European Journal of Pharmaceutics and Biopharmaceutics 139 (2019) 161–167

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Covalently binding mucoadhesive polymers: N-hydroxysuccinimide grafted polyacrylates

Claudia Menzel[a], Moritz Hauser[a], Amelie Frey[a], Max Jelkmann[a], Flavia Laffleur[a], Sina K. Götzfried[b], Ronald Gust[b], Andreas Bernkop-Schnürch[a,*]

[a] Center for Chemistry and Biomedicine, Department of Pharmaceutical Technology, Institute of Pharmacy, University of Innsbruck, Innrain 80/82, 6020 Innsbruck, Austria

[b] Center for Chemistry and Biomedicine, Department of Pharmaceutical Chemistry, Institute of Pharmacy, University of Innsbruck, Innrain 80/82, 6020 Innsbruck, Austria

A B S T R A C T

Aim: The aim of the study was to establish a novel type of covalently mucus-binding polymers by targeting selectively amino groups within mucus glycoproteins.

Methods: N-Hydroxysuccinimide (NHS) was attached to carboxylic groups of polyacrylic acid (PAA). The reaction was mediated by the coupling reagent N,N′-dicyclohexylcarbodiimide (DCC) achieving polymeric NHS esters being able to form amide bonds with free amino groups. The chemical structure of the obtained conjugates was characterized via FTIR- and UV spectroscopy. Reactivity towards mucosal amino groups was evaluated UV spectrometrically upon addition of L-glycine. Furthermore, tensile force evaluations on intestinal mucosa as well as rheological experiments with mucus were performed in order to prove mucoadhesive potential.

Results: Depending on the amount of NHS added to the synthesis, coupling rates of 876 to 1820 μmol NHS per gram polymer were obtained. Kinetic studies of amide bond formation showed a substrate dependent reaction velocity. Rheological synergism of PAA-NHS was proven by a 7.9-fold increased mucus viscosity compared to the control polymer. In further mucoadhesion studies PAA-NHS showed a 5.5-fold improved adhesion time compared to unmodified PAA. Tensile force evaluation confirmed these results with a 1.7-fold higher maximum detachment force (MDF) and 2.7-fold increased total work adhesion (TWA) for PAA-NHS compared to the unmodified control polymer.

Conclusion: The results of the present study provide strong evidence that coupling NHS to polymers could be a promising tool for the development of novel mucoadhesive excipients.

1. Introduction

Mucoadhesive drug delivery systems possess the ability to prolong the contact time between mucosal tissues and dosage forms facilitating improved uptake of drugs [1]. During the last decades of research in the field of mucosal drug delivery, various mucoadhesive auxiliaries have been introduced. Mucoadhesive polymers interacting or binding to mucus glycoproteins showed great potential as mucoadhesive excipients. In particular, polymers that are able to form covalent bonds with mucus showing much higher adhesive properties compared to polymers that interact with the mucosa just via hydrogen bonding, ionic or lipophilic interactions have entered the limelight of research. The most prominent representatives of this group are polymers bearing thiol groups on their backbone, so-called thiomers forming disulfide bonds with cysteine-rich subdomains of mucin, the main component of mucus [2]. As they interact with sulfhydryl groups and/or disulfide bonds, however, they are inappropriate for the application of drugs bearing these substructures, such as captopril, tiopronin, omapatrilat and desmopressin just to name a few. Furthermore, unprotected thiomers are oxidized at pH values above 5. This can result on the one hand in a loss of reactivity towards mucus glycoproteins and on the other hand in an in-situ increase in viscosity preventing the polymer from penetrating the mucus gel layer and reaching the underlying tissue [3].

Therefore it was the aim of this study to introduce an alternative covalent binding mucoadhesive polymer without these mentioned shortcomings. Besides thiol groups of cysteine-rich subdomains, mucus bears also amino groups on lysine and arginine substructures of mucin glycoproteins [4]. The objective was to target these amino groups by a novel type of covalently mucus-binding polymers being able to form amide bonds with these substructures. This was realized by coupling N-hydroxysuccinimide to the polymeric backbone of polyacrylic acid as illustrated in Fig. 1. In the present study, mucoadhesive properties of NHS grafted polymers have been tested for the first time. Polymeric NHS esters have already been applied for wound closing applications [5–7]. However, they have never been investigated as excipients for mucosal drug delivery systems. Due to the esterification, reactivity of

* Corresponding author.
E-mail address: andreas.bernkop@uibk.ac.at (A. Bernkop-Schnürch).

https://doi.org/10.1016/j.ejpb.2019.02.017
Received 15 December 2017; Received in revised form 21 February 2019; Accepted 21 February 2019
Available online 19 March 2019
0939-6411/

