Institute of Pharmaceutical Technology and Biopharmaceutics[1], Center of Pharmacy, University of Vienna, Austria and Faculty of Pharmacy[2], Mahidol University, Bangkok, Thailand

Design and *in vitro* evaluation of a mucoadhesive oral delivery system for a model polypeptide antigen

M. K. Marschütz[1], S. Puttipipatkhachorn[2] and A. Bernkop-Schnürch[1]

A novel mucoadhesive drug carrier system has been generated which protects a model polypeptide antigen from degradation by the most abundant intestinal proteases. The enzyme inhibitors antipain, chymostatin and elastatinal, respectively, were covalently attached to the mucoadhesive polymer sodium carboxymethylcellulose (NaCMC) and the inhibitory efficacy of the resulting polymer-inhibitor conjugates was evaluated *in vitro*. When these inhibitor conjugates were combined with the thiolated polymer polycarbophil-cysteine (PCP-Cys), 95.8 ± 3.8% (mean ± SD, n = 3) of the incorporated model antigen ovalbumin (OVA) was protected from enzymatic degradation within 90 min incubation in the presence of an artificial intestinal fluid containing the pancreatic serine proteases trypsin, chymotrypsin and elastase. Replacing the CMC-inhibitor conjugates in the dosage form by unmodified CMC significantly reduced the protective effect to 78.8 ± 4.7% (mean ± SD, n = 3), whereas incorporation of the model antigen in a CMC dosage form omitting PCP-Cys protected 72.5 ± 3.2% (mean ± SD, n = 3) of OVA from degradation within a 90 min incubation period. Further, the incorporation of PCP-Cys resulted in higher cohesiveness within the dosage form and controlled drug release of the antigen for a time period of more than 9 h. Results suggest that a delivery system combining thiolated polymer and polymer-inhibitor conjugates improves the metabolic stability of the model polypeptide antigen and may therefore be a useful tool for oral protein vaccination.

1. Introduction

Due to the great progress in biotechnology and genetic engineering, the industry is capable of producing not only a large number of (poly)peptide drugs, but also (poly)peptide vaccines based on antigens found on the surface of various infectious micro-organisms and viruses. The oral delivery of (poly)peptide vaccines would offer several advantages, including high potential patient acceptance and compliance, less pain and discomfort during application, and low costs of production and administration, as trained staff would not be required to carry out immunizations. Further, oral administration of vaccines may result in improved efficacy, since oral immunization can stimulate mucosal immunity at the sites at which many pathogens initially infect the host. This might prove particularly advantageous in older patients, as unlike systemic immunity, mucosal immunity does not appear to be subject for associated dysfunction. Oral immunization might also be attractive to infants, because mucosal immunity appears to develop earlier than systemic immunity. However, immune responses following oral protein antigen administration are generally of low titer and short-lived, due to the antigen being denatured in the acid environment of the stomach and rapidly degraded by the proteases of the gastrointestinal tract [1, 2].

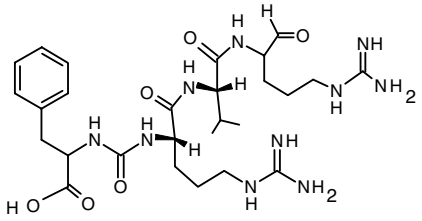
To overcome this barrier a number of delivery systems have been developed, including microparticulate carriers and liposomes [3–6]. Recently, the co-administration of enzyme inhibitors has been shown to enhance the immune responses to microparticulated antigen [7]. However, the unintended disturbed digestion of nutritive proteins, inhibitor induced pancreatic hypersecretion caused by a luminal feedback regulation, and also systemic side effects, can be expected by their co-administration [8, 9]. To overcome these problems, we focused our research work in recent years on the covalent attachment of enzyme inhibitors to unabsorbable mucoadhesive polymer matrices [10, 11]. The covalent attachment maintains the inhibitors concentrated on the drug delivery system thereby avoiding local and systemic toxicity. It is further believed that these matrices act only in a small area of the intestine from which the therapeutic agent is directly released to the mucosa.

Within the present study, a mucoadhesive oral drug delivery system for the model polypeptide antigen ovalbumin (OVA) was designed, which should protect from enzymatic attack. The specific enzyme inhibitors antipain, chymostatin and elastatinal have therefore been covalently attached to the mucoadhesive polymer sodium carboxymethylcellulose (NaCMC). The inhibitory properties of the resulting polymer-inhibitor conjugates have been evaluated. The combination with polycarbophil-cysteine (PCP-Cys), a novel thiolated polymer [12], should provide improved cohesiveness of the dosage form and a controlled release of OVA. The protective effect of the delivery system for OVA towards enzymatic attack from the most abundant intestinal proteases, namely trypsin, chymotrypsin and elastase, has been evaluated *in vitro* as well.

