against 1% NaCl and finally against demineralized water. Controls were prepared and isolated in the same way as the polymer conjugate but omitting EDAC and NHS during coupling reaction. Finally, the frozen aqueous polymer solutions (samples and controls) were lyophilized at −50 °C and 0.01 mbar (Lyolab B; Inula, Austria) and stored at 4 °C until further use.

2.3. Cross-linking of hyaluronic acid-cysteine conjugate

Disulfide-cross-linked conjugates were prepared by dissolving HA-Cys in 0.1 M sodium phosphate buffer pH 6.0 with 1% NaCl, in a final concentration of 0.5% (w/v). Afterwards, the samples were oxidized in open air at a room temperature for 1 day.

2.4. Determination of thiol groups and disulfide bonds content

The amount of thiol groups immobilized on HA-Cys conjugate was determined spectrophotometrically using Ellman's reagent quantifying free thiol groups as described previously (Bernkop-Schnürch et al., 1999). The quantity of free thiol groups was calculated from an according standard curve obtained by solutions with increasing concentrations (0.06–1.1 mg/mL) of L-cysteine hydrochloride.

Disulfide content was determined after reduction with $NaBH_4$ and addition of Ellman's reagent as described by Habeeb (1973).

2.5. Differential scanning calorimetry (DSC)

DSC-thermograms were recorded with a DSC 7 system (Perkin-Elmer, Norwalk, CT, USA) using the Pyris 2.0 software. Samples of approximately 2–3 mg (weights controlled to ±0.0005 mg using UM3 ultramicrobalance, Mettler, Greifensee, CH) were weighed into aluminium pans (25 μL) with perforated covers. Dry nitrogen was used as a purge gas (purge: 20 mL min^{-1}). The instrument was calibrated for temperature with pure benzophenone (mp 48.0 °C) and caffeine (mp 236.2 °C) and the energy calibration was performed with pure indium (purity 99.999%, mp 156.6 °C, heat of fusion 28.45 J g^{-1}). Heating rates of 10 °C min^{-1} were used.

2.6. Decrease in the thiol group content within the polymer conjugate

HA-Cys conjugates were dissolved in 100 mM phosphate buffer pH 6.0 and 7.0 with 1% NaCl, in a final concentration of 0.5% (w/v). All samples were incubated at 37 °C under continuous shaking. At predetermined time points, aliquots of 200 μL were withdrawn and 50 μL of 1 M HCl was added in order to stop any further reactions. The amount of remaining thiol groups was determined via Ellman's reagent.

2.7. Tablets manufacture

Lyophilized HA-Cys conjugate and controls were compressed into 30 mg, 5.0 mm diameter flat-faced tablets (single punch eccentric press-Korsch EK, Germany). The compaction pressure (force of 11 kN) was kept constant during the preparation of all 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 calculated using the following equation (Felt and Newton, 1968):

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

where P is the crushing strength (N), d 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 then calculated.

2.8. Evaluation of the swelling behavior

The water-absorbing capacity was determined by a gravimetric method. Test tablets were fixed to a needle and immersed in a beaker containing 0.1 M phosphate buffer pH 6.8 with 1% NaCl, at 37 °C. At scheduled time intervals, the swollen tablets were taken out of the incubation medium, excess water was removed, and the amount of water uptake was determined gravimetrically (Kast and Bernkop-Schnürch, 2001). The swelling ratio was then calculated according to the following equation:

$$\text{Swelling ratio} = \frac{W_{ut}}{W_0}$$

where W_{ut} is the weight of uptaken water at time t and W_0 is the initial weight of the dry tablet.

2.9. In vitro mucoadhesive studies with the rotating cylinder method

Thirty milligrams of HA-Cys and control tablets were attached to a freshly excised intestinal porcine mucosa, which was fixed on a stainless steel cylinder (diameter: 4.4 cm; height 5.1 cm; apparatus 4-cylinder, USP). Thereafter, the cylinder was placed in the dissolution apparatus according to the USP, entirely immersed with 500 mL of 100 mM phosphate buffer pH 6.8 with 1% NaCl, at 37 °C and agitated with 125 rpm. The detachment of the test tablets was determined visually during an observation time of 48 h (Bernkop-Schnürch et al., 2003).

2.10. In vitro enzymatic degradation

2.10.1. Viscosity measurement

First, 0.5% (w/v) polymer solution (9.0 mL) was prepared in 100 mM phosphate buffer pH 6.0, at 25 °C. Then, 0.5 mg/mL (150 U/mL) hyaluronidase solution (1.0 mL) was added. At predetermined time points, the viscosity of 1.0 mL aliquots of the mixtures was measured at 25 ± 0.5 °C with a Rheolab MC 1 (Paar Physica GmbH, Stuttgart, Germany) cone-plate rheometer. Indicated viscosity was determined at a shear rate of 10 s^{-1}. The change of viscosity at time t was normalized by the viscosity at time zero (values before the addition of enzyme solution). The percent viscosity loss was calculated from the following equation:

$$\text{Percent } \eta_1 = \frac{\eta_o - \eta_t}{\eta_o} \times 100$$

where percent η_1 is the percent viscosity lost after time t, η_t the final viscosity at time t and η_o is the initial viscosity of solution.

2.10.2. Turbidimetric method

The turbidimetric measurement of hyaluronic acid was carried out according to the method described by Tolksdorf (1954). The turbidimetric assay is based on the ability of the remaining HA to form turbidity with an acid albumin solution after incubation with hyaluronidase.

