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Controlled Drug Delivery Systems Based on Thiolated Chitosan Microspheres

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ABSTRACT The aim of the present study was to verify the potential of chitosan-thio-butyl-amidine (TBA) microspheres as carrier systems for controlled drug delivery. In this study microspheres were prepared utilizing water in oil (w/o) emulsification solvent evaporation technique. A concentration of 0.5% of chitosan-TBA conjugate displaying 100 μM thiol groups per gram polymer was used in the aqueous phase of the emulsion in order to prepare microspheres. The obtained non-aggregated free-flowing microspheres were examined with conventional light microscope as well as scanning electron microscopy (SEM). The microscopic images indicated that the prepared chitosan-TBA microspheres were of spherical shape and smooth surface while microparticles obtained from the unmodified chitosan were of porous structure and non-spherical shape. Particle size distribution was determined to be in the range from 1 to 59 μm. The free thiol group content of chitosan-TBA microspheres prepared with an aqueous phase of pH 2, 5, and 6.5 were determined to be 71.4, 49.4, and 8.2 μM/g polymer, respectively. Furthermore, results attained from in vitro release studies with fluorescein isothiocyanate labelled dextran (FITC-dextran) loaded chitosan-TBA microspheres showed a controlled release rate for more than three hours while the control reached the maximum peak level of release already within an hour. According to these results, chitosan-TBA microspheres seem to be a promising tool in transmucosal drug delivery for poorly absorbed therapeutic agents.

KEYWORDS Thiomers, Chitosan-TBA, Microspheres, Drug delivery systems (DOS), Controlled drug release

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INTRODUCTION

Drug carrier technology is facing a real challenge in order to improve bioavailability of non-invasively administered peptide and protein drugs such as insulin, calcitonin, and desmopressin. Particulate drug delivery systems such as nanoparticles, microspheres, and liposomes have received significant interest due to their multifaceted advantages for mucosal controlled and

targeted drug release (Edman et al., 1992; O'Hagan, 1998; van der Lubben et al., 2001). However, the success of these particulate delivery systems is limited by their short residence time at the site of drug absorption. Since the introduction of the concept of mucoadhesion in 1984 by Park and Robinson (1984), many researchers have been using this concept to improve the potential of drug delivery systems. Mucoadhesive particulate drug delivery systems are one of the promising approaches leading to a prolonged residence time at the mucosal site of drug absorption (Jameela et al., 1994) and/or a controlled drug release rate (Ko et al., 2002). Moreover, particulate systems could also deliver the drug to a specific site of absorption (Lorenzo-Lamosa et al., 1998). Numerous attempts have been undertaken to improve mucoadhesive characteristics of drug carrier matrices such as the use of chitosans and polyacrylates. Chitosan, which is a de-acetylated product of chitin—the second naturally most abundant polysaccharides on earth, is gaining increasing interest in the pharmaceutical industry (Bernkop-Schnürch, 2000; Felt et al., 1998) due to its non-toxicity, biodegradability, and biocompatibility (Singla & Chawala, 2001). Many modifications of chitosan have been done in order to improve its potential characteristics in drug delivery (Kotze et al., 1997; Shantha & Harding, 2002). Recently, chitosan was chemically modified by introduction of sulfhydryl groups. In this way, a new generation of mucoadhesive polymers bearing thiol groups on their backbone have been provided, the so-called thiomers (Bernkop-Schnürch et al., 1999). The overall mechanism of the improvement of drug carrier properties of thiomers is attributed to the formation of disulfide bonds between thiol moieties of thiomers and sulfhydryl groups of cysteine-rich subdomains of mucus glycoproteins providing prolonged residence time at the absorbing membranes (Leitner et al., 2003). Introduction of thiomers has opened attractive prospects for the use of these polymers in transmucosal routes of drug administration such as peroral (Guggi et al., 2003), buccal (Langoth et al., 2003), nasal (Leitner et al., 2004), ocular (Hornof et al., 2003), and vaginal routes (Kast et al., 2002). The development of particulate delivery systems being based on thiolated chitosans seems therefore to be a promising strategy. Accordingly, it was the purpose of this study to evaluate the potential of chitosan-TBA microspheres as controlled drug delivery systems. For release studies from these microspheres, a FITC–labelled dextran was used, as it exhibits regarding size and hydrophilicity characteristics very similar to those of hydrophilic macromolecular drugs such as heparins, peptides, and proteins. The chitosan-TBA microspheres were prepared using emulsification/solvent evaporation technique. The obtained microparticles were in vitro characterized regarding their shape, size, and drug release properties. Microparticles of unmodified chitosan were prepared under the same conditions to serve as control.

