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Development and In Vitro Characterization of Liposomes Coated with Thiolated Poly(Acrylic Acid) for Oral Drug Delivery

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Mucoadhesive drug delivery systems offer promising opportunities for oral drug delivery. The aim of this study was to investigate the feasibility of preparing liposomes that are coated with the multifunctional polymer poly(acrylic acid)-cysteine (PAA-Cys). Cationic multilamellar vesicles (MLV) as well as cationic submicron-sized liposomes (ssLip) were prepared and coated with PAA-Cys. Size, zeta potential, amount of free thiol groups, aggregation behavior, drug-loading, and drug release of these novel carriers were evaluated. A switch of the initial positive zeta potential to a negative value after coating indicated the successful coating procedure. In both size ranges, MLV and ssLip, the amount of free thiol groups was comparable to that in a PAA-Cys solution of the same concentration. Drug loading of the hydrophilic marker fluorescence-isothiocyanate 4 kDa (FD4) was higher in PAA-Cys liposomes in comparison to noncoated liposomes, but lower in comparison to liposomes coated with unmodified poly(acrylic acid) (PAA). Only a minor ssLip or no increase MLV of the drug-loading was observed when using carboxyfluorescein (CF). These effects were attributed to interactions between the markers and the poly(acrylates). Coating of liposomes with PAA-Cys and PAA did not influence the release profile of FD4 and CF, whereas the release profile was affected by the molecular mass of the marker and the liposome size. In conclusion, the feasibility of coating liposomes with PAA-Cys was demonstrated, and it could be shown that this novel carrier system fulfills the basic requirements for an intended use in oral drug delivery.

Keywords oral drug delivery; polymer-coated liposomes; oral peptide delivery; thiomers; PAA-Cys

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INTRODUCTION

Various therapeutic agents are poorly absorbed after oral administration and are therefore administered as injections only. Especially in the case of chronic diseases, oral delivery systems would be highly beneficial for patients. The oral application route offers advantages such as painless administration and improved patient compliance. Moreover, it is believed that patients would start earlier with an oral treatment in comparison to a therapy requiring injections. Besides hydrophilic macromolecules including peptides and proteins as well as pDNA or siRNA, various other drugs are only poorly absorbed. This is due to either their physico-chemical properties such as low water solubility or aggregation (Swarbrick and Boylan, 2002), and/or to the barriers that are connected to oral administration. These barriers include low pH and enzymatic degradation mediated by pepsin in the stomach, the occurrence of various luminally secreted and membrane-bound enzymes in the intestine (Woodley, 1994), the physical barrier of the mucus (Bernkop-Schnürch & Fragner, 1996) and the permeation barrier.

Many delivery systems have been developed to improve oral drug absorption; however, especially the delivery of hydrophilic macromolecules remains one of the major challenges for pharmaceutical technologists. Mucoadhesive delivery systems can prolong the residence time of the dosage form on the intestinal membrane, which consequently leads to improved drug plasma levels following oral administration (Chary, Vani, & Rao, 1999). Among the different mucoadhesive delivery systems, two attempts are notably promising: thiolated polymers and polymer-coated liposomes. Thiolated polymers, or the so-called thiomers, constitute of a polymeric backbone which has been modified to display free thiol groups on the surface. Polymeric backbones include for instance chitosan or poly(acrylates). The schematic presentation of a

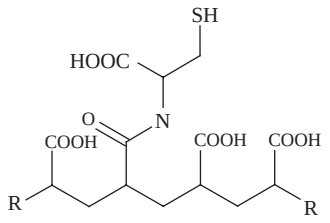

FIGURE 1. Chemical substructure of a poly(acrylate) backbone which has been modified with cysteine.

thiomer, which is based on poly(acrylates) and which has been modified with the thiol group bearing amino acid cysteine is depicted in Figure 1. It has been demonstrated in numerous studies that thiomers display strong mucoadhesive properties (Grabovac, Guggi, & Bernkop-Schnürch, 2005), that they can improve drug permeation (Bernkop-Schnürch, Kast, & Guggi, 2003), provide controlled drug release (Bernkop-Schnürch, Scholler, & Biebel, 2000) and are capable of inhibiting proteolytic enzymes and/or efflux pumps (Bernkop-Schnürch, Walker, & Zarti, 2001; Werle & Hoffer, 2006). Data of in vivo studies showing an improved drug uptake mediated by thiomers are available (Föger, Schmitz, & Bernkop-Schnürch, 2006; Guggi, Kast, & Bernkop-Schnürch, 2003). Another promising approach is the coating of liposomes with mucoadhesive polymers such as chitosan or poly(acrylates). Such coatings have been evaluated to greatly improve the oral uptake of therapeutic proteins including insulin and calcitonin (Takeuchi, Yamamoto, Niwa, Hino, & Kawashima, 1996; Takeuchi, Matsui, Yamamoto, & Kawashima, 2003). This effect is believed to be caused by the improved mucoadhesive properties of such liposomes.

