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Preparation of Thiomer Microparticles and In Vitro Evaluation of Parameters Influencing Their Mucoadhesive Properties

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ABSTRACT It was the aim of this study to develop mucoadhesive microparticulate delivery systems based on thiomers and to investigate parameters influencing their mucoadhesive properties. Microparticles were prepared via coazervation of thiolated or unmodified polycarbophil with fluorescein-diacetate as marker. The protective effect of the polymers toward enzymatic hydrolysis by intestinal enzymes was investigated. Mucoadhesion studies with microparticles, applied in dry and prehydrated form, were performed by ascertaining their residence time on intestinal mucosa. Furthermore, the influence of the amount of thiol groups on mucoadhesion was studied in vitro. Results showed that in comparison to unmodified polycarbophil, thiolated polycarbophil provided a more than 3-fold higher protective effect for the incorporated marker fluorescein-diacetate toward hydrolysis. When being applied in dry form 23.4 ± 4.8% of the fluorescence marker being embedded in thiomer microparticles remained adhering to the intestinal mucosa within 3 h. In contrast, only 11.6 ± 2.0% of the marker remained on the mucosa, when the thiomer microparticles were applied in prehydrated form. In addition, tests performed to assess the impact of the amount of thiol groups pointed out that a high amount of thiol groups is advantageous in order to further improve mucoadhesive properties. This knowledge should contribute to the design of highly efficient drug delivery systems being based on thiomer microparticles.

KEYWORDS Microparticulate delivery system, Thiomers, Polycarbophil-cysteine, Water uptake, Amount of thiol groups, Mucoadhesion

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INTRODUCTION

Since the introduction of mucoadhesive polymers in the pharmaceutical literature in the 1980s their potential was shown in buccal (Korbonits et al., 2004), nasal (Tafaghodi et al., 2004), and ocular drug delivery systems (Hornof et al., 2003), where significantly prolonged residence times could be achieved. In oral

drug delivery, mucoadhesive delivery systems have so far not reached their full potential, because adhesion of such delivery systems in the gastrointestinal (GI) tract is in most cases insufficient to provide a prolonged residence time in the stomach or small intestine (Khosla & Davis, 1987; Harris et al., 1990). To improve the residence time on mucosal membranes and especially provide mucoadhesion in the GI tract, comparatively more effective mucoadhesive delivery systems are in high demand.

A promising strategy to improve mucoadhesion has been introduced in the form of thiolated polymers or designated *thiomers*. Disulfide bonds, the most commonly bridging structure in biological systems, are thereby used to improve adhesion of polymeric carrier systems on mucosal membranes. Thiomers are believed to interact with cysteine-rich subdomains of mucus glycoproteins forming disulfide bonds between the mucoadhesive polymer and the mucus gel layer (Leitner et al., 2003). Due to the immobilization of thiol groups on poly(acrylic acid), for instance, its mucoadhesive properties were even 100-fold improved (Mar-schütz & Bernkop-Schnürch, 2002).

On the other hand, particulate delivery systems can also lead to a prolonged residence time on mucosal membranes (Ponchel & Irache, 1998; Takeuchi et al., 2001). By diffusing into the mucus gel layer their gastrointestinal residence time is significantly prolonged even without exhibiting any mucoadhesive properties. Coupe et al. (1991), for example, showed that in human volunteers particulate formulations have an increased small intestinal transit time in comparison to single unit dosage forms.

To combine both promising strategies, the use of thiomers on the one hand and particulate formulations on the other hand, it was the aim of this study to prepare and characterize microparticulate delivery systems based on thiomers. Polycarbophil-cysteine (PCP-Cys), a poly(acrylic acid)-cysteine derivate of high molecular weight, was chosen as model thiomer because of its strong mucoadhesive properties. In addition, it was an aim of this study to establish a simple and sensitive method that allows the evaluation of mucoadhesive properties of particulate delivery systems.

MATERIALS AND METHODS

Materials

Polycarbophil (PCP, Noveon® AA1) was obtained from BF Goodrich. L-Cysteine HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), 2,4,6-trinitrobenzenesulfonic acid (TNBS-reagent), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), sodium borohydride, and fluorescein-diacetate (FDA) were purchased from Sigma-Aldrich (Vienna, Austria). The solvents dimethyl-sulfoxide (DMSO) and isopropanol were obtained from Acros Organics (Geel, Belgium). All other chemicals were used of analytical grade. Water was distilled.

