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Development and In Vitro Evaluation of Surface Modified Poly(lactide-co-glycolide) Nanoparticles with Chitosan-4-Thiobutylamidine

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ABSTRACT The objective of this study was to develop a nanoparticulate drug delivery system based on the surface modification of poly(lactide-co-glycolide) (PLGA) nanoparticles with a thiolated chitosan. PLGA nanoparticles were prepared by the emulsification-solvent evaporation method. Immobilization of chitosan to the surface of PLGA nanoparticles via amide bonds was mediated by a carbodiimide. Thiol groups were covalently bound to the chitosan surface of particles by reaction with 2-iminothiolane. Obtained nanoparticles were characterized in vitro regarding size, zeta potential, thiol group content, stability at different pH values, mucoadhesion, and drug release. Results demonstrated that the surface modification of PLGA nanoparticles with thiolated chitosan (chitosan-TBA) leads to nanoparticles of a mean diameter of 889.5 ± 72 nm and positive zeta potential of + 24.74 mV. The modified nanoparticles contained 7.32 ± 0.24 μmol thiol groups per gram nanoparticles. The size of nanoparticles was strongly influenced by the pH of the surrounding medium, being 925.0 ± 76.3 nm at pH 2 and 577.8 ± 66.7 nm at pH 7.4. Thiolated nanoparticles showed a 3.3-fold prolonged residence time on the mucosa and an unchanged release profile in comparison to unmodified PLGA nanoparticles. These data suggest that surface modified chitosan-TBA conjugate PLGA nanoparticles have the potential to be used as mucoadhesive drug delivery system.

KEYWORDS Nanoparticles, PLGA, Chitosan, Mucoadhesion, Drug delivery

INTRODUCTION

In the past decades micro- and nanospheres have been recognized as potential carriers for the transport of various therapeutics across mucosal barriers. Recently, poly(lactide-co-glycolide acid) (PLGA) nanoparticles have gained a considerable attention as vehicles for transmucosal drug delivery systems due to their biocompatibility and changeable biodegradability. PLGA provides both protection to the encapsulated therapeutic agent from enzymatic degradation

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(Goldberg, 2003) and controlled release of the drug. Nevertheless, due to short residence time on the mucosa, absorption of the incorporated drug is strongly limited (Ponchel, 1998). By improving the nanoparticulate delivery systems by modification of the surface with mucoadhesive agents, a prolonged residence time on mucosal surfaces can be achieved (Kawashima, 2000). Such complex mucoadhesive nanoparticulate delivery systems would offer efficient absorption and improved bioavailability of drugs due to the high surface-to-volume ratio and intimate contact with the mucus layer (Chickering III, 1999).

Chitosan has gained an increased importance in the pharmaceutical field owing to its good biocompatibility, non-toxicity, biodegradability, and mucoadhesion (Lehr, 1992). Excellent mucoadhesive properties are result of ionic interactions between positively charged primary amino groups and negatively charged sialic and sulfonic acid substructures of the mucus (Bernkop-Schnürch, 2000). The use of chitosan for coating PLGA nanoparticles in order to improve their mucoadhesion and drug release profile has been successfully demonstrated by various authors (Yamamoto, 2005; Zheng, 2004).

Further on the mucoadhesive properties of chitosan can be improved by immobilization of thiol groups on chitosan backbone (Roldo, 2004). Chitosan coupled with 2-iminothiolane resulting in chitosan-4-thiobuthyimidine (chitosan-TBA) shows a 140-fold improved residence time on the mucosa in comparison to unmodified chitosan (Bernkop-Schnürch, 2003).

The goal of this study was to improve the mucoadhesion of chitosan–PLGA nanoparticles by thiolation of chitosan with 2-iminothiolane and consequently to provide a longer residence time on the mucosa. An improvement in mucoadhesive properties was evaluated by measuring the residence time of the nanoparticulate delivery system on porcine small intestinal mucosa, being used as representative mucosa for mucosal membranes in general. Further evaluations included characterization of particles in terms of size, zeta potential, influence of the pH value of the surrounding medium, and in vitro release of the incorporated drug.

