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HEC-cysteamine conjugates: Influence of degree of thiolation on efflux pump inhibitory and permeation enhancing properties

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ABSTRACT

Within the present study hydroxyethyl cellulose-cysteamine conjugates are investigated regarding biocompatibility, in situ gelling, permeation enhancing and efflux pump inhibitory properties. For this purpose, a series of concentrations of sodium periodate was prepared to oxidize HEC leading to ring opening of glucose subunits. The resulting polymers showing varying degrees of oxidation (DO) were then conjugated with cysteamine stabilized via reductive amination. Consequently, HEC-cysteamine conjugates with increasing degree in thiolation were obtained. Since the conjugates are positively charged, potency of cytotoxicity was tested by resazurin assay. In situ gelling properties of the conjugates were studied to investigate change of their viscosity due to inter- and/or intramolecular crosslinking via disulfide bonds. The influence of the presence of the conjugates on transport of rhodamine 123 and fluoresceinisothiocyanate-dextran 4 (FD4) representing model compounds for P-glycoprotein (P-gp) inhibition and permeation enhancing studies, respectively, across Caco-2 cell monolayers was determined. The conjugates showed a degree of thiolation in the range of 316–2158 μmol/g. Within 30 min, dynamic viscosity of the conjugate with the lowest degree of thiolation 0.5% (m/v) increased up to 300-fold. The conjugates showed a degree of thiolation-dependent increase in cytotoxicity but they all were found comparatively low cytotoxic. The addition of the conjugate with thiol group content of 1670 μmol/g resulted in the highest improvement in the transport of both rhodamine 123 and FD4 as compared to buffer control. Accordingly, the degree of thiolation strongly influences the properties of the conjugates and the modulation of the degree of thiolation could be exploited for development of various drug delivery systems.

1. Introduction

Over the last decade, chemically modified polymers have been extensively investigated for their use in the field of drug delivery systems. In particular, polymers exhibiting permeation enhancing and efflux pump inhibitory properties have been developed. These polymers have gained much interest for drug delivery systems due to their capability to improve bioavailability of formulated drugs.

Oral bioavailability of drugs can be modulated by transporters at the absorption site along the gastrointestinal tract (Wacher et al., 1998). Thereby, one of the major obstacles involved in limiting drug absorption is the presence of multidrug resistance (MDR) transporter P-glycoprotein (P-gp) which pumps drugs out from the intestinal mucosa into the intestinal lumen (Shen et al., 2006). Moreover, the nature of drugs such as molecular size and charge also influence the capability of drugs to be transported across intestinal mucosa via paracellular pathway. In case of peptides, their molecular weight is more important for the transport than their charge (Pauletti et al., 1997). Various drugs have been incorporated in polymers displaying P-gp inhibition and permeation enhancing capability in order to improve their oral bioavailability.

The use of thiomers to overcome poor oral bioavailability of drugs has been extensively investigated. Thiomers are potential pharmaceutical excipients for inhibition of P-gp activity and opening of tight junction (Sakloetsakun et al., 2011). Thiomers derived from naturally occurring polymers such as chitosan and alginate have been successfully synthesized and have been evaluated regarding their capability to enhance oral bioavailability (Sakloetsakun et al., 2011; Miyazaki et al., 2000; Bernkop-Schnürch et al., 2001). In addition, it has been reported that they show in situ gelling properties (Miyazaki et al., 2000; Sakloetsakun et al., 2009).

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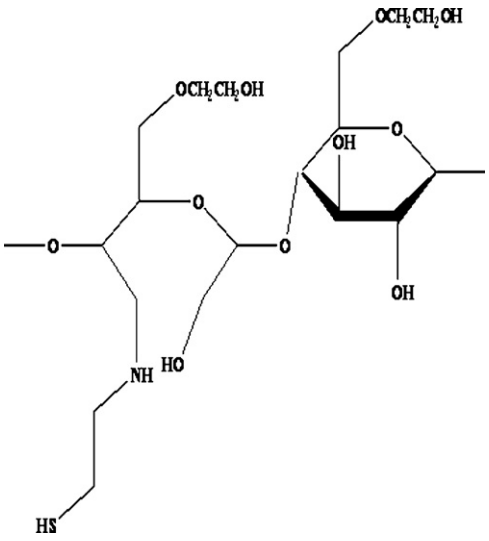

Fig. 1. HEC-cysteamine.

Recently, the thiolation of hydroxyethyl cellulose (HEC) using cysteamine as thiol bearing compound has been successfully achieved by oxidation of HEC followed by reductive amination (Rahmat et al., 2011). Thiolation of oxidized HEC with different degree of oxidation (DO) led to thiolated HEC conjugates with different degree of thiolation. In principle, a permeation enhancing effect of HEC-cysteamine conjugate (Fig. 1) has already been shown (Rahmat et al., 2011). However, it was not investigated whether this improved membrane uptake of a model compound was based on permeation enhancing or efflux pump inhibitory properties. In addition, the impact of the degree of thiolation was not evaluated.

