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# Betaine-modified hydroxyethyl cellulose (HEC): A biodegradable mucoadhesive polysaccharide exhibiting quaternary ammonium substructures

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ABSTRACT

The aim of this study was to improve the mucoadhesive properties of hydroxyethyl cellulose (HEC) *via* the covalent attachment of betaine. Synthesis was carried out through esterification of HEC utilizing N-chlorobetainyl chloride. Betaine-modified HEC was characterized *via* FTIR and NMR analyses, ester quantification and zeta potential measurements. Enzymatic degradation and cell viability were also investigated. Moreover, rheological and mucoadhesive properties were evaluated. FTIR and NMR analyses confirmed the covalent attachment of betaine to HEC. Betaine-modified HEC contained 228.45±11.63 μmol/g ester bonds and its zeta potential was 0.37±0.19 mV. Enzymatic degradation studies showed the ability of lipase to cleave off betaine from HEC. Cytotoxicity studies demonstrated that betaine-modified HEC is up to a concentration of 0.3% not toxic. In comparison to unmodified HEC, betaine-modified HEC showed with mucus a 2.3- and 4-fold higher viscosity within 3 h and 6 h, respectively. Furthermore, betaine-modified HEC exhibited 23.5-fold higher mucoadhesive properties on porcine intestinal mucosa compared to unmodified HEC. In conclusion, betaine-modified HEC might be a useful biodegradable mucoadhesive polymer.

## 1. Introduction

The capability of mucoadhesive polymers to adhere to mucosal membranes provides many advantages for drug delivery including a prolonged residence time of locally acting drugs on the target mucosa and an improved systemic uptake of drugs that are administrated to mucosal membranes (Bernkop-Schnürch, 2002; Mahmood et al., 2016; Netsomboon and Bernkop-Schnürch, 2016). Several types of bonds are involved in the mucoadhesion process of polymers such as hydrogen bonds, electrostatic interactions, hydrophobic interactions and the formation of covalent bonds such as disulfide bond (Andrews et al., 2009; Grabovac et al., 2005; Leitner et al., 2003; Perrone et al., 2018; Roy et al., 2009). In order to improve the mucoadhesive properties of polymers, various ligands can be covalently attached to them providing additional bonds to the mucus gel layer. In this study, modification of polymer, namely hydroxyethyl cellulose (HEC) was carried out by forming a covalent bond with N-chlorobetainyl chloride. HEC as a non-ionic polymer exhibits weak interaction with mucin glycoproteins resulting in poor mucoadhesive properties (Khutoryanskiy, 2011; Ludwig, 2005). The adhesion mechanism of non-ionic polymer is likely based on the interpenetration followed by entanglement of polymer chains (Bernkop-Schnürch, 2002; Khutoryanskiy, 2011). Hence, modification of HEC to increase its mucoadhesive properties is a challenge to be developed. The presence of reactive hydroxyl groups in HEC facilitates modification through interactions with other materials offering benefits for their application in drug delivery. The hydroxyl groups of HEC interact with the C carbonyl of N-chlorobetainyl chloride to form an ester. Oxygen from the C carbonyl ester of betaine-modified HEC interacts with the hydrogen of the oligosaccharide hydroxyl group of mucin glycoproteins leading to the formation of hydrogen bonds resulting in the increase in mucoadhesive properties (Lommerse et al., 1997). In addition, the positive charge originating from the quaternary

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ammonium attaching to the HEC resulted in ionic interactions with the negative charge of the sialic acid and sulfonic acid substructures amplifying in enhancing their mucoadhesive properties (Griffin et al., 2016).

So far, however, the development of betaine-modified HEC through esterification leading to the increase in mucoadhesive properties and formation of environmentally friendly biodegradable materials has not been investigated. Therefore, it was the aim of this study to develop betaine-modified HEC in order to improve the mucoadhesive properties of HEC. Synthesis of betaine-modified HEC was conducted by reacting HEC and N-chlorobetainyl chloride as a reagent and pyridine was used as a catalyst prior to characterization of product including FTIR and NMR. Other properties of the betaine-modified HEC such as ester quantification as well as zeta potential were also evaluated. Degradation studies were carried out to confirm the cleavage ability of ester product by enzyme. Cytotoxicity of betaine-modified HEC was evaluated on Caco-2 cells. Furthermore, rheological and rotating cylinder studies of betaine-modified HEC were also performed to prove the effect of modification on mucoadhesive properties.

## 2. Materials and methods

### 2.1. Materials

Hydroxyethyl cellulose (MW∼90 kDa), hydroxylamine HCl and lipase (from porcine pancreas), Type II, ≥125 units/mg were supplied from Sigma-Aldrich, Germany. N-Chlorobetainyl chloride (90%) was provided by abcr GmbH, Germany. Pyridine (99%) was obtained from Thermo Fisher Scientific, Germany. Diethyl ether was supplied by VWR, Germany. Porcine intestinal mucosa was supplied from slaughterhouse, Innsbruck, Austria.

### 2.2. Methods

#### 2.2.1. Synthesis of betaine-modified HEC

HEC was esterified with N-chlorobetainyl chloride according to the method published by Sievänen et al. (2015) with some modifications. Briefly, one gram of hydroxyethyl cellulose was dispersed in 40 mL of pyridine. Subsequently, 0.4 g (2.32 mmol) of N-chlorobetainyl chloride was added to the solution. This mixture was stirred overnight at room temperature. The solution was precipitated in diethyl ether and the precipitate was washed with ether several times, followed by drying to constant weight under reduced pressure at 50 °C.

#### 2.2.2. Characterization of betaine-modified HEC

*2.2.2.1. Fourier transform infrared spectroscopy (FTIR) and H nuclear magnetic resonance ($^1$H-NMR) analysis of betaine-modified HEC.* In order to confirm the functional group and structure as well as the purity of betaine-modified HEC, FTIR and NMR analyses were performed. FTIR analyses was conducted with a Spectrum Two™ spectrometer (Perkin Elmer, Beaconsfield, United Kingdom) recording the average of four scans measured from wavenumber of 4000 to 400 $cm^{-1}$ at a resolution of 1 $cm^{-1}$. $^1$H NMR measurements were performed on a "Mars" 400 MHz Avance 4 Neo spectrometer from Bruker Corporation (Billerica, MA, USA, 400 MHz) in dimethyl sulfoxide-d6 (DMSO-$d_6$) solution. Concisely, 5 mg of dry sample was dissolved in 0.7 mL of deuterated solvent. Chemical shifts were reported in parts per million, and deuterated solvent, DMSO-*d6*, served as internal standard (δ 2.5 ppm).

