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Design and synthesis of a novel cationic thiolated polymer

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ABSTRACT

The purpose of this study was to design and characterize a novel cationic thiolated polymer. In this regard a hydroxyethylcellulose-cysteamine conjugate (HEC-cysteamine) was synthesized. Oxidative ring opening with periodate and reductive amination with cysteamine were performed in order to immobilize free thiol groups to HEC. The resulting HEC-cysteamine displayed 2035 ± 162 μmol immobilized free thiol groups and 185 ± 64 μmol disulfide bonds per gram of polymer being soluble in both acidic and basic conditions. Unlike the unmodified HEC, in case of HEC-cysteamine, a three-fold increase in the viscosity was observed when equal volumes of the polymer were mixed with mucin solution. Tablets based on HEC-cysteamine remained attached on freshly excised porcine mucosa for 80 h and displayed increased disintegration time of 2 h. Swelling behavior of HEC-cysteamine tablets in 0.1 M phosphate buffer pH 6.8 indicated swelling ratio of 19 within 8 h. In contrast, tablets comprising unmodified HEC detached from the mucosa within few seconds and immediately disintegrated. In addition, they did not exhibit swelling behavior. The transport of rhodamine 123 across freshly excised rat intestine enhanced by a value of approximately 1.6-fold (p-value = 0.0024) in the presence of 0.5% (m/v) HEC-cysteamine as compared to buffer control. Result from cytotoxicity test of HEC-cysteamine applied to Caco-2 cells in concentration of 0.5% (m/v) revealed 82.4 ± 4.60% cell viability. According to these results, HEC-cysteamine seems to be a promising polymer for various pharmaceutical applications especially for intestinal drug delivery.

1. Introduction

Polymeric excipients can be classified as non-ionic, cationic and anionic. Among cationic polymers, poly(L-lysine), polyethylenimine (PEI), polyamidoamine (PAMAM) dendrimers and chitosan seem to be the most commonly used. These polymers have been shown to have a significant application in gene and oligonucleotide delivery against tumors. Polymers based on PEI effectively complex DNA molecules leading to homogeneous spherical particles with a size ≤ 100 nm. These homogenous particles can transfect cells efficiently in vitro and in vivo. The higher charge density and more efficient complexation of DNA and/or oligonucleotides afford significantly enhanced protection against degradation by nucleases when compared to other polycations like poly(L-lysine). Chitosan polymers in the molecular weight range of 30–170 kDa have been shown to provide gene expression levels similar to PEI (Merdan et al., 2002; Jevprasesphant et al., 2003). On the other end, PAMAM dendrimers can mimic globular proteins and biological lipid bilayer membranes. The DNA–PAMAM dendrimer complexes have been reported to show high stability, as well as enhanced gene expression during in vitro transfections (Esfand and Tomalia, 2001; Tang et al., 1996).

While PEI, poly(L-lysine) and PAMAM dendrimers exhibit high cytotoxicity, chitosan shows a significantly better biocompatibility (Merdan et al., 2002; Jevprasesphant et al., 2003; Tang and Szoka, 1997). Many studies described the usage of chitosan for various drug delivery systems such as oral, parenteral and nasal administration because it demonstrates permeation enhancing and mucoadhesive properties (Dodane and Vilivalam, 1998). Recently, it has been demonstrated that the introduction of thiol groups on chitosan can lead to even further improved mucoadhesive, cohesive and permeation enhancing properties. The permeation enhancing and the mucoadhesive properties effect of thiolated chitosans are approximately 80-fold and 140-fold higher, respectively, in comparison to unmodified chitosan (Sakloetsakun and Bernkop-Schnürch, 2010; Roldo et al., 2004).

However, chitosan and subsequently thiolated chitosans bear the disadvantage of precipitation in aqueous media of pH above 6.5. Consequently, they do not reach their full potential as permeation enhancer on mucosal membranes where pH is above 6.5 (Kotzé

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et al., 1999). It was therefore the aim of this study to design and synthesize an alternative thiolated cationic polysaccharide showing similar properties as thiolated chitosan but being soluble at a broad pH range.

As non-ionic polysaccharide, hydroxyethylcellulose (HEC) is one of the obvious choices for pharmaceutical application. HEC is inert cellulose and its properties such as biocompatibility, physiochemical stability and solubility in water make it suitable excipient for drug delivery. Moreover, HEC is widely used as an effective polymer for rate control of drug release (Taviera et al., 2009; Roy and Rohera, 2002).

Hydroxyethylcelullose (HEC) was therefore converted to a positively charged polymer using a reductive amination reaction on oxidized HEC. Ring opening reaction of HEC was done to oxidize the proximal hydroxyl groups thereby forming aldehyde groups. Afterwards, cysteamine as primary amine ligand was reacted with the oxidized HEC to design a novel cationic thiolated polysaccharide. The synthetic pathway is shown in Fig. 1. The novel polymer is characterized in terms of swelling behavior, disintegration behavior, mucoadhesive properties, permeation enhancing properties, cytotoxicity and solubility.

2. Material and methods

2.1. Materials

2-(*N*-Morpholino)ethanesulfonic acid (MES hydrate), trinitrobenzensulfonic acid (TNBS), cysteamine, ethylene glycol, *t*-butyl carbazate, rhodamine 123, sodium periodate, mucin from porcine stomach (type II: crude), sodium borohydride, sodium cyanoborohydride and dialysis tubing cellulose membrane (molecular weight cut-off of 12 kDa) were all purchased from Sigma–Aldrich, Vienna, Austria, whereas hydroxyethyl cellulose (~145 mPa S 1% in H_2O at 20 °C, molecular weight: ~250,000) was obtained from Fluka, Buchs, Switzerland.