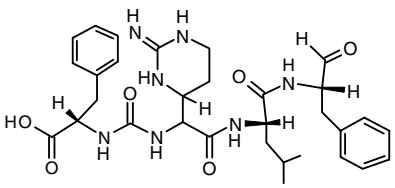
2. Investigations and results

2.1. CMC-inhibitor conjugates

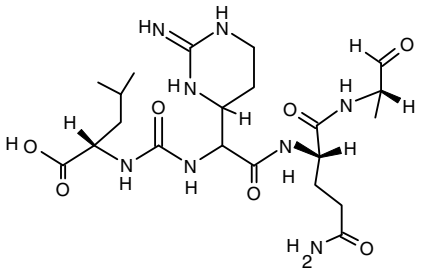
The terminally located aldehyde function of antipain, chymostatin and elastatinal is essential for their inhibitory activity. This moiety was kept uninfluenced, as the reactive carboxylic acid group of the inhibitors is located at the opposite end of the molecules (Scheme).



Antipain (MW: 604.7 Da)



Chymostatin (MW: 607.7 Da)



Elastatinal (MW: 512.6 Da)

All CMC-inhibitor conjugates resulting from different inhibitor ratios during the coupling reaction – namely 10 : 1, 20 : 1 and 40 : 1 conjugates – showed significant enzyme inhibitory properties compared to their control polymers, verifying the efficacy of the method used for purification. Nevertheless, the protective effect within each type of inhibitor-conjugate was not significantly different. Results of the inhibition studies are shown in Figs. 1–3.

As the precipitation of the inhibitor-conjugates in an organic solvent may influence the activity of the inhibitors, the precipitated conjugates used for the preparation of the dosage form have also been evaluated with regard to their inhibitory efficacy. The results revealed that the precipitation of the CMC-inhibitor conjugates had no substantial influence on the inhibitory activity of the inhibitor attached to the polymer, as the inhibitory effect was in exactly the same range as that of the corresponding lyophilized conjugates (data not shown).

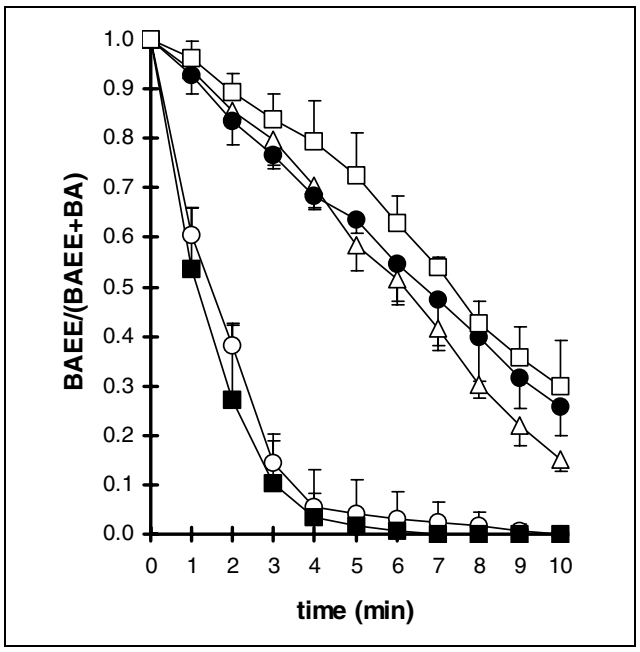
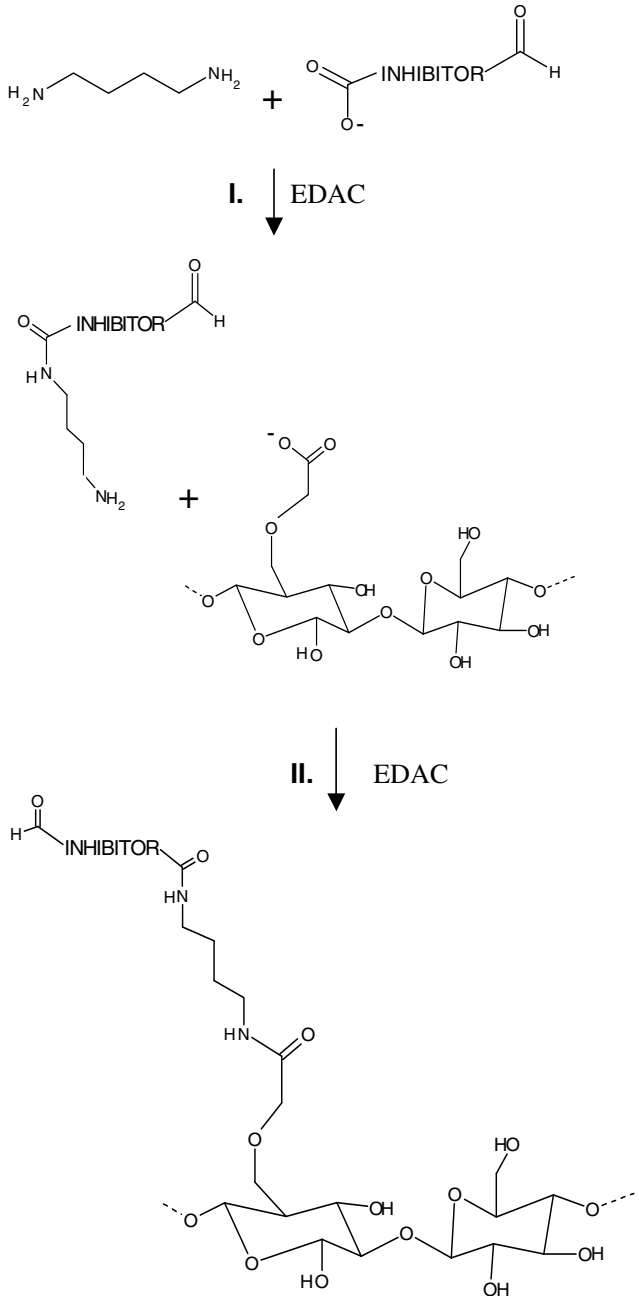


