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Research paper

# Thiomers: development and in vitro evaluation of a peroral microparticulate peptide delivery system

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**Abstract**

The aim of this study was to develop a peroral mucoadhesive microparticulate delivery system for peptide drugs. Microparticles containing either the mucoadhesive polymer poly(acrylic acid)–cysteine (PAA–Cys) or unmodified PAA, 15% insulin used as model peptide drug and 0, 30, 50 and 70% Eudragit RS® (MP-RS0, MP-RS30, MP-RS50 and MP-RS70) were prepared by the emulsification solvent evaporation technique. Particle size distribution, release of incorporated insulin, mucoadhesive and swelling properties were examined. During preparation inter- and intramolecular cross-linking occurred, which could be quantified by the amount of disulfide bonds within the resulting particles; this was determined to be 69.2% of the total amount of thiol groups. This cross-linking led to a higher stability of the particles. Microparticles were spherical displaying a rough surface. The particle diameter was in the range of 1–110 μm in the following rank order beginning with the largest: MP-RS30 > MP-RS50 > MP-RS70 = MP-RS0. The higher the ratio of Eudragit RS® in the microparticles, the more prolonged was the release of insulin. In the case of MP-RS70, a sustained release over a time period of at least 60 min was achieved. Mucoadhesive properties and the capacity of water uptake followed the rank order: MP-RS0 > MP-RS30 > MP-RS50 > MP-RS70. Compared to particles comprising unmodified PAA, the mucoadhesive properties of the thiolated microparticulate systems were up to 14-fold improved. According to these results PAA–Cys–Eudragit RS® microparticles might be a promising tool for the peroral administration of peptide drugs.


*Keywords:* Thiomers; Microparticles; Insulin; Eudragit RS®; Mucoadhesion; Peptide drugs

## 1. Introduction

Peroral bioavailability of most peptide drugs is comparatively very low because of the proteolytic activity of the gut and the low permeability of the intestinal epithelium for these therapeutic agents. Strategies to overcome these barriers for perorally administered peptides include the addition of enzyme inhibitors and/or permeation enhancers or a colon targeting of the drug delivery system where the enzymatic activity is relatively low. Another promising strategy is the use of multifunctional polymers exhibiting permeation enhancing, enzyme inhibitory and mucoadhesive properties. Among these features of multifunctional polymers mucoadhesion seems to be most important, as presystemic metabolism between the delivery system and the mucosa can be excluded by an intimate contact of the dosage form with the absorption membrane. Moreover, a steep concentration gradient on the absorption membrane representing the driving force for passive drug uptake can be provided.

A promising strategy to improve mucoadhesion is the use of *thiomers* (I) [1]—polymers with thiol groups, that are believed to form disulfide bonds with cysteine-rich subdomains of mucus glycoproteins [2]. On the other hand, the use of microparticles might contribute to the improvement of the mucoadhesive properties of delivery systems, as it has been demonstrated that particulate delivery systems display a more prolonged gastrointestinal transit time compared to single-unit dosage forms [3]. Recent studies of microparticles comprising poly(acrylic acid)–cysteine (PAA–Cys)—a polymer displaying more

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than 20-fold improved mucoadhesive properties compared to the unmodified polymer [4,5]—showed a rapid release of the therapeutic peptide insulin within a few minutes. High mucoadhesive properties, however, are senseless, if the incorporated drug is already released before the particles have adhered to the mucosa.

The aim of this study was therefore to prepare PAA–Cys microparticles providing a prolonged release of the model peptide drug insulin, in order to guarantee that the main fraction of the drug will be released after adhesion to the mucosa. To achieve this goal, PAA–Cys was mixed with Eudragit RS®—an acrylic/methacrylic copolymer of low water permeability—in different concentrations. The release kinetics of incorporated insulin were evaluated. Additionally, the new microparticles were investigated regarding their thiol/disulfide content, size distribution and mucoadhesive properties as well as swelling behavior.