It was therefore, the aim of the research herein presented to investigate the structure–properties relationship of a series of HEC-cysteamine conjugates. The investigation was based on the hypothesis that the degree of thiolation could influence the properties of the conjugates namely biocompatibility, in situ gelling, permeation enhancing and efflux pump inhibitory properties.

2. Material and methods

2.1. Material

2-(*N*-Morpholino)ethanesulfonic acid (MES hydrate), trinitrobenzensulfonic acid (TNBS), cysteamine, ethylene glycol, *t*-butyl carbazate, rhodamine 123, fluoreceinisothiocyanate-dextran 4 (FD4), sodium periodate, sodium borohydride, sodium cyanoborohydride, dialysis tubing cellulose membrane (molecular weight cut-off of 12 kDa), triton X-100, penicillin and streptomycin were obtained from Sigma–Aldrich. Fetal bovine serum (FBS) and Minimum Essential Medium (MEM) with Earle's salts were purchased from PAA laboratories, whereas hydroxyethyl cellulose (~145 mPa S 1% in H_2O at 20 °C, molecular weight: ~250,000) was obtained from Fluka, Buchs, Switzerland. Pluronic P85 was donated by BASF Corp. (USA).

2.2. Oxidation of HEC

Briefly, increasing amounts (0.4–1.6 g) of sodium periodate were added to 300 mL of 1% (m/v) aqueous unmodified HEC solution. The reaction mixtures were incubated in the dark for 24 h at room temperature under constant stirring. The reaction was stopped by addition of 400 μL of ethylene glycol and then left for 1 h at room temperature. The resulting oxidized HEC (HEC–CHO) polymers were dialyzed for 3 days against water (molecular weight cut-off of 12 kDa; dialysis tubing cellulose membrane; Sigma, St Louis, MO). Afterwards, the polymers were lyophilized by drying frozen aqueous polymer solutions (−78 °C, 0.01 mbar, VirTis, Gardiner, ME) and stored at 4 °C (Ito et al., 2007).

2.3. Aldehyde assay

The degree of oxidation (DO) of HEC–CHO is defined as the number of oxidized residues per 100 glucose residues and measured according to a method as described previously (Rahmat et al., 2011; Bouhadir et al., 1999). In brief, aqueous solutions of HEC–CHO (0.5 mL, 0.12% m/v) were reacted with t-butyl carbazate (0.5 mL, 0.01 M) in 1% (m/v) aqueous trichloroacetic acid for 24 h at room temperature. A volume of 200 μL of the mixture solution and aqueous trinitrobenzensulfonic acid (TNBS) solution (2 mL, 4 mM) in borate buffer (0.01 M, pH 8) were mixed and allowed to react for 30 min at room temperature. The absorbance of the final mixtures was measured at 334 nm.

2.4. Conjugation of aldehyde polymers to cysteamine

Aqueous HEC–CHO (with various DO) solutions were prepared in a concentration of 2% (m/v) and cysteamine was added in a final concentration of 129.62 mM to each polymer solution. The solutions were buffered at pH 5 using 0.1 M MES hydrate. The reaction mixtures were incubated under permanent stirring for 3 h. Afterwards $NaCNBH_3$ was added to the mixtures in a final concentration of 8% (m/v) and stirred for 72 h. 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. For control, the same procedure was performed but omitting $NaCNBH_3$ (Rahmat et al., 2011).

2.5. Determination of free thiol group content

The amount of free thiol groups immobilized on HEC-cysteamine conjugates were measured photometrically by using Ellman's reagent quantifying free thiol groups as described previously (Hombach et al., 2009). The disulfide content was determined using $NaBH_4$ for reduction and Ellman's reagent as described by Habeeb (1973).

2.6. In situ gelling properties of conjugates

The studies were carried out to investigate influence of in situ gelling properties on the transport of both rhodamine 123 and FD4. Thereby, the transport medium was used to dissolve the tested polymers and the incubation condition of the transport studies was applied during the course of the experimental. Briefly, HEC-cysteamine conjugates derived from HEC–CHO with various DO were hydrated in MEM without phenol red (pH 7.4) at room temperature to give a concentration of 0.2 and 0.5% (m/v). All samples were incubated in a humidified chamber at 37 °C, 5% CO_2 for 180 min and 700 μL samples were transferred immediately after each time point of incubation (15, 30, 60, 90, 120, 150 and 180 min) to a cone-plate viscometer (RotoVisco RT20, Haake GmbH, Karlsruhe, Germany). The temperature on the plate was adjusted to 37 °C. Dynamic oscillatory tests were performed within the linear viscoelasticity range. The storage modulus (G') and loss modulus

(G'') were determined at 1.0 Hz frequency. The apparent viscosity was determined at a shear rate of 50 s^{-1} (Millotti et al., 2009).