*2.2.2.2. Quantification of ester substructures.* Quantification of ester substructures in the polymer was conducted based on a method previously published by Aimanant and Ziemann (2013). Concisely, 40 mg of sample was dissolved in 1 mL of water. Thereafter, 1 mL of 2.4 M KOH in 50% (v/v) ethanol and 1 mL of 2 M hydroxylamine HCl in 50% (v/v) ethanol were added. To dissolve the precipitate formed during the reaction, 0.6 mL of water was added, followed by mixing at a speed of 300 rpm at room temperature for one hour. Afterwards, 1 mL of a mixture of 5 M HCl in 50% (v/v) ethanol and 0.44 M $FeCl_3.6H_2O$ in 0.6 M HCl in ethanol (1:1) was added. Subsequently, 0.4 mL of water was added prior to be vortexed for 30 s. For ester quantification, absorbance of samples was immediately measured using a microplate reader (Tecan Spark® Multimode Microplate Reader, Switzerland) at a wavelength of 540 nm (Aimanant and Ziemann, 2013).

*2.2.2.3. Measurements of zeta potential.* Zeta potential was characterized using a Zetasizer (Nano-ZSP, Malvern P analitycal, UK). Measurement was performed for both HEC before and after modification by dispersing the sample (1:100) in water at 25 °C.

*2.2.2.4. Enzymatic degradation study of betaine-modified HEC.* Biodegradability of betaine-modified HEC was determined with pre-activated lipase in a digestive medium consisting of 10 mM tris buffer pH 7.0 containing 5 mM $CaCl_2$ and 150 mM NaCl. Briefly, one gram of lipase was added to 10 mL of digestive medium followed by centrifugation at a speed of 13,400 rpm at 4 °C for 20 min. Subsequently, the supernatant was stored at 4 °C for further use. On the day of experiment, 60 mg of sample was dissolved in 3 mL of digestion medium with a pH of 7.0 having been adjusted with 0.1 M NaOH prior to the addition 3 mL of lipase solution. The mixture was incubated at 37 °C for ester cleavage by lipase resulting in a pH drop in the reaction mixture. In order to maintain the pH at 7.0, titration with 0.1 M NaOH was conducted at several predetermined time points for 360 min. Incubation was continued for up to 24 h to determine the betaine content in the sample. The amount of NaOH required was equal to the release of betaine. As negative control served unmodified HEC (Shahzadi et al., 2020).

#### 2.2.3. Cytotoxicity studies of betaine-modified HEC on Caco-2 cells

Cytotoxicity studies of betaine-modified HEC were carried out using resazurin assay. Caco-2 cells were cultivated in media containing red minimum essential medium (red MEM) by feeding the cells every two days. Cell culture was conducted in an incubator containing 5% of $CO_2$ at 37 °C. After the cell monolayer was formed for about 14 days, cells were rinsed with sterile HBS (HBSS), followed by incubating the cells using HBSS at 37 ºC for 30 min. Thereafter, the buffer was exchanged with 500 μL of sample dispersion in HBSS and incubated at 37 ºC. As positive and negative controls Triton-X and HBSS were used, respectively. After 4 h of incubation, samples were withdrawn and cells were washed utilizing HBSS prior to the addition of 250 μL of 2.2 mM resazurin solution. Subsequently, cells were incubated at 37 °C for 3 h. Fluorescein intensity of samples was quantified at an excitation and emission wavelength of 540 nm and 590 nm, respectively, using a microplate reader (Tecan Spark® Multimode Microplate Reader, Switzerland).

#### 2.2.4. Rheological studies of betaine-modified HEC

Rheological properties of betaine-modified HEC were evaluated by mixing the polymer with purified intestinal mucus. Mucus supplied from a slaughterhouse was collected from porcine small intestine prior to be purified with 0.1 M NaCl. The purification process was performed as follow, one gram of mucus was added to 5 mL of 1 M NaCl followed by slowly stirring at 4 °C for one hour. Afterwards, mucus was centrifuged at a speed of 12,000 rpm at 4 °C for two hours prior to the collection of the precipitated mucus. This process was repeated once more, followed by rheological analyses using a cone-plate Haake Mars rheometer (Thermo Scientific, Vienna, Austria). Briefly, 500 mg of mucus was mixed with 250 μL of 1% polymer solution including betaine-modified HEC or unmodified HEC as a control in 50 mM phosphate buffer (PB) pH 6.8 prior to incubation at 37 °C for 0, 3 and 6 h. Thereafter, the dynamic viscosity of each sample was evaluated by measuring in the