In this study, we combined the two strategies by coating positively charged liposomes with thiolated poly(acrylic acid) with a molecular mass of 450 kDa (PAA-Cys [poly(acrylic acid)-cysteine]). The aim of the study was to investigate the feasibility of such a coating and to characterize this proposed novel delivery system. Size, zeta potential, amount of free thiol groups, aggregation, drug-loading capacity, and release properties of PAA-Cys-coated liposomes have been evaluated and compared with the properties of PAA-coated liposomes as well as noncoated liposomes.

MATERIALS AND METHODS

Materials

L-α-distearolyphosphatidylcholine (DSPC, Nippon Oil and Fats Co., Japan), cholesterol (Chol, Sigma, Japan), stearylamine (SA, Tokyo Kasei, Japan), poly(acrylic acid) 450 kDa (PAA, Sigma, Japan), fluorescence-isothiocyanate 4 kDa (FD4, Sigma, Japan), carboxyfluorescein (CF, Sigma, Japan), 5,5′-dithiobis(2-nitro-benzoic acid) (Ellman's reagent, Sigma, Japan), *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDAC, Sigma, Japan), and reduced glutathione (GSH, Sigma, Japan) were used as received. All used reagents were of analytical grade.

Synthesis and Characterization of Poly(Acrylic Acid)-Cysteine Conjugate

The synthesis and characterization of PAA-Cys has been described in detail previously (Marschütz & Bernkop-Schnürch, 2002). In brief, *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDAC) was used to activate the carboxylic groups of PAA. Accordingly, a solution of L-cysteine was added. After incubation, the polymer-conjugate was dialyzed to remove unbound cysteine and EDAC. PAA-Cys was lyophilized and the amount of covalently bound thiol groups was evaluated using the Ellman's test as described previously (Kafedjiiski, Föger, Werle, & Bernkop-Schnürch, 2005).

Preparation of Liposomes and Coated Liposomes

Preparation of liposomes coated with the poly(acrylate) carbopol has been described previously (Takeuchi et al., 2003). Cationic multilamellar liposomes (MLV) consisting of DSPC, SA, and Chol (molar ratio: 8:0.2:1) were prepared using the thin-film method. DSPC, SA, and Chol were dissolved in chloroform and a thin-film lipid layer was obtained by evaporating the organic solvent for 3 h at 40°C and water jet vacuum. Afterwards, the obtained thin film was dried in a vacuum oven overnight to ensure complete removal of chloroform. Hydration was performed with HEPES buffer pH 7.4 by repeated gentle heating and vortexing. Finally, the liposome suspension was incubated for 30 min at 10°C. Submicron-sized liposomes (ssLip) were prepared by sonicating the MLV suspension for 3 min (Sonifier 250, Branson).

Polymer coating was performed by mixing an aliquot of the described liposomal suspensions with a 0.2% polymer solution in HEPES pH 7.4 (for thiol group determination a 0.6% PAA-Cys solution was used in addition) and vortexing. The coated liposomes were incubated for 30 min at 10°C. The size of MLV was determined using a LDSA 2400A particle size analyzer (Tohnichi Computer Co., Ltd., Tokyo, Japan) and the size of ssLip was determined by dynamic light scattering (Zetasizer 3000 HSa, Malvern, Worcestershire, UK). The zeta potential was measured with suspensions that were prepared by adding 20 μL of the liposomal suspension to 8 mL of purified water. For control experiments with noncoated liposomes, a 1:1 dilution of the liposomal suspension with buffer was performed in order to achieve same concentrations as with coated liposomes.

Determination of Thiol Groups

Thiol groups of PAA-Cys in solution as well as of liposomal suspension of MLV and ssLip coated with PAA-Cys were determined using Ellman's reagent. After preparation of the coated liposomes, each suspension was diluted 1:10 with

Ellman's buffer (0.5 M phosphate buffer, pH 8.0). To 500 μL of the diluted MLV and ssLip suspensions, 500 μL of Ellman's reagent solution (0.3 mg Ellman's reagent per mL Ellman's buffer pH 8.0) was added. In addition, control samples were prepared by adding 500 μL of Ellman's buffer to 500 μL of MLV and ssLip, respectively. To quantify the free thiol groups, a calibration curve was performed with GSH. Samples were incubated for 60 min in the dark and ultracentrifuged for 45 min at 23,100 × *g* and 4°C. Finally, the fluorescence of the supernatant was determined using a spectrophotometer (Fluostar Galaxy, BMG Labtech, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Results are expressed as percent of thiol groups of PAA-Cys in solution.