Polymer Synthesis and Determination of Thiol Group Content

The PCP-Cys conjugate was synthesized according to a method described previously (Marschütz & Bernkop-Schnürch, 2002). In brief, cysteine was covalently attached to PCP via the formation of amide bonds between the primary amino group of cysteine and a carboxylic acid group of the polymer. The reaction was mediated by EDAC. For purification, the PCP-Cys conjugate was precipitated with isopropanol at –20°C. PCP prepared and isolated in exactly the same way but omitting EDAC served as control. After rehydration in demineralized water, the amount of remaining unbound cysteine was determined with TNBS-reagent (Bernkop-Schnürch et al., 2001). The quantity of thiol groups on and within the PCP-Cys conjugate was measured spectrophotometrically after incubation with Ellman's reagent (Bernkop-Schnürch et al., 1999). The disulfide content was ascertained after reduction with sodium borohydride and addition of Ellman's reagent as described by Habeeb (1973).

Preparation of Particles

First, 50 mg of the marker FDA were dissolved in 60 mL of DMSO, and 20 mL of demineralized water was added in aliquots of 1 mL so that the temperature of the solution remained below 40°C. After cooling the solution to 8°C, isopropanol of –20°C was added in a final concentration of 60% (v/v). This solution was added under stirring to 1 g of PCP-Cys or 1 g of unmodified PCP, hydrated in 100 mL of demineralized water at 8°C, which led to a spontaneous coazervation of the polymer and the marker (PCP-Cys/FDA). The precipitate was removed by centrifugation and dried in a desiccator under vacuum for 24 h at room

temperature. Resulting aggregates were mechanically disintegrated and pulverized in a mortar. Drug load of ensuing microparticles was determined by incubating 1 mg of PCP-Cys/FDA with 2 mL of 5 M NaOH for 20 min at 37°C, to hydrolyze the FDA to the fluorescent sodium fluorescein as shown in Fig. 1. Increasing amounts of FDA, incubated in 2 mL of 5 M NaOH, served as standard curve.

Enzymatic Degradation of FDA

First, 8 mg of FDA was transferred to 25 cm^2 of porcine intestinal mucosa. The mucosa was then kept shaking at 37°C with 25 mL of 100 mM phosphate buffer pH 6.5. After 0, 1, 2, 3, and 4 h the fluorescence of each sample was determined. The same amount of FDA incorporated in PCP and PCP-Cys was treated in the same way to evaluate the extent of protective properties of the polymers against enzymatic hydrolysis. A sodium fluorescein standard curve was used to calculate the degradation of FDA to the fluorescent sodium fluorescein on porcine intestinal mucosa.