The test polymer substrate (4.0 mg) was dissolved in 10 mL HSE buffer (0.1 M sodium phosphate buffer pH 5.3 with 0.15 M sodium chloride) and incubated at 37 °C for 4–5 min to achieve temperature equilibrium. Then, 10 mL enzyme solution (0.05 mg/mL in HSE buffer) was added and incubated for 60 min. At predetermined time points, 1.0 mL aliquots of the mixture were taken and cooled in an ice bath to a room temperature. Afterwards, 9.0 mL of albumin reagent (0.25%, w/v bovine serum albumin in sodium acetate buffer, pH 3.0) was added to the aliquot and incubated at a room temperature for 10 min. Finally, the absorbance was measured at 540 nm (spectrophotometer UV-1202, Shimadzu, Japan). The amount of substrate, remaining after digestion, was determined from the standard curves of HA and the thiomer in the concentration range of 0.04–0.32 mg/mL. The amount of hydrolyzed polymer was calculated by subtracting the digested polymer from the initial polymer quantity used in the enzymatic reaction. The degradation rates were calculated from the initial linear slope of the hydrolyzed polymer mass versus time plots (time up to 20 min).

2.11. Release studies

Release studies were performed with fluorescein isothiocyanate-dextrans with different molecular weights: FD-20 (average MW 20,000), FD-40 (average MW 40,000) and FD-70S (average MW 70,000).

First, 75 mg of HA-Cys conjugate or HA was dissolved in 15 ml of demineralized water and homogenized with 15 mg of FD, dissolved in 3 ml of demineralized water. Then the samples were lyophilized at −30 °C and 0.01 mbar. FD 5 mg tablets were

compressed out of the lyophilized polymer/FD mixture (30 mg mass, 5 mm diameter).

The release rate of these model compounds from tablets comprised either HA-Cys conjugate or unmodified HA was analyzed *in vitro*. Tablets were placed in a beaker containing 10 mL of release medium (100 mM phosphate buffer pH 6.8 containing 1% NaCl, at 37 ± 0.5 °C). Beakers were closed and then placed on an oscillating water bath (GFL 1092; 100 rev/min) and incubated at 37 ± 0.5 °C. Aliquots of 200 μL were withdrawn at 1 h intervals within 24 h and replaced with an equal volume of release medium equilibrated at 37 °C. Sink conditions were maintained throughout this study. The release rate of different molecular weight fluorescein isothiocyanate-dextrans was assayed by measuring the fluorescence intensity within each sample with a microtitration plate reader (excitation wavelength (λ_{exc}): 485 nm; emission wavelength (λ_{em}): 535 nm; Fluostar Galaxy, Offenburg, Germany). The amount of released model compounds was calculated by interpolation from standard curves containing increasing concentrations of fluorescein isothiocyanate-dextrans (15–500 μg/mL).

2.12. Statistical data analysis

Statistical data analysis was performed using the Student's *t*-test with $p < 0.05$ as the minimal level of significance. Statistical comparisons (*p*-values) were made using Student's unpaired *t*-test, two-sided. Calculations were done using the software Sigma Plot version 9.0.

3. Results

3.1. Synthesis and basic characterization of HA-Cys conjugate

The primary role in the mechanism of mucoadhesion of thiomers plays the activity of the thiol groups of the conjugate. Their activity is determined from the chemical structure and the corresponding pK_a, which is favorable for the formation of sufficient concentration of thiolate anions in the physiological medium. The selection of L-cysteine ethyl ester ligand was based upon its thiol pK_a value (6.7), which is lower than the pK_a value of the used so far ligand L-cysteine (8.3). The studies demonstrated a direct correlation between the thiol groups content and the mucoadhesive properties. Therefore, the aim of the conjugate synthesis was to determine the reaction conditions which could lead to a maximum degree of thiol modification.

A novel HA-Cys conjugate was synthesized by the amide bond formation between carboxylic acid groups of hyaluronic acid and amine groups of cysteine ethyl ester. When EDAC is solely used in the coupling reaction, the fast hydrolysis of O-urea derivative reduced the yield of thiol moieties, sample HA-Cys 1 and 2 (Table 1). Recent results indicated that the addition of NHS to EDAC-mediated coupling reaction significantly improved the coupling yields. NHS esters hydrolyze very slowly in comparison to their rates of reaction with primary amino groups. The content of immobilized thiol groups is strongly dependant on the EDAC/NHS concentration and the molar ratio used, sample HA-Cys 3. The efficacy of the purification method utilized here could be verified by controls which were prepared in exactly the same way as the polymer conjugate but omitting EDAC/NHS during coupling reaction, resulting in a negligible amount of thiol groups. The sample HA-Cys 3 exhibited a maximum of 201.3 ± 18.7 μmol immobilized free thiol groups and 85.7 ± 22.3 μmol disulfide bonds per gram polymer. The obtained conjugate appeared as a white, odorless powder of fibrous structure. Oxidation under mild conditions, as described above, led to the formation of disulfide-cross-linked conjugates (Fig. 1). During that process, at about 50–60% of available thiol groups were transformed to disulfide bonds, sample HA-Cys 3 (cross-linked). Further on, we chose samples HA-Cys 3 in order to characterize the HA-Cys conjugates *in vitro*.

3.2. Thermal analysis

DSC curves are illustrated in Fig. 2, showing the heating rate influence on the thermal behavior of HA and HA-Cys 3 conjugate, respectively. Under nitrogen atmosphere, HA decomposition showed two peaks. The first endothermic peak suggests that a dehydration process occurred at around 106 °C. Afterwards, an exothermic peak at 239 °C was observed. Such process was attributed to the decomposition of the polymer, resulting in a carbonized residue. The same behavior was observed with HA-Cys conjugate. The endothermic process took place at 108 °C, followed by an exothermic peak at 228 °C, which corresponded to the conjugate decomposition.