MATERIALS AND METHODS

Materials

Fluorescein sodium and chitosan exhibiting properties including Mw 150 kDa, degree of de-acetylation 79.5% w/w, viscosity, ~100 mPa.s (1% in 1% acetic acid, 20°C) were purchased from Fluka Chemie (Fluka Chemie, Buchs, Switzerland). Traut's reagent (2-iminothiolane HCI) (Mw 137.63 Da) was supplied from Pierce, (Pierce, Oud Beijerland, NL). Ellman's reagent (DTNB), 5,5′-dithiobis(2-nitrobenzoic acid), Tris buffer (Tris[hydroxymethyl] aminomethane) and fluorescein isothiocyanate-dextran (FITC-Dextran, Mw 4.3 kDa) were obtained from Sigma (Sigma, St. Louis, MO). L-Cysteine hydrochloride anhydrous was delivered from Sigma (Sigma-Aldrich, Steinheim, Germany). All other chemicals were of analytical grade.

Methods

Synthesis of Chitosan-TBA Conjugates

Chitosan-TBA was synthesized as described previously (Bernkop-Schnürch et al., 2003a). In Fig. 1 the pathway of chemical synthesis of chitosan-TBA is shown. Briefly, 1 g of chitosan was dissolved in 1% v/v acetic acid solution and the pH was adjusted to 6.5 using 5 M NaOH. Thereafter, 400 mg of Traut's reagent (2-iminothiolane HCI) was added to the chitosan solution. The reaction mixture was incubated for 24 h at room temperature under permanent stirring.

In order to eliminate the remaining uncoupled 2-iminothiolane HCI and to isolate the polymer conjugates, the reaction mixture was dialyzed in dialysis

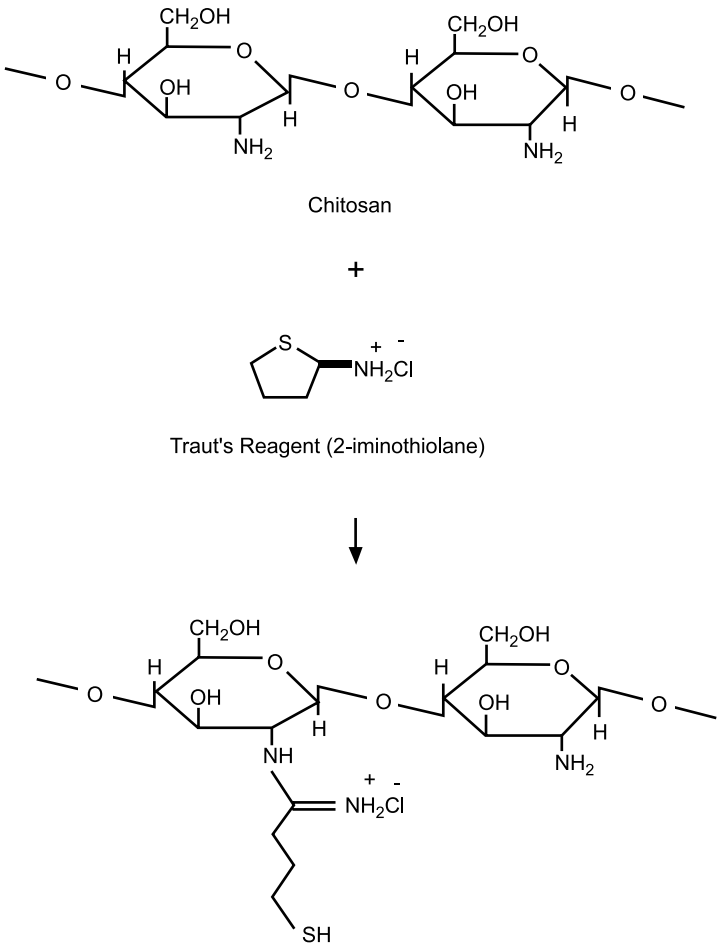


FIGURE 1 **Chemical Modification of Chitosan with Traut's Reagent (2-Iminothiolane).**