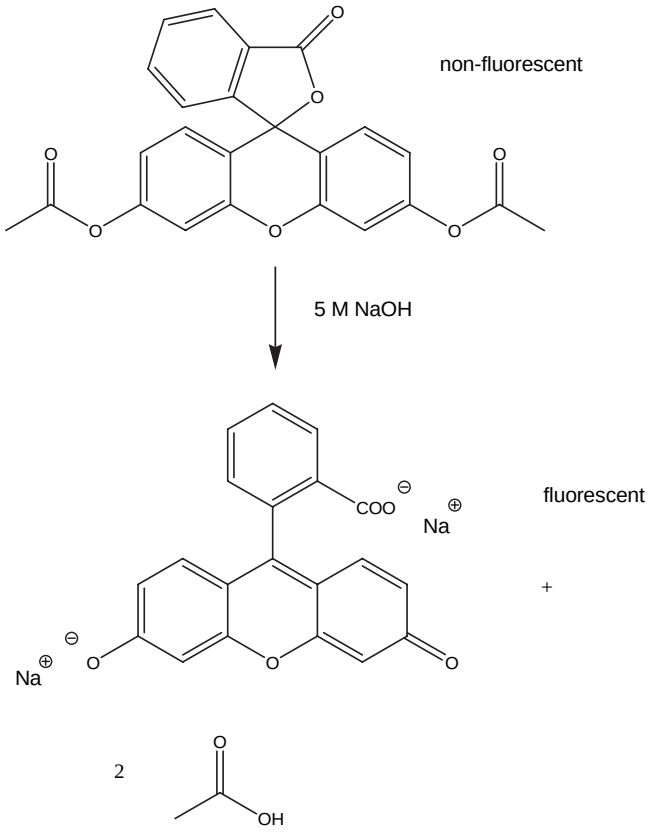

FIGURE 1 **Chemical Structure of the Lipophilic Marker Fluorescein-Diacetate and Its Hydrolization to the Hydrophilic and Fluorescent Sodium Fluorescein Due to the Addition of 5 M**

Particle Size and Zeta Potential Determination

Particle size was specified using a laser diffraction particle size analyzer (FRITSCH Analysette22 Particle Sizer, Idar-Oberstein, Germany). The size of particles was determined in silicone oil (AK10, Wacker, Germany) as a nondissolving and nonswelling dispersion medium within a determination range of 0.3–300 μm. Particles were resuspended via sonification before the measurement.

To determine the swelling behavior of particles, the particle size was measured in demineralized water using microscopic analysis.

The zeta potential was measured by microelectrophoresis in demineralized water at 25°C (Zeta Potential/Particle Sizer; Nicomp TM380 ZLS, Tokyo, Japan). The viscosity and refraction index of demineralized water were used as calculation parameters.

Scanning Electron Microscopy

Surface morphology of particles and the morphological changes produced through immobilization of cysteine on and within the polymer were investigated and documented using scanning electron microscopy (SEM) operating at 10–115 keV (JSM 53IOLV; Jeol, Japan). Particle samples were analyzed under SEM at 350×, 1000×, and 1500×. Images of 1000× and 1500× provided clarification of surface morphology of the particles.

Mucoadhesion Studies

Mucoadhesion studies of thiolated and unmodified polymer microparticles were performed on porcine intestinal mucosa using the experimental setup established by Rango Rao & Buri (1989). An analytical method to quantify the amount of remaining marker being incorporated in the polymer microparticles was developed by our research group.

Porcine intestinal mucosa was mounted on a half pipe and placed in an incubating cupboard with 100% humidity and a temperature of 37°C in an angle of 45°, as shown in Fig. 2. The mucosa was then continuously rinsed with 100 mM phosphate buffer pH 6.5, which served as artificial intestinal fluid. To humidify the mucosa, an equilibration period of 5 min was allowed before administering the particles. During the whole experiment, the temperature of the phosphate buffer was kept at 37°C. A constant flow rate of 1 mL/min

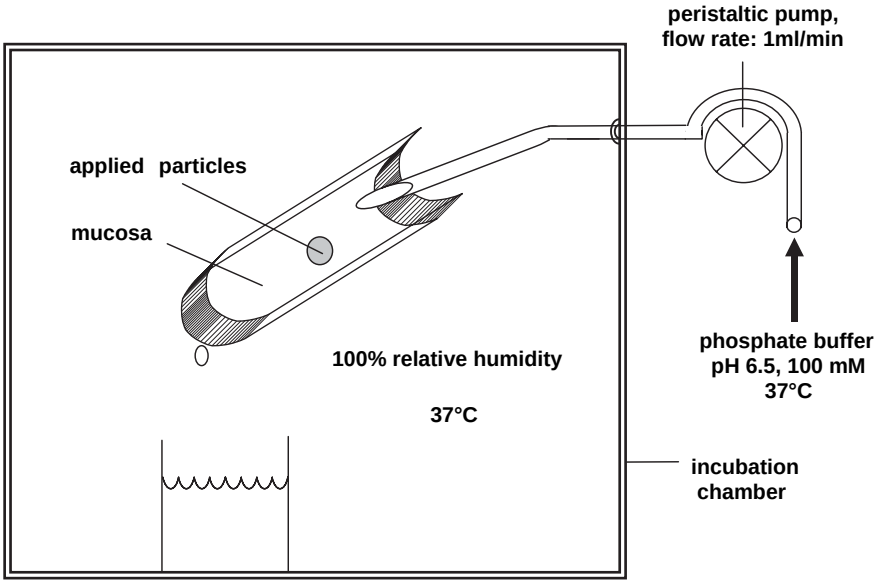

FIGURE 2 **Experimental Setup for Mucoadhesion Studies According to Rango Rao & Buri, 1989.**