The comparison given in Fig. 2 showed that the thermal behavior of HA-Cys conjugate differed mainly in the intensity of the released heat during decomposition and also in the intensity of the peak maxima. As a result, the modification of HA by L-cysteine ethyl ester caused a shift of the exothermic peak to lower decomposition temperatures in comparison with HA (difference of 11 °C).

Table 1
Amount of thiol/disulfide groups immobilized on HA-Cys conjugate

No of the conjugate	Sodium hyaluronate (g/50 mL)	L-Cysteine ethyl ester hydrochloride (g)	EDAC (mM)	NHS (mM)	Thiol/disulfide groups (μmol/g polymer ± SD; $n = 3$)
HA-Cys 1	0.200	0.250	50	–	17.8 ± 4.8/14.1 ± 9.7
HA-Cys 2	0.200	0.250	200	–	26.5 ± 6.8/17.9 ± 8.3
HA-Cys 3	0.200	0.250	50	50	201.3 ± 18.7/85.7 ± 22.3
HA-Cys 3 (cross-linked)	0.200	0.250	50	50	102.6 ± 18.6/178 ± 28.4
Control	0.200	0.250	–	–	8.2 ± 0.8/5.7 ± 1.3

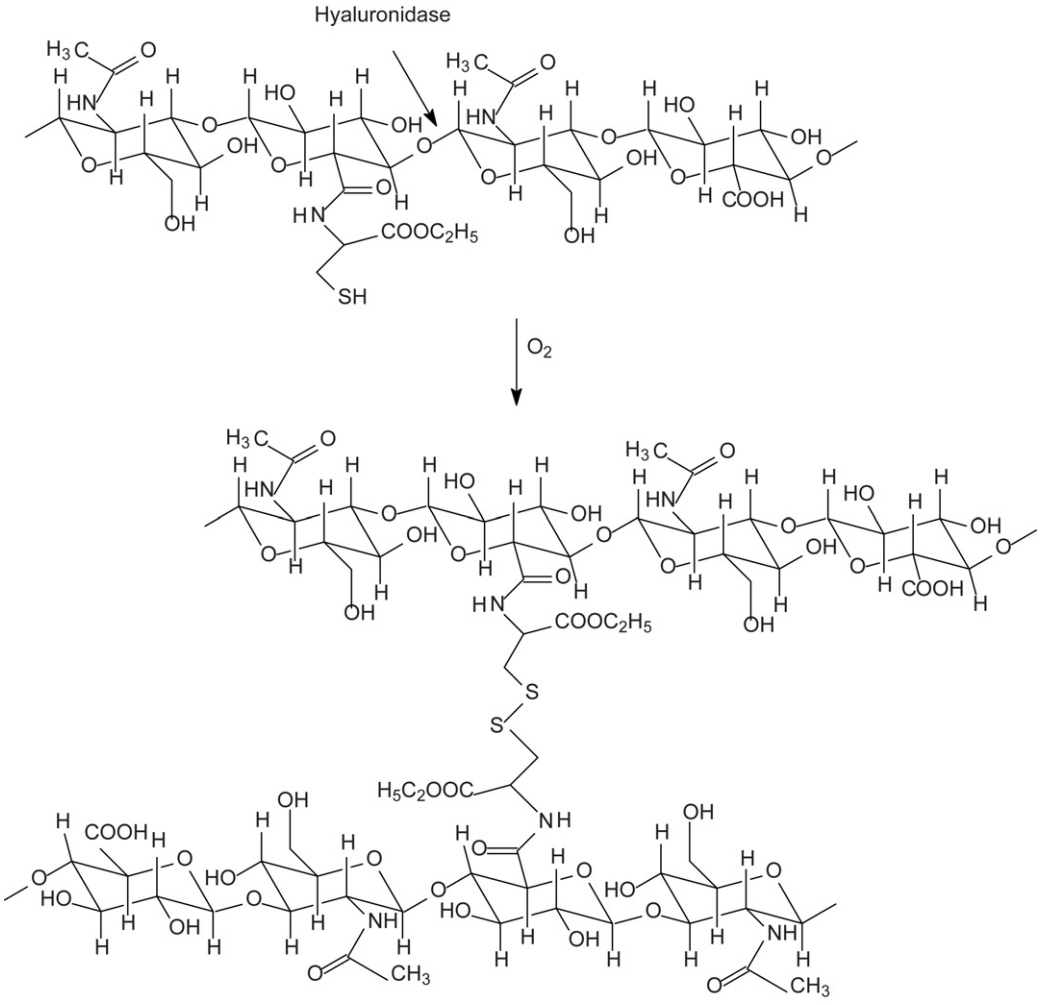

Fig. 1. Presumptive chemical substructure of HA-Cys conjugates.

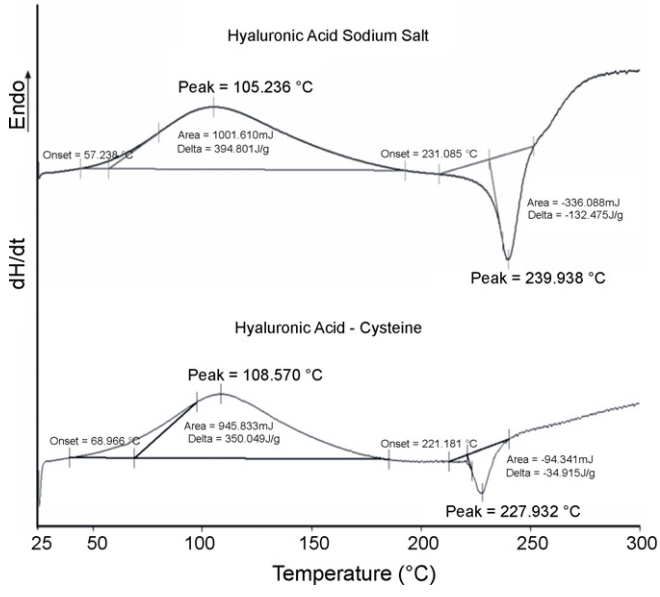

Fig. 2. DSC curves of hyaluronic acid sodium salt and hyaluronic acid-cysteine (HA-Cys 3) conjugate (heating rate: 10 °C min^{-1}).