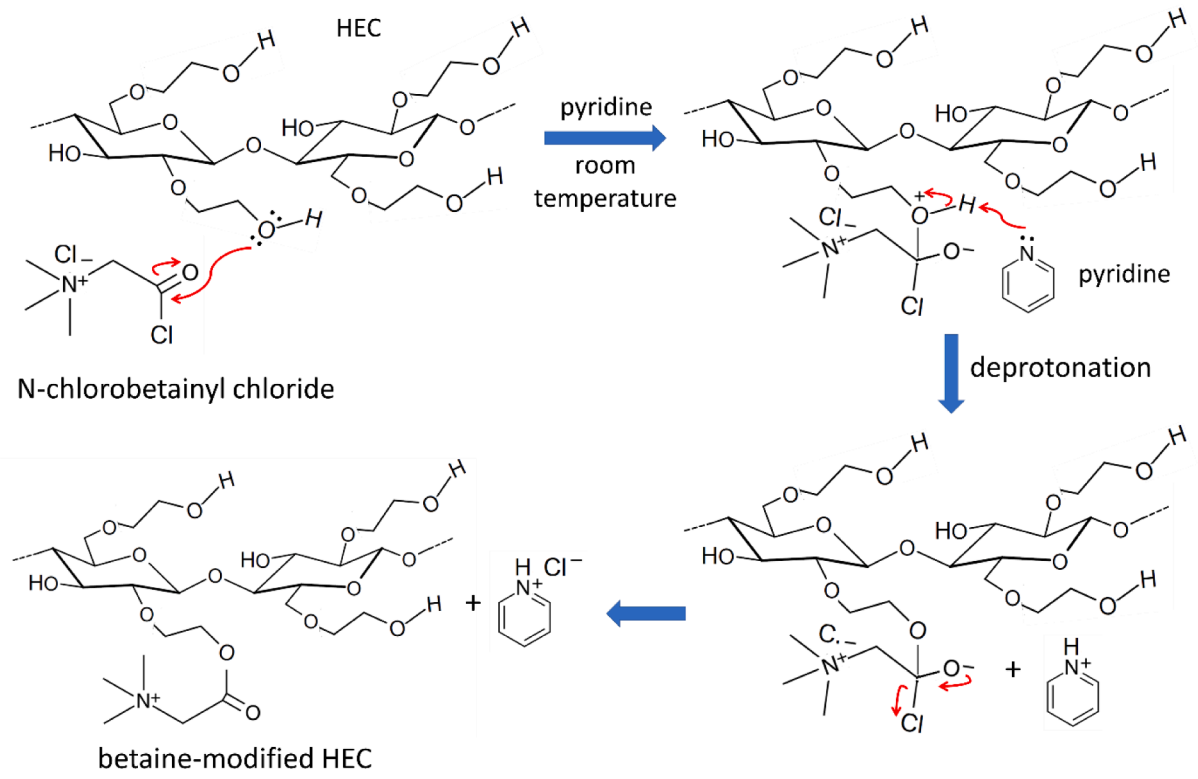


**Fig. 1.** Schematic pathway for the preparation of betaine-modified HEC. The synthesis was performed with the reaction between hydroxyethyl cellulose (HEC) and N-chlorobetainyl chloride in the presence of pyridine as a catalyst at room temperature.

shear rate range of 0.01–50.0 Pa at a frequency of 1 Hz. The experiment was conducted for mucus omitting polymer, unmodified HEC as well as betaine-modified HEC (Fürst et al., 2019; Marschütz and Bernkop-Schnürch, 2002).

#### 2.2.5. Rotating cylinder studies of betaine-modified HEC

For the purpose of rotating cylinder studies, the test disks were prepared by compressing the polymer utilizing a single punch eccentric press (Paul-Otto Weber, Germany). Each disk contained the polymer with the addition of 20% Avicel in order to obtain a compact disk. The disks were formed under a pressure of 11 kN for 60 s of compression with weight and diameter of 30 mg and 5.0 mm, respectively. Mucoadhesion study was carried out utilizing the rotating cylinder method adopted from Bernkop-Schnürch and Steininger (2000) with slight modification. Succinctly, the porcine intestine with a size of 4 cmx6 cm was attached to a stainless-steel cylinder using glue with mucosa facing up, followed by holding it in the dissolution tester (Erweka, Germany). The disks including control and modified polymer were attached on the surface of the intestinal mucosa before being introduced into the dissolution medium consisting of 900 mL of 50 mM PB pH 6.8. The experiment was performed at a cylinder rotation speed of 50 rpm at 37 °C. The time required for the disk to detach and/or erode completely from the intestinal mucosa was recorded (Bernkop-Schnürch and Steininger, 2000).

#### 2.2.6. Statistical data analysis

ANOVA was used to analyse the effect of polymer concentration on cell viability and to compare the viscosity of mucus and polymer mixtures after a predetermined time of incubation. Independent sample t-test was used in the rotating cylinder study to compare the mucoadhesive properties between betaine-modified HEC and the unmodified one as a control.

## 3. Results and discussion

### 3.1. Synthesis and characterization of betaine-modified HEC

Cationic hydroxyethyl cellulose development is a challenge due to its ability to provide various benefits in biomedical applications. Cationic hydroxyethyl cellulose can be prepared by reacting the hydroxyethyl cellulose with quaternary ammonium providing positive charges. Previous study exhibited the synthesis of cationic HEC *via* modification of HEC with glycidyl trimethyl ammonium chloride (GTAC) under alkaline conditions with the addition of NaOH. The synthesis was carried out through a reaction between the epoxy group of quaternary ammonium GTAC with the hydroxyl group of HEC resulting in an increase in the hydrophilicity of polymer (Wang and Ye, 2010). Others types of cationic HEC are polyquaternium-10-cellulose and polyquaternium-4-cellulose, called PC-10 and PC-4, respectively (Samal et al., 2012). PQ-10 is a quaternary amine modified hydroxyethyl cellulose providing a wide range of solubility including water, alcohol as well as non-polar organic solvents and has the ability to increase viscosity as well as bioadhesivity. The preparation of PQ-10 was conducted through the reaction between HEC and trimethyl ammonium substituted epoxide. PQ-10 is commonly used in cosmetics such as in make-up and hair care. In addition, PQ-10 was also applied in drug delivery, namely efavirenz as an HIV treatment through the formation of a polyelectrolyte complex (PEC) between humic acid and PQ-10 where PQ-10 acts as a swelling agent (Samal et al., 2012; Siyawamwaya et al., 2016). In PQ-10, the quaternary ammonium is attached to the end of the PEG chain, while in PQ-4 the position of the quaternary ammonium is directly linked to the cellulose backbone. PQ-4 is prepared based on the reaction between HEC and diallyldimethyl ammonium chloride. In previous study, both PQ-10 and PQ-4 were used as carriers of plasmid DNA (Fayazpour et al., 2006).