was provided by using a peristaltic pump (Ismatec IPC, Wertheim, Germany). After the equilibration period, 8 mg of microparticles was transferred to the mucosa and rinsed continuously with phosphate buffer. After 1, 2, or 3 h the mucosa with the remaining marked particles on it was incubated in 25 mL of 5 M NaOH for 20 min at 37°C under shaking to quantitatively hydrolyze FDA to sodium fluorescein. After centrifugation (13,400 rpm; 5 min), fluorescence of each sample was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm with a microplate reader (Fluostar Galaxy, BMG, Austria). For the calibration curve increasing amounts of PCP-Cys/FDA and PCP/FDA were transferred to intestinal mucosa, which was rinsed for 3 h with artificial intestinal fluid before application of the microparticles. After incubating for 20 min at 37°C in 5 M NaOH the standard samples were centrifuged and their fluorescence was determined.

For experiments performed to assess the influence of water uptake (water uptake = water [mg]/thiomer [mg]) the same experimental setup was used. PCP-Cys/FDA with 615 ± 4.8 μmol/g thiol groups was prehydrated with increasing portions of 100 mM phosphate buffer pH 6.5 (dry, 100 μL, 200 μL, and 300 μL) and transferred to the mucosa, which was subsequently rinsed with buffer (1 mL/min). After 3 h the mucosa with the remaining marked particles was incubated in 25 mL of 5 M NaOH for 20 min and the fluorescence of each sample was determined.

For mucoadhesion studies investigating the impact of the amount of thiol groups on mucoadhesive properties of thiomer microparticles the same method was used and PCP-Cys/FDA samples with increasing amounts of thiol groups (0, 389 ± 5.8 μmol/g, 615 ± 4.8 μmol/g, and 1114 ± 7.2 μmol/g) were transferred to the mucosa. Subsequently the fluorescence of each sample was measured after 3 h of continuous rinsing (1 mL/min) with phosphate buffer.

Statistical Data Analysis

Each experiment was performed in triplicate. Statistical data analyses were performed using Student's *t*-test with $p < 0.01$ as minimal level of significance.

RESULTS

Enzymatic Degradation of FDA

To ensure that FDA is not hydrolyzed by intestinal enzymes on the mucosa during the experiment and before measurement, enzyme-mediated hydrolysis of FDA was examined. In Fig. 3 it is shown that FDA without any formulation is hydrolyzed by 2.33 ± 0.37%, whereas FDA embedded in PCP was hydrolyzed significantly ($p < 0.01$) less by 0.71 ± 0.20% to sodium fluorescein within 4 h. PCP-Cys provided even better protection toward intestinal enzymes, where incorporated FDA was degraded significantly ($p < 0.001$) less by 0.019 ± 0.029%.

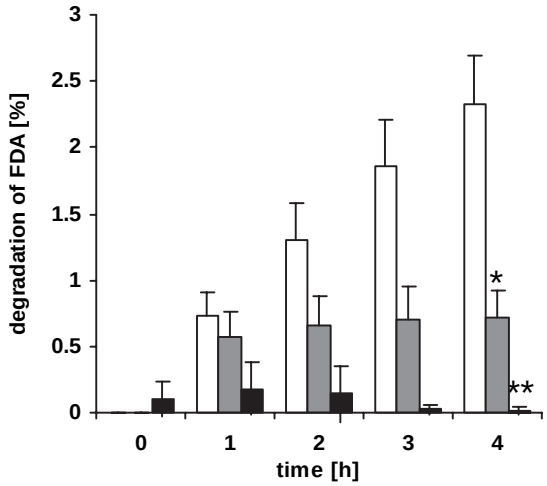

FIGURE 3 **Enzymatic Hydrolysis of Fluorescein-Diacetate on Small Intestinal Mucosa at 37°C Within 4 h; FDA Without Any Formulation (White Bars) Compared to FDA Being Incorporated in Polycarbophil (Gray Bars) and Being Incorporated in Polycarbophil-Cysteine (Black Bars); Indicated Values Are Means ± SD of at Least 3 Experiments. *Differs From FDA Without Any Formulation, $p < 0.01$. **Differs From FDA Without Any Formulation, $p < 0.001$.**

Preparation and Characterization of Particles

PCP-Cys conjugates with increasing amounts of immobilized thiol groups were synthesized. Different degrees of modification with cysteine-HCl were achieved, dependent on the quantity of cysteine-HCl added during the coupling reaction.