Aggregation Studies

To determine a possible aggregation of PAA-Cys-coated liposomes, coated MLV and ssLip were incubated at room temperature in open vessels to allow oxidation for a period of 5 days. Samples were analyzed regarding size and zeta potential after 30 min, 1, 4, 8, 24, and 120 h. Additional experiments were performed with noncoated MLV and ssLip as well as PAA-coated MLV and ssLip for data comparison.

Drug-Loading Studies

First, a thin-film lipid layer was prepared as described above. After removal of the organic solvent, the lipid layer was hydrated with either a 1 mg/mL solution of FD4 or a 1 mg/mL solution of CF in HEPES buffer pH 7.4. Liposomes coated with PAA and PAA-Cys were prepared according to the method described above. Samples were ultracentrifuged for 45 min at 23,100 × *g* and 4°C and the fluorescence of the supernatant was measured using a Fluostar Galaxy (excitation = 485 nm, emission = 538 nm). A calibration curve was prepared with the initial fluorescence marker solution and the results were expressed as percentage of the initial drug amount.

Release Studies

To investigate whether liposome coating with PAA-Cys leads to an alteration in drug release in comparison to coating with PAA or noncoated liposomes, the release of the fluorescence markers FD4 and CF from the coated liposomes was determined. Liposomes were prepared and coated with PAA and PAA-Cys, respectively, as described above. The used hydration medium was 1 mg/mL FD4 and 1 mg/mL CF in HEPES pH 7.4, respectively. Liposomal suspensions of 1,000 μL (MLV and ssLip coated with PAA and PAA-Cys, respectively as well as noncoated MLV and ssLip) were transferred into dialysis tubings (Spectra/Por, MWCO 12–14,000 Da, Spectrum Laboratories Inc., CA, USA). Then the tubings were placed into vessels containg 50 mL HEPES pH 7.4 and incubated at room temperature with gentle stirring. Additional experiments were performed in exactly the same way as described by using solutions of the same concentrations of FD4 and CF in HEPES pH 7.4. Samples were withdrawn after predetermined time points and analyzed with a spectrophotometer (Fluostar Galaxy; excitation = 485 nm, emission = 538 nm).

Statistical Data Analyses

Statistical data analyses were performed using the Student's *t* test with $p < .05$ as the minimal level of significance. Calculations were done using the online calculation programme at http://www.physics.csbsju.edu/stats/t-test_bulk_form.html.

RESULTS AND DISCUSSION

Synthesis of PAA-Cys

A detailed description of the synthesis of PAA-Cys and of the characterization of the amount of free thiol groups and disulfide bonds has been published previously (Marschütz & Bernkop-Schnürch, 2002). In this study, the used PAA-Cys conjugate displayed 353 ± 42 μmol free thiol groups per gram polymer as determined using the Ellman's test. This amount of thiol groups corresponds with data of previous studies and has been shown to affect parameters such as mucoadhesion and permeation (Leitner, Marschütz, & Bernkop-Schnürch, 2003).

Preparation of Noncoated and Coated Liposomes

To prepare PAA and PAA-Cys-coated liposomes, cationic liposomes were prepared in an initial step by incorporating the cationic compound stearylamine into the liposomes based on DSPC and Chol. These liposomes have been used as cationic core liposomes for coatings with the anionic poly(acrylate) carbopol (Takeuchi et al., 2003). The prepared cationic MLV had a size of around 6.8 μm and a zeta potential of around +65. After coating with unmodified PAA, the zeta potential switched to around −70, whereas the size slightly but not significantly increased. PAA-Cys-coated MLV displayed the largest size (not significant), of around 7.4 μm and a zeta potential of around −60. Generally, a switch of the zeta potential of cationic liposomes to a negative value after mixing with anionic polymers indicates successful coating (Takeuchi et al., 2003).