2.7. Cytotoxicity studies

Cytotoxicity studies were conducted using resazurin assay. To assess the toxicity of the tested polymers in Caco-2 cells, 1×10^5 Caco-2 cells were seeded per well in 24-well plates. Afterwards, the cells were incubated in a humidified chamber at 37 °C, 5% CO_2. The cytotoxicity of the tested polymers was evaluated at concentration of 0.2 and 0.5% (m/v). MEM without phenol red and 5% (v/v) Triton X-100 plus medium served as low and high control, respectively. After the cells were incubated with the tested polymers in MEM without phenol red for 72 h, the medium was replaced from each well with 0.5 mL of 44 μM of resazurin solution. The plate was then incubated at 37 °C, 5% CO_2 for 3 h and fluorescence was measured at 540 nm excitation and 590 nm emission wave length with a microplate fluorescence using a Spectrophotometer DU® Series 600 (Jennings et al., 2007). Viability of cells was calculated based on the following equation:

$$\text{Cell viability}\,(\%) = \frac{\text{Average absorbance value of each sample}}{\text{Average absorbance value of low control}} \times 100$$

2.8. Transport studies

Transport studies were performed across Caco-2 cells seeded at approximately $1 \times 10^5/cm^2$ in polystyrene membrane transwell plates (12 mm diameter, 0.4 μm pore size, Transwell®, COSTAR). Rhodamine 123 and FD4 were used at concentrations of 0.001 and 0.1% (m/v), respectively, as fluorescence markers. The tests were carried out using cells passages between 12 and 20. Caco-2 cells were grown in MEM with Earle's salts and L-glutamine (with phenol red) supplemented with 20% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified chamber (37 °C, 5% CO_2), changing media every second day for 15 days. To investigate that P-gp is present in Caco-2 cells, the plates were placed in a humidified chamber (37 °C, 5% CO_2) and refrigerator (4 °C), respectively. The transport of rhodamine 123 and FD4 was assessed either in medium only or in combination with unmodified HEC and HEC-cysteamine (0.2 and 0.5% m/v). A standard efflux pump inhibitor Pluronic P85 was used as positive control at the same concentration as the tested polymers. After 30 min of cells incubation, an appropriate transport medium containing either the test compounds or Pluronic P85 was added to the cells. The cells were then incubated for 180 min and 100 μL samples were taken repeatedly (0, 60, 120 and 180 min) from the acceptor chamber. Afterwards, an equal volume of transport medium was immediately added. Rhodamine 123 and FD4 content were analyzed using fluorescent spectrophotometry and compared with a standard curve (Sakloetsakun et al., 2011). Apparent permeability coefficients (P_{app}) for rhodamine 123 and FD4 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 (1.13 cm^2), c is the initial concentration of the model compounds in the donor compartment (μg/cm^3), and t is the whole time of experiments (s). Thereby, improvement ratio can be calculated as a ratio between the absorptive P_{app} of tested polymers over the absorptive P_{app} of control (Hombach and Bernkop-Schnürch, 2009).

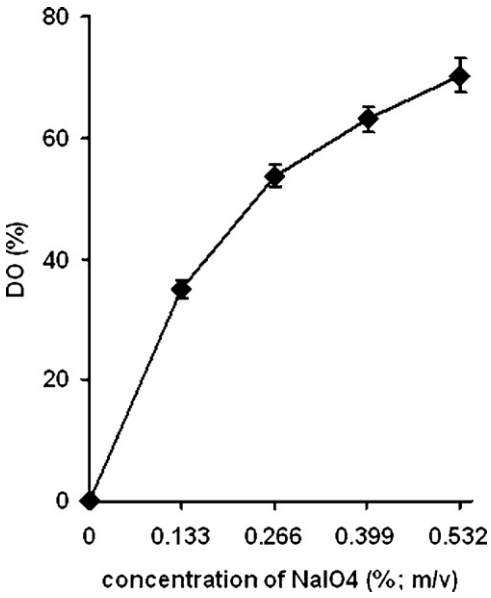

Fig. 2. DO (degree of oxidation) of HEC–CHO obtained by the reaction of 1% (m/v) unmodified HEC with varying amounts of sodium periodate. Indicated values are means of three experiments ± SD.

2.9. Statistical and data analysis

The results are expressed as means ± SD. The differences between groups were tested by student t-test with p-value < 0.05 as the minimal level of significant difference.