MATERIAL AND METHODS

Materials

Resomer® RG 504H (poly(D,L-lactide-co-glycolide), lactide:glycolide 50:50, PLGA) was a kind gift from Boehringer Ingelheim Pharma GmbH, Ingelheim am Rhein, Germany. Polyvinyl alcohol (MW 13,000–23,000; hydrolyzation grade 87–89%), dichlormethane (analytical grade), chitosan low molecular weight (viscosity 20–200 cps, 75–85% deacetylation grade), 2-iminothiolane, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), Ellman's reagent [DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)], fluoresceine diacetate, and curcumin were purchased from Sigma-Aldrich, Austria. Polysorbitane-80-oleate (Tween 80) was purchased from Gatt-Koller, Austria.

Methods

Preparation of PLGA-Chitosan-TBA Nanoparticles

Preparation of PLGA Nanoparticles

PLGA nanoparticles were prepared by the emulsification solvent evaporation method (Mu, 2002). First, 200 mg of PLGA was dissolved in 6 mL of dichlormethane. The organic phase was slowly poured into the stirred aqueous phase containing poly(vinyl alcohol) (PVA, 0.5% w/v) and sonicated with energy output of 40 W in a pulse mode. The formed oil-in-water (O/W) emulsion was gently stirred at room temperature by using a magnetic stirrer at a rate of 120 rpm overnight to evaporate the organic solvent. The resultant sample was collected by centrifugation at 4500 rpm, at 15°C for 15 min and washed with distilled water at least four times to remove PVA. The produced suspension was freeze-dried at 0.08 Pa and –78°C to obtain the dry powder of nanoparticles. In order to obtain nanoparticles loaded with fluoresceine diacetate (FDA) or curcumin, optionally 120 mg of FDA or curcumin were co-dissolved in dichlormethane.

Depolymerization of Chitosan

Chitosan was depolymerized in a way as previously described by Bravo-Osuna et al. (Bravo-Osuna et al., 2006). First, 6 g of chitosan was dissolved in 100 mL of 5 M hydrochloric acid. Thereafter, it was depolymerized at room temperature under vigorous stirring with 10 mL of $NaNO_3$ (8% w/v). After 1 hr of reaction, chitosan was precipitated by the raise in the pH to 9.0 with 5 M NaOH. The solid was filtrated, extensively washed with acetone and redissolved in 20–30 mL of 1 M hydrochloric acid. Purification was carried out by subsequent dialyses against deionized water as described

previously (Bravo-Osuna, 2006). Dialyzed product was freeze-dried and the yellowish lyophilizate was stored at 4°C until use.

Immobilization of Chitosan and 2-iminothiolane on the PLGA Surface

Lyophilized PLGA nanoparticles (180 mg) were dissolved in 3.0 mL of 0.1 M phosphate buffer, pH 4.5. The carboxylic groups on the surface of PLGA nanoparticles were activated by addition of 75 mg of EDAC and dissolved in 2.5 mL of the same buffer. The mixture was incubated at room temperature for 6 hr. To the suspension containing activated nanoparticles 115 mg of depolymerized chitosan, dissolved in 2.0 mL 5 mM HCl pH 3.5, was added. The mixture was stirred vigorously at room temperature for 12 hr. Conjugated nanoparticles were centrifuged and washed five times with 5 mM HCl pH 3.5 in order to remove free chitosan and EDAC. After resuspending the nanoparticles in 3.0 mL phosphate buffer, 75 mg of 2-iminiothiolane was added and the whole mixture was stirred gently for 24 hr. The particles were centrifuged again, washed with 5 mM HCl, and freeze-dried. Dry nanoparticles were evaluated regarding their size, size distribution, and zeta potential.

