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Comparative *in vitro* study of different chitosan-complexing agent conjugates

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In this study we analysed the bioadhesive properties and the enzyme inhibitory effects of different chitosan-complexing agent conjugates. Etylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA), respectively, were covalently attached to chitosan by the formation of amide bonds between the primary amino group of the polymer and the carboxylic acid groups of the complexing agents. Whereas almost each primary amino groups of chitosan could be modified by EDTA, DTPA was bound to only $63.8 \pm 5.8\%$ (n = 3; ±SD) of the amino groups of chitosan. The remaining primary amino groups of the chitosan-DTPA conjugate lead to strongly reduced adhesive properties, with a maximum detachment force of 3.0 ± 1.3 mN in contrast to the chitosan-EDTA conjugate with 81.7 ± 9.9 mN in the tensil studies described here (n = 4; ±SD). However, both polymer conjugates displayed an inhibitory effect towards the zinc-dependent proteases carboxypeptidase A (EC 3.4.17.1) and aminopeptidase N (EC 3.4.11.2). The results of this comparative study should provide substantial knowledge for the development of bioadhesive polymers as auxiliary agents for the peroral administration of peptide and protein drugs.

1. Introduction

A sufficiently high bioavailability of perorally administered therapeutic peptides and proteins is unquestionably based on very sophisticated drug delivery systems. Such dosage forms should guarantee on the one hand a penetration enhancing effect and on the other hand a protective effect towards a presystemic metabolism mainly caused by luminally secreted and brush border membrane bound enzymes. Many delivery systems, which fulfil these demands, are based on bioadhesive polymers [1]. Polycarbophil and chitosan, for instance, display a penetration enhancing capability [2]. In addition, due to the immobilisation of competitive enzyme inhibitors to bioadhesive polymers a strong protective effect towards pancreatic serine proteases can be achieved (see e.g. [3–6]). In contrast to luminally secreted proteases, brush border membrane bound enzymes cannot be inhibited by polymer competitive inhibitor conjugates as the mucus gel layer between the membrane and the bioadhesive polymer conjugate restricts polymer attached inhibitors from a direct interaction with membrane bound proteases. On the other hand, the coadministration of not immobilised inhibitors which are able to permeate the mucus gel layer can lead to severe systemic toxic side effects [7]. A likely solution of this problem might be the use of bioadhesive polymers displaying strong complexing properties for divalent cations. As most brush border membrane bound enzymes are zinc-dependent proteases, they can be inhibited even without any direct interaction by bioadhesive polymers with complexing capability due to the deprivation of this essential divalent cation out of the enzyme structure [8]. In order to substantiate our knowledge for the development of very effective bioadhesive polymers displaying complexing properties, it was the aim of this study to do a comparative study of different polymer-complexing agent conjugates – in particular of chitosan-DTPA and -EDTA conjugates – which should contribute to our understanding concerning structure-function relations.

2. Investigations, results and discussion

2.1. Adhesive properties

The hydratability of bioadhesive polymers in the intestinal milieu with a determined pH-range between 4.5–7.8 [9] represents an important parameter for the practical use of these auxiliary agents. In contrast to unmodified chitosan which is only hydratable in an acidic milieu, the chitosan-DTPA and chitosan-EDTA conjugates were rapidly swellable also at pH-values above 7.0. A reason for this observation can be seen in the introduction of carboxylic acid moieties in the former exclusive cationic polymer structure by the covalent attachment of the complexing agents. The degree of modification was calculated by determining the amount of remaining primary amino residues on the polymer. Although DTPA was used in a two times higher concentration than EDTA at the coupling reaction, the amount of DTPA which was bound to chitosan was significantly lower. Whereas the synthesis of chitosan-EDTA conjugates leads to an almost quantitative modification of all primary amino groups [10], only $63.8 \pm 5.8\%$ (n = 3; ±SD) of the amino groups were modified by DTPA. A reason for this observation can be seen in a possible sterical hindrance caused by already covalently bound DTPA which might restrict the linkage of another DTPA molecule to the vicinally located primary amino group of the polymer. The presumptive structure of the chitosan-DTPA conjugate is shown in Fig. 1. Hence, chitosan-DTPA in contrast to chitosan-EDTA represents a polymer of cationic as well as anionic sub-structures making it easily swellable in the acid and alkaline milieu. As a matter of fact that these moieties seem to compensate their effects on the mucosa, however, the adhesive properties of this polymer conjugate were strongly reduced. The results of bioadhesion studies shown in the Table demonstrate that both the exclusive cationic polymer chitosan and the mainly anionic polymer chitosan-EDTA exhibit a significantly higher maximum detachment force than the cationic as well as anionic polymer chitosan-DTPA. On one hand, mucoadhesion of the cationic polymer chitosan is referred to be caused by electrostatic interactions with negatively charged mucosal surfaces [11]. On the other hand for anionic polymers such as chitosan-EDTA mucoadhesion can be explained by the formation of hydrogen bonds of car-