In this study, synthesis of cationic HEC was performed via the formation of ester HEC in order to improve its mucoadhesive properties. N-chlorobetainyl chloride was used for the formation of betaine-modified

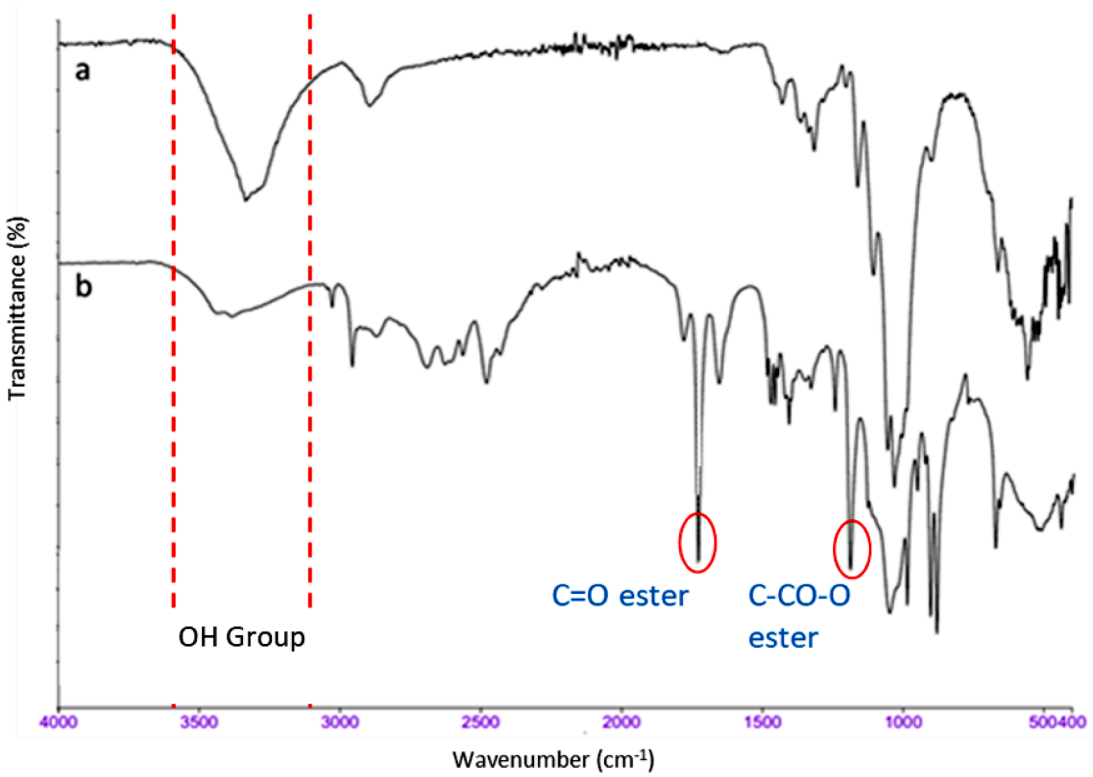


**Fig. 2.** FTIR of (a) unmodified hydroxyethyl cellulose (HEC) and of (b) betaine-modified HEC. After modification with N-chlorobetainyl chloride, there are strong and sharp peaks of betaine-modified HEC at 1732 $cm^{-1}$ and 1250 $cm^{-1}$ indicated the C=O carbonyl and C-CO-O asymmetric stretching of ester, respectively.

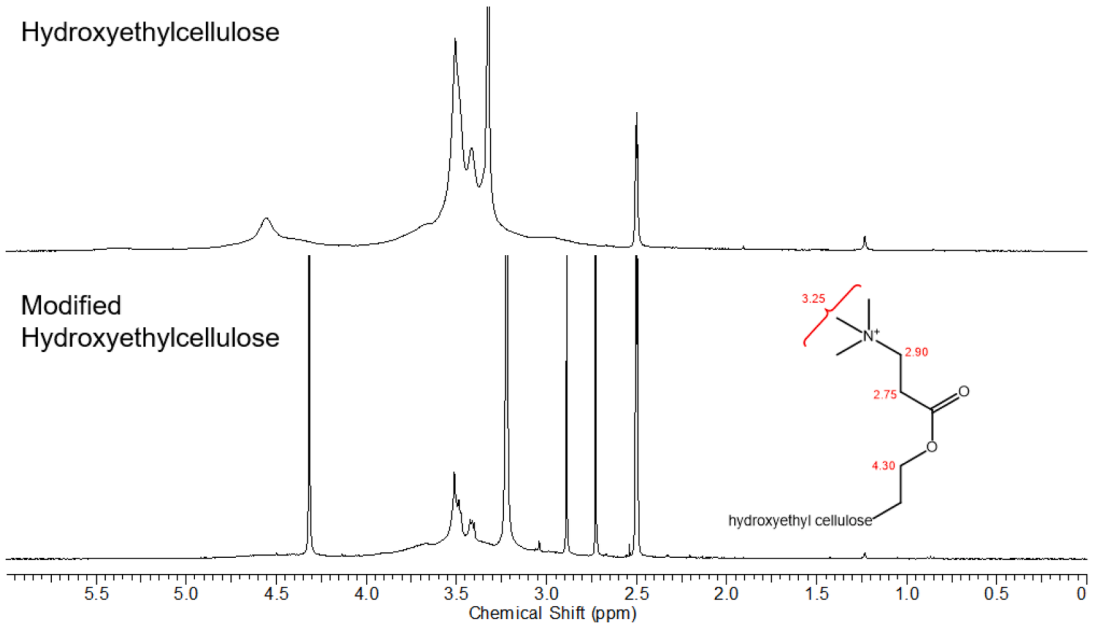


**Fig. 3.** 400 MHz $^{1}$H-NMR spectrum of hydroxyethyl cellulose (top-view) and betaine-modified hydroxyethyl cellulose (down-view) in DMSO-*d6*.

HEC. The C carbonyl of N-chlorobetainyl chloride interacted with the oxygen of HEC hydroxyl groups in the presence of pyridine as a catalyst for chloride release resulting in the formation of betaine-modified HEC as shown in Fig. 1. The hydroxyl groups of HEC could be either directly bound to the C ring of HEC or from the OH of ether alkyl side chain of HEC.