Nanoparticles Characterization

Measurement of Size, Size Distribution, and Zeta Potential

The size, size distribution, and zeta potential of the nanoparticles were determined by photon correlation spectroscopy and laser Doppler anemometry, respectively, using Zeta Potential/Particlesizer NICOMP 380 ZLS® (PSS NICOMP, Santa Barbara, CA). Measurements were performed at wavelength of 632.8 nm with scattered angle fixed at 90° at room temperature (23–25°C). Dried nanoparticles powder was suspended in deionized water and sonicated for 4 hr prior to measurements.

Determination of Thiol Group Content

A number of thiol groups i.e., a degree of modification of thiolated chitosan was determined by Ellman's reaction as described previously (Bernkop-Schnürch, 2003).

Oxidation of Thiol Groups

In order to determine an influence of pH of the surrounding medium on chemical stability of thiol groups, samples containing 1 mg of PLGA-chitosan-TBA nanoparticles were hydrated in 1 mL of 50 mM acetate buffer pH 5.5; 0.1 M phosphate buffer pH 4.0, pH 6.8, and pH 7.4 and sonicated at 37°C for 3 hr. Thereafter, 200 µl of 1 M HCl was added in order to quench any further oxidation. Samples were frozen at –20°C and freeze dried. The amount of remaining free thiol groups was determined by using Ellman's reagent as described above.

Influence of pH on the Particle Size and Size Distribution

Dry nanoparticles were incubated and sonicated for 1 hr in solutions of different pH values: pH 3 (50 mM HCl), pH 4.5 (0.1 M phosphate buffer), pH 5.5 (50 mM acetate buffer), pH 6.8 (0.1 M phosphate buffer), pH 7.4 (0.1 M phosphate buffer), and pH 8.0 (0.5 mM NaOH).

Mucoadhesion Studies

For this study nanoparticles loaded with fluorescein diacetate as fluorescent marker were used. The mucoadhesive properties of PLGA-chitosan-TBA nanoparticles were evaluated by measuring the residence time of nanoparticles on porcine small intestinal mucosa using an experimental set-up established by Rango Rao et al. (1989). In brief, freshly excised porcine intestinal mucosa was mounted on a half pipe and placed in an angle of 45° in an incubation chamber providing 100% humidity and temperature of 37°C. The mucosa was then continuously rinsed with 0.1 M phosphate buffer pH 6.8. To humidify the mucosa, an equilibration period of 5 min was allowed prior to applying the nanoparticles. Throughout the whole experiment a temperature of the phosphate buffer was kept at 37°C. A constant flow rate of 2.5 mL/min was provided by utilizing a peristaltic pump. After an equilibration period, 0.5 to 1.0 mg of lyophilized nanoparticles were transferred on the mucosa in dry form and continuously rinsed with the phosphate buffer pH 6.8. At predetermined time points the mucosa with the remaining nanoparticles was displaced in plastic containers containing 25 mL of 5 M NaOH and vigorously shaken in a waterbath provided with orbital shaker for 20 min, in order to quantitatively hydrolyze FDA to sodium fluorescein (Albrecht, 2006). After centrifugation (13,400 rpm; 5 min) fluorescence of each sample was determined.

Determination of Drug Load

In order to determine the FDA entrapment, 0.5–1.0 mg FDA loaded dry nanoparticles were dissolved in 25 mL of 5 M NaOH containing dichlormethane (10%, v/v). An amount of FDA was determined by measuring fluorescence at E_{em} 514 nm and E_{ex} 490 nm by fluorescence-absorbance reader (Fluostar Galaxy, BMG Labtechnologies, Offenburg, Germany). To determine the entrapment of approximately 1 mg of curcumin loaded nanopaticles particles were dissolved in 1 mL dichlormethane followed by addition of 26 mL of 0.1 M phosphate buffer pH 5.0 containing 0.5% Tween 80 and stirred overnight. The content of curcumin was determined by measuring the absorbance at 450 nm. Drug load was calculated using the following equation:

$$\text{Drug Load}(\%) = \frac{\text{Weight of curcumin or FDA}}{\text{Weight of nanoparticles}} \times 100$$