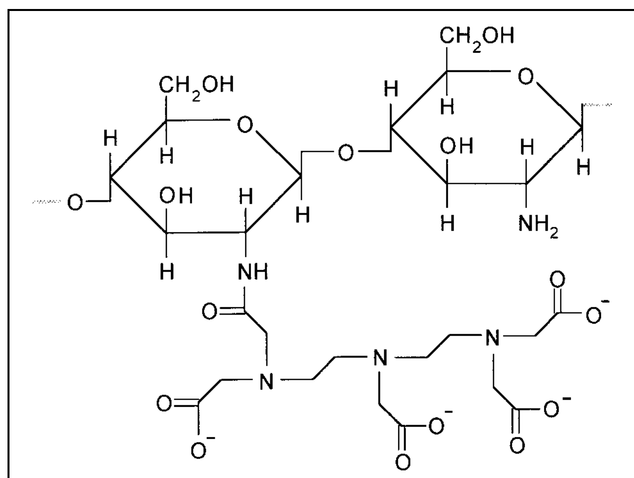

Fig. 1: Presumptive structure of the chitosan-DTPA conjugate; covalent attachment of the complexing agent was achieved by the constitution of an amide bond between a primary amino group of the polymer and a carboxylic acid group of DTPA

boxylic groups with the mucus gel layer [12]. The combination of anionic as well as cationic moieties in the same polymer might compensate both effects. These results are in good accordance with earlier investigations demonstrating that the adhesive properties of chitosan-EDTA conjugates are also strongly reduced if there are remaining primary amino groups on the polymer conjugate [10]. Lueßen et al. could demonstrate a strongly increased intestinal buserelin bioavailability in rats using chitosan hydrochloride as auxiliary agent. A mixture of this cationic polymer with the anionic polymer carbomer, however, leads to a significantly reduced bioavailability of the therapeutic peptide [13]. Reasons for this observation can be seen in reduced absorption enhancing properties based on a reduced charge density which has also to be taken into consideration for the chitosan-DTPA conjugate.

Strong adhesive properties of the polymeric carrier system seem to be a prerequisite for oral (poly)peptide delivery systems. Due to an intimate contact between the dosage form and the intestinal mucosa, a presystemic metabolism of the therapeutic peptide or protein should be excluded. Moreover, a markedly inhibition of membrane bound proteases by polymers with complexing properties can only be achieved if the delivery system can be located as close as possible to the absorption membrane. In contrast to chitosan-DTPA, chitosan-EDTA seems to fulfil these demands.

2.2. *Inhibitory effect*

Various *in vivo* studies could demonstrate a significantly improved bioavailability of orally administered therapeutic peptides and proteins due to the inhibition of gastrointestinal proteases (see e.g. [14–17]). The enzyme inhibitory capability of polymeric excipients for the peroral

Table: Comparison of the adhesive strength of tablets consisting of polymers

Polymer	Maximum detachment force (mN) ±SD, n = 4–5
Chitosan HCl	32.4 ± 14.5
Chitosan-DTPA	3.0 ± 1.3
Chitosan-EDTA	81.7 ± 9.9
Control (no disc)	1.3 ± 0.1