In order to confirm the betaine-modified HEC formation after the reaction between HEC and N-chlorobetainyl chloride, analyses using FTIR and NMR were carried out. FTIR analyses showed a different spectrum of betaine-modified HEC compared to the unmodified one. As shown in Fig. 2, HEC provide the broader peak at a wavelength of 3400 $cm^{-1}$ compared to betaine-modified HEC due to the reduction of HEC hydroxyl groups as a consequent of the ester formation between HEC and N-chlorobetainyl chloride. This was in line with the strong and sharp peak of betaine-modified HEC at 1732 $cm^{-1}$ as a peak of C=O carbonyl ester indicating the formation of an ester after the reaction

**Table 1**
Characterization of betaine-modified HEC including ester quantification and zeta potential measurements. The ester content was quantified by hydrolysing the ester to produce a carboxylic acid which is equivalent with the amount of ester. Zeta potential was measured by dispersing the sample (1:100) in water at 25 °C.

| Materials | Ester content(μmol/g) | Zeta potential(mV) |
|---|---|---|
| Unmodified HEC | – | -0.7 ± 0.14 |
| Betaine-modified HEC | 228.45 ± 11.63 | 0.37 ± 0.19 |

which was not found in the spectrum of unmodified HEC. In addition, a sharp peak at about 1250 $cm^{-1}$ indicated the C-CO-O asymmetric stretching from the ester group (Günzler and Hans-Ulrich, 2002). The $^{1}H$ NMR spectrum of the product, presented in Fig. 3, showed a new signal at around 4.30 ppm, for the methylene protons next to the ester bond, an intense signal at 3.25 ppm for the methyl groups of the quaternary ammonium, and two chemical shifts at 2.75 and 2.90 ppm, for the methylene protons of the dangling betaine moieties. Based on FTIR and NMR spectrum, the formation of betaine-modified HEC through ester formation was confirmed.

The success of the synthesis of betaine-modified HEC was also confirmed with the ester quantification as shown in Table 1. The amount of ester in the product could be quantified *via* the hydrolysis of esters using hydroxylamine hydrochloride as a catalyst in an alkaline reaction environment to produce carboxylic acids forming a complex with $FeCl_3$ (Kapoor et al., 1988). The release of carboxylic acids is equivalent to the amount of esters. Another characterization of betaine-modified HEC was based on zeta potential measurements as depicted in Table 1. After esterification of HEC, there was a change in zeta potential from negative to positive.

Enzymatic degradation studies illustrated in Fig. 4 showed that about 80% of betaine is cleaved from HEC by lipase within 6 h. In the first 30

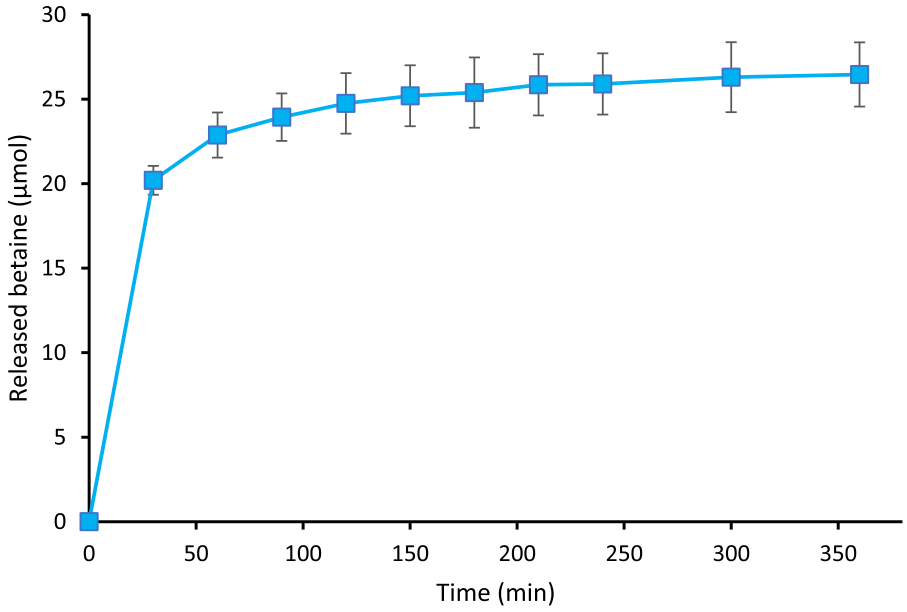


**Fig. 4.** Enzymatic degradation studies of betaine-modified HEC (10 mg/mL). Biodegradation studies were conducted using pre-activated lipase (6250 U/mL) in a digestive medium consisting of 10 mM tris buffer pH 7.0 containing 5 mM $CaCl_2$ and 150 mM NaCl. Data are presented as mean ± SD ($n$=3).

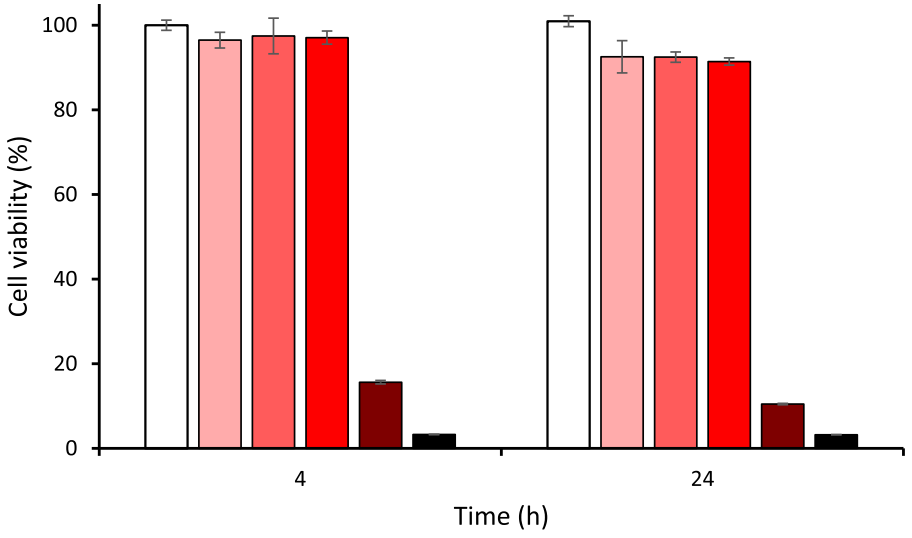


**Fig. 5.** Cell viability of Caco-2 cells incubated with betaine-modified HEC in 24 well plates for 4 h and 24 h. White, very light red, light red, red, dark red and black bars represent negative control, 0.1%, 0.2%, 0.3%, 1% of samples and positive control, respectively. Data are presented as mean ± SD ($n$=3).