After sonicating the MLV for 3 min, the average size of ssLip was around 220 nm and a slight but not significant increase of the size in the ranking PAA-Cys ssLip > PAA ssLip > noncoated ssLip could be observed. In the distribution pattern, two peaks, one at around 150 nm and one at around 500 nm was observed. In general, submicron-sized liposomes with more uniform size distribution can be prepared by using, for example, extrusion or by modifying the conditions of the sonication. The initial positive zeta potential of the core-ssLip of around +40 was switched to around −65 after coating with PAA and to around −40 after coating with PAA-Cys. Another research group, who investigated the effect of chitosan and

thiolated chitosan coating of poly(isobutyl cyanoacrylate) nanoparticles observed a similar difference in the zeta potential between unmodified and thiolated chitosan coated particles. In that case, both polymers displayed the same amount of amino groups, which is due to the formation of an amidine following the reaction of chitosan and 2-iminothiolane to chitosan–thiobutylamidine. If the same amount of amino groups occurs in both polymers, also a similar zeta potential might be expected. As this was not the case, the authors suggested that the difference of the zeta potential (around +40 in the case of unmodified chitosan and around +25 in the case of thiolated chitosan) was most likely not caused by the chemical modification itself, but rather by changes of the viscosity of the surrounding gel layer (Bravo-Osuna, Vauthier, Farabollini, Palmieri, & Ponchel, 2007). Therefore, it can be anticipated that the difference of the zeta potential of unmodified and thiolated PAA observed in this study is most likely also caused by changes of the viscosity. Another possible explanation for this observation is that the different structure of the coating could change the accessibility of the charged groups to ions in the surrounding medium.

Amount of Free Thiol Groups on the Surface of PAA-Cys Coated MLV and ssLip

In the case of thiomers, free thiol groups are essential for the mucoadhesive, enzyme-inhibitory and permeation-enhancing properties. Therefore, the amount of free thiol groups of PAA-Cys in solution as well as of MLV and ssLip coated with PAA-Cys was determined using the Ellman's reagent. This reagent has been used in various studies for the determination of thiol groups, and in particular for the determination of thiol groups in thiolated polymers. The amount of free thiol groups in suspensions containing PAA-Cys-coated liposomes was in the same range as the amount of free thiol groups of PAA-Cys in solution (Table 1). On the one hand, this shows that the coating procedure does not lead to any loss of active thiol groups that are responsible for the improved features of PAA-Cys in comparison to PAA. Therefore, PAA-Cys-coated liposomes prepared by the described method might also possess these beneficial properties. On the other hand, it raises the question, if information regarding the formation of the polymer coating can be derived from these data. Generally, if the liposomes are coated with several polymer layers, a decrease in the amount of free thiol groups might be anticipated. No difference in the percentage of free thiol groups was monitored using two different concentrations of thiomers for the coating (final PAA-Cys concentration 0.1 and 0.3%, see Table 1) The possibility that the low molecular mass Ellman's reagent (MW = 396 g/mol) can entirely diffuse into the polymer layer within the 1-h incubation period to react with every thiol group including the ones which are located within the polymer layers cannot be excluded. This might explain why 100% of the free thiol groups were detected after the coating process, regardless of the PAA-Cys concentrations used.

TABLE 1
Percentage of Free Thiol Groups of PAA-Cys in Solution, PAA-Cys-Coated MLV, and PAA-Cys-Coated ssLip Using Different Concentrations (Conc.) of PAA-Cys; Each Point Represents the Mean ± *SD* of at Least Three Experiments

Formulation	Conc. (%)	Free Thiol Groups (%)
PAA-Cys solution	0.1	100.0 ± 1.5
PAA-Cys MLV	0.1	107.2 ± 4.0
PAA-Cys ssLip	0.1	111.1 ± 2.2
PAA-Cys solution	0.3	100.0 ± 1.4
PAA-Cys MLV	0.3	95.1 ± 2.8
PAA-Cys ssLip	0.3	106.7 ± 0.9

Aggregation

In Tables 2 and 3, the size and zeta potential of noncoated as well as PAA and PAA-Cys-coated MLV and ssLip 30 min after preparation and 5 days afterward are shown. No significant changes of the liposome size or the zeta potential could be observed, regardless of liposome size (MLV/ssLip) and coating, demonstrating the stability of the investigated liposomes in the suspension. Gained data revealed low deviation of size and zeta potential during the observation period (data not shown). Also the polydispersity index (PI) in the case of ssLip did not markedly increase. It might be argued, that if PAA-Cys-coated liposomes do not tend to aggregate during the in vitro experiments then also a formation of disulfide bonds might not take place in vivo. The reason for the stability of PAA-Cys-coated liposomes in suspension can most likely be explained by the defined and constant conditions during the storage period as well as the strong negative zeta potential of the liposomes. This negative