Drug Release

Drug release profile of curcumin from the nanoparticles was determined in medium containing 0.1 M phosphate buffer pH 6.8 and 0.5% Tween 80 as a solubility enhancer for poorly water soluble curcumin. First, 0.5 to 1.5 mg of curcumin-loaded nanoparticles were suspended in 27 mL of buffer solution and placed in a waterbath provided with orbital shaker at 37°C. At predetermined time intervals, the reaction tubes were taken out of the waterbath and centrifuged at 4500 rpm for 5 min. Samples containing 1 mL of supernatant were taken for the analysis and the volume was replaced. Cumulative corrections were made for the previously removed samples. Sink conditions were maintained throughout the whole experiment.

Statistical Data Analysis

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

RESULTS AND DISCUSSION

Preparation of PLGA-chitosan-TBA Nanoparticles

Within this study low molecular mass chitosan was depolymerized according to the method described previously by Bravo-Osuna et al. (2006) resulting in chitosan of molecular mass of 9400 g/mol. The important criteria for choosing the method of depolymerization were preservation of the chitosan structure and free amino groups. This method of depolymerization is reported not to induce structural changes of chitosan (Fischer, 2003), which is important as free amino groups of chitosan were necessary for the further coupling reactions. Moreover, this range of molecular mass might favor the access of the amino groups of chitosan to the carboxylic moieties on the PLGA surface. The PLGA nanoparticles were prepared by emulsification solvent evaporation method using PVA as emulsifier since PVA generates uniform particles with relatively small size distribution (Murakami, 1999). Throughout the whole synthesis the pH of the mixture was maintained at pH 4.5 using phosphate buffer since it provides both electrostatic stability of the unmodified PLGA nanoparticles due to steric repulsions (Stolnik, 1994), optimal pH for EDAC activity, and entity of free amino groups of chitosan (Zheng, 2004). The carboxylic moieties of PLGA nanoparticles were activated by EDAC forming *o*-acylurea derivative as intermediate product which reacts with the primary amino groups of chitosan (Kast, 2001). Chitosan was attached covalently to the carboxylic acid groups under formation of amide bonds. Non-covalently attached chitosan and EDAC were eliminated by centrifugation and washing four times with 1 mM hydrochloric acid (pH 3.5). In order to immobilize thiol groups to the surface of chitosan coated PLGA nanoparticles, 2-iminothiolane was attached covalently to the remaining free amino groups of chitosan. The modification of chitosan gives rise to the cationic amidine substructure, whereby the positive character of chitosan coated nanoparticles will be additionally emphasized. According to Hassan et al. (1990), cationic character of chitosan as polymeric drug carrier is responsible for ionic interactions with anionic substructures of the mucus layer such as sialic acid and sulfonic acid, which contribute to its mucoadhesiveness. The proposed synthetic pathway of the nanoparticles is shown in Fig. 1.

Characterisation of Nanoparticles

The physicochemical characteristics of obtained nanoparticles such as particle size and zeta potential are summarized in Table 1. Both unmodified and