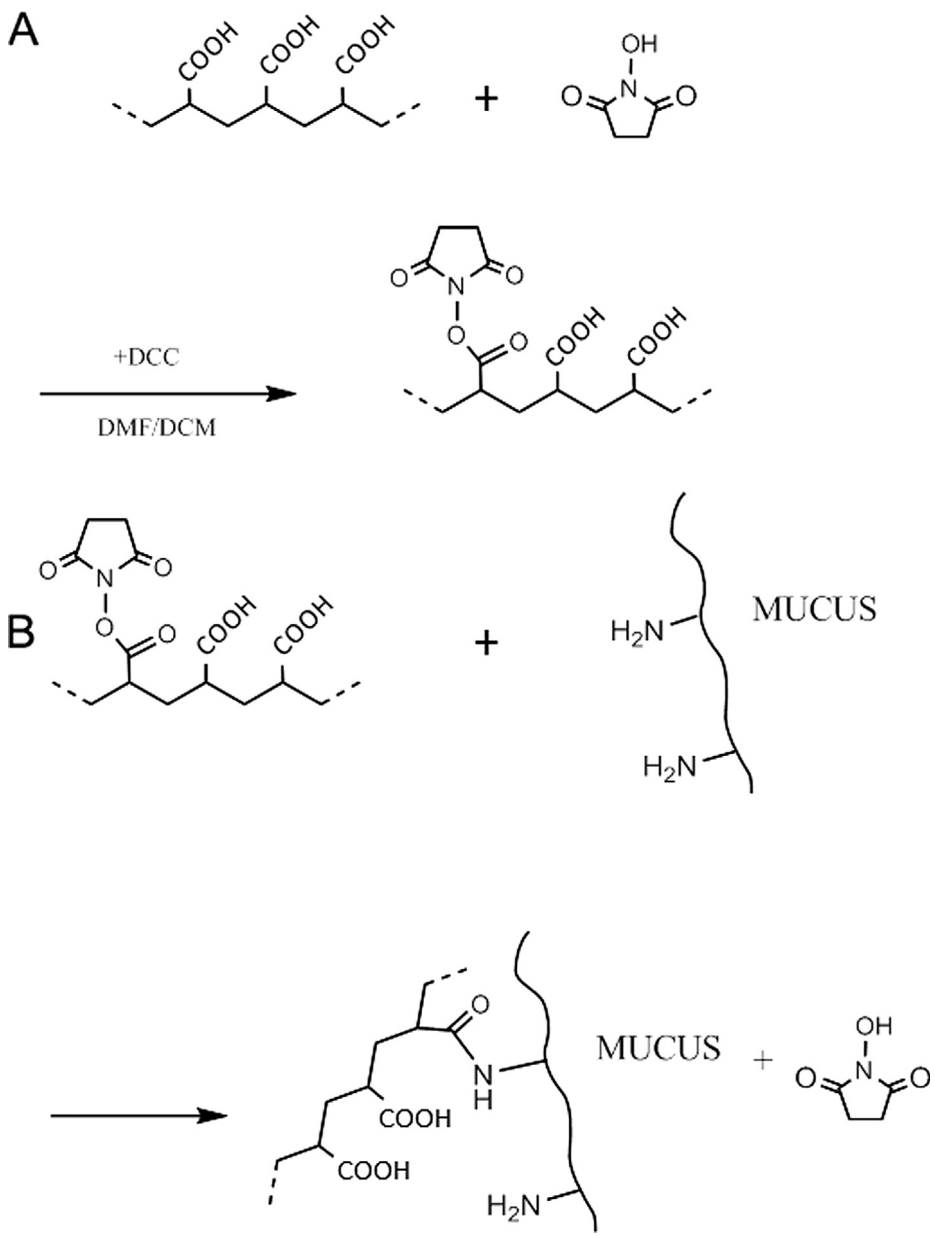

Fig. 1. (A) Synthetic pathway for the generation of PAA-NHS. (B) Reaction of PAA-NHS with amino groups of mucus glycoproteins via amide bond formation.

polymeric carboxylic groups towards mucosal amino groups is strongly increased facilitating covalent attachment under physiological conditions [8]. The chemical structure of this synthesized polymer was analyzed in order to prove successful coupling of the ligand. The reactivity towards amino groups was evaluated photometrically. Furthermore, physicochemical and mucoadhesive properties as well as the influence on cell viability were investigated depending on different coupling rates.

2. Materials and methods

2.1. Materials

Dichloromethane and dimethylformamide were obtained from VWR chemicals (Radnor, PA, USA). Aceton was purchased from Donauchem GmbH (Vienna, Austria). Minimum essential medium (MEM) was purchased from Biochrom AG (Germany). Polyacrylic acid (450 kDa), N-hydroxysuccinimide, 2,4,6-trinitrobenzenesulfonic acid (TNBS), L-glycine, dicyclohexylcarbodiimide (DCC), resazurin sodium salt, Triton™ X-100, all buffers and salts were purchased from Sigma-Aldrich (Vienna, Austria).

2.2. Methods

2.2.1. Synthesis of PAA-NHS

NHS was attached to the polymeric backbone of PAA (450 kDa) mediated by DCC used as coupling reagent. First, 1 g of the polymer was dissolved in dichloromethane (DCM)/dimethylformamide (DMF) (1 + 1) under ice cooling. Then, 2.06 g of DCC and 1.15 g of NHS for a lower coupling rate and 4.13 g of DCC and 2.30 g of NHS for a higher coupling rate were predissolved separately in DCM/DMF (1 + 1). DCC was added dropwise to the reaction mixture and 5 min later NHS was added in the same manner. After stirring for 1 h under ice cooling the reaction mixture was further stirred at 22 °C for 12 h. During the reaction, DCC forms dicyclohexylurea (DCU) which is insoluble in most organic solvents and precipitates in the reaction mixture. DCU was then removed by filtration and the organic solvents were evaporated under reduced pressure. The obtained product was subsequently purified by

washing several times with acetone and then with DCM and dried under vacuum.

2.2.2. Characterization of polymeric NHS esters

The degree of modification of PAA-low NHS and PAA-high NHS was determined photometrically based on a method described previously [9]. First, 1 mg of each polymer was dissolved in 2 ml of phosphate buffer (50 mM, pH 6.8). Then, 200 µl of 1.0 M NaOH were added to each test sample and absorption was measured at 260 nm. Quantification of NHS was evaluated by means of a NHS calibration curve.