2.2. Preparation of aldehyde polymer

Unmodified HEC was modified to aldehyde forms, HEC-CHO with a slight modification according to the procedure described by Ito et al. (2007). Briefly, in 500 mL Erlenmeyer flask wrapped with aluminium foil, 1.5 g of HEC was dissolved in 140 mL water, then 10 mL of solution containing 800 mg of sodium periodate was added, and stirred for 2 h at room temperature. Two hundred micro liter of ethylene glycol was added to inactivate any unreacted periodate. The reaction was stirred for 1 h at room temperature and then dialyzed exhaustively (molecular weight cut-off of 12 kDa; dialysis tubing cellulose membrane; Sigma, St Louis, MO) against water for 3 days. The water was changed at least three times a day. The purified product was freeze dried (−78 °C, 0.01 mbar, VirTis, Gardiner, ME) and kept at 4 °C.

2.3. Aldehyde assay

t-Butyl carbazate was used to quantify percent oxidation of HEC-CHO. In brief, solutions of HEC-CHO (0.5 mL, 0.12%, m/v) and *t*-butyl carbazate (0.5 mL, 0.01 M) in 1% (m/v) aqueous trichloroacetic acid were mixed and allowed to react for 24 h at room temperature. A volume of 200 μL of the previous solution was mixed with aqueous trinitrobenzensulfonic acid (TNBS) solution (2 mL, 4 mM, 0.01 M borate buffer, pH 8) contained in a disposable scintillation vial. The mixture was allowed to react for 30 min at room temperature, followed by dilution with 0.5 M aqueous HCl. The absorbance of the mixture was measured at 334 nm. A calibration curve was prepared with aqueous *t*-butyl carbazate solutions as standards. The blank

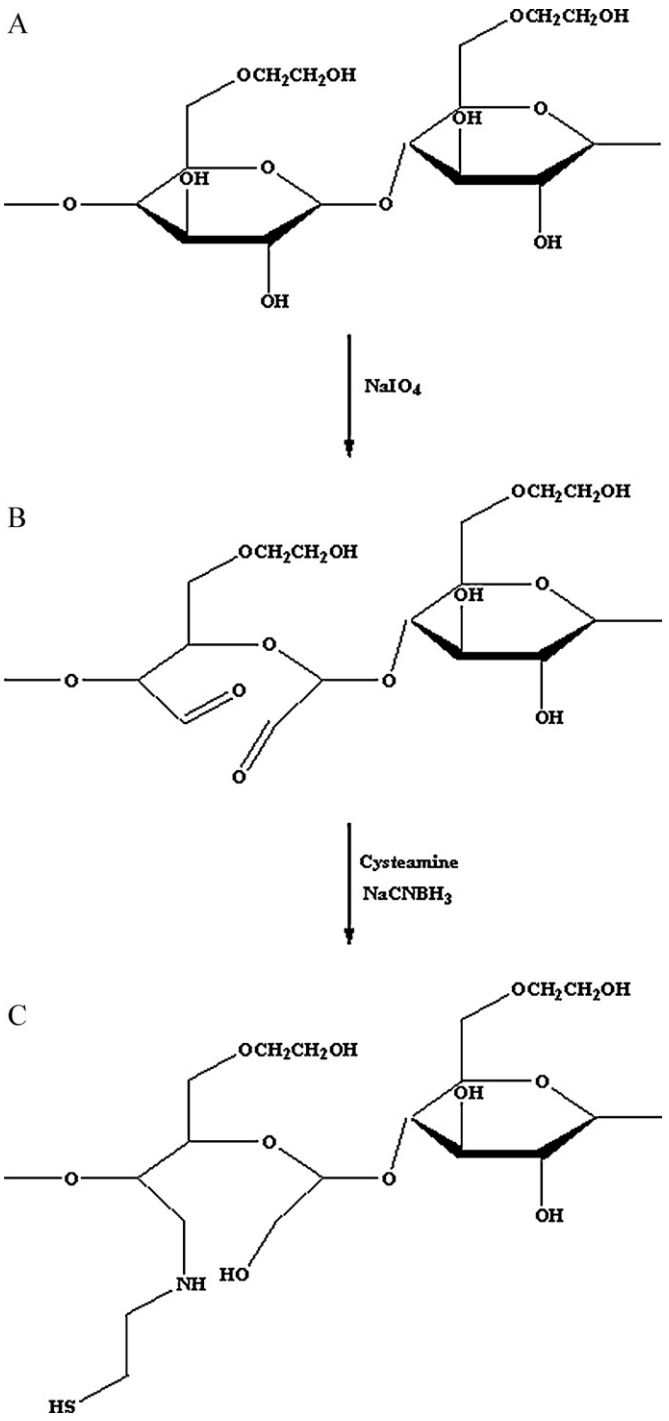

Fig. 1. Hydroxyethylcellulose (HEC). (A) Unmodified HEC. (B) Periodate oxidation (to HEC-CHO). (C) Reductive amination/thiolation (to HEC-cysteamine).

comprised of 0.5 mL H_2O and aqueous trichloroacetic acid (0.5 mL, 1%, m/v) (Bouhadir et al., 1999)