Fig. 1: Comparison of the inhibitory activity of the CMC-antipain conjugates towards trypsin; Hydrolysis of N-α-benzoyl-arginine ethylester (BAEE) to N-α-benzoyl-arginine (BA) by trypsin (56.25 BAEE U/ml) which has been incubated for 30 min at room temperature in the presence of 0.1% unmodified NaCMC (○), 0.1% CMC-antipain control (■), 0.1% CMC-antipain conjugates 40 : 1 (△), 0.1% CMC-antipain conjugates 20 : 1 (●) and 0.1% CMC-antipain conjugates 10 : 1 (□). Each point represents the mean ± SD of at least three determinations

Scheme

Synthetic scheme for the polymer-inhibitor conjugates. Covalent attachment of the spacer putrescine to the inhibitor was mediated by a carbodiimide (EDAC). The spacer-inhibitor conjugate was then immobilized to the polymer by the formation of an amide bond between the free primary amino group of the spacer and a carboxylic acid group of CMC. The reaction was mediated by EDAC.



2.2. *Characterization of the polymer-cysteine conjugate*

The purified polycarbophil-cysteine conjugate displayed 66.8 ± 9.9 μM (mean ± SD of three experiments) sulfhydryl groups per gram polymer. Omitting the coupling re-

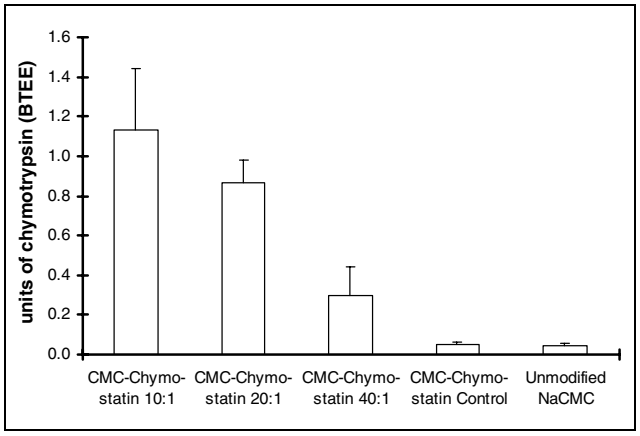


Fig. 2: Comparison of the inhibitory activity of the CMC-chymostatin conjugates towards α-chymotrypsin. As differences in the inhibitory effect of the polymer-inhibitor conjugates towards chymotrypsin could not be evaluated at just one enzyme level, it was impossible to show more informative reaction kinetics of enzyme inhibition for this comparison. Inhibitory activity was therefore measured in units of α-chymotrypsin (BTEE), which have to be added to 0.086% unmodified NaCMC, 0.086% CMC-chymostatin control, 0.086% CMC-chymostatin conjugates 40 : 1, 0.086% CMC-chymostatin conjugates 20 : 1, and 0.086% CMC-chymostatin conjugates 10 : 1 in order to obtain a 50% inhibition of proteolytic activity. The assay was performed at room temperature. Each bar represents the mean ± SD of at least three determinations

agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) during the reaction led to a polymer exhibiting a negligible amount of sulfhydryl groups. The stability, swelling behavior, and mucoadhesive properties of various polycarbophil-cysteine conjugates have already been evaluated [12, 13]. The features of the polymer-cysteine conjugate described here were in good accordance with them.