FTIR studies were carried out with PAA, PAA-high NHS and NHS using a Perkin Elmer Spectrum 100 ATR-IR spectrometer (Perkin Elmer, Waltham, MA) in an arrangement with the software version 6.3.1.0134 (Perkin Elmer). Spectra were recorded with 10 scans in a wave number range from 4000 to 650 cm^{-1} and a resolution of 1 cm^{-1}. Measurements were performed at 22 °C [10].

The polymer's ability to react with amino acids was tested by adding L-glycine to PAA-high NHS in a ratio of 5:1 (NHS:NH_2) in 50 mM phosphate buffer pH 6.8 followed by reaction with 2,4,6-trinitrobenzenesulfonic acid and detection at 340 nm in order to quantify unreacted amino acid [2].

2.2.3. Assessment of cell viability

Effects of polymers on cell viability were examined via resazurin assay on Caco-2 cells. The test was performed as described previously and is based on the conversion of resazurin, which is blue and non-fluorescent, into its red and fluorescent form resorufin [11,12]. Cells were treated with 0.5% (m/v) test solutions of PAA, PAA-low NHS and PAA-high NHS, negative control (MEM without phenol red) and positive control (1% (m/v) Triton X-100). The incubation time of cells with polymer solutions was 24 h. Afterwards cells were incubated with resazurin solution for 3 h and the supernatant was subsequently analyzed at 540 nm with background subtraction at 590 nm using a fluorimeter (Tecan infinite, M200 spectrometer, Grödig, Austria).

2.2.4. Rheological measurements

Rheological investigations were performed with PAA, PAA-low NHS and PAA-high NHS with and without the addition of porcine intestinal mucus. The mucus was obtained from a freshly slaughtered pig by scratching it off from the small intestinal tissue. Purification and homogenization were performed by addition of 5 ml 0.1 M sodium chloride solution per gram mucus followed by stirring at 10 °C for 1 h. The mixture was in the following centrifuged at 4 °C and 13,000*g* for 2 h. After discarding the supernatant, the mucus was separated from granular material, which sedimented at the bottom of the centrifugation tube. Measurements were performed with a plate-plate viscometer (RotoViscoTM 158 RT20, Haake GmbH, Karlsruhe, Germany). Therefore, 300 µl of 4% (m/v) solutions (50 mM phosphate buffer, pH 6.8) were combined with 1 g of porcine mucus. Controls of polymer solutions without mucus were mixed with 1 g of 50 mM phosphate buffer pH 6.8. After incubation times of 30 min and 120 min, 750 µl of the samples were transferred to the plate of the viscometer and the dynamic viscosity was measured at a frequency of 1 Hz and a sheer stress range of 0.01 Pa * s–20 Pa * s. The rheological parameters were calculated by subtracting the particular reference value from each measured viscosity of the particular mucus-polymer mixtures.

2.2.5. Preparation of test discs

Samples each polymer (PAA, PAA-low NHS and PAA-high NHS) were lyophilized at −30 °C and 0.01 mbar (Christ Beta; Germany) (primary drying conditions of 23 h, beginning with 3 h at −30 °C, augmenting to −15 °C for 3 h , up to −10 °C for 2 h, following 17 h at 0 °C; secondary conditions of 8 h, starting with 1 h at + 20 °C and 7 h at + 25 °C) and finally stored at 4 °C until further use. Lyophilisates of each polymer (PAA, PAA-low NHS and PAA-high NHS) were compressed into flat faced discs of 30 mg and 5.0 mm diameter using a single punch excentric press (Paul Weber, Remshalden-Grünbach, Germany). During the manufacturing process the compaction pressure of 10 kN was kept constant. The test discs were stored under dry conditions until further use.

2.2.6. Rotating cylinder

In addition to the rheological measurements, the attachment time of discs on the mucosa was evaluated using the rotating cylinder method as described previously by our research group [13]. In brief, discs were attached to a piece of porcine intestinal mucosa which was fixed on a stainless steel cylinder. The cylinder was placed in a dissolution tester (Erweka GmbH, Heusenstamm, Germany) according to the European Pharmacopeia (Paddle Apparatus). The vessels were filled with 900 ml of a 50 mM phosphate buffer, pH 6.8, at 37 °C. Then, the cylinders were rotated with a speed of 100 rpm and the detachment of the test discs was observed visually.

2.2.7. Tensile force evaluation

Tensile studies were performed with freshly excised porcine intestinal mucosa obtained from a local abattoir. The mucosal tissues were cut into pieces of approximately 2 cm^2 and fixed on the bottom of a beaker that was placed on a balance. Test discs were fixed to a stainless steel flat cylinder (8 mm in diameter, 0.3 g of weight in the system) that was hung from a stand with a thread. Then, the balance was carefully raised until the mucosa came into contact with the tablet. After 20 min incubation, the platform was lowered at a rate of 0.1 mm/s until the tablet was completely detached from the mucosa. The maximum detachment force (MDF) and the total work of adhesion (TWA) were calculated from the obtained data.

2.2.8. Water uptake studies

The swelling behavior of polymer discs was analyzed by determining the water-uptake capacity gravimetrically as described previously [14]. In brief, discs were fixed on a needle, weighed and incubated in 50 mM phosphate buffer pH 6.8 at 37 °C. At predetermined time points the discs were taken out of the medium and weighed again.

2.2.9. Reactivity

For reactivity studies PAA-NHS was predissolved in phosphate buffer (50 mM, pH 6.8) in a final concentration of 1 mg/mL contributing to a concentration of 1800 µmol NHS groups per mL. In order to investigate the kinetic of amide bond formation in presence of amino groups, L-glycine was added in a final concentration of 0.9 µmol/mL. This ratio of interacting groups was chosen according to a previous study [15]. Immediately after the addition of L-glycine, the absorption was measured photometrically at a wavelength of 260 nm in order to detect released NHS due to amide bond formation. Furthermore, the same experiment was performed with an excess of amino acid of 4.5 µmol/mL.