2.4. Conjugation of aldehyde polymers to cysteamine

First, one gram of HEC-CHO was hydrated in 40 mL of distilled water and MES hydrate was added in a final concentration of 0.1 M. Then, cysteamine was added in different amounts (0.125, 0.25, 0.5 g). In order to evaluate the influence of the pH-value on the reaction, the pH was adjusted using 1 M HCl to pH 5, 6, 7 and 8, respectively. The final volume of the mixture was adjusted to 50 mL

with distilled water. After 3 h of incubation under stirring at room temperature, four gram of $NaCNBH_3$ was added into the solution and stirred for 2, 4, 8, 24, 36, 48, 60 and 72 h at room temperature. In order to eliminate unreacted product and to isolate the polymer conjugates, the reaction mixtures were dialyzed six times in tubing (molecular weight cut-off of 12 kDa; dialysis tubing cellulose membrane; Sigma, St Louis, MO) at 10 °C in the dark. In detail they were dialyzed one time against distilled water, one time against 0.2 mM HCl, then two times against the same medium but containing 1% NaCl to quench ionic interactions between the cationic polymer and the ionic sulfhydryl compound. Then, the samples were dialyzed exhaustively two times against 0.2 mM HCl. Finally, the frozen aqueous polymer solutions were lyophilized (−78 °C, 0.01 mbar, VirTis, Gardiner, ME) and stored at 4 °C until further use. Controls were prepared in exactly the same way but omitting $NaCNBH_3$ during the reaction.

2.5. Determination of the thiol group content

Ellman's reagent was used to quantify free thiol groups immobilized on HEC-cysteamine as described previously (Hombach et al., 2009). First, 0.5 mg of each conjugate and the control were hydrated in 500 μL of 0.5 M phosphate buffer pH 8.0 followed by 500 μL of Ellman's reagent (5 mg dissolved in 10 mL of 0.5 M phosphate buffer pH 8.0). The hydrated conjugate and the control were light protected and incubated at room temperature for 2 h. Centrifugation was performed at 13,400 rpm for 5 min (Minispin, Eppendorf, Vienna, Austria) and 300 μL of each sample was transferred into a microplate to measure absorbance at 450 nm using a microplate reader (FluoStar Galaxy, BMG, Offenburg, Germany). The amount of thiol groups were estimated from a standard curve of cysteamine prepared in exactly the same way as the samples. Disulfide content was determined after reduction with $NaBH_4$ and addition of Ellman's reagent (Habeeb, 1973).

2.6. Fourier transport infrared spectroscopy (FTIR) studies

FTIR spectra were recorded with a BRUKER Vertex 70 spectrometer, equipped with a MIRacle ATR-diamond unit (*A*ttenuated *T*otal *R*eflection) in the range 600–5500 cm^{-1}, spectral resolution of ~4 cm^{-1}. Samples were pressed onto the diamond window by a plane steel cone. 64 scans for the sample and the background were acquired. All displayed spectra were cut in the range 2400–2800 cm^{-1}, when background correction was applied third order polynoms were fit to the spectra minima using the OPUS 6.5 software.

2.7. Tablets manufacture

Unmodified HEC, HEC-CHO and HEC-cysteamine were compressed into 30 mg, 5.0 mm diameter flat-faced tablets (single punch eccentric press-Korsch EK, Germany). The compaction pressure (force of 10 kN) was kept constant during the preparation of all tablets.

2.8. Disintegration and swelling behavior studies

In order to determine the disintegration behavior of the tablets, disintegration test was performed in 0.1 M phosphate buffer pH 6.8 at 37 °C and analyzed with a disintegration test apparatus according to the European Pharmacopoeia. The oscillating frequency was adjusted to 0.5 s^{-1}. The swelling behavior studies were carried out to determine the water-absorbing capacity of the tablets using a gravimetric method. Test tablets were fixed to a needle and immersed in a beaker containing 0.1 M phosphate buffer pH 6.8 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. Rheological studies

Rheological studies were carried out by hydrating 4 g of porcine mucin in 25 mL of demineralized water with continuous stirring. Further the pH was adjusted to 6.8 by the addition of 1 M NaOH and the mucin solution was diluted to a final volume of 50 mL with 200 mM phosphate buffer pH 6.8. This 8% (m/v) mucin stock solution was stored at 4 °C until further use. Unmodified HEC, HEC-CHO and HEC-cysteamine, respectively, were hydrated in demineralized water to reach a final concentration of 6% (m/v). After complete hydration, the obtained polymer solutions were mixed vigorously with an equal volume of 8% (m/v) mucin stock solution. Solutions containing either 3% (m/v) polymer or 4% (m/v) mucin in 50 mM phosphate buffer pH 6.8 were used as references. Following sample preparation, each mixture was allowed to equilibrate for 20 min (Marschütz and Bernkop-Schnüch, 2002). Dynamic oscillatory test within the linear viscoelasticity region were performed at 1 Hz frequency. The parameters obtained thereby were the phase angle (δ) and the complex modulus (G^*). The elastic modulus (G'), the viscous modulus (G'') and the dynamic viscosity (η^*) were calculated by:

$$G' = G^* \cos \delta$$

$$G'' = G^* \sin \delta$$

$$\eta^* = \frac{G''}{2\pi\nu}$$

where ν is the oscillatory frequency. Loss tangent ($tan\,\delta$), a parameter that represents the ratio between the viscous and the elastic properties of the polymer was also calculated ($tan\,\delta = G''/G'$) (Bernkop-Schnürch et al., 2003a).

2.10. Mucoadhesive studies

Tablets made of 30 mg of unmodified HEC, HEC-CHO and HEC-cysteamine 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 at 37 °C and agitated with 125 rpm. The detachment of the test tablets was determined visually during an observation time of 90 h (Bernkop-Schnürch et al., 2003a).