tubings (molecular weight cut off 12 kDa, dialysis tubings, cellulose membrane; Sigma, St. Louis, MO) for 3 days at 10°C in the dark against 5 mM HCI and two times against the same medium but containing 1% NaCI. Thereafter, the samples were dialyzed exhaustively against 1 mM HCI to adjust the pH of the polymer to 4–5. Following dialysis, the aqueous polymer solution was freeze-dried at −30°C and 0.1 mbar (Christ Beta 1 -8K, Osterode am Harz, Germany) and stored at 4°C until further use.

Determination of the Thiol Group Content in the Polymer Conjugates

The amount of thiol groups on the thiolated chitosan was determined spectrophotometrically using Ellman's reagent (DTNB), 5,5′-dithiobis(2-nitrobenzoic acid). First, 0.5 mg of the polymer was swollen for 2 h in 0.25 ml of demineralized water. Then 0.25 ml of 0.5 M phosphate buffer pH 8.0 was added. Thereafter, 0.5 ml of 0.03% w/v DTNB dissolved in 0.5 M phosphate buffer pH 8.0 was added to the polymer suspension and samples were incubated for 2 h at room temperature. The precipitated polymer was removed by centrifugation (13500 rev·min^{-1}, 5 min). Samples of 0.3 ml were transferred to a 96 well microtitration-plate and the absorbance was measured at 450 nm with a 96 well microtitration-plate reader (Anthos Reader 2001, Salzburg, Austria). The amount of thiol moieties on the polymer was calculated from a standard curve of L-cysteine hydrochloride anhydrous in 0.5 M phosphate buffer pH 8.0 in a concentration of 0.8–28 μg/ml.

Preparation of Chitosan-TBA Microspheres

Medium molecular mass chitosan-TBA microparticles were prepared according to the emulsification/

solvent evaporation technique. A pH 2, 5, and 6.5 aqueous solution of 0.5% (m/m) freshly prepared chitosan-TBA (aqueous phase) was dropped into paraffin oil (viscosity: 11–230 mPa s) (oil phase) containing 0.3% Span 20 as emulsifying agent. Emulsification process was achieved by utilizing an Ultraturrax (Omni 5000, Omni International, Marietta, USA) (continuous phase/dispersed phase ratio 9:1 v/v). The emulsion was thermostated at 37°C, stirred by a rotating paddle system at 300 rpm. Based on a simple oxidation process, disulfide bonds were formed within chitosan-TBA microparticles. Then methanol was added into the emulsion (methanol/chitosan-TBA solution ratio 1:4 v/v). The dispersed aqueous phase was completely evaporated by blowing air bubbles (5 L/min) and raising the emulsion temperature to 40°C. The emulsion was kept under stirring for 14 h. Petroleum ether (20 ml) was added and the evaporation process continued for additional 15 min. The formed microspheres were separated from the oil phase by centrifugation (Sorvall RC, Vienna, Austria; 3000 rpm; 5 min), washed several times with petroleum ether to remove the remaining traces of paraffin oil, and freeze dried at −30°C and 0.01 mbar (Christ Beta 1 -8K, Germany). Unmodified chitosan microspheres were prepared under the same conditions to serve as a control.

Determination of the Oxidized Disulfide Bonds

The degree of oxidation of the thiol moieties during the preparation of microspheres was determined. The reaction with Ellman's reagent was performed after reducing disulfides with sodium borohydride. To 0.5 mg of the polymers, 0.25 ml of demineralized water was added. After a hydration time of 30 min, 0.75 ml of 0.05 M Tris buffer pH 6.8 and 1 ml 4% (w/v) sodium borohydride solution were added. The samples were incubated for 1 h at 38°C. Then the remaining sodium borohydride was inactivated by addition of 0.20 ml of 5 M HCl. The pH of the reaction mixture was adjusted to 8 with 1 ml of 1 M phosphate buffer pH 8. After addition of 0.10 ml of Ellman's reagent (40 mg DTNB, 5,5′-dithiobis(2-nitrobenzoic acid) in 10 ml of 1 M phosphate buffer pH 8) the samples were incubated for 2 h at room temperature. Measurements of the absorbency and quantification of thiol groups were performed as described previously. The percentage of the disulfide bonds on the conjugate can be calculated after subtraction of the free thiol content.