2.2.10. Statistical data analysis

The software Sigma Plot version 12.3 was used for the statistical data analysis. One way ANOVA and Bonferroni *t*-test were performed with $P < 0.05$ as the minimal level of significance.

3. Results and discussion

3.1. Synthesis and characterization of PAA-NHS

The mucoadhesive polymer was generated by attaching NHS to the polymeric backbone of PAA via a carbodiimide mediated esterification in anhydrous environment (Fig. 1). The obtained conjugates turned out slightly yellow, odorless and of fibrous structure. Depending on the amount of NHS and DCC used in the reaction, different coupling rates could be obtained: The coupling rate of the lower modified PAA conjugate was 876 ± 23 µmol and of the higher modified conjugate

1820 ± 48 µmol per gram polymer.

The reaction of PAA with NHS can be confirmed by FTIR spectroscopy. The polyacrylic acid displays typical C—H stretching vibrations at about 2940 cm^{-1}. The carboxyl group is characterized by a wide OH stretch from 3176 to 2583 cm^{-1}, which superimpose the C—H stretching bands. The C═O stretch of the carboxylic acid appears at 1699 cm^{-1} those of C—O exists at 1235 cm^{-1}. In the spectra of NHS characteristic bands are 3107.13 cm^{-1} (O—H), 2975.29 and 2867.78 cm^{-1} (C—H), 1677.29 cm^{-1} (C═O) and the out of plane bending band of the OH-group at 647.65 cm^{-1}.

PAA-low NHS and PAA-high NHS show nearly identical spectra. However, the still detectable broad OH band of PAA-low NHS is an indication of a higher number of free carboxylic groups. On the other hand, this band is not detectable for PAA-high NHS and shows that most COOH groups are esterified with NHS. With this reaction a characteristic shift of the C═O vibration from 1699.99 to 1729.98 cm^{-1} takes place. The binding of NHS is further confirmed by the signals for the CH2-groups at 2926.73 and 2848.72 cm^{-1}, which are shifted to lower frequencies upon esterification. Furthermore, at 1623.35 cm^{-1} the amide I (C═O—stretching) band is detectable. Interestingly, a new peak at 3320.88 cm^{-1} arises, which could not be assigned to a characteristic group frequency at the moment.

Quantification of bound NHS was performed based on a method described by Miron and Wilcheck [9]. After hydrolyzation and release of NHS, the absorbance maximum of the molecule shows a bathochromic shift and can be detected at 260 nm. The polymer's ability to react with amino acids was tested by the addition of an excess amount of L-glycine to PAA-high NHS followed by quantification of unreacted amino acid. Results revealed that 41% of activated carboxylic groups reacted with the amino groups of L-glycine under amide bond formation confirming the assumption that the polymeric NHS esters are able to form covalent bonds with mucus.

3.2. Assessment of cell viability

Fig. 2 shows the results of cell viability studies. After 24 h, cell viability was significantly affected by PAA, PAA-NHS and PAA-NHS ($P < 0.05$). Safety of PAA was confirmed by numerous studies. No significant difference in cell viability between PAA, PAA-low NHS and PAA-high NHS could be observed after 24 h. The results from this study suggest that modification of the polymers with NHS does not result in

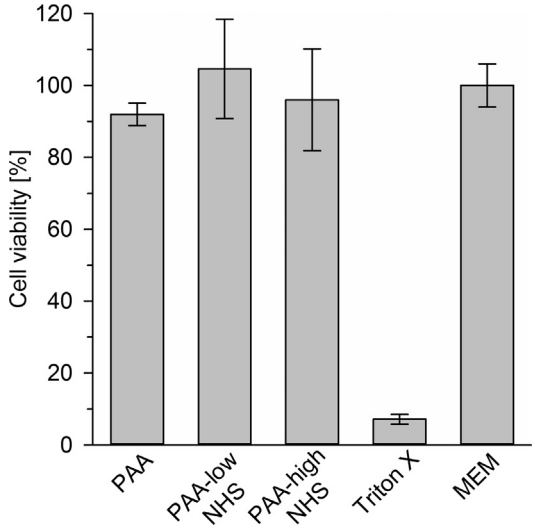

Fig. 2. Cell viability. The histogram shows influence on cell viability of PAA, PAA-low NHS, PAA-high NHS and TritonX in [%] referred to cells treated with Minimum essential media (MEM). Indicated values are means ± standard deviation of at least six experiments.

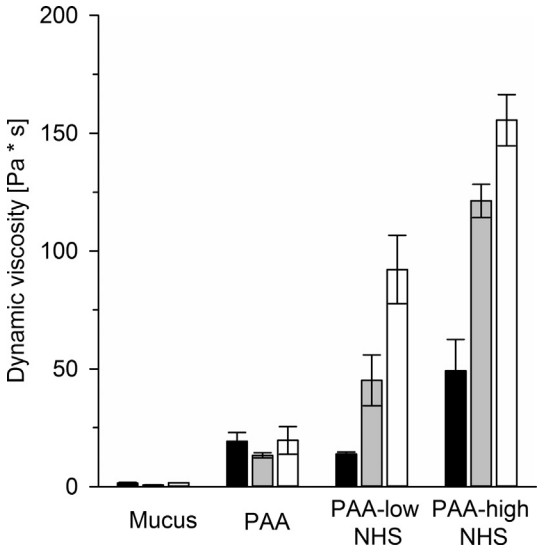

Fig. 3. Dynamic viscosity of mucus mixed with 50 mM phosphate buffer pH 6.8 or with indicated polymer solutions in the same buffer after 1 min (black), 1 h (grey) and 3 h (white) of incubation time at 37 °C. Values show the mean of at least three measurements and the standard deviation.

any increase in cell toxicity compared to the unmodified control polymer. In situ released NHS also does not show any toxic effects as shown from published data [16]. Additionally, NHS does not have any acute, mutagenic, teratogenic or carcinogenic effects according to the safety data sheets based on CLP regulations and provided by the manufacturer. Hence, polymeric NHS ester can be considered safe in their application as drug carrier systems.