2.11. Cytotoxicity studies

Lactate dehydrogenase (LDH) test was performed to determine the cytotoxicity of the polymers and the result was confirmed using MTT assay. Gels (1%; m/v) were prepared using the polymers to be tested. In brief, 1×10^5 Caco-2 cells per well were plated in 24 well plates till a monolayer was obtained. The confluent cells were washed two times with 1 × phosphate buffer saline (PBS) after aspirating the culture medium. Subsequently, test solution containing 250 μL of gels and 250 μL of MEM without phenol red was added to different culture wells. Cells incubated with MEM medium and

Table 1
Quantification of thiol groups on the conjugates.

HEC-CHO (g)	Cysteamine (g)	pH	$NaCNBH_3$ (g)	Reaction time (h)	–SH (μmol/g polymer)	–S–S– (μmol/g polymer)
1	0.5	5	4	72	2035 ± 162	185 ± 64
1	0.25	5	4	72	1743 ± 104	122 ± 32
1	0.125	5	4	72	1316 ± 94	107 ± 14

with 5% (v/v) Triton-X 100 plus medium served as controls. Supernatant from samples (100 μL) collected at 0 and 3 h was measured at 492 nm using a spectrophotometer (DU® Series 600) and cell viability (%) was calculated based on the following equation:

$$\%\text{cell viability} = 100 - \left[\frac{\text{average absorbance value of each triplicate} - \text{negative control}}{\text{positive control} - \text{negative control}}\right] \times 100$$

Cytotoxicity of the systems was further quantitatively assessed by MTT assay. MTT solution was prepared by dissolving MTT in serum-free MEM without phenol red at a concentration of 0.5 mg/mL. The solution was sterilized by passing through 0.20 μm filter and stored at 2–8 °C. Caco-2 cells were seeded in 24 wells plates as reported for the LDH assay. Cells were incubated for 3 h with the test solution containing 250 μL of gels and 250 μL of MEM without phenol red. The medium was then aspired and rinsed with 1 × PBS three times in order to remove traces of the gels. Five hundred micro liter of prepared MTT solution was then transferred to each well and incubated for 3 h at 37 °C in 5% CO_2 atmosphere. Thereafter, the supernatants were discarded. The converted dye was solubilized with 500 μL DMSO and mixed thoroughly to dissolve the blue-violet crystals. The dye solution was then transferred to 2 mL tubes and centrifuged at 13,400 rpm for 2 min. The absorbance of the resulting solution was recorded immediately at 570 nm. Percentage of cell viability was calculated by comparison to 0% viability (positive control) and 100% viability of untreated cells. All experiments were performed in triplicate (Sakloetsakun et al., 2009).

2.12. Permeation studies

Permeation studies were carried out in vertical Ussing type chambers with a volume of 1 mL in the donor and acceptor-chamber and a permeation area of 0.64 cm^2. Immediately after sacrificing the rat, the small intestine (duodenum) was excised and mounted in the Ussing chamber. The small intestine was bathed in a solution containing 250 mM NaCl, 2.6 mM $MgSO_4$, 10 mM KCl, 40 mM glucose and 50 mM $NaHCO_3$ buffered with 40 mM HEPES, pH 7.4. Transepithelial electrical resistance (TEER) of rat intestinal mucosa was measured with the EVOM instrument (World Precision Instruments, Sarasota, FL). All experiments were performed in an atmosphere of 95% O_2 and 5% CO_2 at 37 °C. After 15–20 min of preincubation with the artificial intestinal fluid, the incubation medium of the donor compartment was substituted by either 0.5% (m/v) HEC-cysteamine solution or the control solution (0.5%; m/v, unmodified HEC and HEC-CHO). Furthermore, each sample contained 0.001% (m/v) rhodamine 123 as a model compound. Samples of 100 μL were withdrawn from the acceptor compartment every 30 min over a time period of 3 h. Samples were immediately replaced by 100 μL artificial intestinal fluid equilibrated at 37 °C. The amount of permeated rhodamine 123 was determined using a fluorometer (SLT, Spectra Fluor, Tecan, Austria) at an extinction wavelength of 485 nm and an emission wavelength of 520 nm (Palmberger et al., 2008). Apparent permeability coefficients (P_{app}) for rhodamine 123 were calculated according to the following equation:

$$P_{app} = \frac{Q}{A.c.t}$$

where P_{app} is the apparent permeability coefficient (cm/s), Q is the total amount permeated over the incubation period (μg), A is the diffusion area (0.64 cm^2), c is the initial concentration of the model drug in the donor compartment (μg/cm^3), and t is the whole time of experiments (s). Improvement ratios were calculated from the ratio between the absorptive P_{app} of tested compounds over the absorptive P_{app} of buffer control.

2.13. Solubility test

Solubility test was carried out to investigate the precipitation of the polymer solution over a wide range of pH (1–14). Briefly, 0.5 g of HEC-cysteamine was dissolved in 50 mL of distilled water and the pH was adjusted to acidic condition by using 1 M HCl and to basic condition by using 1 M NaOH.

2.14. Statistical data analysis

The results are expressed as the mean of at least 3 experiments ± SD. Statistical data analysis was performed using the student t-test, two tails with 95% confident interval (p-value < 0.05) as the minimal level of significance.

3. Results

3.1. Synthesis of HEC-cysteamine

Unmodified HEC available commercially was oxidized with sodium periodate to prepare HEC-CHO. The viscosity of the reaction mixture decreased after 2 h. The degree of oxidation (DO) of oxidized HEC was determined to be 70%. The same result was obtained when the reaction was allowed to proceed for 24 h. Maia et al. (2005) found that the percentage of doubly oxidized glucose of dextran increased with the sodium periodate concentration. The double oxidation of the glucose unit of dextran can be detected by the formic acid released during oxidation reaction. Result from pH study demonstrated that there was no change in pH of the solution during the reaction. It means that undesired reactions did not occur. The thiolation of unmodified HEC depicted in Fig. 1 took place at room temperature.