Preparation of FITC-Dextran Loaded Chitosan-TBA Microspheres

The FITC-dextran loaded chitosan-TBA microspheres were prepared as follows: 7.5 mg of FITC-dextran were added to 10 ml of 1% aqueous solution of chitosan-TBA. The solution was mixed and homogenized for 1 h and microspheres were prepared as described above. Unmodified FITC-dextran loaded chitosan microspheres were prepared under the same conditions to serve as a control. The samples were stored at 4°C until further use.

Total Drug Content Determination

The amount of FITC-dextran encapsulated was determined by dissolving the microspheres in 1% acetic acid. The absorbency of each sample was measured fluorimetrically at an excitation wavelength of 485 nm and an emission wavelength of 535 nm (Biolise, Spectrafluor, Austria). Determination of the percentage of FITC-dextran encapsulation was calculated according to standard curves obtained from FITC-dextran dissolved in 1% acetic acid.

FITC-Dextran Release Profile

Release profile of FITC-dextran was obtained by suspending 5 mg chitosan-TBA microspheres loaded with FITC-dextran in 1 ml 100 mM acetate buffer pH 5.5 serving as a release medium in an Eppendorf tube incubated in an oscillating water bath (GFL 1092; 100 rpm) at 37°C. Samples were withdrawn every 15 min to a microtitration plate and substituted with the same volume of 100 mM acetate buffer pH 5.5 pre-equilibrated at 37°C. Release profile could be estimated according to a standard curve of FITC-dextran dissolved in 100 mM acetate buffer pH 5.5.

Particle Size Determination

Paraffin oil (viscosity: 25–80 mPa s) was used as a non-dissolving dispersion medium. The particle diameter was determined using a laser diffraction particles size analyser (Shimadzu SALD 1100). Microspheres were suspended by sonification and magnetic stirring during the measurement.

Scanning Electron Microscopy (SEM)

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, The Netherlands).

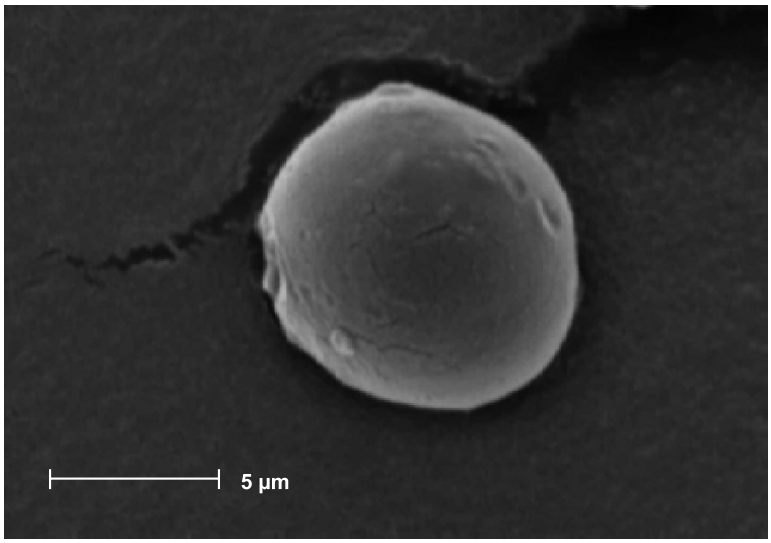
Statistical Data Analysis

Each experiment was performed in triplicate. 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).