3.3. Rheological investigations

Rheological synergism proved to be a reliable parameter to assess the mucoadhesive potential of polymers [17,18]. The ability of a polymer to interact with mucus glycoproteins leads to increased viscosity due to formation of a polymer-mucus network structure [19]. These interactions occur in form of ionic and lipophilic interactions or the formation of covalent bonds. Thiomers form this network building disulfide bonds with cysteine-rich substructures of mucus [2]. Polymeric NHS-esters, on the other hand, are able to form covalent amide bonds with lysine and arginine subdomains of mucin glycoproteins. As can be seen in Fig. 3, unmodified PAA did not show a significant increase in viscosity over the testing period. This has also been observed by former studies and can be explained by repulsive forces between the negatively charged carboxylic groups and the negative net charge of mucus avoiding the formation of a polymer-mucus-network. On the other hand, a rheological synergism could be observed for polymer mucus mixtures of both polymeric NHS esters. After 2 h of incubation mixtures of PAA-low NHS with mucus showed a 4.7 higher dynamic viscosity and mixtures of PAA-high NHS with mucus an even 7.9-fold increased viscosity compared to the control polymer ($P < 0.05$). These results confirm the assumption that polymeric NHS esters are able to form gel-like structures with mucin. Furthermore, a correlation between the coupling rate and mucoadhesive potential could be observed. Another potential mechanism could be the increased lipophilicity of NHS-esters compared to the unmodified polymer. Even the part of activated groups that does not react with mucus under amide bond formation could thereby increase the mucoadhesive interaction possibilities by lipophilic interactions with fatty acid substructures [20]. In contrast to thiomers, the rheological synergism of polymeric NHS esters is solely based the direct interactions between polymer and mucus. Unlike thiomers, NHS esters do not undergo intramolecular oxidation reactions. This is on the one hand advantageous as they do not lose

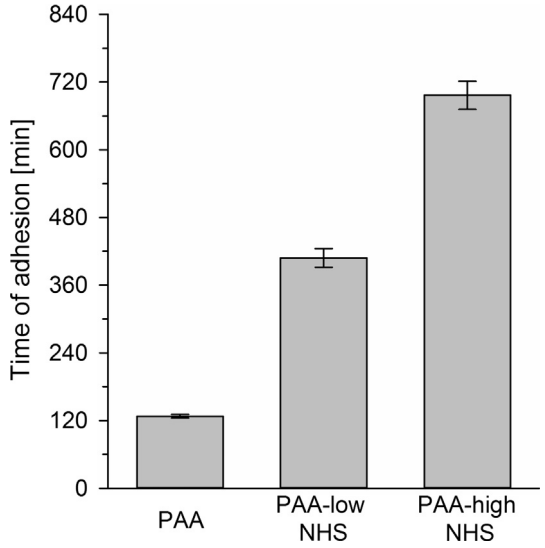

Fig. 4. Rotating cylinder. Mucoadhesive properties of PAA, PAA-low NHS and PAA-high NHS. The time of adhesion on freshly excised porcine intestinal mucosa was determined via rotating cylinder in 50 mM phosphate buffer pH 6.8. The graph shows the means of at least three experiments ± standard deviation.

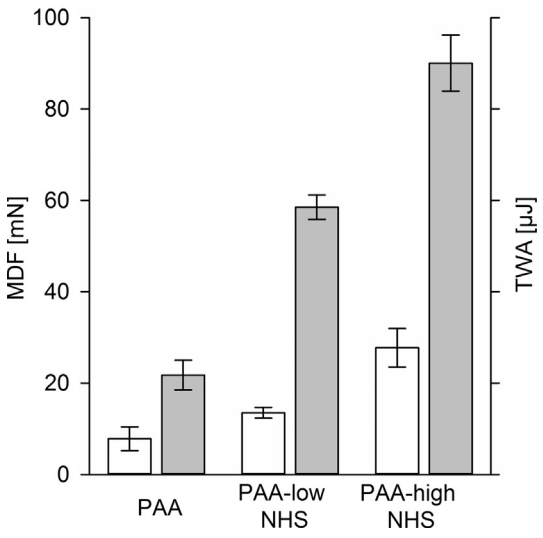

Fig. 5. Tensile studies. The graph displays the mucoadhesive properties of PAA, PAA-low NHS and PAA-high NHS, determined via tensile studies. White bars show the maximum detachment force (MDF) and grey bars represent the total work of adhesion (TWA). Values are means ± standard deviation of at least three experiments.

their mucoadhesive potential in aqueous environment, but bears on the other hand the downside, that the polymers do not build an intramolecular network. As the amide bond formation is a non-reversible reaction under physiological conditions, the mucoadhesive interactions last for a whole mucus turnover cycle. In contrast, thiomer-mucus interactions in form of disulfide bonds are reversible and can be reduced in situ by glutathione, which makes them probably less stable.