Table 1
Compositions of poly(acrylic acid)–cysteine–Eudragit RS® and poly (acrylic acid)–Eudragit RS® microparticle formulations (MP-RS0, MP-RS30, MP-RS50 and MP-RS70)

| Components of microparticles | MP-RS0 | MP-RS30 | MP-RS50 | MP-RS70 |
|---|---|---|---|---|
| PAA–Cys or unmodified PAA (mg) | 50 (42.5*) | 35 (29.75*) | 25 (21.25*) | 15 (12.75*) |
| Eudragit RS® (mg) | 0 (0*) | 15 (12.75*) | 25 (21.25*) | 35 (29.75*) |
| Insulin (mg) | 7.5* | 7.5* | 7.5* | 7.5* |

Amounts marked with an asterisk were used for the preparation of insulin-loaded particles. Microparticles were prepared via the water-in-oil (w/o) emulsification solvent evaporation technique at pH 6.

## 2. Materials and methods

### 2.1. Polymer synthesis

The PAA–Cys conjugate was purchased from Muco-Biomer (Leobendorf, Austria). The amount of remaining unbound cysteine in the polymer was determined with 2,4,6-trinitrobenzenesulfonic acid (TNBS, Sigma, St. Louis, MO) reagent as described previously [6].

Fluorescence-labeled polycarbophil was generated as follows: first, 1 g of polycarbophil (Noveon, Raubling, Germany) was hydrated in 100 ml of demineralized water and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC; Sigma, St. Louis, MO) was added in a final concentration of 40 mM. The reaction mixture was incubated for 20 min at room temperature in order to activate the carboxylic acid moieties of the polymer. Then, 20 mg of 6-amino-fluorescein (Sigma, St. Louis, MO) were dissolved in 2 ml of 1 M NaOH and added to the polymer solution. The reaction was allowed to proceed at pH 5 for 3 h at room temperature. To remove the unbound 6-amino-fluorescein, the polymer was dialyzed (Sigma, St. Louis, MO, cellulose membrane dialyzing tubing with molecular weight cutoff of 12 kDa) against 0.2 mM HCl, twice against the same medium also containing 1% NaCl, then four times exhaustively against 0.2 mM HCl and finally against demineralized water. After dialysis the aqueous polymer solution was freeze-dried (−30 °C, 0.1 mbar; Christ Beta 1-8; Osterode am Harz, Germany) and the conjugate was stored in air tight containers at 4 °C until further use.

### 2.2. Preparation of poly(acrylic acid)–cysteine–Eudragit RS® microparticles

Microparticles were prepared by a water-in-oil (w/o) emulsification solvent evaporation technique. The PAA–Cys conjugate was hydrated in 3 ml demineralized water in amounts as listed in Table 1. For drug release studies a 0.75% solution of insulin (from bovine pancreas; Sigma, St. Louis, MO) was added to each polymer solution. The pH was adjusted to 6 with 1 M NaOH. Then the solutions were added dropwise to 90 g of paraffin oil (viscosity 11–230 mPa s) containing 0.25% Span 20 as emulsifying agent. The emulsion was obtained by utilizing an ultraturax (Omni 5000; Omni International). Eudragit RS® 100 (polymethacrylate containing neutral methacrylic acid ester groups and trimethylammoniummethyl methacrylate chloride groups, where the molar ratio of esters/ammonium groups is 40:1; Röhm Pharma, Darmstadt, Germany) was dissolved in methanol and added to the emulsion according to Table 1 to obtain a total polymer concentration in the aqueous phase of 1% (m/m); in the presence of insulin the total polymer concentration was 0.85% (m/m). Once the emulsion was formed, the dispersed aqueous phase was completely evaporated by maintaining the temperature at 25–35 °C. Additionally the emulsion was bubbled with air (5 l/min) and stirred with a paddle at 300 rev./min for 8 h. In this time period the aqueous phase was totally evaporated. Petroleum ether (20 ml) was added and mixed with the oil phase for 10 min. The microparticles were separated from the oil phase by centrifugation (Sorvall RC; 3000 rev./min; 5 min), washed several times with petroleum ether to remove remaining traces of paraffin oil and freeze-dried at −30 °C and 0.01 mbar (Christ Beta 1-8K, Germany). Microparticles containing 0, 30, 50 and 70% Eudragit RS® referred to the polymer fraction were called MP-RS0, MP-RS30, MP-RS50 and MP-RS70 microparticles, respectively.