min, the release was very rapid, i.e., about 40 micromoles/h, followed by a slow first-order release from one hour to 6 h at about 0.6 micromole/h. This two-steps time course of ester hydrolysis by lipase was in agreement with previous studies (Knezevic et al., 1998; Lykidis et al., 1995). The HEC produced by this hydrolysis process is a substrate for lysozyme, a common endogenous depolymerase enzyme (Callewaert and Michiels, 2010). HEC glycosidic bond cleavage was studied previously in our research group published by Leonaviciute and co-workers, revealing that with 3 h of incubation with lysozyme at a concentration of 500 U/ml, about 60% of HEC was completely degraded to glucose, the HEC monomer (Leonaviciute et al., 2016). A bioprocess combining 1 h incubation of betaine-modified HEC with lipase as carried out in this study and 3 h with lysozyme to degrade the polymer is likely faster than trimethyl chitosan (TMC) biodegradation. Since TMC contains neither an ester functional group nor an ester derivative, lipase cannot degrade TMC. However, its O-glycosidic bonds can be cleaved by lysozyme. TMC depolymerization using lysozyme was previously evaluated by Verhaul et al., using 40 kDa TMC at an enzyme concentration of 794 U/ml. After 4 h of incubation, this biodegradation process slightly reduced the molecular weight of the polymer to an average of 35 kDa. The degradation process was still incomplete even though the incubation time was extended to about 120 h, as indicated by the molecular weight of the remaining TMC polymer which was around 30 kDa (Verheul et al., 2009).

### 3.2. Cytotoxicity of betaine-modified HEC

Betaine-modified HEC cytotoxicity studies as shown in Fig. 5 revealed for polymer concentration ≤0.3% cell viability of more than 96% and 91% for incubation times of 4 h and 24 h, respectively. Increasing the concentration up to 1% showed a significant decrease ($p<0.05$) in cell viability i.e., only 15.6% and 10.5% for 4 h and 24 h incubation, respectively. Therefore, samples up to a concentration of 0.3% were not toxic as indicated by a cell viability of >85% (López-García et al., 2014). Betaine-modified HEC exhibited less cytotoxicity in studies on Caco-2 cells compared to TMC. Study on the cytotoxicity test of quaternary ammonium modified chitosan, namely TMC, showed that the degree of quaternization (DQ) and polymer concentration had a significant impact on cell survival where increasing DQ led to an increase in toxicity at all levels of solution concentration (Verheul et al., 2008).

The concentration of quaternary ammonium in the tested solution, which is determined by DQ and the sample concentration, has a significant impact on cytotoxicity. The toxicity of TMC on Caco2 cells showing the DQ of 33% was compared to betaine-modified HEC in this study. TMC solution prepared at 0.17 mM quaternary ammonium concentration showed 75% cell viability after 2.5 h incubation (Verheul et al., 2008) indicating that TMC was toxic (<85%) (López-García et al., 2014), whereas betaine-modified HEC at a higher quaternary ammonium concentration of 0.23 mM, even at an incubation time of 24 h, showed a cell viability of 96%. In addition, TMC also exhibited cytotoxicity in tests with other cells such as HEK23 and L929 with the incubation time of 24 h at quaternary ammonium concentrations of 0.1 and 0.2 mM, respectively. The results showed that the test with HEK23 and L929 cells provided cell viability of 60% and 65%, respectively (Li et al., 2010). The toxicity of cationic polymers may be due to the positive charge in the polymer molecules leading to the interactions with cells. The higher the DQ, the higher the number of positive TMC charges that can bind to the negative charge of the cell (Kean et al., 2005).

Another cationic polymer also showed high toxicity, namely poly (ethylene imine) (PEI), a cationic polymer for gene delivery. In order to compare its cytotoxicity with betaine-modified HEC, the concentration of ammonium in the tested solution determined by the amine content in the polymer and the degree of protonation should be considered. In branched PEI solutions, ammonium ions are produced by protonation of primary, secondary and tertiary amine groups. Linear PEI containing only secondary amine, about 55% of amine group is protonated under physiological conditions (Ziebarth and Wang, 2010), whereas branched PEI containing not only secondary amine but also primary and tertiary amines, degree of protonation is determined by the degree of branching. It was reported that a commercial 10 kDa branched PEI was protonated no more than 50% at physiological pH (Nagaya et al., 1996). PEI was more toxic than betaine-modified HEC in studies using Caco2 cells, since in the viability test of PEI solution at a concentration of 0.004% which is equivalent to an ammonium concentration of 0.4 mM, about 68% of cells were still alive during 24 h incubation (Rufino et al., 2021), whereas betaine-modified HEC in 0.7 mM ammonium concentration showed 91% cell viability. The toxicity of PEI was also tested against other tumor cell lines including 4T1 and A549. PEI solution at a concentration of 0.01% showed high toxicity, i.e., more than 90% of the cells died after 48 h of incubation (Xin et al., 2017). In addition, PEI also showed cytotoxicity in mammalian cell, for example L929 where 0.01% PEI solution killed more than 95% of cells at 24 h incubation time (Fischer et al., 2003). The positive charge of PEI is responsible for the