3.4. Rotating cylinder

In this study the adhesion time of test discs to the small intestinal porcine mucosa was evaluated in order to investigate cohesive as well as adhesive properties of polymeric NHS esters depending on their degree of modification. The main advantage of this experimental setup is a close in vivo/in vitro correlation allowing investigation of cohesive as well as adhesive properties of polymers [21]. Several former studies showed a correlation between the adhesion time of compressed polymeric tablets and the polymers mucoadhesiveness [21,22]. Results revealed a significant enhancement in mucosal adhesion time for polymeric NHS esters (Fig. 4). PAA-low NHS tablets showed a 3.2-fold prolonged retention time and tablets of PAA-high NHS an even 5.5-fold improved adhesion time compared to unmodified PAA ($P < 0.05$). The significant positive correlation between mucoadhesion and the amount of attached ligand was also observed for thiomers [23]. All tablets converted into a shapeless jelly mass during the experimental period proving swelling properties for both NHS derivatives. As tablets are applied directly, the pH value of the mucosal surface is particularly important as the reactivity of NHS esters and hence the amide bond formation has its optimum in the alkaline pH range. On the other hand, the rate of hydrolysis is also increased with increasing pH values. According to the literature, NHS esters have a half-life of 4–5 h at pH 7, 1 h at pH 8 and only 10 min at pH 8.6 [24,8,25]. On the other hand, the pH optimum for the amide bond formation is in the range of 7–9, as the formation of a negatively charged nucleophile is needed for the reaction. Depending on the small intestinal segment the pH gradually increases from pH 5 to about pH 7.4 offering an appropriate environment for the reaction in favor of amide bond formation vs hydrolysis [26]. Moreover, mucus shows a transverse pH gradient from the luminal site of the mucosa to the epithelial cell layer where pH is almost neutral [27]. Due to this condition, polymeric NHS esters might penetrate the more acidic parts of the mucus layer on the luminal site and form covalent amide bonds in more basic environment as soon as they have reached the apical epithelium. This could be an advantage towards thiomers building covalent disulfide bonds even under mild acidic conditions at pH values above 5 preventing them from reaching the underlying epithelium.

3.5. Tensile studies

A third mucoadhesion study was performed not only to confirm results of the two other studies, but also to investigate the mechanism of mucoadhesion in more detail. While rheological experiments mainly focus on structure properties and network building due to mucoadhesive interaction of polymer mucus mixtures, rotating cylinder studies asses the actual prolongation of retention time of the mucoadhesive dosage form. On the other hand, tensile force tests evaluate the force that has to be applied to detach a dosage form from the mucosa positively correlating with binding forces between polymer surface and mucosa [28]. The maximum detachment force as well as the total work of adhesion serve thereby as measures for mucoadhesion and are provided in Fig. 5. PAA-low NHS performed superiorly compared to the unmodified control polymer exhibiting 1.7-fold higher MDF and 2.7-fold increased TWA ($P < 0.05$). A further improvement in mucoadhesion showed PAA-high NHS with a 3.5-fold higher MDF and a 4.1-fold higher TWA. Interestingly, these results are in the same order of magnitude as the outcomes of studies with preactivated thiomers as state-of-the-art mucoadhesive polymers despite their different mechanism of action [29]. Since the polymer comes in direct contact with the mucosa instead of building a gel-like mixture with mucus, the inability to form intramolecular covalent bonds seems to play a minor role in the experimental setup with test discs.

3.6. Water uptake studies

The water uptake of tablet disks as percentage of their initial weight is shown in Fig. 6. During the experiment, all test disks increased their weight due to swelling in aqueous environment. Modified polymers showed a higher water uptake being a multiple of their initial weight, whereas the control disks were subject of immediate swelling and early disruption. PAA-high NHS showed a prolonged swelling time compared

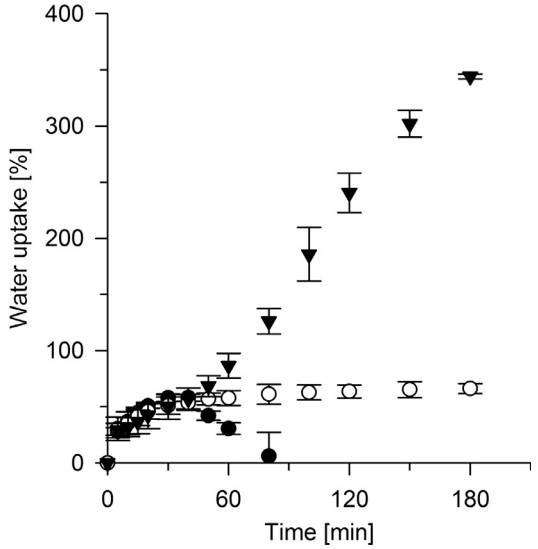

Fig. 6. Swelling behavior. Time dependent water uptake of PAA (●), PAA-low NHS (▼) and PAA-high NHS (○) test disks in 50 mM phosphate buffer pH 6.8. Values are the means of at least three experiments ± standard deviation.