3.3. Oxidation of thiol groups

To characterize the cross-linking process, the decrease of thiol groups was quantified as a function of time. The oxidation experiments were carried out under physiological pH 6.0 and 7.0, which is prevalent on the surface of mucosal membranes. Results of this study are presented in Fig. 3. A rapid formation of disulfide bonds occurred within the first 8 h of incubation, which was followed by a comparatively lower oxidation process within the next hours. At pH 7.0, the cross-linking process took place even more rapidly. At the end of the process, the viscosity of the thiomer solution was increased, but at least 35–40% of thiol groups remained available to react.

3.4. Swelling behavior

The water uptake of HA-Cys 3 conjugate and unmodified HA is shown in Fig. 4. Tablets comprising the respective polymers started to swell immediately after being immersed in the aforementioned buffer and continued swelling at approximately constant rate, thus, reaching a maximum weight and swelling ratio of 16.4 after 120 min. In this part of the curves,

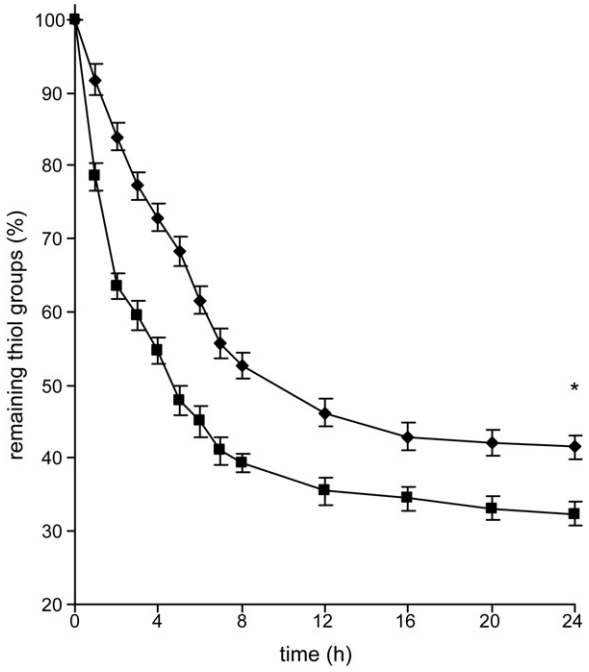

Fig. 3. Decrease of the thiol groups content within aqueous 0.5% (w/v) HA-Cys 3 conjugate solutions at pH 6 (♦) and pH 7 (■), respectively, at 37 °C. Indicated values are means (±S.D.) of at least three experiments; *Differs from conjugate at pH 7 $p < 0.002$.

the covalent attachment of cysteine ethyl ester has no significant influence on the swelling behavior of HA. At the end of the experiment, the swollen thiomer tablet retained its integrity and swelling ratio even after 3 h. In contrast, a gradual decrease

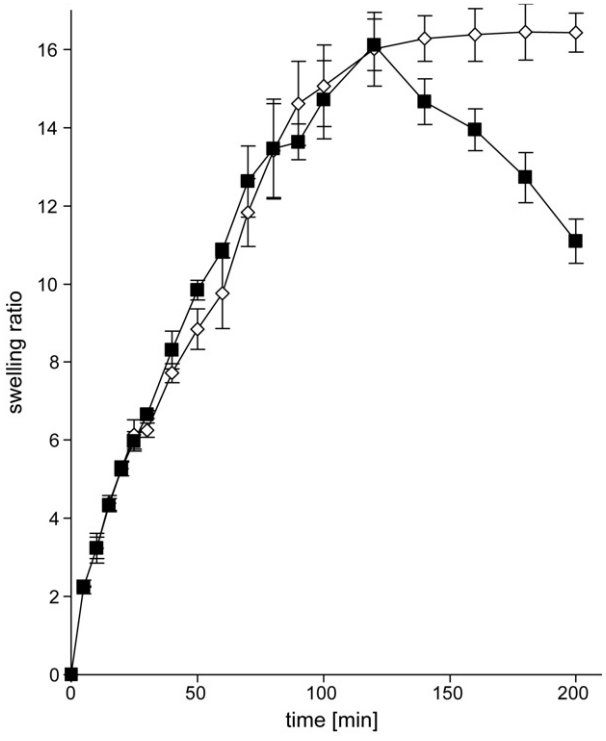

Fig. 4. Swelling behavior of tablets comprising HA-Cys 3 (◊) and unmodified sodium hyaluronate (■) in 0.1 M phosphate buffer pH 6.8 with 1% NaCl at 37 °C; indicated values are means (±S.D.) of at least three experiments.