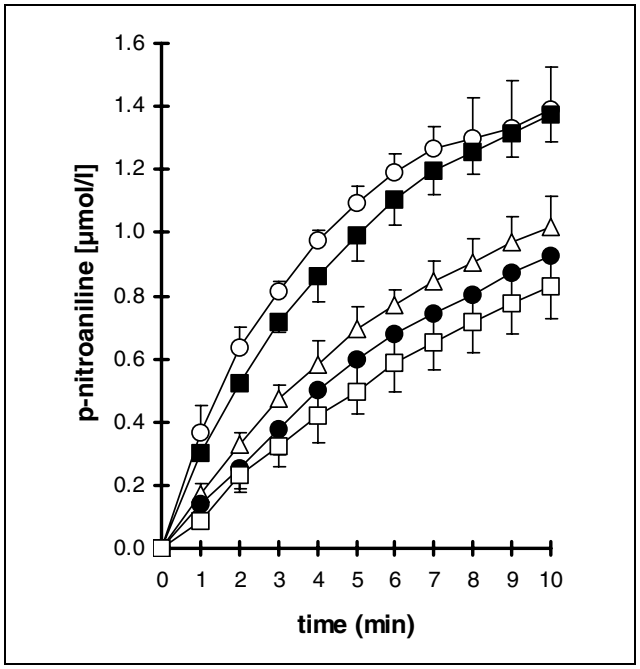


Fig. 3: Comparison of the inhibitory activity of the CMC-elastatinal conjugates towards elastase; Hydrolysis of N-succinyl-(L-alanyl)$_3$-4-nitroanilide to p-nitroaniline by elastase (0.03 units/ml) which has been incubated for 30 min at 37 °C with 0.1% unmodified NaCMC (○), 0.1% CMC-elastatinal control (■), 0.1% CMC-elastatinal conjugates 40 : 1 (△), 0.1% CMC-elastatinal conjugates 20 : 1 (●) and 0.1% CMC-elastatinal conjugates 10 : 1 (□). Each point represents the mean ± SD of at least three determinations

Table 1: Composition of dosage forms A, B and C

Components	Dosage forms		
	A	B	C
OVA-FITC	0.6 mg	0.6 mg	0.6 mg
CMC-antipain 20 : 1	–	–	0.6 mg
CMC-chymostatin 20 : 1	–	–	0.6 mg
CMC-elastatinal 20 : 1	–	–	0.6 mg
unmodified CMC	5.4 mg	1.8 mg	–
PCP-Cys	–	3.6 mg	3.6 mg

2.3. Release profile of the dosage form

The dosage form exclusively consisting of unmodified CMC (formulation A, Table 1) showed a release of approximately 10% OVA-FITC within the first hour and then 20% per h. Disintegration occurred within the first 2 h of the dissolution study.

In contrast, the PCP-Cys delivery system containing unmodified CMC (formulation B, Table 1) showed a release rate of approximately 10% OVA-FITC per h within the first 5 h and then 5% per h. A plateau phase was reached after 12 h of incubation when 76.4 ± 5.6% (mean ± SD, n = 3) of the incorporated drug were dissolved out of this delivery system. Release rates obtained from tablets containing also CMC-inhibitor conjugates (formulation C, Table 1) were not significantly different to tablets B. Both delivery systems containing PCP-Cys remained stable for more than 12 h under the conditions of the dissolution study.

The release profiles of OVA-FITC from the dosage forms A, B and C during the first 9 h of incubation are illustrated in Fig. 4.

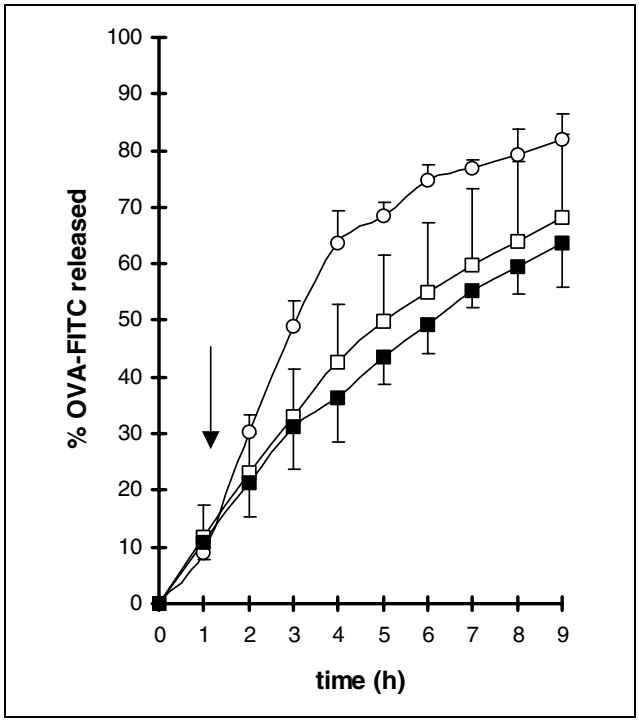


Fig. 4: Release of OVA-FITC from tablets (6 mg) based on unmodified CMC (A; ○), PCP-Cys/CMC-control (B; □) and PCP-Cys/CMC-inhibitor conjugates (C, ■) respectively; Studies were carried out in 100 mM phosphate buffer pH 7.1 at 37 °C. Indicated values are means ± SD of at least three experiments. The arrow indicates the point of disintegration of the CMC dosage form (○)