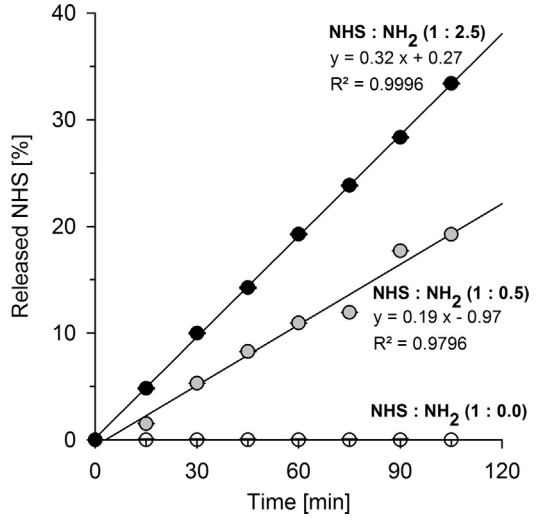

Fig. 7. Reactivity of esterified carboxylic groups. Illustration of time dependent NHS release from PAA-NHS after addition of L-glycine in different concentrations at pH 6.8. Indicated values are the means of at least six experiments ± standard deviation.

to PAA-low NHS ($P < 0.05$) indicating a positive correlation between substitution degree and decelerated water uptake. These results indicate improved cohesiveness due to modification showing a similar swelling behavior compared to preactivated thiomers [15]. Since polymeric NHS esters do not undergo intramolecular covalent bond formation as thiomers do, the mechanism behind is most likely based on lipophilic interactions between NHS groups as well as due to increased overall lipophilicity. The greater water uptake of PAA-low NHS vs PAA-high NHS might be due to the higher percentage of carboxylic groups with the ability to interact with water molecules in form of hydrogen bond formation. To conclude, the coexistence of carboxylic groups as well as NHS groups might be responsible for enhanced swelling behavior and cohesiveness of NHS esters. Tests which were performed with almost completely modified PAA-NHS showed unsatisfying results in swelling, solubility and rheological synergism (data not shown) owing to the small COOH/NHS ratio.

3.7. Reactivity

The velocity of reaction with mucosal subdomains was evaluated. Therefore, the presence of mucosal primary amino groups was simulated by addition of L-glycine to the polymer. This amino acid has been chosen as an amino group donor because of the clear allocation of its reactive amino group. In contrast, lysine and arginine as natural parts of mucin glycoproteins both exhibit more than one domain for potential reactions. In order to prove that the reaction velocity is dependent on substrate concentration, two different concentrations of amino acid were tested. The measure of parameter in this experiment is the increase in absorption over time due to release of NHS causing a bathochromic absorption shift and facilitating detection at 260 nm [9]. In a preliminary study it was proven, that NHS is stable under the chosen conditions during and beyond the testing period. Fig. 7 shows the linear range of the time dependent NHS release after addition of glycine in a molar ratio of reacting groups of 1:0.5 ($NHS:NH_2$) and of 1:2.5 ($NHS:NH_2$) as well as of the polymer without glycine. By increasing the amount of amino acid a 2-fold increase in reaction velocity was achieved indicating substrate dependent reaction kinetics. The control without the presence of NH_2 groups showed no increase in absorption over time proving sufficient in situ stability of the polymer in aqueous environment. Hence, the reaction takes place in situ as soon as the polymer comes in contact with amino acids of the target mucosa. Evaluating these results in view of data from a previous study, the reaction velocity was 3-fold higher compared to entirely preactivated thiomers and in the same range as that of highly reactive preactivated thiomers [15].

4. Conclusion

In the present study a novel concept of mucoadhesion for polymeric excipients has been established. So far, reactive polymeric NHS esters have never been examined in the context of mucoadhesion. The esterification with NHS resulted in improved adhesion on mucosal tissues. The obtained polymeric NHS esters react thereby selectively with mucosal amino groups and showed sufficient stability in buffered solutions. There are two possible mechanisms of mucoadhesion: either by interacting with the mucus gel layer or by attaching to the mucosa itself [30]. Thiomers need the presence of sulfhydryl groups to exploit their adhesive potential. Hence their application is limited to tissues covered by a cysteine-rich mucus layer. Polymeric NHS esters, on the other hand, could be used for various surfaces as for example notoriously lysine-rich connective tissues and muscle tissues [31]. These results as well as the comparatively high in situ reactivity and the health safety indicate that polymeric NHS esters could be a promising excipient for mucosal drug delivery.

References

[1] S. Cohen, G. Coué, D. Beno, R. Korenstein, J.F.J. Engbersen, Bioreducible poly (amidoamine)s as carriers for intracellular protein delivery to intestinal cells, Biomaterials 33 (2012) 614–623.
[2] A. Bernkop-Schnürch, V. Schwarz, S. Steininger, Polymers with thiol groups: a new generation of mucoadhesive polymers? Pharm. Res. 16 (1999) 876–881.
[3] F. Hintzen, S. Hauptstein, G. Perera, A. Bernkop-Schnürch, Synthesis and in vitro characterization of entirely S-protected thiolated pectin for drug delivery, Eur. J. Pharm. Biopharm. 85 (2013) 1266–1273.
[4] A. Borgert, J. Heimburg-Molinaro, X. Song, Y. Lasanajak, T. Ju, M. Liu, P. Thompson, G. Ragupathi, G. Barany, D.F. Smith, R.D. Cummings, D. Live, Deciphering structural elements of mucin glycoprotein recognition, ACS Chem. Biol. 7 (2012) 1031–1039.
[5] A. Shefy-Peleg, M. Foox, B. Cohen, M. Zilberman, Novel antibiotic-eluting gelatin-alginate soft tissue adhesives for various wound closing applications, Int. J. Polym. Mater. Polym. Biomater. 63 (2014) 699–707.
[6] L. Sanders, R. Stone, K. Webb, T. Mefford, J. Nagatomi, Mechanical characterization of a bifunctional Tetronic hydrogel adhesive for soft tissues, J. Biomed. Mater. Res. A 103 (2015) 861–868.
[7] V. Bhagat, M.L. Becker, Degradable adhesives for surgery and tissue engineering, Biomacromolecules 18 (2017) 3009–3039.