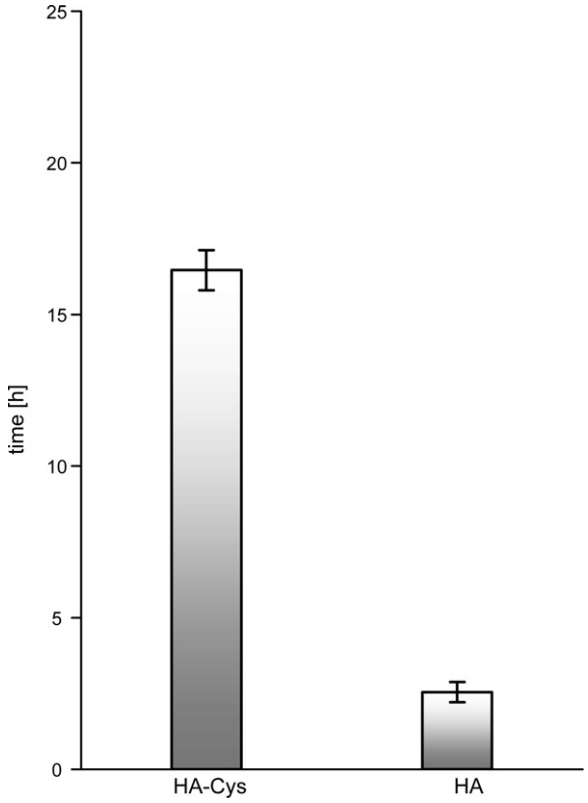

Fig. 5. Comparison of the adhesion time of HA-Cys 3 conjugate and unmodified HA on the rotating cylinder. Studies were performed in 0.1 M phosphate buffer pH 6.8 with 1% NaCl at 37 °C. The indicated time of adhesion represents the mean (±S.D.) of at least three experiments.

in the tablet weight of unmodified HA was observed followed by a complete dissolution–erosion after 3 h. The thiomer and HA tablets showed a tensile strength of 0.441 ± 0.018 and 0.473 ± 0.019 MPa, respectively.

3.5. Mucoadhesion studies

The mucoadhesion studies with HA and HA-Cys 3 conjugates were carried out with the rotating cylinder method (Fig. 5). This method is supposed to correlate better with the *in vivo* situation, as it concurrently imitates the adhesion and cohesiveness of the polymer in physiological medium (Kafedjiiski et al., 2005a). The adhesion time or the duration of adhesion of HA-Cys was around 17 h, which means that more than 6.5-fold increase in the adhesion time was obtained compared with unmodified HA. The improvement ratio was calculated by the adhesion time of thiolated HA versus adhesion time of HA (Kast and Bernkop-Schnürch, 2001).

3.6. In vitro enzymatic degradation

One of the most sensitive methods to study the kinetics of the enzymatic degradation of hyaluronan is viscosimetry (Vercruysse et al., 1995). By combining viscosimetry measurements and gel-permeation chromatography, Vercruysse et al. have demonstrated that HA is degraded at random way in the initial stage of the reaction (Vercruysse et al., 1994). The effect of

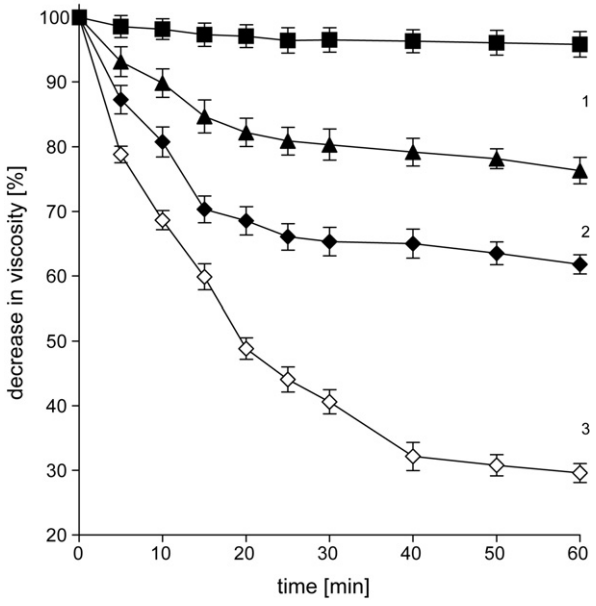

Fig. 6. Comparison of the decrease in viscosity of sodium hyaluronate (HA) and HA-Cys 3 conjugates by hyaluronidase (0.5 mg/mL) at pH 6.0: HA without hyaluronidase (■), HA-Cys 3 (cross-linked) (▲), HA-Cys 3 (♦) and HA (◊). Indicated values are means (±S.D.) of at least three experiments: 1 and 2, differ from 3, $p < 0.00001$; 1, differs from 2, $p < 0.0006$.

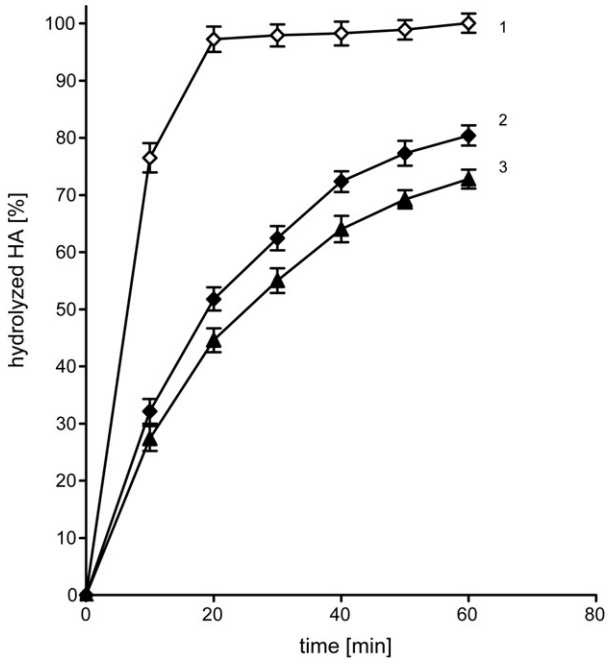

Fig. 7. Comparison of the enzymatic hydrolysis of sodium hyaluronate (HA) and HA-Cys 3 conjugates by turbidimetric measurement in 0.1 M sodium phosphate buffer pH 5.3 with 0.15 M sodium chloride: HA (◊), HA-Cys 3 (♦) and HA-Cys 3 (cross-linked) (▲). Indicated values are means (± S.D.) of at least three experiments: 3 and 2, differ from 1, $p < 0.0001$; 3, differs from 2, $p < 0.005$.