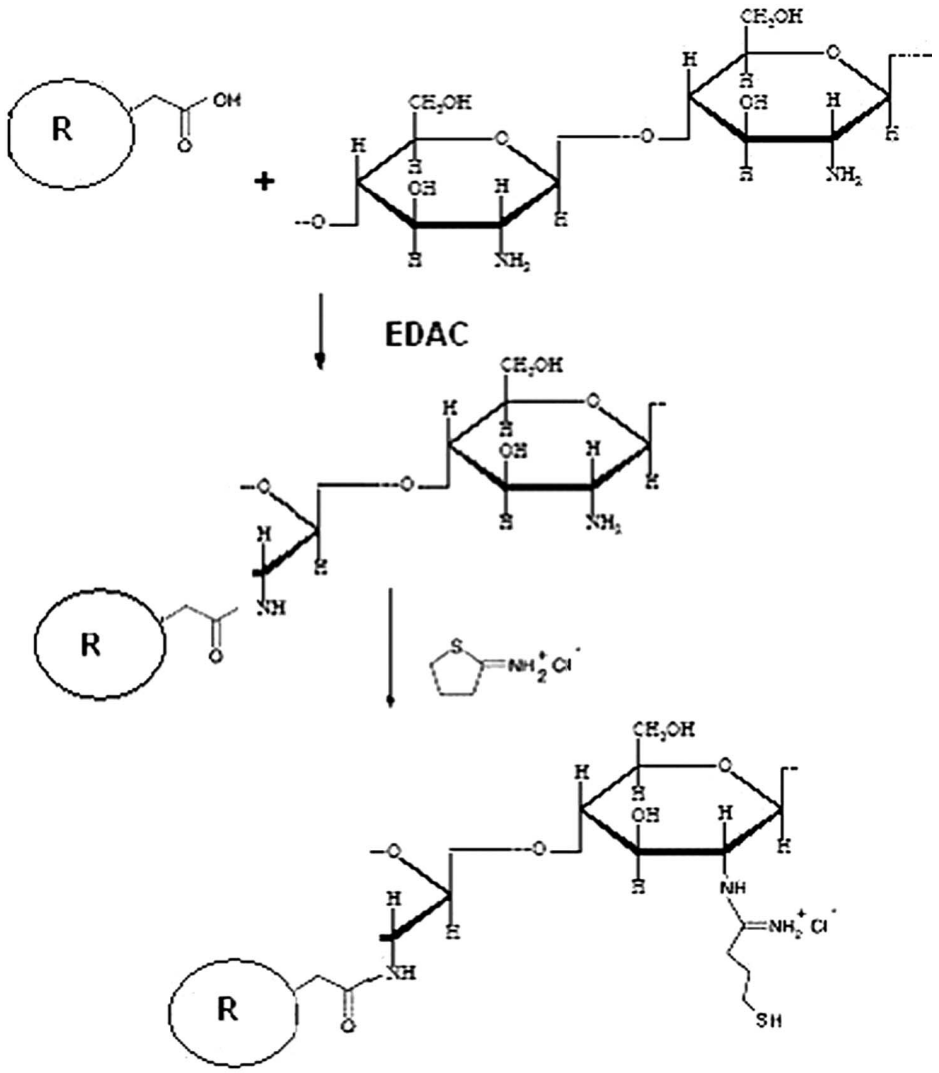

FIGURE 1 Synthetic pathway for the modification of PLGA nanoparticles with chitosan and 2-iminothiolane. R = $(CH_2)_2$-COO $(CH_2CH_2O)_n$-$(CO\text{-}CHCH_3\text{-}O\text{-}OCO\text{-}CH\text{-}CH_3)_m$-OH.

TABLE 1 Main characteristics of the PLGA and PLGA-chitosan-TBA nanoparticles

Nanopartculate Formulation	Mean Particle Diameter	Zeta Potential (mV)	Thiol Groups (μmol/g polymer)	FDA Entrapment (%)	Cucurmin Entrapment (%)
PLGA	352.5 ± 68.3	–22.91 ± 1.35	1.03 ± 0.12	31.6 ± 0.7	55.9 ± 3.9
PLGA-chitosan-TBA	889.5 ± 72	+24.75 ± 1.55	7.3.2 ± 0.24	19.9 ± 2.6	27.6 ± 4.7

surface-modified nanoparticles demonstrated unimodal size distribution (data not shown). A size range of the uncoated PLGA nanoparticles was 284.2–420.8 nm, whereas that of the chitosan coated nanoparticles was 817.0–961.5 nm. These results clearly indicate an attachment of chitosan. Moreover, the attachment of chitosan was confirmed by the measurement of zeta potential. Whereas uncoated nanoparticles showed negative zeta potential of –22.9 ± 1.35, zeta potential of the coated thiolated nanoparticles was +24.74 ± 1.55. This inversion of zeta potential towards positive values indicates an immobilization of cationic chitosan at the surface of nanoparticles and under suppression of original negative charge of PLGA.