Fluorescence marked microparticles were prepared via coazervation of the thiomer and unmodified polymer with FDA dissolved in DMSO/demineralized water using isopropanol temperate to –20°C as nonsolvent. The drug load was determined to be approximately 8 ± 0.7%. During the preparation process, PCP-Cys showed a decrease of around 23% thiol groups per gram of polymer due to the formation of disulfide bonds within the polymeric network. Because of this in situ cross-linking the resulting particles were stable but swelled in aqueous solution, whereas particles of unmodified PCP dissolved in aqueous medium. Generally the mean size of unmodified PCP and PCP-Cys particles in silicone oil was determined to be 66.1 ± 3.8 µm and 57.1 ± 2.9 µm, respectively. Size distribution studies demonstrated no significant change in particle size as a result of the immobilization of thiol groups. The particle surface could be investigated by scanning electron microscopy images, as shown in Fig. 4. Particles were of cubic shape. PCP/FDA particles displayed a relatively smooth and even surface, which is shown in Fig. 4 (A), whereas PCP-Cys/FDA particles exhibited a porous and rough structure, as shown in Fig. 4 (B).

The swelling behavior of PCP-Cys/FDA microparticles was investigated via microscopical analysis. In comparison to their size in silicone oil, an 8-fold greater particle size was determined in demineralized water, whereas an immediate swelling within few seconds was observed. As PCP particles dissolved immediately in

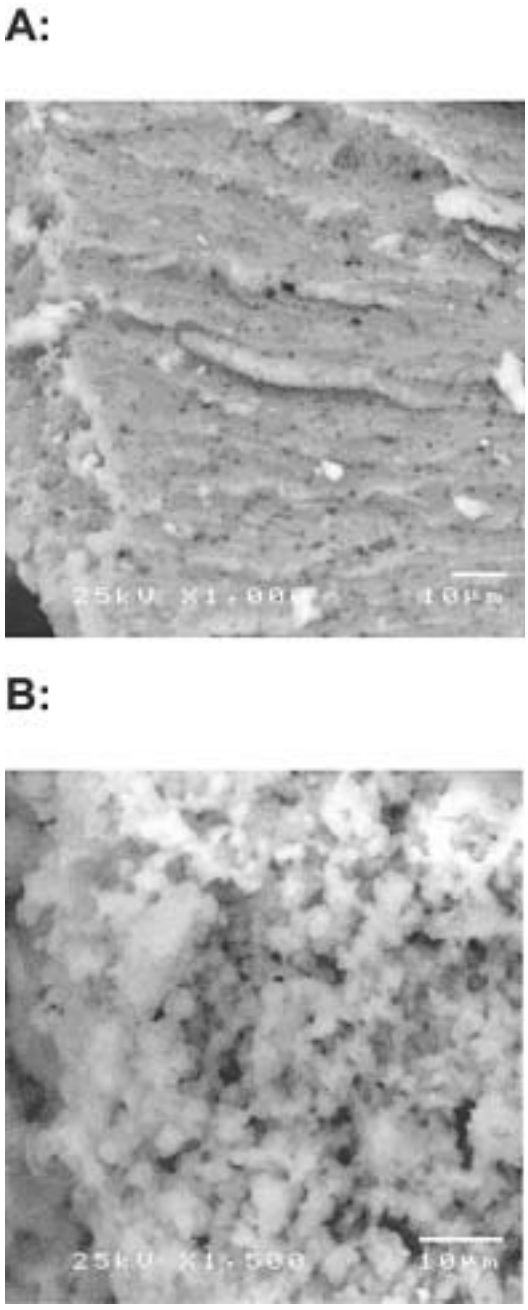

FIGURE 4 **SEM Images of the Surface of Unthiolated Polycarbophil Particles (A) and Thiolated Polycarbophil Particles (B).**

aqueous solutions, only the zeta potential of PCP-Cys particles could be determined to be –20.71 ± 1.6 mV.