TABLE 2
Size and Zeta Potential of MLV 30 min After Coating and 5 Days After Coating

Liposomes	Size (μm)	Zeta Potential (mV)
After 30 min		
Noncoated	6.8 ± 2.7	+65.3 ± 6.3
PAA coated	7.1 ± 3.1	−72.4 ± 7.1*
PAA-Cys coated	7.4 ± 3.5	−61.4 ± 8.6*
After 5 days		
Noncoated	6.6 ± 3.2	+67.2 ± 8.6
PAA coated	7.2 ± 3.6	−71.8 ± 8.1*
PAA-Cys coated	7.3 ± 2.9	−63.2 ± 9.1*

*Differs significantly ($p < .005$) from noncoated liposomes; each value represents the mean ± *SD* of at least three experiments.

TABLE 3
Size, PI, and Zeta Potential of ssLip 30 min After Coating and 5 Days After Coating

Liposomes	Size (nm)	PI	Zeta Potential (mV)
After 30 min			
Noncoated	221 ± 55	0.58 ± 14	+41.0 ± 9.7
PAA coated	235 ± 68	0.58 ± 14	–66.0 ± 9.1*
PAA-Cys coated	256 ± 73	0.46 ± 15	–38.2 ± 8.9*
After 5 days			
Noncoated	242 ± 48	0.56 ± 12	+49.2 ± 8.3
PAA coated	248 ± 62	0.69 ± 15	–63.6 ± 9.4*
PAA-Cys coated	272 ± 61	0.88 ± 11	–37.1 ± 8.4*

*Differs significantly ($p < .005$) from noncoated liposomes; each value represents the mean ± *SD* of at least three experiments.

charge might lead to repulsion among the liposomes, so that the free thiol groups might not be able to form disulfide bonds. However, in the vivo situation there are many factors which might influence the aggregation behavior observed in vitro, including varying pH and ion concentrations as well as gut motility during gastrointestinal passage.

Drug-Loading Studies

Drug-loading studies were performed with the hydrophilic marker FD4, which has an average molecular mass of 4 kDa and which is frequently used as a model drug for peptides and proteins, as well as with the hydrophilic low molecular mass fluorescence marker CF (376 Da). As shown in Table 4, the FD4 loading of both, PAA and PAA-Cys-coated liposomes did strongly increase in comparison to noncoated liposomes, whereas only a minor increase was observed in the case of CF. As the coating is performed after the hydration step, it can be anticipated that the amount of the hydrophilic markers entrapped inside of the hydrophilic core of the liposomes remains the same. Interactions of FD4 with the polymer coating are the most likely cause of the observed increase in drug loading. In orientating studies (data not shown), we investigated this theory by incubating liposomes (noncoated and PAA-coated MLV) containing no fluorescence marker in a FD4 solution. In addition, the "drug loading" of a PAA solution was assessed by using the centrifugation method described above. Results indicate that the increased drug loading is entirely due to interactions of FD4 with PAA. As this phenomenon was not observed in the case of the low molecular mass marker CF, the FD4-polymer interactions are most likely mediated by the high molecular mass of FD4. Independent of the size of the liposomes, the FD4 loading of liposomes coated with PAA was higher than that of liposomes coated with PAA-Cys. However, this observation does not consequently imply that drug loading of PAA-Cys-coated liposomes is in any case

TABLE 4
Drug-Loading of Noncoated Cationic Liposomes, Liposomes Coated with PAA, and Liposomes Coated with PAA-Cys Containing the Hydrophilic Model Drugs FD4 or CF

Liposomes	%	mg/g Lipid
MLV—FD4		
Noncoated	7.1 ± 4.0	2.1 ± 1.2
PAA coated	76.2 ± 3.7*	22.1 ± 1.1*
PAA-Cys coated	35.0 ± 2.0*	10.2 ± 0.6*
ssLip—FD4		
Noncoated	9.5 ± 2.5	2.8 ± 0.7
PAA coated	58.6 ± 2.7*	17.0 ± 0.8*
PAA-Cys coated	27.2 ± 1.4*	7.9 ± 0.4*
MLV—CF		
Noncoated	8.7 ± 3.3	2.5 ± 1.0
PAA coated	12.3 ± 2.6	3.6 ± 0.8
PAA-Cys coated	12.5 ± 2.6	3.6 ± 0.8
ssLip—CF		
Noncoated	8.3 ± 1.1	2.4 ± 0.3
PAA coated	13.5 ± 1.9*	3.9 ± 0.6*
PAA-Cys coated	15.4 ± 3.4*	4.5 ± 1.0*

Values are presented in percent of the initial fluorescence marker concentration used (%) as well as in mg drug per g liposomes (mg/g lipid).