3. Results

3.1. Preparation of aldehyde polymer

The oxidation of HEC with increasing amounts of sodium periodate resulted in HEC–CHO polymers with increasing DO (Fig. 2). In principle, to determine DO HEC–CHO was reacted with an excess of t-butyl carbazate to form carbazones. Afterwards, the quantification of the unreacted carbazate was carried out by reaction with TNBS (Bouhadir et al., 1999). As depicted in Fig. 2, the increase in DO was not linear with the increase in the amount of added periodate. Faster oxidation of terminal residues of HEC might occur and consume comparatively higher quantity of periodate (Kristiansen et al., 2010).

3.2. Conjugation of aldehyde polymers to cysteamine

The conjugation of cysteamine to HEC–CHO was stabilized by sodium cyanoborohydride. The coupling rate depends on the pH value of the reaction mixture, the amount of cysteamine and the reaction time (Rahmat et al., 2011). The resulting polymers were white, odorless and of fibrous-like structure. All conjugates had an amount of free thiol groups in the range of 316–2158 μmol/g polymers as shown in Table 1. The degree of thiolation is significantly increased at a DO > 50%. The HEC oxidation with various amount of periodates resulted in HEC–CHO polymers with different chain flexibility properties (Kristiansen et al., 2010). It seems that at a DO > 50% the chain flexibility properties changed drastically and influenced the ability of HEC–CHO polymers to interact with cysteamine.

Table 1
Comparison of different DO of HEC–CHO utilized for thiolation of unmodified HEC.

Oxidized unmodified HEC	Thiolated HEC	Σ-SH (μmol/g)	Σ-S-S- (μmol/g)
HEC–CHO 1 (DO = 34.97%)	HEC-cysteamine 1	316 ± 31	79 ± 3
HEC–CHO 2 (DO = 53.64%)	HEC-cysteamine 2	1670 ± 79	63 ± 3
HEC–CHO 3 (DO = 63.05%)	HEC-cysteamine 3	1765 ± 78	45 ± 4
HEC–CHO 4 (DO = 70.23%)	HEC-cysteamine 4	2158 ± 106	158 ± 9

Table 2
Rheological properties of 0.5% (w/v) HEC-cysteamine 1 in MEM without phenol red (pH 7.4).

Time (min)	G' (mPa)	G'' (mPa)
0	–	94 ± 6
15	17,790 ± 989	995 ± 136
30	16,553 ± 260	1829 ± 178
60	16,290 ± 868	1064 ± 229
90	16,083 ± 875	1769 ± 215
120	20,177 ± 460	1606 ± 264
150	20,603 ± 558	1417 ± 198
180	16,350 ± 736	967 ± 94

3.3. In situ gelling properties

Only 0.5% (m/v) HEC-cysteamine 1 gellated within 15 min and showed a significant 300-fold increase in viscosity compared to the initial value within 30 min (Fig. 3). This phenomenon can be explained by an inter- and/or intramolecular crosslinking process via disulfide bond formation. In contrast, an increase in viscosity was not observed for the other conjugates. Since pKa-value of cysteamine is 8.6, negative thiolate anion ($-S^-$) was available at pH 7.4. This could result in a thiol/disulfide exchange reaction and/or an oxidation process. In addition, air oxidation could mediate a sol–gel transition of HEC-cysteamine which can be evaluated by the value of loss tangent ($\tan\delta = G''/G' < 1$ (Table 2).

3.4. Cytotoxicity studies

In these studies, a comparative in vitro cytotoxicity study of polymers with similar structure was performed using resazurin assay. The cytotoxicity of HEC-cysteamine conjugates was examined on Caco-2 cells at concentration of 0.2 and 0.5% (m/v). Resazurin is reduced to resorufin (pink color) which can be measured by fluorometer at 540 nm excitation and 590 nm emission wave length (Jennings et al., 2007; O'Brien et al., 2000).

HEC-cysteamine conjugates are positively charged polymers which are usually found to be more toxic than neutral polymers. All conjugates displayed more than 80% cell viability and the following ranking of the conjugates regarding cytotoxicity for both tested concentrations was found: HEC-cysteamine 4 > HEC-cysteamine 3 > HEC-cysteamine 2 > HEC-cysteamine 1 > unmodified HEC (Fig. 4). Amine functional groups of cysteamine can be protonated to confer positive charge on the conjugates. The higher the degree of thiolation, the more positively the polymers are charged. Since HEC-cysteamine 4 is the most positively charged, it showed the highest cytotoxicity.