Mucoadhesion Studies

To evaluate mucoadhesive properties of microparticles and examine the influence of various parameters on mucoadhesion, a new analytical method has been established. FDA was chosen as marker, because sodium fluorescein obtained by hydrolyzation of FDA can be detected in concentrations as low as 50 ng/mL.

Studies focusing on the validity of this new method demonstrated that a direct correlation between the amount of microparticles adhering to the mucosa and the subsequently determined concentration of fluorescence marker on this mucosal area is provided with a regression of $R = 0.9985$.

Influence of the Thiolation of PCP on Mucoadhesion

After application of the microparticles in dry form, the marker being incorporated in thiolated PCP remained to a significantly ($p < 0.001$) higher extent on the mucosa than being embedded in the unmodified polymer; 23.4 ± 4.8% of PCP-Cys/FDA microparticles remained on the mucosal surface after 3 h of elution, compared to only 4.5 ± 3.0% of PCP/FDA microparticles. For this study PCP-Cys with 615 ± 4.8 μmol/g thiol groups was used and results are shown in Fig. 5.

Influence of Prehydration on Mucoadhesion

When microparticles were applied to the mucosa in prehydrated form, 11.6 ± 2.0% of the fluorescence marker being embedded in PCP-Cys remained on the mucosal surface, whereas only 4.2 ± 1.8% of FDA being incorporated in unmodified PCP adhered to the mucosa within 3 h. For this study PCP-Cys with 615 ± 4.8 μmol/g thiol groups was used and the results are shown in Fig. 6.

Influence of Water Uptake on Mucoadhesion of Thiomer Particles

To point out whether the water uptake (water uptake = water [mg]/thiomer [mg]) of thiomer-microparticles is of importance for their mucoadhesive properties, PCP-Cys/FDA samples were prehydrated with different portions of 100 mM phosphate buffer ph 6.5. At 37.5-fold water uptake, what turned out to be the maximum degree of hydration, lowest mucoadhesive properties were measured. Even at minor water uptake, mucoadhesive features of microparticles strongly declined

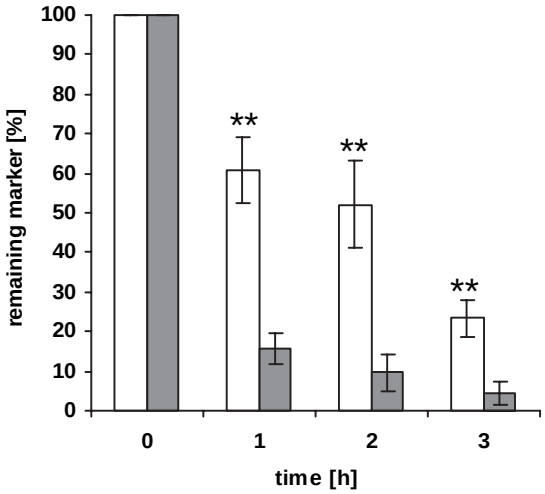

FIGURE 5 **Comparison of the Amount of Marker Being Incorporated in Polycarbophil-Cysteine Microparticles With 615 ± 4.8 μmol/g Thiol Groups (White Bars) and Polycarbophil (Gray Bars) Remaining on Intestinal Mucosa as a Function of Time. Both Types of Particles Were Applied to the Mucosa in Dry Form. Indicated Values Are Means ± SD of at Least 3 Experiments. **Differs From Polycarbophil, $p < 0.001$.**