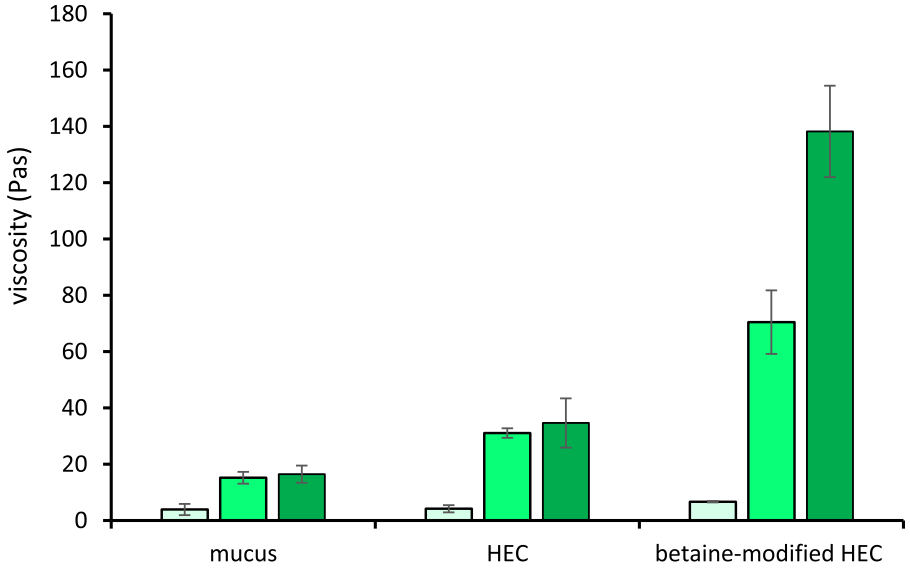


**Fig. 6.** Dynamic viscosity of mucus mixed with 50 mM phosphate buffer pH 6.8 or with indicated 2% polymer solutions in the same buffer after 0 h (light green bar), 3 h (green bar) and 6 h (dark green bar) of incubation time at 37 °C. Data are presented as mean ± SD ($n$=3).

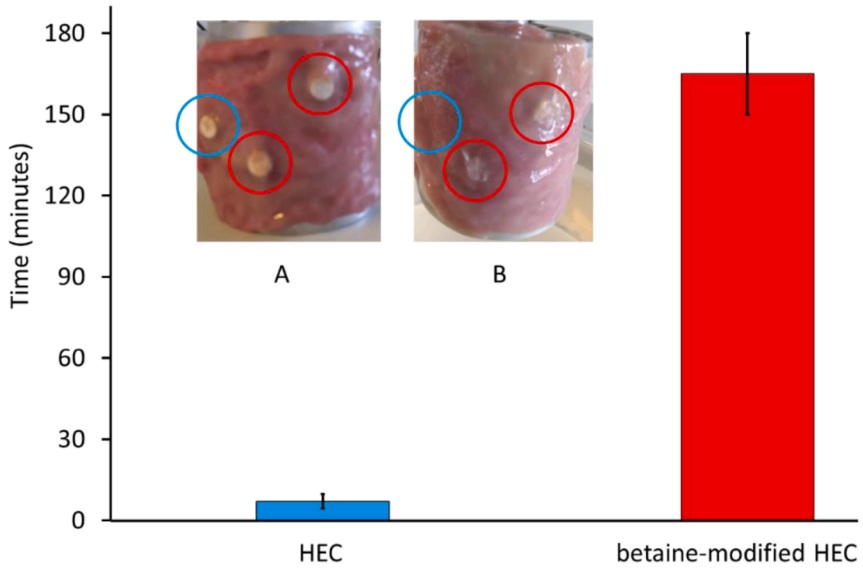


**Fig. 7.** Adhesion time of 30 mg disks of unmodified HEC and betaine-modified HEC on intestinal porcine mucosa using rotating cylinder method. The cylinders were immersed in 50 mM phosphate buffer pH 6.8 and rotated at 50 rpm. Fig. 7A and B indicated the performance of disks containing unmodified HEC (blue circle) as well as betaine-modified HEC (red circles) at time point 0 and after 3 h of rotation, respectively. Data are presented as mean ± SD ($n$=3).

electrostatic interaction with the cell membrane leading to an increase in cytotoxicity. A more positive zeta potential leads to a higher charge density resulting in the enhancement of cytotoxicity. In the case of solution, the molecular weight and flexibility of the PEI chains affect the cytotoxicity, whereas in the dispersion of PEI nanoparticles, the additional properties such as particle size and zeta potential greatly affect the cytotoxicity (Kunath et al., 2003).

### 3.3. Rheological measurement of betaine-modified HC

Attachment of materials containing quaternary ammonium substructures to the polymer leads to the formation of a modified polymer providing polyelectrolytes imparting typical properties due to the presence of positive charges attaching on the polymer backbone. This modified polymer impact on the formed interaction with other substances, biological membrane as well as its solubility especially in aqueous medium (Dizman et al., 2006; Jaeger et al., 2010). The attachment of quaternary ammonium substructures namely N-chlorobetainyl chloride as performed in this study provides benefits including (1) low toxicity and biodegradable properties, (2) preferable mucoadhesive properties compared to unmodified HEC, as shown in the following rheological and rotating cylinder studies.

Rheological properties of betaine-modified HEC and porcine intestinal mucus mixtures were evaluated in order to investigate the mucoadhesive properties of this polymer. The increase in viscosity of modified polymer with mucus characterizes the mucoadhesive properties of the polymer, namely the greater the viscosity, the greater the mucoadhesive properties (Caramella et al., 1994; Mortazavi et al., 1992; Rossi et al., 1995). The high viscosity of betaine-modified HEC can be explained by interactions between the polymer and intestinal mucus including electrostatic interactions and hydrogen bond formation as shown in Fig. 8. Although the difference of zeta potential between unmodified and betaine-modified HEC was low, electrostatic interactions between the positive charge of quaternary ammonium substructures of betaine-modified HEC with the negative charge of sialic and sulphonic acid of mucins are responsible for this increase in viscosity. In addition, the hydrogen bonds formation between oxygen of ester with the hydroxyl group of mucins dominated the enhancement of viscosity (Mortazavi, 1995). At time point zero, the viscosity of all samples including only mucus, unmodified HEC as well as betaine-modified HEC was almost the same, whereas the longer the incubation time was the more viscosity of the polymer with mucus mixture increased. As shown in Fig. 6, the viscosity of betaine-modified HEC was significantly higher ($p<0.05$) than that of unmodified HEC as a control with a 2.27- and 4-fold increase at 3 h and 6 h, respectively. When using mucus only no change in viscosity at 3 h and 6 h was observed.