[8] J.V. Staros, R.W. Wright, D.M. Swingle, Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions, Anal. Biochem. 156 (1986) 220–222.
[9] T. Miron, M. Wilchek, A spectrophotometric assay for soluble and immobilized N-hydroxysuccinimide esters, Anal. Biochem. 126 (1982) 433–435.
[10] S.A. Schonbichler, L.K. Bittner, J.D. Pallua, M. Popp, G. Abel, G.K. Bonn, C.W. Huck, Simultaneous quantification of verbenalin and verbascoside in Verbena officinalis by ATR-IR and NIR spectroscopy, J. Pharm. Biomed. Anal. 84 (2013) 97–102.
[11] P. Jennings, C. Koppelstaetter, S. Aydin, T. Abberger, A.M. Wolf, G. Mayer, W. Pfaller, Cyclosporine A induces senescence in renal tubular epithelial cells, Am. J. Physiol. Renal Physiol. 293 (2007) F831–838.
[12] A. Vetter, G. Perera, K. Leithner, G. Klima, A. Bernkop-Schnürch, Development and in vivo bioavailability study of an oral fondaparinux delivery system, Eur. J. Pharm. Sci. 41 (2010) 489–497.
[13] A. Bernkop-Schnürch, S. Steininger, Synthesis and characterisation of mucoadhesive thiolated polymers, Int. J. Pharm. 194 (2000) 239–247.
[14] C.E. Kast, A. Bernkop-Schnürch, Thiolated polymers — thiomers: development and in vitro evaluation of chitosan–thioglycolic acid conjugates, Biomaterials 22 (2001) 2345–2352.
[15] C. Menzel, J. Silbernagl, F. Laffleur, C. Leichner, M. Jelkmann, C.W. Huck, S. Hussain, A. Bernkop-Schnürch, 2,2′Dithiodinicotinyl ligands: key to more reactive thiomers, Int. J. Pharm. 503 (2016) 199–206.
[16] H. Dannenberg, N-Hydroxy-succinimid, eine nicht krebserzeugende N-Hydroxy-Verbindung, Zeitschrift für Krebsforschung und Klinische Onkologie 76 (1971) 216–218.
[17] F. Madsen, K. Eberth, J.D. Smart, A rheological examination of the mucoadhesive/mucus interaction: the effect of mucoadhesive type and concentration, J. Control. Release 50 (1998) 167–178.
[18] S.A. Mortazavi, J.D. Smart, Factors influencing gel-strengthening at the mucoadhesive-mucus interface, J. Pharm. Pharmacol. 46 (1994) 86–90.
[19] P.C. Griffiths, P. Occhipinti, C. Morris, R.K. Heenan, S.M. King, M. Gumbleton, PGSE-NMR and SANS studies of the interaction of model polymer therapeutics with mucin, Biomacromolecules 11 (2010) 120–125.
[20] G.P. Andrews, T.P. Laverty, D.S. Jones, Mucoadhesive polymeric platforms for controlled drug delivery, Eur. J. Pharm. Biopharm. 71 (2009) 505–518.
[21] S. Bonengel, S. Haupstein, G. Perera, A. Bernkop-Schnürch, Thiolated and S-protected hydrophobically modified cross-linked poly(acrylic acid) – A new generation of multifunctional polymers, Eur. J. Pharm. Biopharm. 88 (2014) 390–396.
[22] J. Nowak, F. Laffleur, A. Bernkop-Schnürch, Preactivated hyaluronic acid: a potential mucoadhesive polymer for vaginal delivery, Int. J. Pharm. 478 (2015) 383–389.
[23] T.F. Palmberger, K. Albrecht, B. Loretz, A. Bernkop-Schnürch, Thiolated polymers: evaluation of the influence of the amount of covalently attached l-cysteine to poly (acrylic acid), Eur. J. Pharm. Biopharm. 66 (2007) 405–412.
[24] A.J. Lomant, G. Fairbanks, Chemical probes of extended biological structures: synthesis and properties of the cleavable protein cross-linking reagent [35S]dithiobis(succinimidyl propionate), J. Mol. Biol. 104 (1976) 243–261.
[25] P. Cuatrecasas, I. Parikh, Adsorbents for affinity chromatography Use of N-hydroxysuccinimide esters of agarose, Biochemistry 11 (1972) 2291–2299.
[26] J. Fallingborg, Intraluminal pH of the human gastrointestinal tract, Dan. Med. Bull. 46 (1999) 183–196.
[27] E. Touitou, B.W. Barry, Enhancement in Drug Delivery, CRC Press, 2006.
[28] S. Dünnhaupt, J. Barthelmes, D. Rahmat, K. Leithner, C.C. Thurner, H. Friedl, A. Bernkop-Schnürch, S-protected thiolated chitosan for oral delivery of hydrophilic macromolecules: evaluation of permeation enhancing and efflux pump inhibitory properties, Mol. Pharm. 9 (2012) 1331–1341.
[29] S. Bonengel, S. Haupstein, G. Perera, A. Bernkop-Schnürch, Thiolated and S-protected hydrophobically modified cross-linked poly(acrylic acid)–a new generation of multifunctional polymers, Eur. J. Pharm. Biopharm. 88 (2014) 390–396.
[30] K. Yoncheva, S. Gómez, M.A. Campanero, C. Gamazo, J.M. Irache, Bioadhesive properties of pegylated nanoparticles, Expert Opin. Drug Deliv. 2 (2005) 205–218.
[31] Q. Nguyen, C.G. Zarkadas, Comparison of the amino acid composition and connective tissue protein contents of selected bovine skeletal muscles, 1989.