### 2.3. Determination of the thiol/disulfide content

The amount of free thiol groups on the PAA–Cys conjugate and within the resulting microparticles was determined via Ellman's reagent (5,5′-dithiobis(nitrobenzoic

acid)) as described previously [6]. Disulfide content was measured after reduction with $NaBH_4$ and addition of 5,5′-dithiobis(nitrobenzoic acid) as described by Habeeb [7].

### 2.4. Particle size determination

The size of microparticles was determined using a laser diffraction particle size analyzer (Shimadzu SALD 1100). The size of the microparticles was determined in paraffin oil (viscosity 5 mPa s) as a non-dissolving dispersion medium. Particles were suspended by sonification and magnetic stirring during the measurement.

### 2.5. Scanning electron microscopy

The microparticles were dried in a vacuum chamber to remove residual water, sputter-coated with a gold layer (Sputter coater AGAR B7340, Stansted, UK) and viewed in a scanning electron microscope (Philips XL20, Eindhoven, NL).

### 2.6. Disintegration studies

The stability of microparticles was analyzed visually in 100 mM phosphate buffer (pH 6.8) at 37 °C over 24 h.

### 2.7. Drug release studies

The release rate from microparticles containing insulin was analyzed in vitro. First, 1 mg of microparticles was placed in an Eppendorf vial containing 1.0 ml of release medium (100 mM phosphate buffer pH 6.8 preequilibrated to 37 °C). The vial was closed, placed on an oscillating water bath (GFL 1092; 100 rev./min) and incubated at 37 °C. At predetermined time points aliquots of 100 μl were withdrawn and replaced by an equal volume of release medium preequilibrated to temperature. Released insulin was assayed by HPLC as described previously [8]. Concentrations were calculated by interpolation from a standard curve.

### 2.8. Mucoadhesion studies

For mucoadhesion studies microparticles of PAA–Cys and of the corresponding unmodified polymer were prepared in the same way as described above, but 20% of the total polymer fraction were substituted by fluorescence-labeled polycarbophil. Mucoadhesion studies were performed on 5 cm × 3 cm pieces of freshly excised porcine intestinal mucosa according to a method previously described [9]. First, 4 mg of microparticles were hydrated in 90 μl of 100 mM phosphate buffer (pH 6.8) preequilibrated to 37 °C. The suspension was then transferred on the porcine mucosa, which was mounted on a platform placed at an angle of 45°. The mucosa was incubated at 37 °C and continuously rinsed with 100 mM phosphate buffer (pH 6.8) (3 ml/h). The amount of adherent particles was investigated visually utilizing a UV-lamp. For quantification, the mucus gel layer and the adherent microparticles were scraped off the mucosa after 3 h and diluted 1:4 with 100 mM phosphate buffer (pH 6.8). Samples were shaken for 5 min and the fluorescence of each sample was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm (Biolise, Spectrafluor, Austria). The amount of adherent particles was calculated by interpolation from a standard curve, which was established from increasing amounts of microparticles being applied to the mucosa and being immediately thereafter scraped off and analyzed as described above. As the fluorescent agent was covalently linked to polycarbophil, it was not at all released during the experiment.

### 2.9. Evaluation of the swelling behavior

The water absorbing capacity was determined by a gravimetric method. First, 0.5 mg of PAA–Cys–Eudragit RS® microparticles were incubated in 200 μl of 100 mM phosphate buffer (pH 6.8) preequilibrated to 37 °C. After 1 h of incubation under continuous shaking on an oscillating water bath (GFL 1092; 100 rev./min) at 37 °C the samples were centrifuged for 5 min at 24,000 × $g$ and the supernatant was removed. The weight of remaining swollen microparticles was determined and the weight of uptaken water was calculated.