3.2. Characterization of HEC-cysteamine

HEC-cysteamine was of white color and odorless. The polymer had a fibrous structure and was hydratable in aqueous solutions. The rate of cysteamine immobilization to HEC-CHO increases with the weight ratio of cysteamine to HEC-CHO, the decrease in pH values from 8 to 5 and the reaction time. A reaction at pH 5 for 72 h led to the highest degree of thiolation (Table 1). A control prepared in the same manner as HEC-cysteamine but omitting $NaCNBH_3$ during the coupling reaction serving as negative control showed only a negligible amount of free thiol groups. HEC-cysteamine with the highest thiol group content (2035 μmol/g) was chosen for further

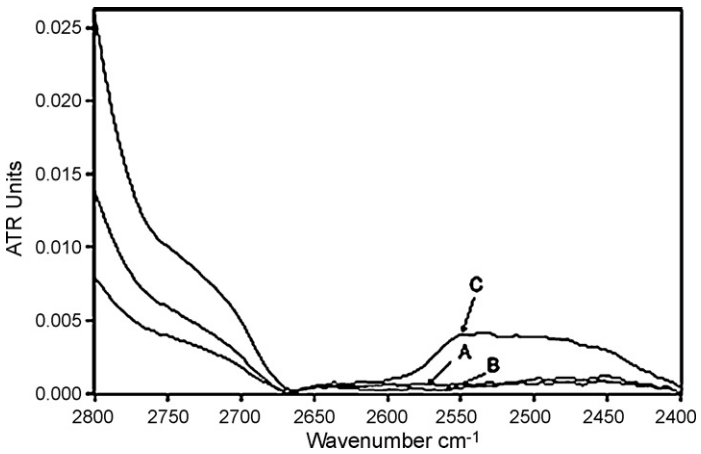

Fig. 2. FTIR spectra with background correction applied to unmodified HEC (A), HEC-CHO (B) and HEC-cysteamine (C).

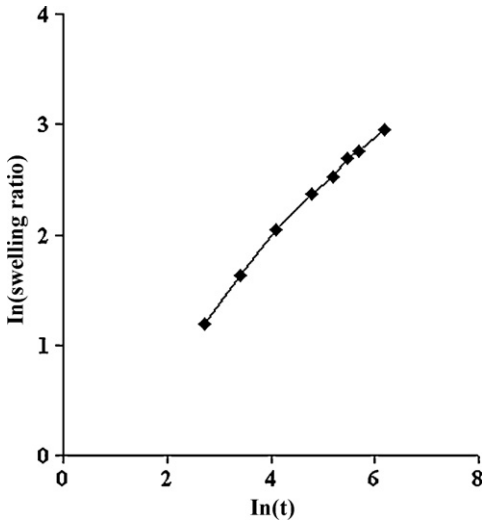

Fig. 3. Swelling behavior of tablets comprising HEC-cysteamine in 0.1 M phosphate buffer pH 6.8 at 37 °C; indicated values are means of three experiments ± SD. Swelling ratio is the ratio W_{ut}/W_0, where W_{ut} is the weight of uptaken water (mg) at time t (min) and W_0 is the initial weight of the dry tablet (mg). In contrast, unmodified HEC and HEC-CHO tablets dissolved immediately thus they did not swell.

evaluations. Chamow et al. (1994) found that the reaction parameters influencing the rate of MePEGylation of CD4-IgG were reaction time, temperature and molar excess of MePEG-CHO over CD4-IgG. Furthermore, they reported that MePEG-CHO incubated with a protein at pH 6–9 results in reversible addition of MePEG to amino groups of the protein via Schiff's base formation. These linkages are converted to stable secondary amines by reduction with sodium cyanoborohydride. Cysteamine with amino groups renders positive charge to the polymer when it is conjugated to HEC-CHO. In addition, unreacted aldehyde groups were reduced to hydroxyl groups.

The spectra of HEC-cysteamine in the range 2400–2800 cm^{-1} shows a weak, broad band around 2550 cm^{-1}, which is absent in the spectra of unmodified HEC and HEC-CHO. The presence of this band is better visible when background correction is applied (Fig. 2). Absorption bands of S–H functional groups are expected in this wavenumber range (Korányi et al., 1997) and thus confirm the presence of S–H in the HEC-cysteamine.

3.3. Disintegration and swelling behavior studies

The influence of thiol groups on the cohesiveness of tablets was investigated by disintegration studies. Unmodified HEC and HEC-CHO tablets of 30 mg disintegrated within 20 s after having been immersed into the buffer solution of 500 mL since they were hydrated and swollen very fast, whereas tablets comprising HEC-cysteamine remained stable for 2 h. This demonstrated an increased cohesiveness and stability of tablets consisting of HEC-cysteamine due to the formation of disulfide bonds within the thiomer. Tablets comprising unmodified HEC and HEC-CHO started to swell and dissolve immediately after being immersed in 0.1 M phosphate buffer pH 6.8, whereas HEC-cysteamine tablets maintained their integrity even within 8 h. These results were in good agreement with disintegration studies performed with tablets comprising unmodified HEC, HEC-CHO and HEC-cysteamine, respectively. The covalent attachment of cysteamine to hydroxyethyl cellulose has a significant impact on the swelling behavior of the polymers. Moreover, neither erosion nor disintegration of HEC-cysteamine tablets could be observed within this time period, demonstrating also the high cohesive properties of the polymer. At the initial stages of swelling, the swelling mechanism was determinated using the following equation:

$$\text{Swelling ratio} = Kt^n$$

where K is the swelling constant, t is the time and n is the swelling exponent that describes the mode of water transport (Karadağ et al., 2002). By plotting ln(*swelling ratio*) versus ln(t) (Fig. 3), the following equation was obtained.

$$y = 0.5x - 0.0966$$

According to the final equation, $n = 0.5$ indicating Fickian diffusion and $K = 90.79 \times 10^{-2}$.