hyaluronidase on the viscosity of HA and HA-Cys 3 conjugate is shown in Fig. 6. Regarding to HA, the viscosity of the reaction mixture decreased rapidly as the reaction proceeded: decrease in viscosity to almost 50% after 20 min reaction time and to less than 30% at 60 min was observed. Concerning thiomers, the initial decrease of viscosity occurred much more slowly compared with unmodified HA, 68% decrease of HA-Cys and 82% of cross-linked HA-Cys at 20 min was found. Moreover, at the end of the experiment, the loss of viscosity of the conjugate and cross-linked thiomer was 38 and 24%, respectively, which was significantly different from the observed with HA, 70%. These findings were confirmed when the enzyme reaction was monitored by the turbidimetric measurement of the polymers concentrations (Fig. 7). Under the test conditions, complete hydrolysis of HA occurred after 20 min. In contrast, HA-Cys 3 and cross-linked HA-Cys 3 underwent 80 and 72% hydrolysis after 60 min. The degradation rate of HA (4.86%/min, $R^2 = 0.901$) was significantly faster in comparison with HA-Cys (2.59%/min, $R^2 = 0.981$) as well as the degradation rate of HA-Cys was more rapid compared with cross-linked HA-Cys (2.23%/min, $R^2 = 0.983$).

3.7. Drug release studies

To assess the delivery of various hydrophilic macromolecular drugs, a homologous series of different molecular weight water-soluble fluorescein isothiocyanate-dextrans were used as model compounds. As shown in Fig. 8, there is little difference between the release patterns of the various unmodified hyaluronate tablets. After 2.5–3 h, at about 50% of FD of all formulations was released. In all cases, a sustained release within 6–7 h was reached. In contrast, formulations comprised HA-Cys 3 conjugate showed a slower release rate with a complete release within 12–24 h, depending on the molecular weight of FD used (Fig. 9). For instance, FD20, displaying the lowest molecular weight, provided the fastest release profile and the

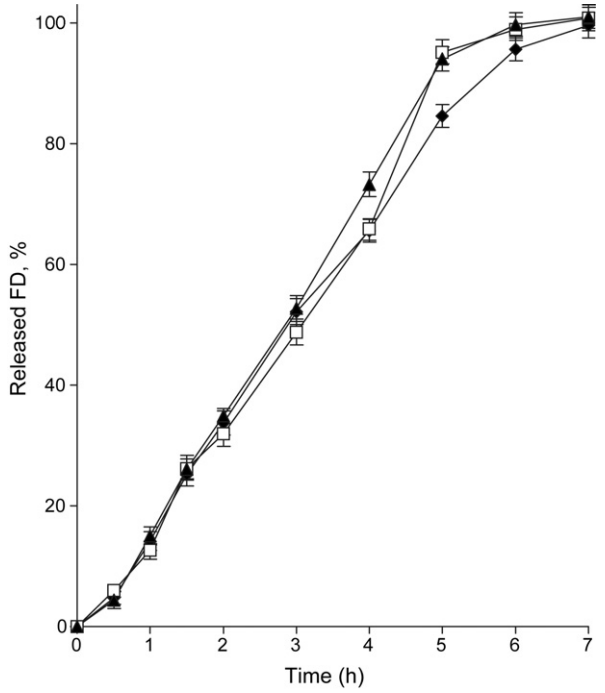

Fig. 8. Cumulative percentage of fluorescein isothiocyanate-dextran (FD) released from different formulations prepared with sodium hyaluronate: FD20 (♦), FD40 (▲), FD70S (□). Indicated values are means (±S.D.) of at least three experiments.

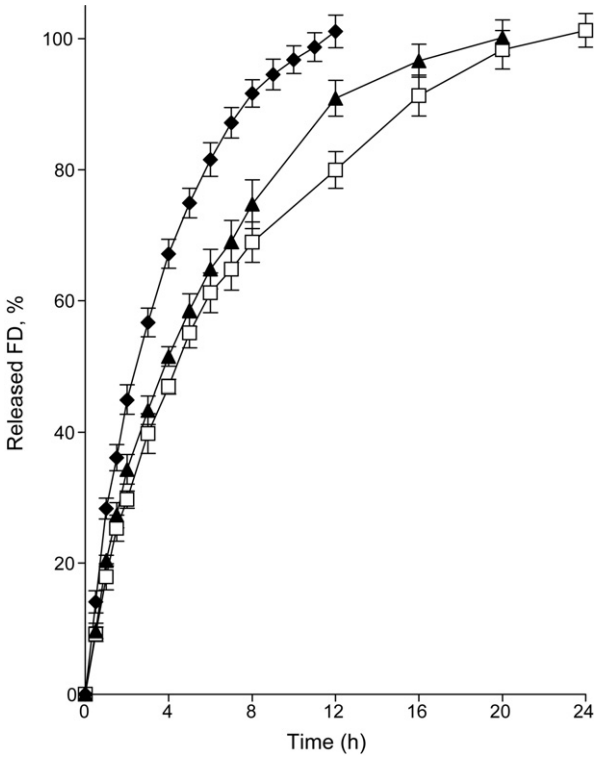

Fig. 9. Cumulative percentage of fluorescein isothiocyanate-dextran (FD) released from different formulations prepared with HA-Cys 3: FD20 (♦), FD40 (▲), FD70S (□). Indicated values are means (± S.D.) of at least three experiments.

shortest controlled release period. Only in the initial part of the release patterns of both polymers, the same rapid burst effect up to 3 h was observed. Consequently, at the beginning of the process the release may be controlled by the swelling behavior of the polymers, which was the same as already assessed in Section 3.4.