### 2.10. Statistical data analysis

Statistical data analyses were performed using the Student's $t$-test with $P < 0.05$ as the minimal level of significance. Calculations were done using the software Xlstat version 5.0 (b8.3).

## 3. Results

### 3.1. Chemical characterization of the polymer

The amount of free thiol groups and disulfide bonds within the PAA–Cys conjugate was quantified via Ellman's reagent; 1607.2 ± 163.3 μmol free thiol groups and 1580.6 ± 313.2 μmol oxidized thiol groups per gram polymer were determined. The amount of unbound free cysteine was determined to be 5.2 ± 0.5 μmol/g polymer.

### 3.2. Particle shape and size

Scanning electron microscopy showed almost spherical microparticles with a rough surface. As all particles had almost the same shape, only RS30 particles are exhibited in Fig. 1 as being representative of all others.

The amount of Eudragit RS® mixed with the PAA–Cys conjugate influenced the size of resulting microparticles:

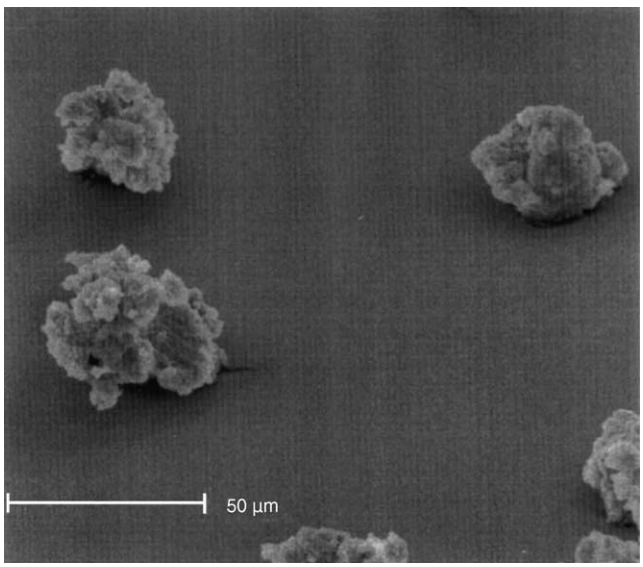


Fig. 1. SEM micrograph of poly(acrylic acid)–cysteine–Eudragit RS® microparticles, MP-RS30-containing 30% Eudragit RS®. Bar represents 50 µm.

RS30 particles exhibited a higher average size than RS0 particles without the acrylic/methacrylic copolymer; more Eudragit RS® as in MP-RS50 led to smaller microparticles compared to MP-RS30. Particles consisting of 70% Eudragit RS® were the smallest Eudragit RS® containing microparticles with almost the same average size as RS0 particles (Fig. 2).

### 3.3. Degree of cross-linking during the preparation process

During the preparation process the thiomer formed inter- and intramolecular disulfide bonds. The PAA–Cys polymer used for microparticle production featured almost as many free thiol groups as oxidized thiol groups as described previously. RS0, RS30, RS50 and RS70 microparticles, in contrast, exhibited 69.2% oxidized thiol groups indicating inter- and intramolecular cross-linking. Therefore an increased stability of the resulting microparticles was obtained as demonstrated by disintegration studies. Generally there was almost no difference in the free thiol group/disulfide bond ratio between RS0, RS30, RS50 and RS70 microparticles.

### 3.4. Disintegration studies

To show the stabilization of the different PAA–Cys–Eudragit RS® microparticles by the formation of disulfide bonds, disintegration studies were performed. RS0, RS30, RS50 and also RS70 microparticles did not disintegrate in physiological buffer within a time period of 24 h, whereas unmodified polymer/Eudragit RS® particles disintegrated within minutes.