Determination of Thiol Groups

Quantification of thiol groups immobilized on the surface of nanoparticles by Ellman's reagent demonstrated on average of 7.32 ± 0.25 μmol thiol groups per gram polymer (Table 1). In contrary, control nanoparticles obtained by omitting EDAC during the synthesis showed negligible amount of thiol groups, due to an inefficient covalent attachment of chitosan, to which thiol moieties are bound. Increasing the time of activation of carboxylic groups appears to favor the attachment of chitosan and chitosan-TBA, respectively, since at activation times less than 3 hr no immobilized thiol groups could be detected (data not shown).

Oxidation of Thiol Groups

Depending on the pH-value of a surrounding medium, thiol groups of the polymer conjugates can undergo oxidation leading to formation of inter- and intramolecular disulfide bonds. A formation of inter- and intramolecular disulfide bonds during an oxidation process results in poor mucoadhesive properties of the polymer (Roldo et al., 2004). It is evident from Fig. 2 that the content of free thiol groups of thiolated PLGA nanoparticles decreases with increasing pH value of the surrounding medium. Whereas at pH 4.0 an amount of thiol groups of PLGA-chitosan–TBA remained constant (Table 1), a significant decrease in thiol group content could be observed at higher pH values. As shown in Fig. 2 at pH 6.8 an amount of thiol groups decreased by 35%, while at pH 7.4 a decrement of thiol groups by 65% could be observed. These results can be explained by a decrease of H+ concentration at higher pH-values leading to a higher concentration of thiolate anions, S^-, representing the active form for oxidation.

Influence of pH on Nanoparticle Stability

In order to determine the impact of pH of physiological fluids on the stability of particles, the PLGA-chitosan-TBA nanoparticles were incubated for 6 hr in series of buffer solutions of different pH values covering the pH situation on all mucosal membranes. As shown in Fig. 3, at pH 5.5–7.4, mean diameter of resulting nanoparticles was in a range between 577.8 ± 66.7

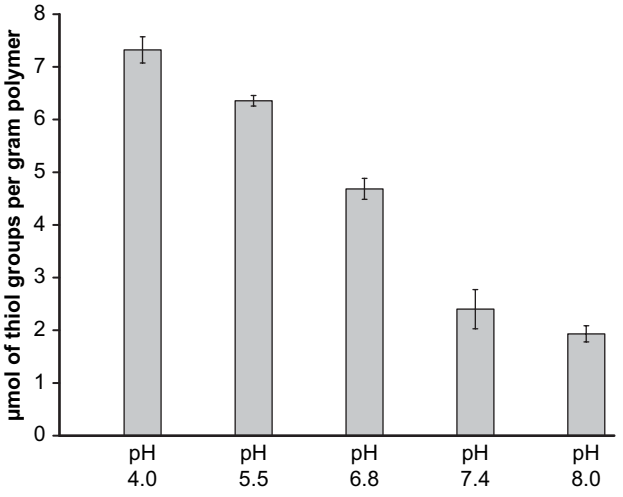

FIGURE 2 **Influence of pH of the surrounding medium on the content of thiol groups of PLGA chitosan-TBA nanoparticles. Nanoparticles were incubated for 3 hr at 37°C. Indicated values are means ± *S.D.* of three experiments.**

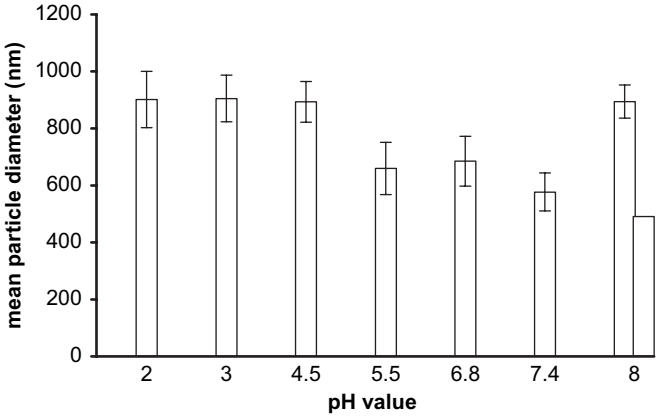

FIGURE 3 **Influence of pH of the surrounding medium on particle size and size distribution. At all tested pH values particles showed 100% unimodal size distribution, except at pH 8 where it was bimodal. At pH 8 white bars 70%, black bars 30%.**