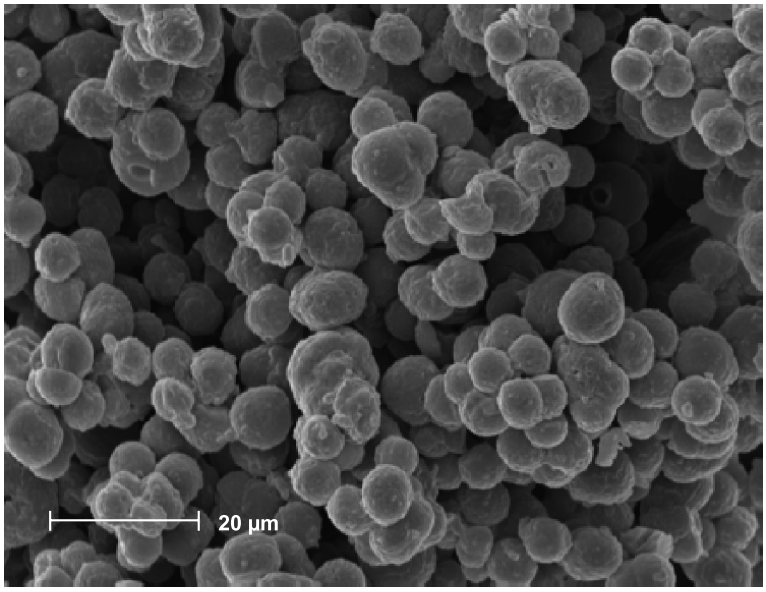
RESULTS AND DISCUSSION

Improved Characteristic of Chitosan-TBA and Microparticles Potential

Due to non-toxicity, biocompatibility, biodegradability, and cheap production costs of chitosans, in



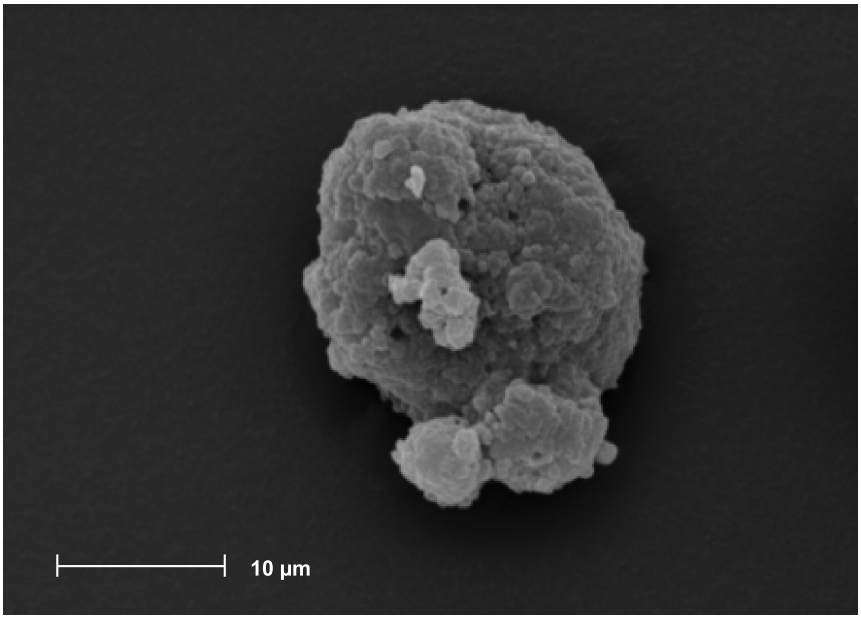
[A]



[B]

FIGURE 2 **Scanning Electronic Microscope (SEM) Micrograph: [A] Image of a Single Chitosan-TBA Microsphere. [B] Image of a Group of Chitosan-TBA Microspheres which Shows Spherical Shape and Smooth Surface Characteristics.**

many studies the advantages of these natural polysaccharides have been investigated. Van der Lubben et al. (2001, 2003), for instance, demonstrated the enhanced immune response against diphtheria toxoid by using chitosan microspheres for oral and nasal vaccination in mice as well as improved particulate antigen uptake by human lymphoid tissue for oral vaccine delivery. In addition to these useful characteristics, much improvement of chitosan properties could be achieved by some chemical modifications. Chitosan-TBA was synthesized using Traut's reagent (2-iminothiolane HCI) which is covalently linked to the primary amino group of chitosan via the formation of amide bonds as shown in Fig. 1. Chitosan-4-thio-butylamidine, the resulting conjugate, exhibits terminal free sulfhydryl groups which are responsible for the