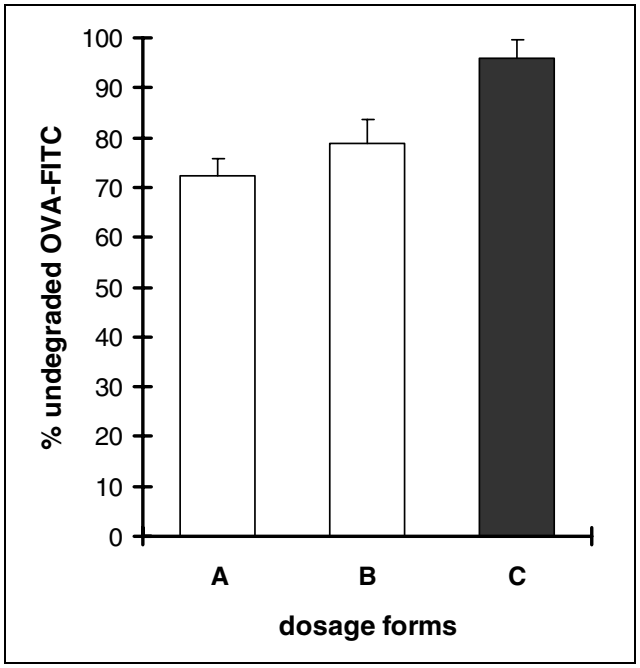


Fig. 5: Comparison of the protective effect of a CMC matrix, a PCP-Cys/CMC matrix and a PCP-Cys/CMC-inhibitor conjugate matrix (formulations A, B and C, according to Table 3). The amount of remaining undegraded polypeptide (%) was determined after 90 min of incubation in 100 mM phosphate buffer pH 7.1 containing trypsin (1400 U/ml), chymotrypsin (8.32 U/ml) and elastase (0.32 U/ml) at 37 ± 0.5 °C. Each bar represents the mean ± SD of at least three experiments

2.4. Protective effect of the delivery system for the model antigen towards enzymatic attack

The protective effect of the delivery system towards enzymatic attack of the pancreatic endopeptidases trypsin, chymotrypsin and elastase, which are mostly responsible for the vast degradation of (poly)peptides in the gut was evaluated. The tablets were thereby incubated for 90 min with an artificial intestinal fluid containing these enzymes. Results of the study are shown in Fig. 5. It could be demonstrated, that the protective effect of the delivery system containing PCP-Cys and CMC-inhibitor conjugates (formulation C, Table 1) was significantly higher ($p < 0.02$) than that of tablets containing PCP-Cys but unmodified CMC (formulation B, Table 1) and tablets containing pure CMC (formulation A, Table 1), respectively.

3. Discussion

To reach sufficient immune response after oral administration of polypeptide antigens the antigen of interest has to be protected whilst in transit through the harsh environment of the gastrointestinal tract. Especially degradation by the luminally secreted proteases trypsin, chymotrypsin and elastase should be prohibited, as these endopeptidases are considered to be mainly responsible for the presystemic metabolism of polypeptide antigens in the gut. They are able to cleave peptide bonds interior to the terminal bonds of the (poly)peptide chain, leading to a vast degradation of the (poly)peptide structure consequently resulting in a loss of antigenicity [14].

A drug carrier matrix in which the novel thiolated polymer PCP-Cys was combined with CMC-protease inhibitor conjugates displayed in this connection several advantages for the model antigen OVA-FITC compared to unmodified CMC.

This may be contributed to the high cohesive properties of PCP-Cys. The formation of inter- and intramolecular disulfide bonds within the polymeric network [13] can provide high stability of the dosage form. Beside these high cohesive properties, PCP-Cys displays also strong mucoadhesive properties. For instance, mucoadhesion of a PCP-Cys conjugate with 5.3 ± 2.4 μM thiol groups per gram polymer was shown to be twice as high than that of unmodified polycarbophil [12].

Due to the mucoadhesive as well as cohesive features of PCP-Cys a prolonged residence time of the delivery system in the intestine seems to be feasible. An increase in drug concentration at the surface of the mucous membrane should thereby lead to improved drug absorption. Therefore, a controlled drug release for an extended period of time becomes very important. Due to the formation of disulfide bonds within matrix tablets based on PCP-Cys during hydration a sustained drug release could be controlled even over a time period of several hours. Nevertheless, the drug load was not completely released from the delivery system. This partly immobilization of OVA-FITC on the carrier matrix may be explained by adsorptive interactions between polymer-matrix and the model antigen. For instance, Tzannis et al. demonstrated that protein-polymer interactions can cause structural changes and aggregation of proteins [15].

Additionally, a number of studies have concluded that polyacrylates per se inhibit the proteolytic activity of trypsin, chymotrypsin, carboxypeptidase A and the cytosolic leucine aminopeptidase [16–18]. More recently, PCP-Cys was shown to have an even higher inhibitory activity towards chymotrypsin and carboxypeptidase A than unmodified PCP [19]. However, significant protection for OVA-FITC from enzymatic attack could only be reached by combining PCP-Cys with CMC-inhibitor conjugates, which provide sufficient inhibitory properties towards trypsin, chymotrypsin and elastase.