*Differs significantly ($p < .05$) from noncoated liposomes; each point represents the mean ± *SD* of at least three experiments.

lower in comparison to PAA-coated liposomes. As the improved drug loading of coated liposomes is due to drug–polymer interactions, these interactions depend on the properties of the drug as well as the polymer, respectively. As the exact interactions between liposomes and oppositely charged polymers are unknown, it remains unclear whether the observed increased drug load is beneficial for oral drug delivery. Besides, it has been demonstrated recently, that in the case of peptide/protein drugs such as calcitonin, drug-loading of more than 90% was achieved in chitosan- and carbopol-coated MLV (Takeuchi et al., 2003).

Release Studies

Due to the inter- and intramolecular crosslinking of thiolated polymer chains, a sustained drug release out of delivery systems based on such polymers has been reported previously (Hoyer, Föger, Kafedjiiski, Loretz, & Bernkop-Schnürch, 2007). Therefore, it was of particular interest to investigate whether coating of liposomes with these modified polymers leads to alterations in the release profile in comparison to noncoated liposomes as well as liposomes coated with the corresponding unmodified polymer. A general observation within the current studies was that the model drug FD4 was released more slowly from MLV

(Figure 2, left) than from ssLip (Figure 2, right). On the contrary, no liposome size-dependent differences in the release profile of CF out of MLV and ssLip was observed (Figure 3). Moreover, CF was released much more rapid (100% in less than 4 h) than FD4 (100% released not before 12 h). Interestingly, no differences between the release profiles of noncoated, PAA- and PAA-Cys-coated liposomes were observed. Notably, there was no difference in the release profile of CF solution in comparison with CF liposomes (Figure 3), whereas such a difference was observed between FD4 solution and FD4 ssLip and in particular between FD4 solution and FD4 MLV (Figure 2). These observations lead to the conclusion, that the polymer coating does not affect the release profile, but that the size of the drug as well as the size of the liposomes are two important factors that influence the release profile. As the actual formation of the polymer layers covering the liposomes is not known, no definite statement can be given that explains the similar drug release from PAA/PAA-Cys-coated liposomes and noncoated liposomes. In general, it is not surprising that a sustained release will be much more pronounced, for example, in matrix tablets or patch systems than in coated liposomes, especially when considering the mechanisms involved in the process. In the case of for example, patch systems (Hoyer et al., 2007), it is likely that most polymer chains of the entire polymeric network are connected with each other via disulfide bonds, but also via hydrogen bonds or interpenetration of polymer chains. In the case of polymer-coated liposomes, each liposome represents a single delivery unit surrounded by a polymer layer. Therefore, the cohesive properties of the polymer layer covering the liposome will most likely not have a pronounced effect on the release profile. Contrary, a sustained release out of polymeric microparticulate systems entirely based on thiomers has been reported (Elhassan Imam & Bernkop-Schnürch, 2005).

In the current study it was shown that cationic liposomes can be coated with the anionic thiomer PAA-Cys, and that the resulting liposomes expose free thiol groups on their surface. Regardless of the size of the liposomes—MLV or ssLip—the novel coated liposomes displayed similar features concerning size, zeta potential, aggregation behavior, drug loading, and drug release like liposomes coated with unmodified PAA. Liposomes coated with another poly(acrylate), namely carbopol, have been evaluated to be a promising tool for the oral delivery of peptide/

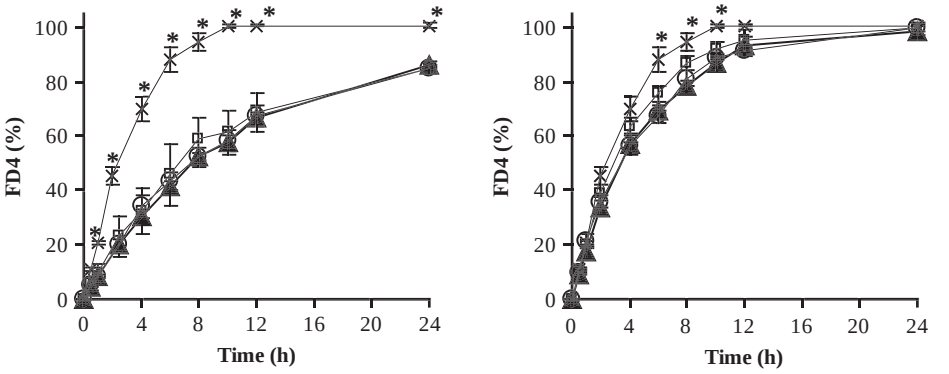

FIGURE 2. FD4 release profiles from MLV (left) and ssLip (right) in HEPES pH 7.4: -×-, FD4 solution; -▲-, uncoated liposomes; -□-, PAA-coated liposomes, -○-, PAA-Cys-coated liposomes; * all liposome formulations differ significantly ($p < .05$) from FD4 solution; each point represents the mean ± *SD* of at least three experiments.