Maximum detachment force was determined in 50 mM Tris HCl buffered saline pH 6.8

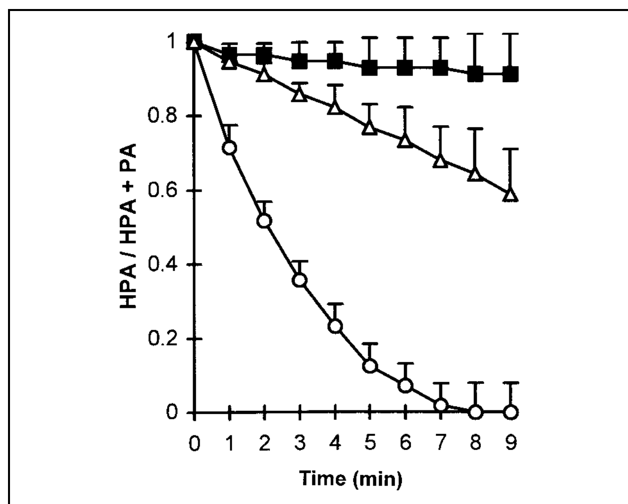

Fig. 2: Comparison of the inhibitory effect of chitosan-DTPA and chitosan-EDTA towards carboxypeptidase A; hydrolysis of hippuryl-L-phenylalanine (HPA) to L-phenylalanine (PA) and hippuric acid by carboxypeptidase A (0.25 units/ml) in the presence of 0.2% chitosan-DTPA (△——△), 0.2% chitosan-EDTA (■——■), and without any chitosan derivative (○——○). Each point represents the mean ±SD of at least three experiments

(poly)peptide administration is therefore an important parameter for their practical use. In order to compare the inhibitory effect of chitosan-EDTA with chitosan-DTPA towards zinc-dependent proteases, the luminally secreted enzyme carboxypeptidase A and the most abundant brush border membrane bound protease aminopeptidase N [18] were chosen as model enzymes. Both polymer conjugates showed a significant inhibitory effect towards these enzymes. This enzyme inhibition by chitosan-complexing agent conjugates can be explained by the deprivation of the essential zinc ion out of the protein structure. Although DTPA displays a higher association constant towards zinc than EDTA determined to be $10^{18.6}$ and $10^{16.5}$, respectively, the inhibitory effect of the chitosan-EDTA conjugate was markedly higher towards both proteases. This observation can be explained by the fact that DTPA

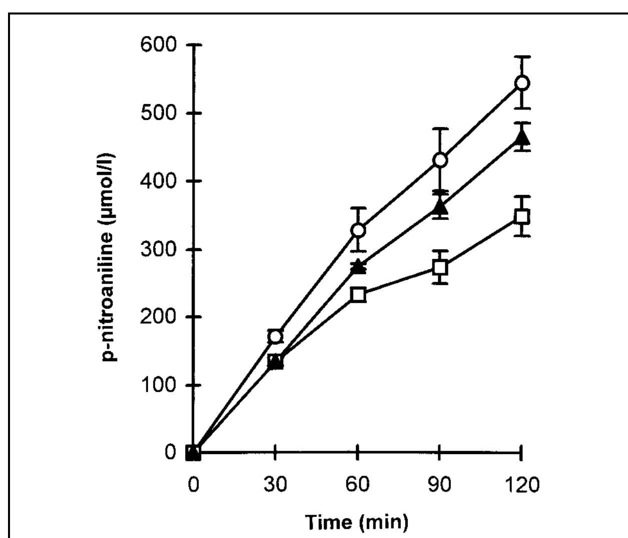

Fig. 3: Comparison of the inhibitory effect of chitosan-EDTA and chitosan-DTPA towards aminopeptidase N; hydrolysis of L-leucine-*p*-nitroanilide by aminopeptidase N (25 mU/ml) in the presence of 0.35% chitosan-DTPA (▲——▲), 0.35% chitoson-EDTA (□——□), and without any polymer (○——○). Each point represents the mean ±SD of at least three experiments

was bound to approximately only each second primary amino group of chitosan, whereas EDTA was bound to almost each amino group of the polymer. Accordingly, on the one hand the binding capacity of the chitosan-DTPA conjugate towards zinc should be comparable lower, and on the other hand the remaining primary amino groups might interfere with the complexation of zinc ions. Results of these studies are shown in Figs. 2 and 3. An interference of the complexation of zinc ions by other divalent cations – in particular by calcium ions with a determined concentration in the intestinal fluid of 0.4–0.7 mM [19] – should be negligible, as the association constant of calcium towards DTPA and EDTA is only $10^{10.9}$ and $10^{10.7}$, respectively. Recent studies demonstrated that chitosan-EDTA displays besides an inhibitory effect towards carboxypeptidase A and aminopeptidase N also an inhibitory effect towards carboxypeptidase B [20]. As most membrane bound enzymes such as carboxypeptidase P, endopeptidase 24.11 and endopeptidase 24.18 are also zinc-dependent proteases [7], the inhibition of these further proteases by chitosan-DTPA and chitosan-EDTA can be expected, but has to be verified by further investigations.