3.4. Rheological studies

Before mixing with mucin, unmodified HEC 3% (m/v) showed the highest viscosity compared to the two other polymers but after 20 min incubation with mucin 4% (m/v) the dynamic viscosity (η^*) of HEC-cysteamine conjugate 3% (m/v) was almost 3-fold higher compared to its initial value (Fig. 4) and even was higher than that of unmodified HEC 3% (m/v). It became more and more viscous during the incubation time due to the formation of disulfide bonds, physical chain entanglements as well as noncovalent intermolecular interaction between HEC-cysteamine and mucin layer. This finding is confirmed by the decrease in $tan\,\delta$ of the HEC-cysteamine conjugate below 1 during the incubation time. In contrast, unmodified HEC and HEC-CHO did not show any increase in viscosity at all as G' and G'' of unmodified HEC and of HEC-CHO were the same as those of the mucin/polymer mixtures.

3.5. Mucoadhesive studies

Mucoadhesion studies with unmodified HEC, HEC-CHO and HEC-cysteamine were performed with the rotating cylinder method. The adhesion time of HEC-cysteamine tablets was around 80 h whereas the mucoadhesive properties of unmodified HEC and HEC-CHO could not be detectable as the tablet disintegrated immediately after being immersed into the buffer solution. It means that thiolation totally changes the initial properties of the polymer. This improved mucoadhesion is presumably due to disulfide bond formation between the thiomer and the cysteine-rich subdomaines of the mucus. This observation is in good correlation with swelling behavior studies. A rapid swelling of HEC-cysteamine contributes to the interdiffusion process between the polymer and the mucus layer leading to stronger adhesive properties (Bernkop-Schnürch, 2005).

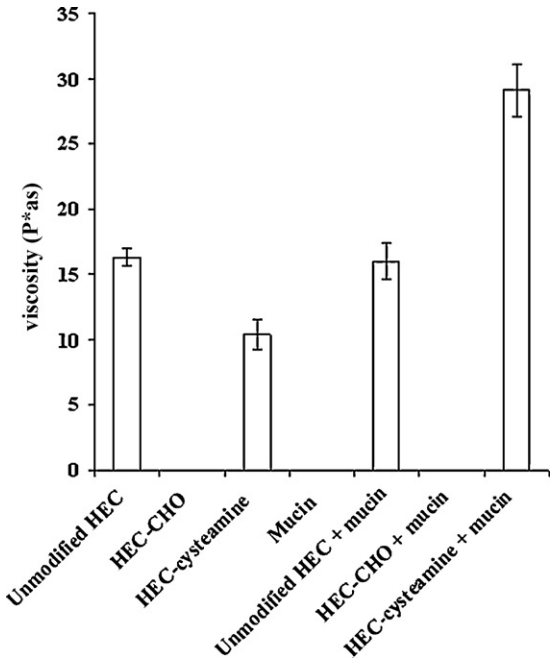

Fig. 4. Viscosity values and rheological synergism for 3% (m/v) unmodified HEC, HEC-CHO and HEC-cysteamine and their corresponding mixtures with mucin; indicated values are means of at least three experiments ± SD.

3.6. Cytotoxicity studies

MTT test was carried out to determine effects of the polymers on metabolic activity of mitochondria of Caco-2 cells, whereas LDH test was performed to assess the damage of cell membranes by quantification of the extracellular concentration of LDH. Generally, all tested polymers did not induce severe cytotoxicity within 3 h. Based on the results obtained from LDH test more than 80% viable cells were determined after incubation with the polymers (Fig. 5). Those results were confirmed by MTT test displaying cell viability of the unmodified HEC, HEC-CHO and HEC-cysteamine of 94.7 ± 3.9, 89.5 ± 4.1 and 82.4 ± 4.60%, respectively (Fig. 6).

3.7. Permeation studies

The permeation enhancing effect of the polymers on the uptake of rhodamine 123 across rat intestinal mucosa was evaluated. A clear and continuous mucus gel covered the surface of rat intestinal mucosa. The influence of unmodified HEC, HEC-CHO and HEC-cysteamine was plotted as cumulative transport over a time period of 180 min. Results demonstrated a significant permeation enhancing effect of HEC-cysteamine whereas the addition of unmodified

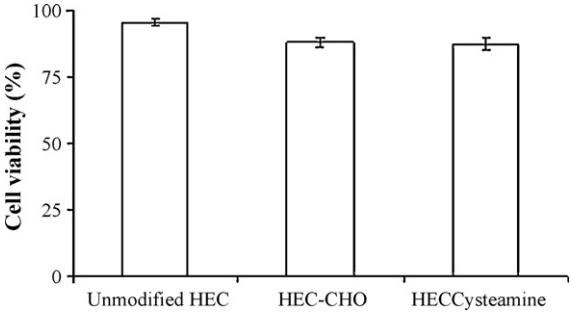

Fig. 5. Viability of Caco-2 cells was determined by LDH assay. The cells were incubated with unmodified HEC, HEC-CHO and HEC-Cysteamine for 3 h of incubation. Each point represents the mean ± SD of three experiments.