Current oral vaccine delivery is commonly targeted to the gut-associated lymphoid tissue (GALT), which is generally organized into aggregates of lymphoid follicles called Peyeŕs Patches, displaying the induction of a secretory immune response to ingested antigens as major physiological role. In this connection, a great deal of research has been focused on the delivery of antigens trapped in microparticulate carriers. A number of animal models could already show the potency of such systems [3]. Nevertheless, exposure of sensitive antigens to organic solvents during the microencapsulation process is often a limiting factor for the formulation. High antigen loads are required in order to induce sufficient immunity response. The delivery system introduced in the present study may be seen as an alternative approach to the oral delivery of vaccines, due to its protective effect (I) for incorporated peptides from intestinal proteases. Further, the mucoadhesive properties of the polymers (II) and a sustained drug release (III) might extend the time period of antigen being available for the immunomodulatory mechanisms of the intestine. Whether these polymer conjugate systems will also prove successful *in vivo* will be subject of ongoing investigations.

4. Experimental

4.1. Synthesis and isolation of the CMC-inhibitor conjugates

The CMC-inhibitor conjugates have been synthesized according to the method recently described by our research group [11]. The inhibitors antipain, chymostatin, and elastatinal (Sigma, St. Louis, MO) were covalently

Table 2: Amounts of reagents used for reaction mixtures in the preparation of protease-inhibitor-spacer conjugates 1 : 1 (molar ratio)

Protease inhibitor-spacer conjugates	Antipain (mg)	α-Chymostatin (mg)	Elastatinal (mg)	EDAC (mg)	Putrescine dihydro-chloride (mg)
Antipain-putrescine control	1	–	–	–	0.27
Antipain-putrescine conjugate	3	–	–	60	0.81
Chymostatin-putrescine control	–	1	–	–	0.26
Chymostatin-putrescine conjugate	–	3	–	60	0.78
Elastatinal-putrescine control	–	–	1	–	0.32
Elastatinal-putrescine conjugate	–	–	3	60	0.96

Table 3: Amounts of reagents used for reaction mixtures in the preparation of CMC-protease inhibitor conjugates with increasing amounts of antipain, chymostatin and elastatinal

CMC-protease inhibitor conjugates	NaCMC (mg; hydrated in 0.5 ml H_2O)	EDAC (mg)	Protease inhibitor-spacer conjugates (ml; equivalent to 1 mg inhibitor); according to Table 2
CMC-antipain control	10	–	1 (control)
CMC-antipain 10 : 1	10	30	1
CMC-antipain 20 : 1	20	30	1
CMC-antipain 40 : 1	40	30	1
CMC-chymostatin control	10	–	1.2 (control)
CMC-chymostatin 10 : 1	10	30	1.2
CMC-chymostatin 20 : 1	20	30	1.2
CMC-chymostatin 40 : 1	40	30	1.2
CMC-elastatinal control	10	–	1 (control)
CMC-elastatinal 10 : 1	10	30	1
CMC-elastatinal 20 : 1	20	30	1
CMC-elastatinal 40 : 1	40	30	1

attached to the mucoadhesive polymer sodium carboxymethylcellulose (NaCMC, average MW: 250000, viscosity of a 2% aqueous solution at 25 °C: 400–800 cps; Kwizda, Vienna, Austria) via the spacer putrescine dihydrochloride (tetramethylendiamine 2 HCl; Sigma, St. Louis, MO, Scheme).

Antipain (3 mg) and elastatinal (3 mg) were dissolved in 1.5 ml of demineralized water, while chymostatin (3 mg) was suspended in a mixture of tetrahydrofuran (0.6 ml) and demineralized water (1.5 ml). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC; Sigma, St. Louis, MO) was added to each preparation in order to activate the carboxylic acid group of the inhibitor, and the reaction mixtures were stirred for 15 min at room temperature. Putrescine (molar ratio 1 : 1 to the inhibitors, see Table 2) was dissolved in 1.5 ml of demineralized water and the pH was adjusted to 6 with 0.1 M NaOH. This solution was added to each activated inhibitor. The resulting reaction mixtures were then incubated for 4 h at room temperature under stirring. An overview concerning the amounts of reagents used for the coupling reaction is given in Table 2.

The resulting spacer-inhibitor conjugates were directly coupled to NaCMC by the same condensation reaction as described above. The remaining NH_2-group of the spacer was thereby covalently bound to a carboxylic acid group of the polymer by the formation of an amide bond. Increasing amounts of NaCMC, as listed in Table 3, were hydrated in 0.5 ml of demineralized water, 30 mg of EDAC were added and mixtures were incubated for 15 min at room temperature in order to activate the carboxylic acid groups of the polymer. The inhibitor-spacer reaction mixture in a volume equivalent to 1 mg of inhibitor was added to each sample and the reaction was allowed to proceed for 4 h with stirring at room temperature. The resulting polymer-inhibitor conjugates were isolated by dialyzing against 0.25 M HCl four times every 8–12 h at 10 °C and then exhaustively against demineralized water for 12 h at room temperature.

Control polymers were prepared and isolated in the same way as for the CMC-inhibitor conjugates, however, EDAC was omitted during the coupling reaction. The isolated CMC-inhibitor conjugates were lyophilized by drying frozen aqueous polymer solutions at −30 °C and 0.01 mbar (Christ Beta 1–8 K; Osterode am Harz, Germany) and stored at −20 °C.