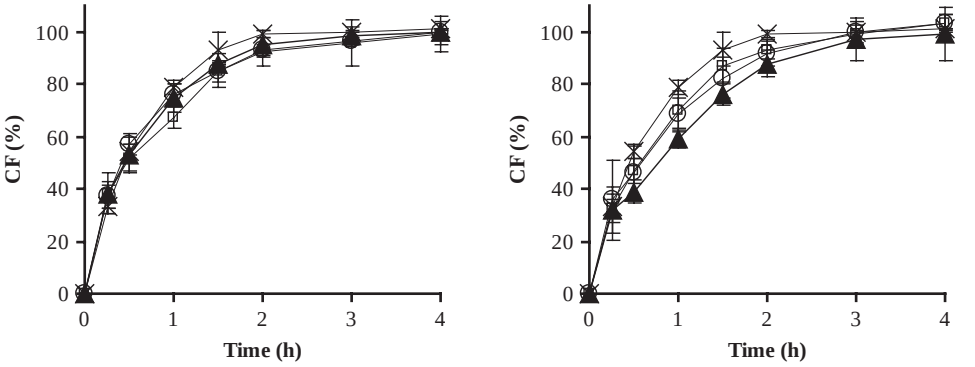

FIGURE 3. CF release profiles from MLV (left) and ssLip (right) in HEPES pH 7.4 -×-, CF solution; -▲-, uncoated liposomes; -□-, PAA-coated liposomes; -○-, PAA-Cys-coated liposomes; each point represents the mean ± *SD* of at least three experiments.

protein drugs. Therefore, the described novel delivery system fulfills the basic requirements for an intended use for the oral delivery of hydrophilic macromolecules.

However, whether PAA-Cys coating leads to an improved efficacy of the liposomes in comparison to a coating with unmodified PAA is the subject of ongoing investigations. In particular, a thorough comparison of the mucoadhesive and the permeation enhancing effects of PAA and PAA-Cys-coated liposomes is necessary to demonstrate the predominance of PAA-Cys-coated liposomes. The strongly improved mucoadhesiveness of thiolated polymers in comparison to unmodified polymers is mediated by the formation of disulfide bonds between the free thiol groups of the thiomer and cysteine-rich subdomains of mucus glycoproteins. It has been demonstrated in this study that the described preparation technique of PAA-Cys-coated liposomes does not lead to a loss of free thiol groups. Therefore, improved mucoadhesion of the novel delivery systems seems generally likely. The permeation-enhancing effect of thiomers has been shown to be due to an inhibition of the enzyme tyrosine phosphatase. A combination of reduced glutathione and thiolated polymers has been found to even further improve the permeation-enhancing effect of thiomers (Clausen, Kast, & Bernkop-Schnürch, 2002). Analog to the mechanism of mucadhesion of thiomers, free thiol groups are essential for the permeation enhancing properties of these polymeric excipients. Taking these considerations into account, the novel delivery system that has been described in this study is believed to further improve the features of so far described polymer-coated liposomes for oral drug delivery.

CONCLUSION

MLV and ssLip were successfully coated with the thiolated polymer PAA-Cys. The amount of active thiol groups in the liposome suspension of both, PAA-Cys-coated MLV and ssLip was in the same range as the amount of PAA-Cys in solution. No significant changes in size and zeta potential were observed during a 5-day incubation period. Polymer coating affected the drug loading, though, it can be anticipated that the drug amount entrapped inside of the liposomes was not altered. The release profile of FD4 and CF entrapped in PAA-Cys-coated liposomes did not differ significantly from the profiles observed with PAA-coated liposomes and noncoated liposomes. In conclusion, it was demonstrated that it is feasible to prepare PAA-Cys-coated liposomes and that they fulfill the basic requirements regarding stability, drug loading, and drug-release for an intended use in oral drug delivery. If PAA-Cys coating leads to improved features of these novel carriers such as improved mucoadhesion and/or improved drug permeation is currently under investigation.