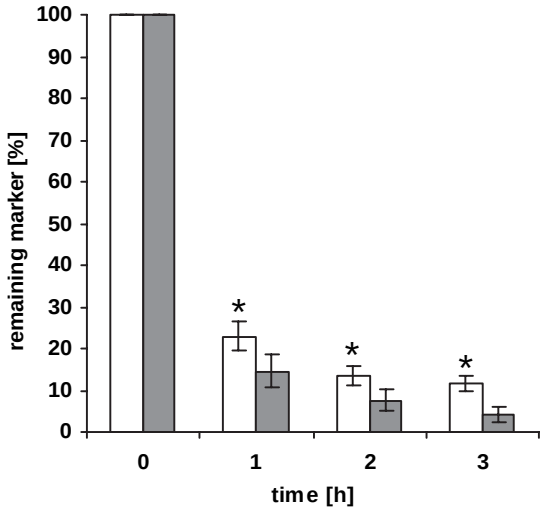

FIGURE 6 **Comparison of the Amount of Marker Being Incorporated in Polycarbophil-Cysteine Particles With 615 ± 4.8 μmol/g Thiol Groups (White Bars) and Polycarbophil (Gray Bars) Remaining on Intestinal Mucosa as a Function of Time. Both Types of Particles Were Applied to the Mucosa in Prehydrated Form. Indicated Values Are Means ± SD of at Least 3 Experiments. *Differs From Polycarbophil, $p < 0.01$.**

compared to dry application. For these experiments PCP-Cys with 615 ± 4.8 μmol/g thiol groups was used and results are shown in Fig. 7.

Influence of the Amount of Thiol Groups on Mucoadhesion of Thiomer Particles

A correlation between the amount of thiol groups immobilized on PCP-Cys/FDA microparticles and their mucoadhesive quality is demonstrated in Fig. 8. The relative highest mucoadhesive properties showed PCP-Cys/FDA microparticles with 1114 ± 7.2 μmol/g thiol groups. When being applied in dry form, 57.4 ± 7.2% of the marker being included in thiomer microparticles remained adhering to the mucosal surface.

DISCUSSION

Within this study an alternative method for the preparation of polyacrylate microparticles has been developed. In contrast to established polymerization techniques for generating poly(acrylic acid) microparticles (Kriwet et al., 1998) this method offers essential advantages. The preparation itself is simpler, as no oxygen free condition has to be provided and no free radicals for initiation of polymerization are needed. The immediate precipitation, which is used for purifying and coazervation, leads to a faster particle preparation. Because the marker FDA could be embedded in the microparticles, the incorporation of various therapeutic agents should also be feasible via this technique.

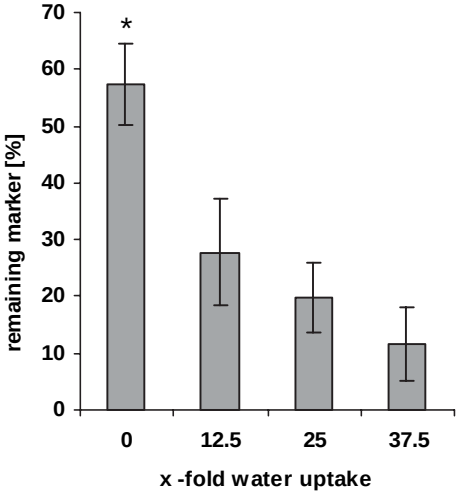

FIGURE 7 **Comparison of the Quantity of Marker Being Embedded in Polycarbophil-Cysteine Microparticles Exhibiting 615 ± 4.8 μmol/g Thiol Groups With Increasing Water Uptake (Water Uptake = Water [mg]/Thiomer [mg]) Remaining on the Mucosa After 3 h; Each Point Represents the Mean ± SD of at Least 3 Experiments. *Differs From PCP-Cys/FDA 37.5-Fold Water Uptake, $p < 0.001$.**

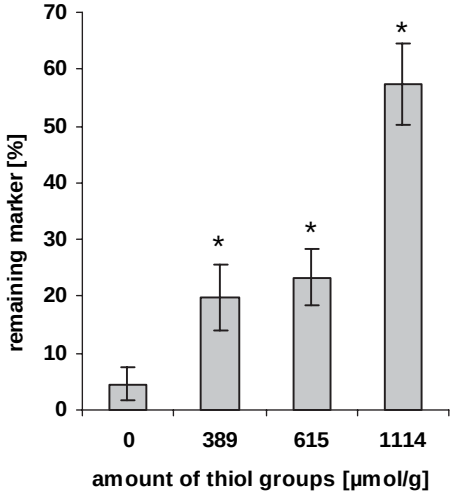

FIGURE 8 **Comparison of the Quantity of Marker Being Incorporated in Polycarbophil-Cysteine Microparticles With Increasing Amounts of Thiol Groups (389 ± 5.8 μmol/g, 615 ± 4.8 μmol/g, and 1114 ± 7.2 μmol/g) Remaining on Intestinal Mucosa After 3 h; the Microparticles Were Applied to the Mucosa in Dry Form; Each Point Represents the Mean ± SD of at Least 3 Experiments. *Differs From PCP-Cys/FDA 0 μmol/g Thiol Groups, $p < 0.001$.**