4.2. Synthesis of the polycarbophil-cysteine conjugate

The mucoadhesive thiolated polymer polycarbophil-cysteine (PCP-Cys) was synthesized according to the method previously described [13]. In brief, 3 g of Na-PCP were hydrated in 600 ml of demineralized water. The carboxylic acid moieties of the polymer were activated for 45 min by adding EDAC at a final concentration of 50 mM. Thereafter, 1 g of L-cysteine (Sigma, St. Louis, MO) was added to the activated Na-PCP. In order to avoid an oxidation of the sulfhydryl groups the pH was adjusted to 5. The reaction mixture was then incubated for 3 h at room temperature with stirring. The resulting conjugate was isolated by dialyzing at 10 °C in the dark against 1 mM HCl containing 2 μM EDTA, then two times against the same medium but containing 1% NaCl and finally exhaustively against 0.5 mM HCl. The dialyzed conjugate was precipitated by pouring the gel in a four-fold excess of methanol under stirring. The pH was adjusted between 5 and 6 by adding 5 M NaOH. The suspension was centrifuged ($9000 \times g$; 10 min) and the precipitate was air-dried, pulverized in a mortar and stored at 4 °C.

The degree of modification was determined by quantifying the amount of thiol moieties on the polymer by iodometric titration (1 mM iodine; indicator: starch) at pH 3 [13].

4.3. In vitro evaluation of the inhibitory effect of the conjugates towards enzymatic degradation

4.3.1. Trypsin (EC 3.4.21.4)

Polymer-antipain conjugates, control or unmodified NaCMC (0.8 mg) were hydrated in 0.5 ml of 80 mM Tris-HCl buffered saline (TBS) pH 6.8. Trypsin (45 spectrophotometric BAEE units; type I, from bovine pancreas, Sigma, St. Louis, MO) dissolved in 100 μl of 80 mM TBS pH 6.8 was added to the hydrated polymer and the samples were incubated for 30 min at room temperature. The synthetic trypsin substrate *N*-α-benzoylarginine ethylester (BAEE, Sigma, St. Louis, MO) was dissolved in 80 mM TBS pH 6.8 (1 mg/ml). To each sample 200 μl of substrate stock were added and the change in absorbance at 260 nm was recorded at 1 min intervals for 10 min, using a UV/Vis-spectrophotometer (Lambda-16, Perkin-Elmer, USA).

4.3.2. α-Chymotrypsin (EC 3.4.21.1)

Polymer-chymostatin conjugates, control or unmodified NaCMC (0.60 mg) were hydrated in 0.3 ml of 50 mM TBS pH 7.1. In order to determine the amount of α-chymotrypsin which is inhibited by 50% because of the tested polymers, increasing amounts of α-chymotrypsin (52 BTEE units/mg; type II, from bovine pancreas, Sigma, St. Louis, MO) dissolved in 100 μl of the same buffer were added in steps of 0.2 units. After an incubation period of 30 min at room temperature, 0.3 ml of the substrate solution [BTEE, *N*-benzoyl-L-tyrosine ethylester (Sigma, St. Louis, MO); 18.5 mg substrate dissolved in 31.7 ml of methanol and 18.3 ml of demineralized water] were added and the change in absorbance at 256 nm was recorded at 1 min intervals for 10 min, using a UV/Vis-spectrophotometer (Lambda-16, Perkin-Elmer, USA).

4.3.3. Elastase (EC 3.4.21.36)

Polymer-elastatinal conjugates, control or unmodified NaCMC (0.3 mg) were hydrated in 160 μl of 50 mM TBS pH 7.1 and transferred to the wells of a microtitration plate (96-well, Greiner Labortechnik, Austria). Elastase (670 μg; type II-A, from porcine pancreas; Sigma, St. Louis, MO) was dissolved in 1 ml of 50 mM TBS pH 7.1 and 10 μl of this stock solution were added to each sample followed by 30 min incubation at 37 °C. Thereafter, 130 μl of the substrate medium [0.2 mg of succinyl-(L-alanyl)$_3$-4-nitroanilide (Sigma, St. Louis, MO)/ml of 50 mM TBS pH 7.1; filtered before use] were added and the increase in absorbance ($\Delta A_{405\,nm}$) caused by the enzymatic reaction at room temperature was recorded at 1 min intervals for 10 min with a microtitration plate reader (Anthos reader 2001; Salzburg, Austria). The concentration of the hydrolyzed substrate was calculated by interpolation from an according standard curve.