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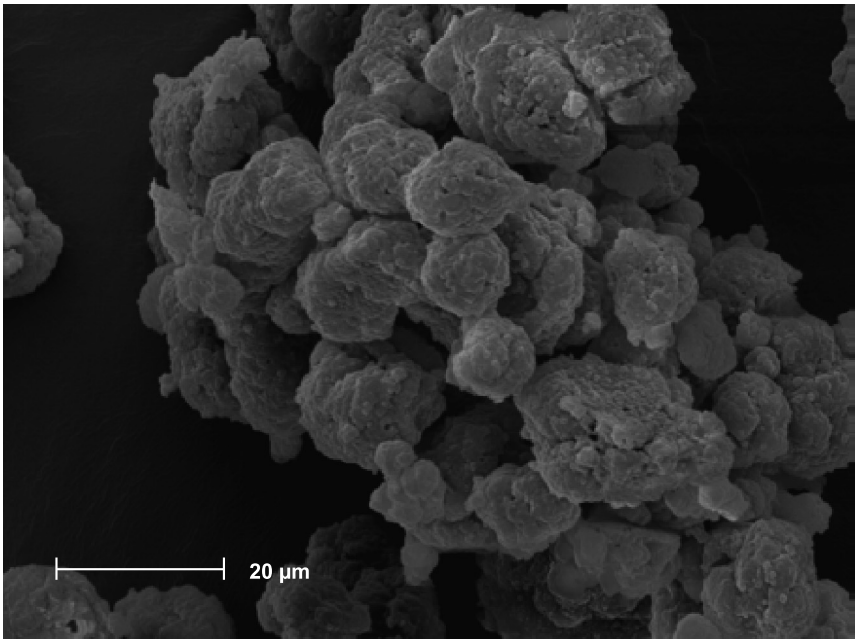

[B]

FIGURE 3 Scanning Electronic Microscope (SEM) Micrograph: [A] Image of a Single Microparticle of Unmodified Chitosan. [B] Image of a Group of Unmodified Chitosan Microparticles which Shows Rough Surface and Porous Particle Characteristics.

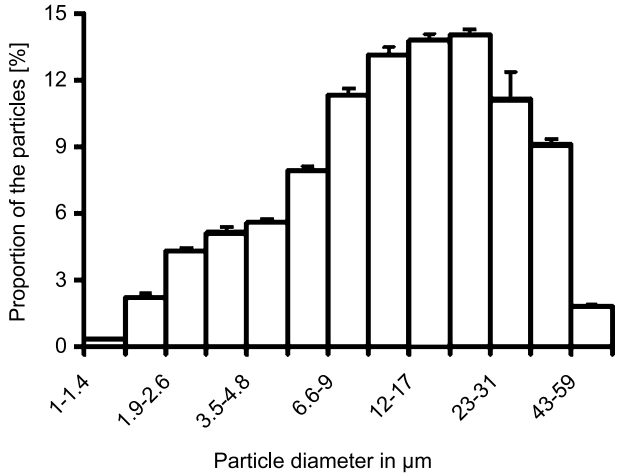

FIGURE 4 **Particle Size Distribution of Chitosan-TBA Microspheres. Data Represent Particle Size Diameter of at Least Three Experiments ± SD.**

improved features of chitosan-TBA as a mucoadhesive excipient. The improved mucoadhesive characteristics of chitosan-TBA should lead to a prolonged residence time at mucosal membranes which favors mucosal drug uptake. The polymer conjugate was analyzed spectrophotometrically using Ellman's reagent and the thiol group content was determined to be 100 µmol/g polymer. By using chitosan-TBA, proteolytic enzymes can be inhibited (Bernkop-Schnürch et al., 2001). Moreover chitosan-TBA displays in situ-gelling features by pH dependent inter- as well as intra-molecular disulfide bonds which consequently guarantee a prolonged controlled drug release. Furthermore, due to the immobilization of thiol groups, chitosan-TBA was assumed to contribute in the opening of epithelial cells tight junctions via inhibition of protein tyrosine phosphatase being responsible for the closing of tight junctions (Bernkop-Schnürch et al., 2003b). Accordingly, chitosan-TBA shows a permeation enhancing effect for hydrophilic drugs via the paracellular pathway.

Referring to these features, the combination of thiolated chitosan and particulate delivery systems could be of great advantage. Therefore, microparticulate drug delivery systems based on chitosan-TBA could be a promising tool for non-invasive delivery of poorly absorbed macromolecules and hydrophilic therapeutic agents.