### 3.5. Drug release studies

The drug release of insulin was determined in vitro. As shown in Fig. 3 PAA–Cys conjugate microparticles without Eudragit RS® displayed a burst release of almost the complete amount of the peptide. The more Eudragit RS® was added to microparticles, the slower insulin was released. This effect seems to be based on the hydrophobic properties of the acrylic/methacrylic copolymer.

### 3.6. Mucoadhesion studies

Results of the mucoadhesion studies as shown in Fig. 4 demonstrated that microparticles containing the thiolated

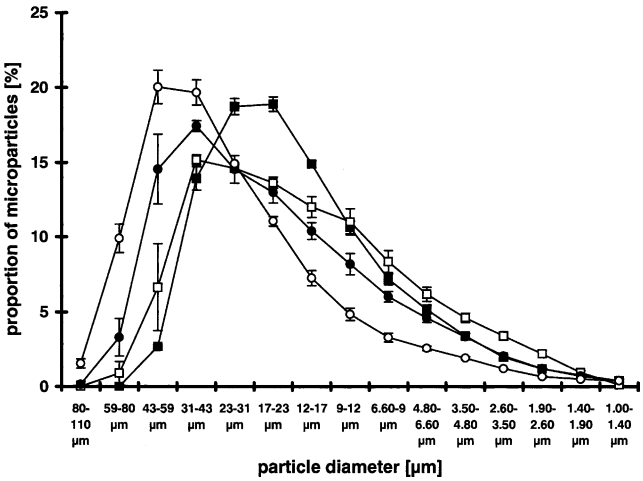


Fig. 2. Size distribution of poly(acrylic acid)–cysteine–Eudragit RS® microparticles [MP-RS0 (■), MP-RS30 (○), MP-RS50 (●) and MP-RS70 (□)], prepared via the water-in-oil (w/o) emulsification solvent evaporation technique. The concentration of the polymers in the aqueous phase was 1% (m/m); the means ($n = 3$) and standard deviation bars are shown.

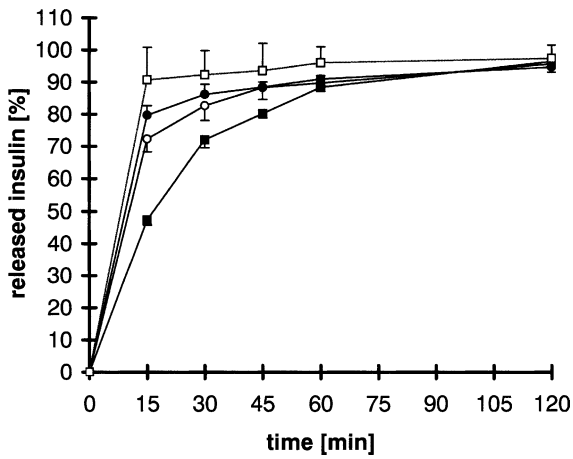


Fig. 3. Release profile of insulin from poly(acrylic acid)–cysteine–Eudragit RS® microparticles [MP-RS0 (□), MP-RS30 (●), MP-RS50 (○) and MP-RS70 (■)]. The release study was performed in 100 mM phosphate buffer pH 6.8 at 37 °C. The means ($n = 3$) and standard deviation bars are shown.