Furthermore, a simple and sensitive analytical method for determining the mucoadhesive properties of microparticles has been established. The accuracy of this method was evidenced by the given correlation between the amount of particles on mucosal areas and the subsequently measured amount of applied marker. FDA was chosen as fluorescence marker because it is insoluble in aqueous media and could consequently not be washed out with the artificial intestinal fluid. After the experiment it was easy to transform FDA into the water soluble sodium fluorescein, which is the virtual fluorescence marker. Regarding the in vivo situation, FDA should be advantageous over other fluorescence markers, as no decline in fluorescence occurs during the experiment.

PCP was selected as model thiomer because of its excellent mucoadhesive properties (Leitner et al., 2003). Results demonstrated that thiolated PCP microparticles are of advantage compared to unmodified PCP microparticles concerning adherence to intestinal mucosa and stability. PCP-Cys particles are stabilized via the in situ formation of intra- and intermolecular disulfide bonds and were therefore stable but swelling in aqueous solutions, whereas PCP particles dissolved.

Furthermore, PCP-Cys is a potent inhibitor of luminally secreted and membrane bound peptidases (Bernkop-Schnürch et al., 2001). Within this study the protective effect toward intestinal enzymes was shown on the basis of FDA hydrolyzation. This protective effect, however, should also be warranted for peptide drugs as already shown in previous studies by using different amino acid derivates (Bernkop-Schnürch & Thaler, 2000). Due to this inhibitory effect, a presystemic metabolism of incorporated peptide drugs that are extensively degraded in the GI tract could be minimized. In addition, the permeation enhancing effect of thiolated polymers could be used to improve bioavailability of higher molecular mass peptide drugs (Clausen et al., 2002; Kast et al., 2003).

Further experiments within this study showed that application of microparticles in dry form led to a 2-fold increased amount of fluorescence marker remaining on the mucosal surface compared to administration of microparticles in prehydrated form. Water uptake and immobilization of different amounts of thiol groups correlated significantly with the mucoadhesive quality of PCP-Cys/FDA. The lower the water uptake and the higher the amount of thiol groups immobilized on the thiomer, the better mucoadhesive properties were obtained.

The interpenetration of mucoadhesive polymers into the mucus gel layer is more pronounced when the polymer is applied in dry form, because of the smaller particle size of unhydrated particles. This better interpenetration leads to a greater surface area of possible interactions such as the formation of disulfide bonds between the thiomer and the mucus layer. In addition, it was shown that particles with rougher surface provide stronger adhesion to biological membranes than particles with a smooth texture (Peppas & Buri, 1985). SEM pictures of PCP-Cys/FDA provided a porous and rough texture in contrast to the smooth and relatively even structure of the PCP/FDA particles, leading perhaps to further improved mucoadhesive properties of thiolated PCP microparticles.

According to these results, thiomer microparticles with a high amount of immobilized thiol groups, reaching the small intestinal mucosa in unhydrated form should lead to the comparatively highest residence time of microparticulate delivery systems. An enteric coating of capsules containing the thiomer particles, for example, should ensure that the microparticles reach the small intestine in dry form.

Microparticles providing FDA as marker could be administered to rats or mice, and after sacrificing these animals, the amount of remaining marked thiomer in each GI segment could be quantified. This technique could be used for various types of polymers and thiomers, not only for microparticles but also for nanoparticles.

CONCLUSION

Within the present study it could be shown for the first time that the amount of thiol groups correlates significantly with mucoadhesive properties of thiomer microparticles. In addition, experiments performed to assess the impact of water uptake on mucoadhesive properties of microparticles demonstrated that microparticles applied in dry form provided the comparatively longest residence time on mucosal surfaces. As a consequence, the investigation of delivery systems, which release thiomer microparticles at once in unhydrated form within the small intestine are in high demand from the oral drug delivery point of view.

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