To study and assess the drug release mechanism, the early-time release data were fitted to the following empirical equation (Peppas, 1985; Grassi and Grassi, 2005):

$$\frac{M_t}{M_\infty} = kt^n$$

where M_t corresponds to the amount of drug released at time t, M_∞ the total amount of drug released after an infinite time, k a constant related to the geometric characteristics of the drug delivery system, and n is the release exponent indicating the drug release mechanism. The obtained values of n and the coefficient of determination R^2 are reported in Table 2.

Table 2
The parameters derived from fitting the model of the controlled release of fluorescein isothiocyanate-dextrans (FD).

Sample	k	n	R^2
Sodium hyaluronate tablets			
FD20	15.8809	1.0407	0.9966
FD40	15.6148	1.1163	0.9984
FD70S	13.6694	1.1838	0.9929
HA-Cys tablets			
FD20	29.2948	0.5663	0.9929
FD40	21.4116	0.6308	0.9948
FD70S	19.2403	0.6118	0.9946

4. Discussion

Most of the thiomers properties (mucoadhesion, cohesion, stability towards oxidation, etc.) are strongly dependant on the reactivity of immobilized thiol groups because thiol/ disulfide exchange reactions in the system underlie their features thus rendering thiolated polymers a promising advanced tool for the oral delivery of various hydrophilic macromolecular drugs (Bernkop-Schnürch et al., 2004). Thiol-disulfide interchange involves the nucleophilic attack of thiolate anion along the S–S bond axis of the disulfide. The apparent rate of the thiol-disulfide reaction reaches its maximum when the thiol pK_a is close to the pH of the surrounding medium (Singh and Whitesides, 1991). Considering this effect, we chose the ligand cysteine ethyl ester, which exhibits pK_a value of 6.7 (Krepela et al., 1999). The pK_a values of all used so far ligands—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 a thiolate anion at pH 6.8. Another observation is that when cysteine ethyl ester is used as a ligand, the undesired Cys–Cys side reaction during synthesis is excluded. These results confirmed that the carbodiimide-mediated coupling of HA to primary amines did not lead efficiently to the formation of amide bonds. In contrast, the addition of NHS to the reaction scheme caused the formation of a non-rearrangable intermediate product and thus improved the coupling yields. The obtained conjugate showed the same thermal behavior like unmodified HA.

The thermal behavior of sodium hyaluronate has already been described (Villetti et al., 2002; Benešová et al., 2006). The data previously published indicated HA thermal resistance at least up to 150 °C. A detailed study of the first endotherm has showed a gradual moisture evaporation proceeding in several steps. This indicated different types of water bound (non-freezing, freezing-bound) on HA molecular skeleton as well as trapped water inside the secondary HA structure. It was suggested that particularly non-freezing water contributes to the structural stability of HA chains. According to the results of Villetti et al., temperatures above 200 °C in a nitrogen atmosphere caused a degradation of sodium hyaluronate. The exothermal peak in the range of 230–280 °C can be attributed to the initial phase of the degradation—the cleavage of β-(1–4) glycosidic bond in the backbone. The comparison given in Fig. 2 showed that the thermal behavior of HA-Cys conjugate differed mainly in the intensity of the released heat during decomposition in this first step and also in the intensity of the peak maxima. Consequently, the modification of HA by L-cysteine ethyl ester caused a shift to lower decomposition onset temperatures in comparison with HA (difference of 11 °C). The thermal analysis is an important method, considering the fact that in industrial processes heat is commonly employed and the thermal decomposition data yield information regarding stability and the residues of decomposition.

Table 3
Comparison of the mucoadhesive properties of various anionic thiomers. Mucoadhesive studies were performed via the rotating cylinder method

Thiomer	Degree of modification (μmol/g ± S.D., $n = 3$)	Adhesion time (h)	Improvement ratio	References
HA-Cys	201.3 ± 18.7	17	6.5[a]	
PAA_{450}-GSH	353.7 ± 41.8	21	14[a]	Kafedjiiski et al. (2005b)
PAA_{450}-Cys	404.1 ± 65.5	22	13[a]	Leitner et al. (2003)
PAA_{250}-Cys	404.1 ± 65.5	15	12.5[a]	Leitner et al. (2003)
Polycarbophil-Cys	404.1 ± 65.5	9	1.7[a]	Leitner et al. (2003)
Carboxymethylcellulose-Cys	22.3	1.7	1.13[a]	Bernkop-Schnürch and Steininger (2000)

[a] Differs from control $p < 0.05$.

The oxidation process of thiol groups of thiomers plays a substantial role in different *in vitro* and *in vivo* test systems, describing the properties of conjugates. Thiol groups within close proximity can be oxidized to disulfides by either an intra- or intermolecular reaction. Disulfide bond formation is controlled by the access of oxygen and by the pH value of aqueous medium. Compared with other anionic thiomers, such as poly(acrylic acid)-glutathione, poly(acrylic acid)-homocysteine and poly(acrylic acid)-cysteine, this new conjugate showed the highest degree and rate of oxidation. This oxidation behavior can be explained by the pK_a of the thiol groups and by the steric factor. Thiol moieties being located closely to each other can form disulfide bonds more rapidly rather than remaining isolated thiol groups (Kafedjiiski et al., 2005a).