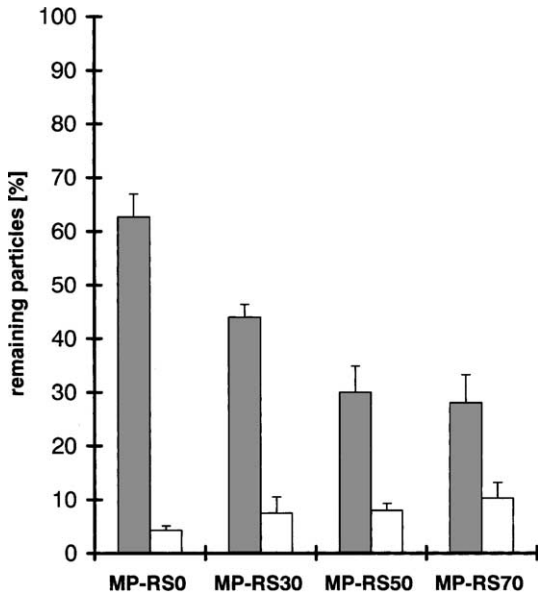


Fig. 4. Mucoadhesion of poly(acrylic acid)–cysteine–Eudragit RS® microparticles (gray bars) and poly(acrylic acid)–Eudragit RS® microparticles (white bars) on freshly excised small intestinal mucosa rinsed with 100 mM phosphate buffer (pH 6.8) at 37 °C. All microparticles were fluorescence-labeled and the percentage of remaining microparticles after 3 h were calculated referred to the amount of microparticles on the mucosa at the beginning of the experiment. The means ($n = 3$) and standard deviation bars are shown.

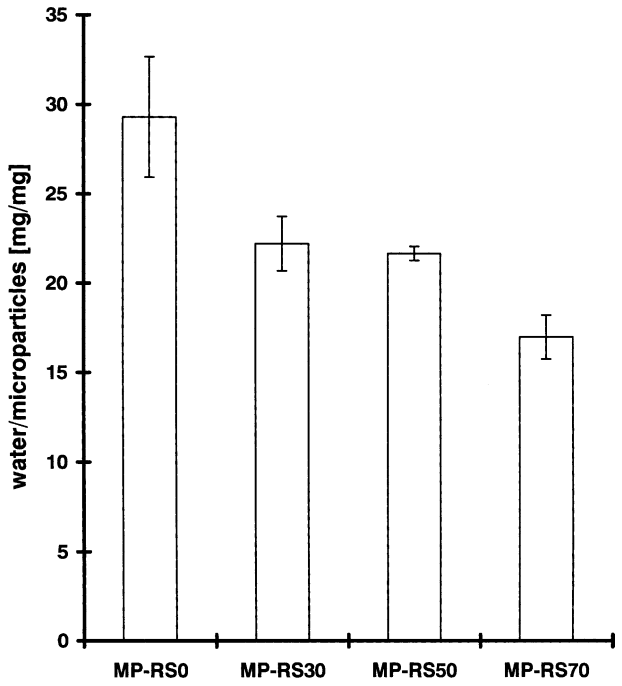


Fig. 5. Swelling behavior of poly(acrylic acid)–cysteine–Eudragit RS® microparticles incubated in a 100 mM phosphate buffer solution (pH 6.8) at 37 °C for 1 h. The means ($n = 3$) and standard deviation bars are shown.

polymer displayed significantly stronger adhesive properties than particles comprising the unmodified polymer. The mucoadhesive properties of unmodified polymer particles were 14-fold improved by thiolation. However, as more Eudragit RS® was added to PAA–Cys conjugate microparticles, fewer particles remained on the mucosa in the first 3 h. An explanation for this observation can be given by the decrease in thiol groups on the microparticles due to the addition of Eudragit RS®.

### *3.7. Evaluation of the swelling behavior*

All microparticulate systems swelled within minutes. MP-RS0 exhibited the highest water uptake, MP-RS30 and MP-RS50 almost the same and MP-RS70 comprising the highest ratio of acrylic/methacrylic copolymer exhibited the lowest water uptake. Results are shown in Fig. 5.

## 4. Discussion

Within this study a new peroral microparticulate peptide delivery system based on a thiolated polymer was developed displaying a prolonged drug release and increased mucoadhesive properties compared to particles comprising the corresponding unmodified polymer.

Kriwet et al. [10] prepared similar microparticles of the mucoadhesive polymer PAA and showed that resulting microparticles featured high mucoadhesive properties. Due to the immobilization of cysteine to PAA the mucoadhesive behavior of PAA particles could be multiplied: PAA–Cys microparticles presented in this study showed a 14-fold improved mucoadhesion compared to the unmodified polymer.