3.5. Transport studies

The transport of rhodamine 123 and FD4 across Caco-2 cell monolayers at different time in the presence of unmodified HEC and thiolated HEC was tested. Results are shown in Figs. 5–7. Significant increase in apical (AP) to basolateral (BL) transport of rhodamine 123 and FD4 was observed when HEC-cysteamine conjugates derived from HEC–CHO with comparatively lower DO were tested (Table 3). Moreover, the effect of degree of thiolation and

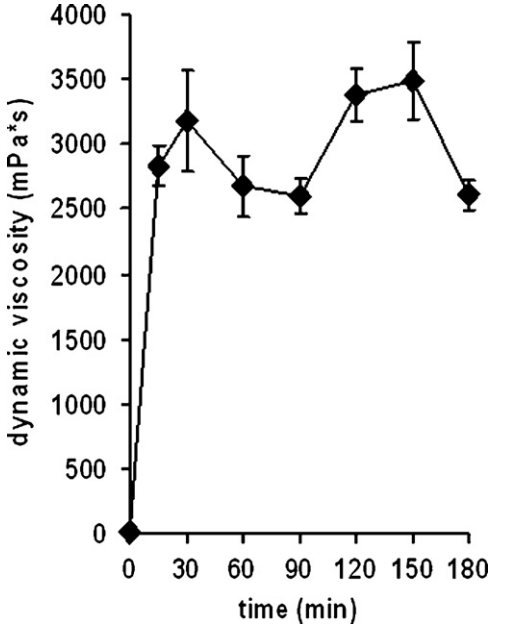

Fig. 3. Dynamic viscosity (η^*) of 0.5% (m/v) HEC-cysteamine 1 in MEM without phenol red (pH 7.4). The means ($n = 3$) and standard deviation of three experiments are shown.

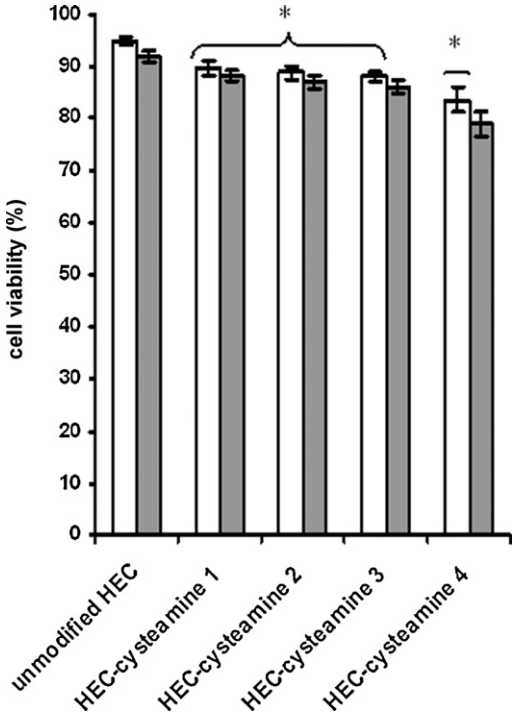

Fig. 4. Results of resazurin assay on Caco-2 cell monolayers after incubation time of 72 h with unmodified HEC and HEC-cysteamine conjugates at the concentration of 0.2% (m/v) (white bars) and 0.5% (m/v) (grey bars). Results are expressed as mean ± S.D. of three experiments. *, differs from unmodified HEC, p-value <0.05.

Table 3
Comparison of the absorptive apparent permeability coefficients (P_{app}) of rhodamine 123 and FD4 across Caco-2 cell monolayers in the presence of indicated test compounds. Each point represents the means ± SD of three experiments.

Substrate	Test compounds	P_{app} ($\times 10^{-6}$ cm/s)	Improvement ratio
Rhodamine 123	Buffer (37 °C)	1.96 ± 0.25	–
	Buffer (4 °C)	4.27 ± 0.60	2.18*
	0.2% (m/v) Pluronic P85	3.95 ± 0.57	2.02*
	0.2% (m/v) unmodified HEC	2.81 ± 0.66	1.43
	0.2% (m/v) HEC-cysteamine 1	3.76 ± 0.46	1.92*
	0.2% (m/v) HEC-cysteamine 2	4.67 ± 0.89	2.38*
	0.2% (m/v) HEC-cysteamine 3	4.26 ± 0.52	2.17*
	0.2% (m/v) HEC-cysteamine 4	3.83 ± 0.70	1.95*
	Buffer (37 °C)	2.00 ± 0.58	–
	0.5% (m/v) Pluronic P85	3.51 ± 0.29	1.76*
	0.5% (m/v) unmodified HEC	3.11 ± 1.23	1.56
	0.5% (m/v) HEC-cysteamine 1	4.54 ± 0.57	2.27*
	0.5% (m/v) HEC-cysteamine 2	7.56 ± 0.68	3.78*
	0.5% (m/v) HEC-cysteamine 3	6.13 ± 0.56	3.07*
	0.5% (m/v) HEC-cysteamine 4	5.10 ± 0.60	2.55*
FD4	Buffer (37 °C)	0.22 ± 0.04	–
	0.5% (m/v) unmodified HEC	0.21 ± 0.03	0.95
	0.5% (m/v) HEC-cysteamine 1	0.29 ± 0.05	1.32
	0.5% (m/v) HEC-cysteamine 2	0.54 ± 0.04	2.45*
	0.5% (m/v) HEC-cysteamine 3	0.42 ± 0.05	1.91
	0.5% (m/v) HEC-cysteamine 4	0.38 ± 0.08	1.73

*p-value < 0.05 compared to buffer control.

viscosity of the conjugates should be taken into account. In the presence of HEC-cysteamine conjugates, rhodamine 123 transport was up to 3.78-fold improved (Table 3).