3. Experimental

3.1. Synthesis of the chitosan-complexing agent conjugates

First 1 g of chitosan (from crab shells; minimum 85% deacetylated; poly-[1→4]-β-D-glucosamine; Sigma, St. Louis, USA) was suspended in 80 ml of demineralised water. The pH-value of this suspension was kept constant at pH 6 by continuously adding of 1 N HCl until the polymer was completely dissolved. Demineralised water was added to make the final volume 100 ml. Thereafter, 36.3 g of DTPA (diethylenetriaminepentaacetic acid; Sigma, St. Louis, USA) were dissolved in 100 ml of demineralised water and the pH-value was adjusted to 6.0 with 1 N NaOH. The two solutions were combined and EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; Sigma, St. Louis, USA) was added in a final concentration of 0.1 M in order to mediate the formation of amide bonds between amino groups of chitosan and carboxyl groups of DTPA. The reaction mixture was incubated at room temperature under permanent stirring for 12 h. The resulting chitosan-DTPA conjugate was isolated by exhaustive dialysing against demineralised water, 0.05 N NaOH and once more against demineralised water. The purified product was precipitated by pouring the dialysed polymer solution rapidly into an unstirred bath of non-solvent (acetone) at a solvent to non-solvent ratio of 1 : 200, washed in acetone, and air-dried. The dried polymer was stored at room temperature until use.
The chitosan-EDTA conjugate was synthesised and purified as described previously [21].

3.2. Degree of modification

The degree of modification of the chitosan-derivatives was determined by measuring the free amino groups of unmodified and modified chitosan, using 2,4,6-trinitrobenzenesulfonic acid reagent. Chitosan-DTPA or chitosan-EDTA (0.80 mg) were swelled in 200 μl demineralised water and incubated with 200 μl of 4% (m/v) sodium bicarbonate and 200 μl of 0.1% (m/v) 2,4,6-trinitrobenzenesulfonic acid for 2 h at 37 °C. Thereafter, 200 μl of 2 N HCl were added before the absorbance at 344 nm was measured (Lambda 16; Perkin-Elmer, Vienna, Austria). The amount of remaining primary amino groups was calculated using a standard curve obtained by the amino-group determination of a series of solutions with increasing amounts of unmodified polymer.

3.3. Bioadhesion studies

First, 40 mg of chitosan HCl, chitosan-DTPA and chitosan-EDTA were compressed (Hanseaten Type El, Hamburg, Germany) into 5.0 mm diameter flat-faced discs. The pressing power was thereby kept constant during the preparation of all discs.
Fresh porcine small intestine was obtained from a slaughter, longitudinally dissected and washed gently with 50 mM Tris HCl buffered saline (TBS) pH 6.8 to remove the intestinal content. The mucosal surface was individually mounted on a platform of 30 mm diameter in TBS pH 6.8 at 37 °C and secured in place with a clamp. It was set on a balance (Mettler PC 4400) which was placed on a moving platform. The discs were individually attached to an over this mucosal surface suspending 1.5 g weight using a cyanoacrylate adhesive. The platform was raised up until the test disc attached to the intestinal mucosa and after 2 min lowered at a rate of 2 mm min^{-1}, until the test disc pulled clear of the membrane. The maximum detachment force at which the adhesive bond failed was recorded.

3.4. Inhibition studies

3.4.1. Inhibition of carboxypeptidase A (EC 3.4.17.1)

First, 3 mg of chitosan-DTPA or chitosan-EDTA and carboxypeptidase A (0.375 units; from bovine pancreas; Sigma, St. Louis, USA) in 750 μl of 25 mM Tris HCl pH 6.8 containing 2.9% NaCl were incubated at room temperature for 30 min. After adding 750 μl of the substrate hippuryl-L-phenylalanine (1 mM) dissolved in the same buffer, the increase in absorbance was measured at 254 nm at one minute intervals at 20 °C.

3.4.2. Inhibition of aminopeptidase N (EC 3.4.11.2)

First, 0.7 mg of chitosan-DTPA or chitosan-EDTA and 5 mU of aminopeptidase N (Sigma, St. Louis, USA) in 150 μl of 50 mM TBS pH 6.8 were incubated in the wells of a microtitration plate for 30 min at 37 °C. Thereafter, 50 μl of L-leucine-*p*-nitroanilide in a final concentration of 1 mM were added and the increase in absorbance ($\Delta A_{405\,nm}$) caused by the enzymatic reaction at 37 °C was recorded at predetermined time points with a microtitration plate reader (Anthos reader 2001, Salzburg, Austria). The concentration of the hydrolysed substrate was calculated by interpolation from an according standard curve.

3.5. Statistical data analysis

Statistical data analysis were performed using the t test with $p < 0.05$ as the minimal level of significance.

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