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REFERENCES

Bernkop-Schnürch, A., & Fragner, R. (1996). Investigations into the diffusion behaviour of polypeptides in native intestinal mucus with regard to their peroral administration. *Pharm. Sci., 2*, 361–363.

Bernkop-Schnürch, A., Kast, C. E., & Guggi, D. (2003). Permeation enhancing polymers in oral delivery of hydrophilic macromolecules: Thiomer/GSH systems. *J. Control. Release, 93*, 95–103.

Bernkop-Schnürch, A., Scholler, S., & Biebel, R. G. (2000). Development of controlled drug release systems based on thiolated polymers. *J. Control. Release, 66*, 39–48.

Bernkop-Schnürch, A., Walker, G., & Zarti, H. (2001). Thiolation of polycarbophil enhances its inhibition of intestinal brush border membrane bound aminopeptidase N. *J. Pharm. Sci., 90*, 1907–1914.

Bravo-Osuna, I., Vauthier, C., Farabollini, A., Palmieri, G. F., & Ponchel, G. (2007). Mucoadhesion mechanism of chitosan and thiolated chitosan-poly(isobutyl cyanoacrylate) core-shell nanoparticles. *Biomaterials, 28*, 2233–2243.

Chary, R. B., Vani, G., & Rao, Y. M. (1999). In vitro and in vivo adhesion testing of mucoadhesive drug delivery systems. *Drug Dev. Ind. Pharm., 25*, 685–690.

Clausen, A. E., Kast, C. E., & Bernkop-Schnürch, A. (2002). The role of glutathione in the permeation enhancing effect of thiolated polymers. *Pharm. Res., 19*, 602–608.

Elhassan Imam, M., & Bernkop-Schnürch, A. (2005). Controlled drug delivery systems based on thiolated chitosan microspheres. *Drug Dev. Ind. Pharm., 31*, 557–565.

Föger, F., Schmitz, T., & Bernkop-Schnürch, A. (2006). In vivo evaluation of an oral delivery system for P-gp substrates based on thiolated chitosan. *Biomaterials, 27*, 4250–4255.

Grabovac, V., Guggi, D., & Bernkop-Schnürch, A. (2005). Comparison of the mucoadhesive properties of various polymers. *Adv. Drug Deliv. Rev., 57*, 1713–1723.

Guggi, D., Kast, C. E., & Bernkop-Schnürch, A. (2003). In vivo evaluation of an oral salmon calcitonin-delivery system based on a thiolated chitosan carrier matrix. *Pharm. Res., 20*, 1989–1994.

Hoyer, H., Föger, F., Kafedjiiski Loretz, K. B., & Bernkop-Schnürch, A. (2007). Design and evaluation of a new gastrointestinal mucoadhesive patch system containing chitosan-glutathione. *Drug Dev. Ind. Pharm., 33*, 1289–1296.

Kafedjiiski, K., Föger, F., Werle, M., & Bernkop-Schnürch, A. (2005). Synthesis and in vitro evaluation of a novel chitosan-glutathione conjugate. *Pharm. Res., 22*, 1480–1488.

Leitner, V. M., Marschütz, M. K., & Bernkop-Schnürch, A. (2003). Mucoadhesive and cohesive properties of poly(acrylic acid)-cysteine conjugates with regard to their molecular mass. *Eur. J. Pharm. Sci., 18*, 89–96.

Marschütz, M. K., & Bernkop-Schnürch, A. (2002). Thiolated polymers: Self-crosslinking properties of thiolated 450 kDa poly(acrylic acid) and their influence on mucoadhesion. *Eur. J. Pharm. Sci., 15*, 387–394.

Swarbrick, J., & Boylan, J. C. (2002). *Encyclopedia of pharmaceutical technology* (2nd ed., p. 885). New York: Marcel Dekker, Inc.

Takeuchi, H., Matsui, Y., Yamamoto, H., & Kawashima, Y. (2003). Mucoadhesive properties of carbopol or chitosan-coated liposomes and their effectiveness in the oral administration of calcitonin to rats. *J. Control. Release, 86*, 235–242.

Takeuchi, H., Yamamoto, H., Niwa, T., Hino, T., & Kawashima, Y. (1996). Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. *Pharm. Res., 13*, 896–901.

Werle, M., & Hoffer, M. (2006). Glutathione and thiolated chitosan inhibit multidrug resistance P-glycoprotein activity in excised small intestine. *J. Control. Release, 111*, 41–46.

Woodley, J. F. (1994). Enzymatic barriers for GI peptide and protein delivery. *Crit. Rev. Ther. Drug Carrier Syst., 11*, 61–95.