Studies comparing the effect of well-known efflux pump inhibitor, Pluronic P85 were also performed in Caco-2 cells. The concentration of Pluronic P85 was used above its critical micelle concentration. As shown in Table 3, the transport of rhodamine 123 was increased in the presence of 0.2 and 0.5% (m/v) Pluronic P85 as compared to buffer only. Pluronic P85 demonstrated the capability of depletion of cellular adenosine triphosphate and membrane impairments as the main mechanism responsible for P-gp inhibition (Föger et al., 2006). 0.5% (m/v) Pluronic P85 showed lower potency in P-gp inhibition in comparison with 0.2% (m/v) Pluronic P85. When 0.5% (m/v) Pluronic P85 was applied, comparatively higher amount of rhodamine 123 could incorporate into the micelle core (Batrakova et al., 1998). Hence, the transport of rhodamine 123 in the presence of 0.5% (m/v) Pluronic P85 was lower than that of 0.5% (m/v) all HEC-cysteamine conjugates.

Prior the studies, the absorptive transport of 0.001% (m/v) rhodamine 123 were carried out at 37 °C and 4 °C, respectively. At 37 °C, the absorptive P_{app} was $1.96 \pm 0.25 \times 10^{-6}$ cm/s, whereas that of at 4 °C was $4.27 \pm 0.60 \times 10^{-6}$ cm/s. The absorptive P_{app} at 37 °C was

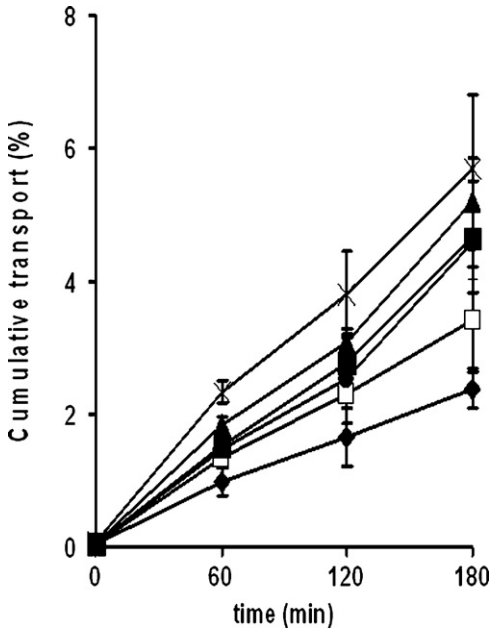

Fig. 5. The absorptive transport studies of rhodamine 123 across Caco-2 cell monolayers. Effect of 0.2% (m/v) unmodified HEC (□), 0.2% (m/v) HEC-cysteamine 1 (•), 0.2% (m/v) HEC-cysteamine 2 (×), 0.2% (m/v) HEC-cysteamine 3 (▲), 0.2% (m/v) HEC-cysteamine 4 (■) in comparison to buffer only (♦). Indicated values are the means ± S.D. of three experiments.

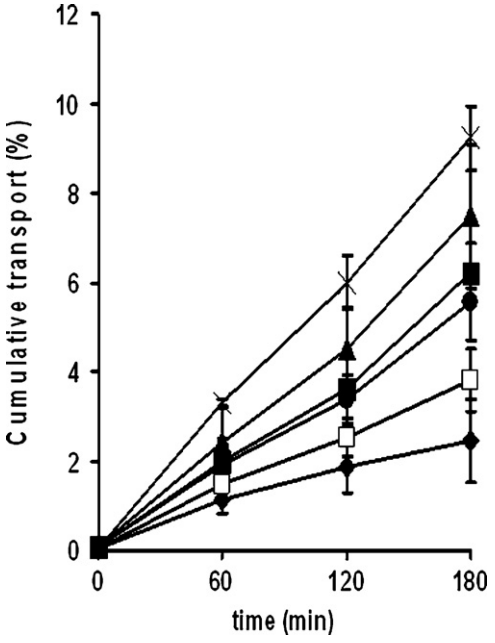

Fig. 6. The absorptive transport studies of rhodamine 123 across Caco-2 cell monolayers. Effect of 0.5% (m/v) unmodified HEC (□), 0.5% (m/v) HEC-cysteamine 1 (•), 0.5% (m/v) HEC-cysteamine 2 (×), 0.5% (m/v) HEC-cysteamine 3 (▲), 0.5% (m/v) HEC-cysteamine 4 (■) in comparison to buffer only (♦). Indicated values are the means ± S.D. of three experiments.