4.4. FITC-labeling of ovalbumin (OVA)

Ovalbumin (OVA; Sigma, St. Louis, MO) was labeled with fluorescein isothiocyanate (FITC; Sigma, St. Louis, MO) [20]. In brief, 40 mg of OVA were dissolved in 20 ml of 0.1 M Na_2CO_3. Thereafter, 2 mg of FITC dissolved in 1 ml of dimethylsulfoxide (DMSO; Merck KGaA, Darmstadt,

Germany) were added step by step under stirring and the coupling reaction was allowed to proceed for at least 8 h at 4 °C. The reaction was stopped by adding NH_4Cl in a final concentration of 50 mM. The resulting OVA-FITC conjugate was isolated by gel filtration (Sephadex G15, Pharmacia Uppsala, Sweden) and lyophilized by drying the frozen conjugate-solutions at −30 °C and 0.01 mbar (Christ Beta 1–8 K; Osterode am Harz, Germany). Based on a standard curve the amount of covalently attached fluorescein was determined by measuring the absorbance of isolated conjugates at 495 nm with a UV/Vis spectrophotometer (Lambda 16, Perkin Elmer; USA).

4.5. Preparation of the drug delivery systems

The CMC-inhibitor conjugates 20 : 1 were chosen for the development of the drug delivery system, as – according to recently performed studies [21, 22] – the slight modification of CMC has no influence on the mucoadhesive properties of the polymer. In order to be able to homogenize the polymer-inhibitor conjugates with the model-drug and the PCP-cysteine conjugate, the initially lyophilized inhibitor conjugates were precipitated. Each inhibitor conjugate (20 mg) was hydrated in 200 µl of demineralized water and 2.8 ml of isopropanol were added. The precipitates were separated by centrifugation (13500 rpm; 5 min), air-dried and pulverized using a mortar and a pestle. Thereafter, as listed in Table 1, ovalbumin-FITC, PCP-Cys and the pulverized CMC-inhibitor conjugates or unmodified CMC were compressed (Hanseaten, Type EI, Hamburg, Germany) into 6 mg tablets (diameter: 3 mm; depth: 1 mm). The compression force was kept constant during the preparation of all tablets.

4.6. In vitro release studies

The small dosage forms (A, B and C, see Table 1) prepared as described above were placed into 5 ml tubes containing 1 ml release medium (100 mM phosphate buffer, pH 7.1). The vessels were closed, placed on an oscillating waterbath (GFL 1092; 60 rpm), and incubated at 37 ± 0.5 °C; sink conditions were maintained throughout the study. Samples of 100 µl were withdrawn at 1 h intervals and replaced with an equal volume of release medium at 37 °C. The amount of OVA-FITC released was evaluated by measuring the fluorescence at 485/535 nm with a fluorimeter (SLT; Spectra Fluor; Tecan, Austria). Concentrations were calculated by interpolation from a standard curve of OVA-FITC. Cumulative corrections were made for the previously removed samples in determining the total amount released.

4.7. Evaluation of the protective effect of the drug delivery systems

Tablets A, B and C (as listed in Table 1), respectively, were incubated with trypsin (1400 U/ml), chymotrypsin (8.32 U/ml) and elastase (0.32 U/ml) in a final volume of 1 ml of 100 mM phosphate-buffer pH 7.1 at 37 ± 0.5 °C in an oscillating water bath (GFL 1092; 60 rpm). Enzyme concentrations correspond thereby to physiological duodenal endopeptidase activity [23]. After 90 min of incubation, samples were boiled at 95 °C for 10 min to terminate the enzymatic reaction. The polymer-content was then removed from the samples by centrifugation (20000 × *g*, 4 °C, Hermle Z 323K) and the supernatant fluids were diluted with an equivalent volume of 1% trifluoroacetic acid (TFA). A portion (20 µl) of these dilutions was directly injected for HPLC analysis (series 200 LC; Perkin-Elmer, USA). OVA-FITC and/or degradation products were separated on a Nucleosil 100-5 C18 column (250 × 4 mm) at 40 °C. Gradient elution was performed as follows: flow rate 1.0 ml/min, 0–22 min; linear gradient from 91% A/9% B to 39% A/61% B (eluent A: 0.1% TFA in water; eluent B: acetonitrile). OVA-FITC and/or degradation products were detected by absorbance at 220 nm with a diode array absorbance detector (Perkin-Elmer 235 C). The metabolism of OVA-FITC was calculated by following the ratio of the integrated peak area of remaining OVA-FITC to the integrated peak areas of degradation products.

4.8. Statistical data analysis

Statistical data analysis was performed using the student t-test with $p < 0.05$ as the minimum level of significance unless indicated otherwise.

Acknowledgments: This work was supported by Grant No. P13085-MOB from the Fonds zur Förderung der wissenschaftlichen Forschung (FWF) to A. Bernkop-Schnürch.
The Austrian Academic Exchange Service and the Ministry of University Affairs, Thailand were also acknowledged for the exchange fellowship under the Thai-Austrian Cooperation in Science and Technology Project to S. Puttipipatkhachorn.

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Received February 12, 2001
Accepted March 6, 2001

Univ.-Prof. Dr. Andreas Bernkop-Schnürch
Institute of Pharmaceutical Technology and Biopharmaceutics
Center of Pharmacy
University of Vienna
Althanstr. 14
1090 Vienna
Austria
andreas.bernkop-schnuerch@univie.ac.at