and 685.5 ± 87.3 nm. At the pH < 4.5 and pH > 8.0, a mean diameter increased, likely due to precipitation of PLGA and chitosan, respectively. At pH 8 the particles showed bimodal distribution, namely, a mean diameter of 30% of particles was 491.5 ± 67.7 nm and of 70% was 894 ± 58.2. This value might represent a threshold at which nanoparticles become unstable and precipitate.

As a mucosal delivery device, nanoparticles can release the incorporated drug directly on the mucosa or can at least to some extent be absorbed from the mucosa per se (Jung, 2000) releasing the drug after

having reached the systemic circulation. It has been found that the uptake of nanoparticles depends on their in vivo stability and size (Florence, 1995). At the pH values 5.5–7.4, which are mostly present at mucosal membranes, the particle diameter was in the size range that can cross the mucosal barriers by being adsorbed by lymphoid tissues (Brooking, 2001; Lefevre, 1978).

Mucoadhesion Studies

Results obtained by in vitro studies on porcine small intestinal mucosa demonstrated that a significant improvement of mucoadhesive properties could be achieved by surface modification of PLGA nanoparticles with chitosan–TBA. In Fig. 4, a comparison in the residence time of the fluorescent marker FDA incorporated into unmodified and modified PLGA nanoparticles, respectively, is shown. After 2 hr of permanent rinsing at a flow rate of 2.5 mL/min, on average 52% of the applied FDA incorporated into the unmodified nanoparticles remained on the mucosa, whereas an amount of PLGA-chitosan-TBA nanoparticles did not change significantly. Although at pH 6.8 free thiol groups are oxidized, strongly improved mucoadhesive properties were still observed. The surface modification or coating of particles with mucoadhesive polymers has turned out to be one of the most promising strategies for designing mucoadhesive particulate systems (Takeuchi, 2001). A contribution of thiol groups in order to improve mucoadhesion has been confirmed and is on the one hand, a result of disulfide exchange reactions within the mucine (Madsen, 1998; Mortazavi, 1992). On the one hand an improvement of the mucoadhesive properties of surface modified PLGA with chitosan–TBA polymer can be ascribed to the positive charge of chitosan–TBA moiety due to ionic interactions between cationic amidine substructure of the conjugate and the anionic substructures of the mucus layer (Hassan, 1990). A feasibility of coating PLGA nanoparticles with mucoadhesive polymers such as chitosan, poly(vinyl alcohol), and poly(acrylic acid) has been demonstrated in various studies (Kawashima, 2000; Takeuchi, 2001). However, herein the improvement of the mucoadhesion properties of the nanoparticles by the thiolation of the polymeric coating is shown for the first time.