It is known, however, that mixtures of anionic and cationic polymers display less mucoadhesive properties and less in vivo effectiveness than the individual excipients [11]. Therefore, a loss of the excellent mucoadhesive behavior of the PAA–Cys polymer could be expected for microparticles being a mixture of the anionic PAA–Cys and the cationic Eudragit RS®. PAA–Cys/Eudragit RS® microparticles in fact exhibited less mucoadhesive properties than PAA–Cys particles without Eudragit RS®; the amount of particles stuck to the mucosa after 3 h was less the more cationic polymer was used. Microparticles containing only 30% of PAA–Cys, however, displayed still 6.5-fold stronger mucoadhesive properties than particles prepared only from unmodified PAA.

Although in vitro mucoadhesion tests cannot mimic all details of the in vivo situation, nevertheless an in vitro–in vivo correlation can be found. Miyazaki et al. obtained similar mucoadhesion results for dextran derivative microspheres determined on excised rat intestinal mucosa and in vivo. In this study more than 80% of microspheres adhered to the mucosa in vitro for 1.5 h and almost the same amount was found in the stomach 1 h after administration to rats [12]. These results are in good accordance with those of

Akiyama et al., who found comparable in vitro and in vivo mucoadhesion for polyglycerol ester of fatty acid/carbopol 934P-microspheres. More than 90% of this microparticulate system adhered to excised stomach and intestinal mucosa and 89.6% were still in the stomach and small intestine 5 h after peroral administration to rats [13]. Because of this in vitro–in vivo mucoadhesion correlation, PAA–Cys–Eudragit RS® microparticles are expected to exhibit strong mucoadhesive properties also in vivo.

These mucoadhesive properties of particulate delivery systems seem to be essential to gain a sufficient high peroral bioavailability of therapeutic peptides. Kawashima et al. [14], for instance, achieved a prolonged and intensified reduction of the blood calcium level in rats after administration of chitosan-coated elcatonin-loaded DL-lactide/glycolide copolymer nanospheres compared to uncoated nanoparticles. Akiyama et al. achieved in another study a 1.8 times larger area under the plasma concentration–time curve for furosemide and urinary recovery was 2.4 times higher for riboflavin with adhesive carboxyvinyl polymer microspheres compared to non-adhesive microspheres applied in human studies [15].

Apart from the mucoadhesive properties and the sustained drug release, microparticles developed within this study should also display permeation enhancing and enzyme inhibitory properties being highly beneficial for peroral peptide delivery.

PAA displays per se a permeation enhancing effect [16], that was significantly improved by the immobilization of thiol groups on it [17]. According to these results, PAA–Cys–Eudragit RS® microparticles will also exhibit strong permeation enhancing properties.

Additionally, PAA shows a strong inhibitory effect towards the endopeptidase trypsin [18] and as a result of the immobilization of thiol groups on PAA, a pronounced inhibitory effect towards exopeptidases such as carboxypeptidase A and B or aminopeptidase N can also be achieved [19,20]. Being incorporated in PAA–Cys–Eudragit RS® microparticles, peptide drugs should therefore be protected towards an enzymatic attack by these enzymes.

## 5. Conclusion

Within this study various insulin-loaded PAA–Cys–Eudragit RS® microparticles were prepared and analyzed regarding drug release and mucoadhesive properties. In particular PAA–Cys–Eudragit RS® microparticles containing a comparatively high ratio of Eudragit RS® displayed a sustained release of insulin over at least 60 min. The mucoadhesive properties of these microparticles were significantly improved compared to unmodified PAA particles. As there is, however, a strong competition between release prolongation and mucoadhesiveness, the best compromise will have to be found by in vivo studies. Since the combination of mucoadhesiveness and sustained drug release seems to be crucial for the efficacy of peroral peptide delivery systems, PAA–Cys–Eudragit RS® microparticles might represent a promising novel tool for the peroral administration of therapeutic peptides.

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