The swelling behavior of mucoadhesive polymers is an essential parameter of the mechanisms, which are responsible for their adhesive and cohesive properties, and drug release as well. Controlling the rate and extent of hydration is required to promote a prolonged adhesion, and strategies such as cross-linking and introduction of hydrophobic entities are used to achieve this purpose (Smart, 2005). Compared with poly(acrylic acid)-glutathione and other generated anionic thiomers, HA-Cys conjugate exhibited two-fold higher swelling ratio. To check the cohesion of the dry tablets, the crushing strength was determined and the tensile strength was calculated. The HA tablets showed 0.473 ± 0.019 MPa tensile strength. In addition, the tensile strength of the thiomer tablets was also in the same range, 0.441 ± 0.018 MPa. The swelling behavior of HA-Cys conjugate could be explained by its retained hydrophilic character and property of hydration, as well as by *in situ* paralleled running process of disulfide-cross-linking of the conjugate. The cross-linking of the thiomer decreases the tablet matrix solubility and increases its cohesiveness, thus preventing the tablet dissolution.

The obtained data from the mucoadhesion studies were in the same range compared with poly(acrylic acid)-glutathione conjugate and poly(acrylic acid)-cysteine derivatives depending on the degree of modification with thiol groups and the molecular mass of poly(acrylic acid)-cysteine (Table 3) (Leitner et al., 2003; Kafedjiiski et al., 2005b). This is the first chemical modification of HA which results in such a significant improvement in the mucoadhesive properties. The new thiolated conjugate displayed a sufficiently prolonged adhesion time on mucosa as a consequence of balanced mucoadhesive and cohesive properties. Based on thiol/disulfide exchange reactions and/or a simple oxidation process, disulfide bonds between thiomers and cysteine-rich subdomains of mucus glycoproteins are formed. Another likely mechanism ascribed to be responsible for the highly improved mucoadhesive properties of these thiolated polymers is based on their *in situ* cross-linking properties. During and after the interpenetration process, disulfide bonds are formed within the polymer matrix, leading to additional anchors via chaining up with the mucus gel layer (Bernkop-Schnürch, 2005).

HA-Cys and cross-linked HA-Cys displayed very slow degradation rates but there was a significant difference between their rates of degradation. The hydrolysis of HA by testicular hyaluronidase was supposed to occur according to the double-displacement substrate assisted mechanism involving the amino acids Asp-147 and Glu-149 (Botzki et al., 2004). Glu-149 acts as a proton donor, while the nucleophile is the acetamido oxygen of the HA substrate probably forming a covalent oxazolinium intermediate. In the next step, the intermediate is hydrolyzed by water molecule. Concerning thiolated HA, the modification of the structure covers only D-glucuronic acid units. Therefore, the polymer remains a substrate susceptible to hydrolysis by hyaluronidase. The rate of degradation of the cross-linked HA-Cys was reduced additionally as a result of a cross-linking process via disulfide bonds. This effect revealed that disulfide bonds comprising both inter- and intramolecular cross-links between conjugate molecules were stable and rendered the thiomer resistant to hydrolysis.

The biodegradability of component materials is critical when designing a drug delivery device. The erosion process occurs either in bulk wherein the matrix degrades uniformly, or at the polymers surface whereby release rates are related to the polymer surface area. Therefore, varying each of these factors allows researchers to adjust the rate of matrix degradation and, subsequently, control the rate of drug delivery. Based on this approach, various polysaccharides have been investigated for a colon-specific drug release. The matrices of polysaccharides are assumed to remain intact in the physiological environment of the stomach and small intestine, but once they reach the colon, they are acted upon by the bacterial polysaccharidases, leading to the matrices degradation. Another interesting application of HA-Cys conjugates will be the development of formulations as topical, injectable and implantable vehicles for the controlled and localized delivery of active molecules.

Thiolated polymers appear to be the most promising for oral delivery of hydrophilic macromolecular drugs. Therefore, a homologous series of high molecular weight water-soluble

fluorescein isothiocyanate dextrans have been used initially for calibration purposes as they are readily available and easily assayed model compounds with molecular weights encompassing the range of 4–70 kDa.

Sodium hyaluronate formulations displayed values of *n* very close to one, which indicates a release approaching zero-order kinetics or case II transport, implying that the drug is released at a constant rate. The release was, thus, controlled by the viscoelastic relaxation of the matrix during solvent penetration, as well as by the diffusion of FD in the gel layer formed as the tablet swelled. These types of matrix systems are known as swelling-controlled release systems. Concerning HA-Cys tablets, the values of *n* are between 0.5 and 0.89 and, therefore, can be regarded as an indicator of non-Fickian or anomalous transport. The anomalous transport occurs due to a coupling of Fickian diffusion and polymer relaxation. A possible explanation for the change in the release behavior of the thiomer could be as follows. Considering the fact that in the system a running process of oxidation of the conjugate matrix takes place *in situ* leading to the formation of disulfide bonds and cross-linking of the polymer, the release of the model substance may be controlled by this additional mechanism. The cross-linking process decreased the velocity of diffusion of macromolecules and contributed to the dependence of the release rate from the molecular weight, respectively from the molecular size.

5. Conclusion

Within this study, the new hyaluronic acid-cysteine ethyl ester conjugate has been synthesized by the amide bond formation in the presence of the double catalytic system EDAC/NHS. The new thiomer offers several advantages as vehicles for the non-invasive administration of peptides and other hydrophilic drugs. By applying the concept of the immobilization of thiol groups on hyaluronic acid, the mucoadhesive properties of the conjugate were 6.5-fold improved. The formation of inter- and intramolecular disulfide bonds *in situ* in the thiomer matrix modulated the release rate, leading to non-Fickian (anomalous) kinetic and a sustained drug release for more than 12 h. The significantly lowered biodegradation rate in comparison to unmodified HA is a prerequisite for future versatile HA-Cys clinical applications in wound healing and tissue repairment. Therefore, this novel thiolated polymer appears to be a very promising excipient for the development of various drug delivery systems.

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