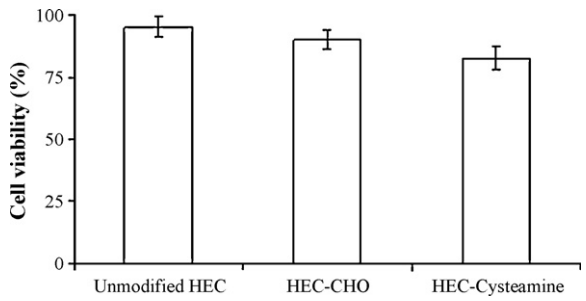

Fig. 6. Viability of Caco-2 cells was determined by MTT assay after 3 h of incubation. The samples were calculated referred to the control. Each point represents the mean ± SD of three experiments.

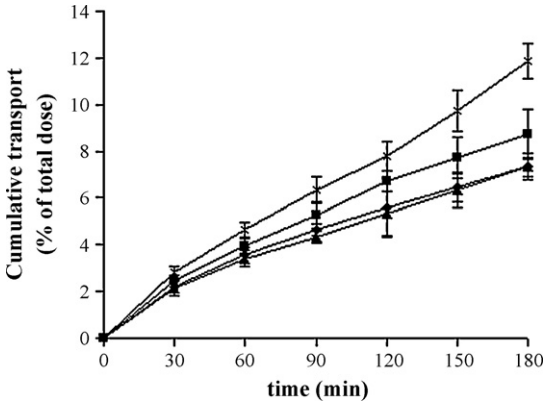

Fig. 7. Transport of rhodamine 123 across rat intestinal mucosa. Transport data are expressed as percentage of the total dose of rhodamine 123 applied to the luminal side of the mucosa. Control without polymer ♦; 0.5% (m/v) unmodified HEC ■; 0.5% (m/v) HEC-CHO ▲; 0.5% (m/v) HEC-cysteamine x; (mean ± SD; $n = 3$).

HEC was found to give a statistically insignificant increase in permeation enhancing effect. In contrast, HEC-CHO did not give any effect on transport of rhodamine 123 across rat intestinal mucosa (Fig. 7). Accordingly, it was demonstrated that immobilization of thiol groups results in increased permeation of rhodamine 123. In comparison to buffer control, the transport of rhodamine 123 across rat intestinal mucosa in the presence of 0.5% (m/v) HEC-cysteamine was 1.6-fold (P-value = 0.0024) improved. The resulting P_{app} values of rhodamine 123 are shown in Table 2. The TEER values were slightly decreased by about 10–20% in comparison to the initial values when the polymers were applied to rat intestinal mucosa.

3.8. Solubility test

Unmodified HEC is a non-ionic water swellable polymer leading to clear aqueous gels. Since the polymer is to a comparatively low degree crosslinked, HEC-cysteamine is soluble in distilled water. The solubility of HEC-cysteamine is influenced by the amount of cysteamine immobilized to HEC-CHO and the subsequent formation of disulfide bonds. When HEC-cysteamine was dissolved in water, its solubility was neither influenced by acidic nor basic pH of

Table 2
Comparison of the absorptive apparent permeability coefficients (P_{app}) of rhodamine 123 across rat intestinal mucosa in the presence of indicated test compounds. Each point represents the means ± SD of three experiments.

Test compounds	P_{app} ($\times 10^{-5}$ cm/s)	Improvement ratio
Buffer	1.05 ± 0.06	–
Unmodified HEC	1.26 ± 1.12	1.19 ± 0.13
HEC-CHO	1.06 ± 0.04	1.01 ± 0.07
HEC-cysteamine	1.71 ± 1.16	1.63 ± 0.10

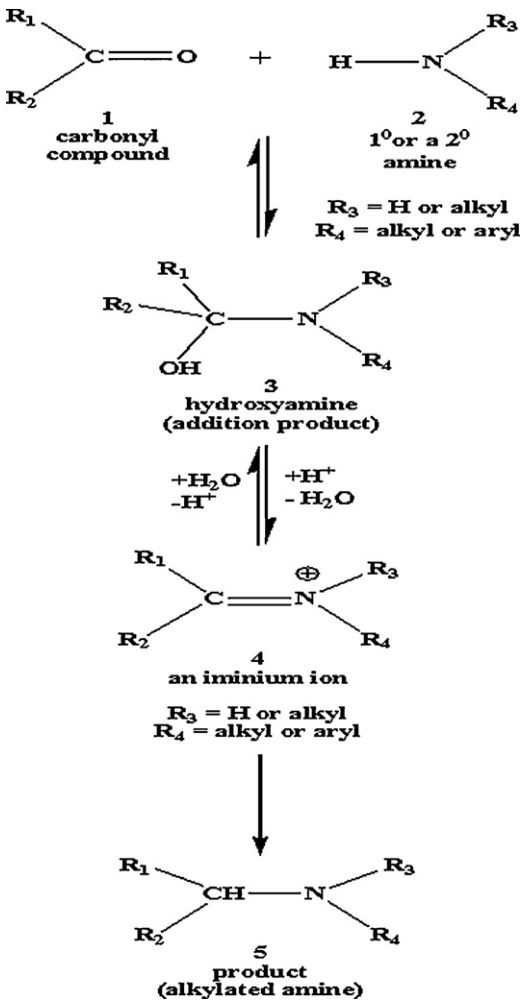

Fig. 8. Reductive aminations (Abdel-Magid et al., 1996).

aqueous solutions as no precipitation occurred at pH 1–14. In contrast to chitosan, which precipitates in aqueous media at pH above 6.5, HEC-cysteamine has the advantage of maintaining swollen over a broad pH range. Accordingly, HEC-cysteamine could, for instance, be applied to improve the permeation of drugs within all segments of intestine.