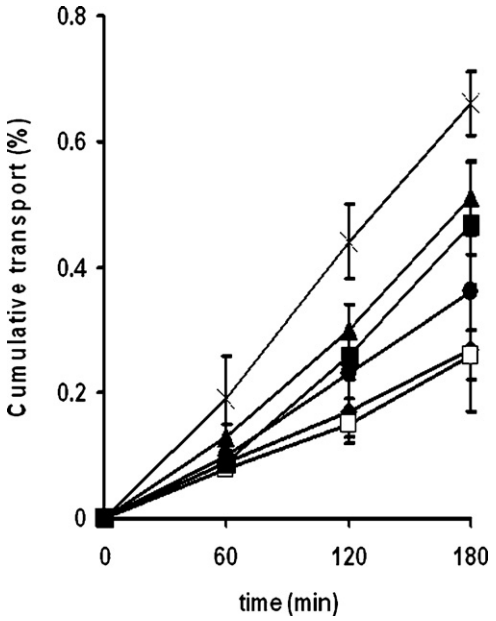

Fig. 7. The absorptive transport studies of FD4 across Caco-2 cell monolayers. Effect of 0.5% (m/v) unmodified HEC (□), 0.5% (m/v) HEC-cysteamine 1 (●), 0.5% (m/v) HEC-cysteamine 2 (×), 0.5% (m/v) HEC-cysteamine 3 (▲), 0.5% (m/v) HEC-cysteamine 4 (■) in comparison to buffer only (♦). Indicated values are the means ± S.D. of three experiments.

lower than at 4 °C as the activity of ATP dependent efflux pump was blocked at 4 °C. The transport of rhodamine 123 was therefore only based on passive diffusion at 4 °C. At 37 °C P-gp was active, thus the absorptive transport of rhodamine 123 was reduced (Varma et al., 2003).

In the presence of 0.2% (m/v) unmodified HEC, P_{app} of rhodamine 123 was $2.81 \pm 0.66 \times 10^{-6}$ cm/s corresponding to an 1.43-fold improvement over the buffer control, whereas due to the addition of 0.2% (m/v) HEC-cysteamine 1, HEC-cysteamine 2, HEC-cysteamine 3 and HEC-cysteamine 4 P_{app} was approximately 1.92-, 2.38-, 2.17- and 1.95-fold increased, respectively. In contrast, 0.2% (m/v) unmodified HEC and 0.2% (m/v) each conjugate did not show any effect on FD4 transport (data not shown).

Rhodamine 123 transports in the presence of 0.5% (m/v) unmodified HEC, HEC-cysteamine 1, HEC-cysteamine 2, HEC-cysteamine 3, HEC-cysteamine 4 was approximately 1.56-, 2.27-, 3.78-, 3.07- and 2.55-fold improved, respectively, in comparison to the buffer control. In addition, FD4 transport in the presence of 0.5% (m/v) HEC-cysteamine 1, HEC-cysteamine 2, HEC-cysteamine 3, HEC-cysteamine 4 was approximately 1.32-, 2.45-, 1.91- and 1.73-fold improved, respectively. In contrast, due to the addition of 0.5% (m/v) unmodified HEC, the transport of FD4 was not increased at all. Among HEC-cysteamine conjugates, 0.5% (m/v) HEC-cysteamine 2 led to the highest increase in transport of both fluorescence markers.

The transport of rhodamine 123 in the presence of 0.5% (m/v) HEC-cysteamine 1 was the lowest in comparison with 0.5% (m/v) the other conjugates but the effect of 0.2% (m/v) HEC-cysteamine 1 on rhodamine 123 transports was similar to that of 0.2% (m/v) HEC-cysteamine 4. This could be explained by the evidence that 0.5% (m/v) HEC-cysteamine 1 gellated within 15 min and displayed the highest viscosity. However, due to the thiolation the transport of both fluorescence markers in the presence of HEC-cysteamine 1 was still higher compared to unmodified HEC and the buffer control.

Related thiolated HEC conjugates showed a different effect on both P-gp inhibition and permeation enhancement although all these conjugates (except HEC-cysteamine 1) exhibited an amount of free thiol groups which was comparatively high and close to each other. Conformational changes might take place after thiolation (oxidation and reductive amination) leading to the differential capability of free thiol groups of each conjugate to interact with cell membranes. Variety in DO of oxidized HEC could impart conformational diversity to conjugates as the ring opening might influence the overall conjugate shape (Kristiansen et al., 2010).