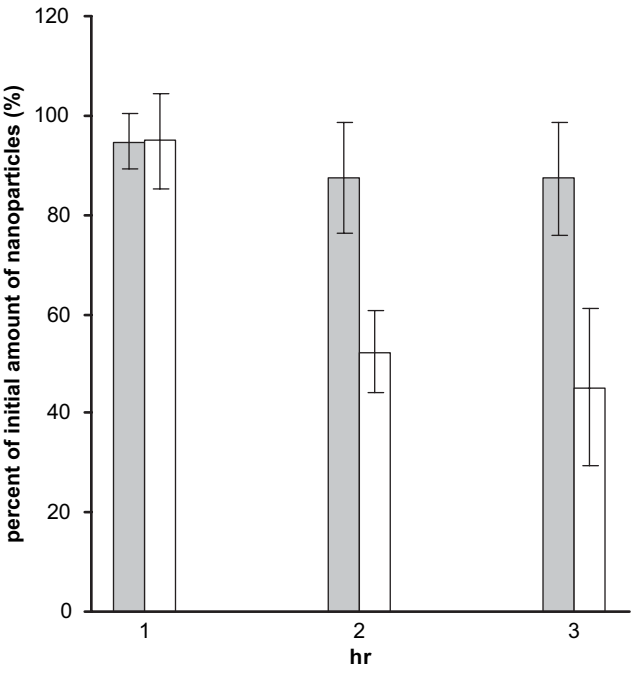

FIGURE 4 The percentage of initial amount of unmodified PLGA (white bars) PLGA-chitosan-TBA (grey bars) nanoparticles remaining on the mucoasa after 1, 2, and 3 hr. Data represent means ± *S.D.* of seven experiments.

In Vitro Drug Release Studies

In Table 1, the drug load of the unmodified and modified nanoparticles is shown. An entrapment efficacy of the unmodified PLGA was higher, compared to the drug entrapment of the modified PLGA nanoparticles, partially due to a longer exposition of the nanoparticles to aqueous solution during the synthesis, leading to a loss of the drug.

In vitro release profiles of the unmodified and surface modified PLGA nanoparticles for cucurmin showed a similar biphasic configuration with an initial burst followed by a sustained release as shown in Fig. 5. Within first 30 min, drug release rate from modified nanoparticles was slightly slower than from unmodified PLGA nanoparticles reaching the maximum release rate of average 80% after 2 hr. Due to the swelling of the thiolated polymer (Roldo et al., 2004) located on the surface of the nanoparticles, a slight improvement of the initial burst could be achieved. The PLGA as a hydrophobic polymer represents an ideal drug carrier for the hydrophobic drugs. However, due to the initial burst the continuous levels of the drug in the target organ cannot be guaranteed. Therefore the hydrophilic polymer coating might

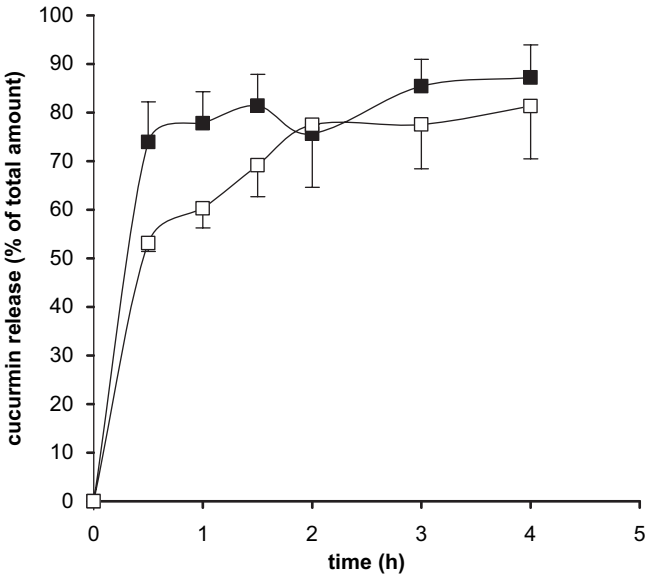

FIGURE 5 **Release of cucurmin from the PLGA (■) and PLGA-chitosan-TBA (□) nanoparticles within 6 hr in 0.1 M phosphate buffer, pH 6.8 containing 0.5% Tween 80 as solubility enhancer for cucurmin. Data represent means ± *S.D.* of three experiments.**

offer an advantage of sustained release and physical stability in aqueous environment.

CONCLUSION

Within this study the surface of PLGA nanoparticles was modified by covalent attachment of chitosan-4-TBA. The attachment of polymeric layer on the surface of nanoparticles was confirmed by increased size and positive zeta potential of nanoparticles. Due to the positive charge and immobilization of thiol groups, surface-modified nanoparticles showed improved mucoadhesive properties, resulting in a prolonged residence time on the mucosa. At pH values present in physiological fluids mucosal surfaces nanoparticles showed stability regarding their size distribution. Based on these findings, modified-surface PLGA nanoparticles might represent a promising transmucosal drug delivery device.

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