4. Discussion

To address the insufficient applications of thiolated chitosans at pH above 6.5, a novel cationic thiomer was prepared from partially oxidized HEC. The periodate oxidation reaction on unmodified HEC involves a periodate ion and a molecule with vicinal diols, and implies the oxidation of the reaction site with the breakage of the C–C bond and subsequent formation of two aldehydic groups able to couple primary amino groups (Le-Tien et al., 2004). The mechanism of the reaction can be depicted in the following way: in the first step, one of the I–O bonds of the periodate attacks one of the two hydroxyl groups of the vicinal diols; the second step is the formation of the planar cyclic ester as part of an octahedral intermediate, the rate of which must depend on the acidity of the OH groups and their relative positions (Sussich and Cesáro, 2000; Price and Knell, 1942).

The reaction of aldehyde polymer (HEC-CHO) with primary amine (cysteamine) produces carbinol amine 3 (Fig. 8) that can dehydrate to form an imine. The imine is protonated to form an iminium ion 4 under weakly acidic to neutral condition. Thereafter, the iminium ion produces the alkylated amine product 5. Among reports about reductive amination only a few suggest a direct reduction of the carbinol amine 3 as a possible pathway leading to 5 (Abdel-Magid et al., 1996; Tadanier et al., 1981). For all these reactions sodium cyanoborohydride ($NaCNBH_3$) was utilized as a reducing agent because of its stability in relatively strong acid conditions (~pH 3) and its different selectivity at different pH values (Borch et al., 1971). In this study, the reaction was performed at pH 5 to generate a high amount of free thiol groups. At pH 8, however, the reaction did not take place at all.

Result from solubility test of HEC-cysteamine showed that the solubility is not affected by high pH value of the solution. In this regards, HEC-cysteamine will not precipitate at pH 1–14 during drug formulation and applying to the target sites.

The improvement in the mucoadhesive properties of HEC-cysteamine was due to the formation of disulfide bonds between thiol-bearing side chains of the polymers and cysteine-rich subdomains of mucus glycoprotein. Comparatively more free thiol groups should remain available for thiol/disulfide exchange reactions with the mucus gel layer, consequently leading to higher mucoadhesive properties of the thiomer (Bernkop-Schnüch et al., 2004). In contrast, unmodified HEC and HEC-CHO detached, either by means of erosion in form of fibrous fragments or simply dissolved on the mucosa. They are capable of rapid water uptake and, since not cross-linked, susceptible to rapid over swelling and disintegration resulting in short adhesion time. Furthermore, the periodate oxidation reaction produced much more porous polymer compared to unmodified polymer (Bashaiwoldu et al., 2004).

HEC-cysteamine is able to interpenetrate into mucin. The disulfide bonds between HEC-cysteamine and mucus are responsible for its enhanced mucoadhesive properties and viscosity. Hassan and Gallo (1990) have described rheological synergy phenomenon as an index for mucoadhesive bond strength. As rheological studies were performed at pH 6.8, mucin carboxylic acid groups (from terminal sialic acids) were in the anion form. Thereby, ionic attraction between cationic HEC-cysteamine and mucin occurred. This ionic attraction along with the other forces of interaction such as polar, non polar or physical interaction caused likely the increase in viscosity.

Permeation studies were performed using rhodamine 123 as the marker compound to evaluate permeation enhancing effect of polymers. HEC-cysteamine 0.5% (m/v) led to a significantly improved permeation of rhodamine 123. This demonstrated that the presence of immobilized thiol moieties on the polymer is a crucial factor for the underlying mechanism of improved permeation (Clausen and Bernkop-Schnürch, 2001).

Thiolated polymers can shift the balance between GSSG (oxidized glutathione) and GSH (reduced glutathione) on the membrane to the side of GSH. A comparatively high concentration of GSH on the membrane should, in turn, lead to an opening of the tight junctions (Bernkop-Schürch et al., 2003b). The molecular and cellular mechanism concerning the gate fence function of the tight junctions is still not completely understood. Opening and closing of the tight junctions seem to be mediated also by various proteins such as occludin and tight junction-associated peripheral membrane proteins zonula occludens 1 (ZO1) and zonula occludens 2 (ZO2). It was reported that ZO1, ZO2 and other tight junction-associated peripheral membrane proteins can form a complex (Yap et al., 1998).

Some researchers demonstrated that improved permeation of the widely used positively charged polymer chitosan is only found in acidic environments in which the pH was less or of the order of the pKa value of chitosan (5.5–6.5). Under such conditions, chitosan has a higher charge density and will have a better solubility and a better possibility for intimate contact with the epithelial membrane. Thereby, chitosan is incapable of enhancing absorption in

more basic environment of small intestine as well as of large intestine and colon due to solubility problem (Borchard et al., 1996; Kotzé et al., 1998). Taking the solubility at basic conditions into consideration, the novel cationic thiomer HEC-cysteamine seems to be more effective to show permeation enhancing properties than chitosan at physiological pH values.

5. Conclusion

An attempt to design and synthesize a new polymer displaying solubility over a broad pH range has been carried out. HEC-cysteamine can be synthesized and designed by ring opening of glucose subunits of unmodified HEC using sodium periodate followed by reductive amination of the oxidized HEC. The conjugate is a novel thiomer with positive charges which can be explored in the pharmaceutical field especially for intestinal drug delivery due to properties such as solubility in basic conditions, biocompatibility, non toxicity, mucoadhesive and permeation enhancing properties.

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