### 3.4. Rotating cylinder study of betaine-modified HEC

The results from the rotating cylinder study were in agreement with rheology studies, in which disks containing betaine-modified HEC showed a significantly ($p<0.05$) longer 23-fold adhesion time compared to the unmodified HEC as a control., as depicted in Fig. 7. Disk containing unmodified HEC detached rapidly from the surface of the intestinal mucosa, whereas disk with betaine-modified HEC adhered properly to the mucosa and detached mainly due to erosion. Quaternary ammonium substructures on betaine-modified HEC are responsible for the change of zeta potential to positive contributing to the increase in mucoadhesive properties.

The synthesis of cationic polymers was also carried out previously by replacing the hydroxyl group of the starch glucose monomer with an amine group to produce a primary amine (Jelkmann et al., 2019). This product also contains carbonyl oxygen because the amination reaction leaves an aldehyde group, a potential hydrogen acceptor group for the formation of hydrogen bonds. In contrast to betaine-modified HEC which is completely ionized to produce ammonium groups, the cationic state in amine-modified starch is produced by protonation of primary amines. The structural similarity of amine-modified starch to glucosamine polymer supports the assumption that less than 20% of amines are protonated under physiological conditions (Burgos-Díaz et al., 2021). The higher increase of mucoadhesion time in betaine-modified HEC compared to amine-modified HEC was 23:7, explaining that electrostatic interactions also play a role in mucus interactions in addition to hydrogen bond formation.

Furthermore, betaine-modified HEC provided stronger hydrogen bonds than unmodified HEC. Polymers containing hydroxyl groups form hydrogen bonds with hydroxyl groups of mucin oligosaccharides playing an important role in mucoadhesion (Serra et al., 2009). The formation of an ester group in betaine-modified HEC causes the addition of two types of oxygen, namely ester and carbonyl oxygens. These two

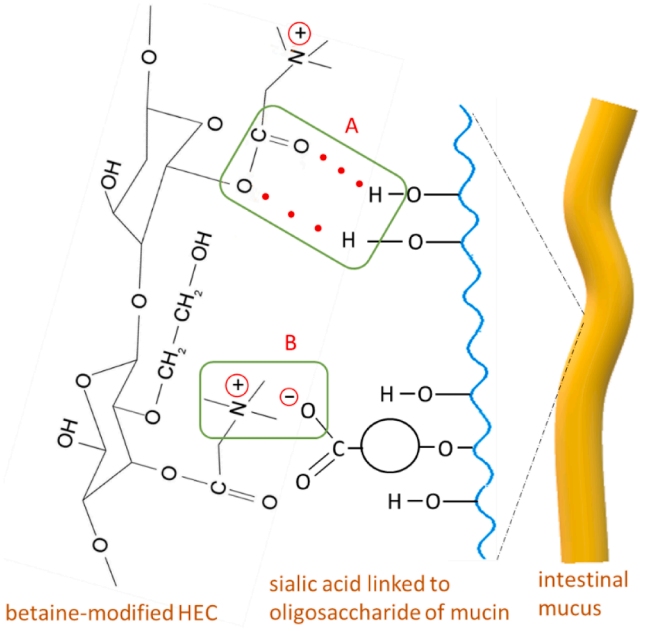


**Fig. 8.** Illustration of mucoadhesive mechanism of betaine-modified HEC. (A) Hydrogen bonds formation between two types of oxygen, namely ester as well as carbonyl oxygens and hydroxyl groups of mucin glycoproteins. (B) Electrostatic interactions between the positive charge of quaternary ammonium substructures of betaine-modified HEC and the negative charge of sialic acid of mucins.

types of oxygen in the ester functional group exhibit hydrogen acceptor properties that form hydrogen bonds with the hydroxyl groups of mucus oligosaccharides as shown in Fig. 8 (Lommerse et al., 1997). To compare the strength of hydrogen bonds formed by unmodified HEC and betaine-modified HEC with the hydroxyl groups of oligosaccharides, two parameters were used, namely length and angle of hydrogen bonds. The more linear and shorter, the stronger the hydrogen bonds (Steiner, 2002). Hydrogen bonds formed between two hydroxyl groups in a compound, for example in sucrose, show an average length and angle of about 2.093 Å and 167.8°, respectively (Lu et al., 2018). The hydrogen bond angle formed from the interaction of carbonyl oxygen (C=O), ether or ester (based on Cambridge Structural Database (CSD) data collection) as a hydrogen acceptor with aliphatic hydroxyl alcohol as a donor is in the range of 167°–180°, slightly more linear than the hydrogen bond of sucrose. In addition, the hydrogen bond length is in the range of 1.8–2.0 Å, lower than sucrose. Both properties are responsible for stronger hydrogen bond interactions also explaining the higher mucoadhesive properties of betaine-modified HEC (Lommerse et al., 1997).

## 4. Conclusion

Development of betaine-modified HEC is a potential strategy to improve the mucoadhesive properties of HEC. The success of the synthesis was indicated by the presence of ester groups and positive zeta potential in the betaine-modified HEC. The ability of betaine-modified HEC to be degraded by lipase and its non-toxic properties make this polymer feasible to be developed. Furthermore, betaine-modified HEC exhibited higher mucoadhesive properties compared to the unmodified HEC due to hydrogen bond formation and electrostatic interactions between betaine-modified HEC and mucin glycoproteins. Therefore, this modified polymer might be suitable for use as a carrier in mucoadhesive drug delivery systems.

## CRediT authorship contribution statement

**Nuri Ari Efiana:** Methodology, Investigation, Writing – original draft, Visualization, Writing – review & editing. **Gergely Kali:** Methodology, Investigation, Visualization. **Andrea Fürst:** Investigation. **Aida Dizdarević:** Visualization. **Andreas Bernkop-Schnürch:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

## Data availability

Data will be made available on request.

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