4. Discussion

Unmodified HEC was oxidized to reactive aldehyde groups using varying amounts of sodium periodate. Cysteamine was directly coupled to the resulting polymers leading to HEC-cysteamine conjugates of various degrees of thiolation (Table 1). The conjugation between aldehyde and amine leads to the formation of a Schiff base intermediate which is rapidly reversed to corresponding aldehyde and amine by hydrolysis unless sodium cyanoborohydride is added in order to reduce the intermediate into a stable secondary amine linkage (Peng et al., 1987).

Since thiol groups on HEC-cysteamine 2, HEC-cysteamine 3 and HEC-cysteamine 4 are located closely to each other because of high degree of thiolation, they could form intrachain disulfide bonds more rapidly than more isolated thiol groups (Kafedjiiski et al., 2005; Dodou et al., 2005) as found on HEC-cysteamine 1. Therefore, only HEC-cysteamine 1 has in situ gelling properties and the highest viscosity in comparison with the other HEC-cysteamine conjugates.

The alamar blue used in cytotoxicity assay is nontoxic to the cells and reduced by the cells into resorufin (the pink fluorophor). This reduction is directly proportional to the number of viable cells (O'Brien et al., 2000). Generally, the magnitude of the cytotoxic effect of the conjugates was found to be cationic charge density-dependent, which is an important parameter for the interaction with the cell membranes. The mechanism of cytotoxicity caused by cationic polymers is not yet fully understood. It is likely that cytotoxic effects are mediated by interactions of cationic polymers with cell membranes, consequently leading to cell damage (Fischer et al., 2003).

In transport studies, structurally related polymers (HEC-cysteamine conjugates) were screened regarding P-gp inhibition and permeation enhancement. To compare the potency of each conjugate, transport studies of two fluorescence markers were performed across Caco-2 cell monolayers. Their transport was assessed via calculation of the P_{app} in the apical (AP) to basolateral (BA) direction. Inhibition of P-gp activity was measured using rhodamine 123 as a substrate of P-gp, whereas FD4 was used to investigate the effect of permeation enhancing properties of the tested polymers. As a hydrophilic macromolecule with partition coefficient of −3.41, FD4 is transported exclusively via the paracellular route across intestinal epithelia. Hence, FD4 is widely used as a fluorescent paracellular marker in Caco-2 cells (Thanou et al., 2007; Tomita et al., 2000).

Unmodified HEC and all HEC-cysteamine conjugates displayed efflux pump inhibitory properties at concentration of 0.2 and 0.5% (m/v). In contrast, significant permeation enhancing properties of the conjugates were only observed at a concentration of 0.5% (m/v). Furthermore, 0.2 and 0.5% (m/v) unmodified HEC did not show any permeation enhancing properties at all.

Since both HEC-cysteamine conjugates and chitosan bear nitrogen atoms that could render positive charges, HEC-cysteamine conjugates will likely follow the same mechanism in opening of tight junction as chitosan. Hence, HEC-cysteamine conjugates could bind to the epithelial membrane through a charge-dependent mechanism, leading to F-actin depolymerization and disbandment of the tight junction protein ZO1 (zonula occludens 1)

(Schipper et al., 1997). This permeation enhancing properties of HEC-cysteamine conjugates are likely further augmented due to the presence of free thiol groups which might be able to form disulfide bonds with the Cysteine 215 of protein tyrosine phosphatase (PTP). Thereby, the activity of the PTP is inhibited. It was reported that the PTP is capable of dephosphorylation of tyrosine residues at the loops of occludin (transmembrane protein) leading to closing of tight junctions (Clausen and Bernkop-Schnürch, 2000). In addition, HEC-cysteamine conjugates could inhibit the P-gp activity through a covalent interaction between thiol groups on the polymers and cysteine residues of P-gp (Iqbal et al., 2010).

Since there are lots of polysaccharides which have been extensively investigated as pharmaceutical excipients, conformational change via the oxidation of polysaccharides could be explored to optimize either efflux pump inhibitory or permeation enhancing properties of thiolated polysaccharides. Moreover, the conformational change could lead to identification of new mechanisms of such polymers in drug-bioavailability enhancement.

5. Conclusion

Within this study, HEC-cysteamine conjugates of various degrees of thiolation generated by oxidation and reductive amination improved the transport of model compounds as compared to the buffer control. Generally, it was assumed that the difference in both P-gp inhibition and permeation enhancing capability was due to the difference in degree of thiolation and viscosity. The conjugate with the lowest degree of thiolation demonstrated in situ gelling properties due to inter- and/or intramolecular crosslinking via disulfide bonds. Although the viability of the conjugates decreased along with increase in degree of thiolation, all the conjugates showed a comparatively low cytotoxicity. According to the achieved results, the conjugates could be a promising tool for delivery of P-gp substrates and peptide/protein drugs to the gastrointestinal tract.

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