Morphological Characteristics of Microparticles

The microparticles obtained from chitosan-TBA aqueous polymer solutions at pH 2, 5, and 6.5 were morphologically characterized according to their shape, surface, and size distribution. Chitosan-TBA microparticles were of smooth surface and spherical shape as shown in Fig. 2. Unlikely, microspheres gained from unmodified chitosan were of non-spherical shape and with rough porous surface (Fig. 3). The particle diameter of chitosan-TBA microparticles was determined to be in the range of 1–59 µm with the center at 10 µm. Results are shown in Fig. 4. In contrast, the particle diameter of unmodified chitosan microparticles was determined to be in the range of 1–71 µm with the center at 16 µm.

Degree of Polymer Thiolation of Microspheres

An oxidation of thiol groups of the polymer into disulfide bonds takes place to some extent during the microparticles preparation process. Results showed that the degree of oxidation was pH dependent. The lower the pH of the aqueous phase of the polymer solution was, the lower was the degree of oxidation. The amount of free thiol groups of the microparticles was 71.4, 49.4, and 8.2 µmol/g polymer for microparticles prepared at pH 2, 5, and 6.5, respectively (results are shown in Fig. 5). The amount of the oxidized thiol moieties were 28.6, 50.6, and 91.8 µmol/g polymer for microparticles prepared at pH 2, 5, and 6.5, respectively. It was demonstrated that free thiol moieties of the microparticles were inversely proportional to the pH value of the aqueous phase. These results were in good agreement with previous results reported by our research group (Roldo et al.,

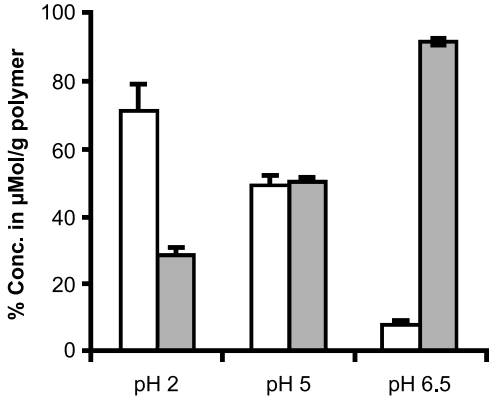

FIGURE 5 **Percentage of Free Thiol Groups (White Bars) and Disulfide Bonds (Grey Bars) on Chitosan-TBA Microspheres Obtained at Indicated pH Values of the Aqueous Phase during the Preparation Process. Data Represent at Least Three Experiments ± SD.**

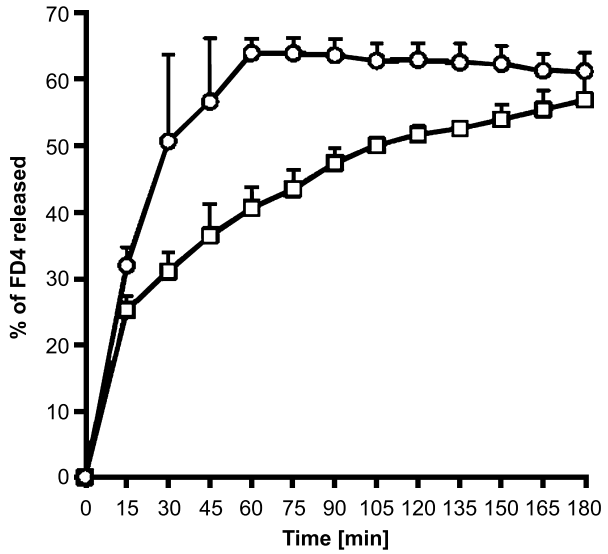

FIGURE 6 **Release Profile of the Model Drug FITC-Dextran (FD4) Obtained from Chitosan-TBA Microparticles [□] Compared with the Release from Microparticles of Unmodified Chitosan [○]. Data Represent Release Profile of at Least Three Experiments ± SD.**

2004). The decrease of thiol content with increase of the pH of the aqueous phase is attributed, on the one hand, to the formation of the interpolymer disulfide bonds which lead to decreased flexibility of the polymeric chains and, on the other hand, to the reduced possibility of disulfide bridging of thiol groups of the polymer with the thiol groups of cysteine-rich subdomains of mucus glycoproteins.

FITC-Dextran Release Profile

As reported by Coupe et al. the small intestinal transit of particulate dosage forms was estimated to be 3 h (Coupe et al., 1991). According to this, and taking into consideration that the absorption of hydrophilic macromolecular drugs from the small intestine is comparatively much higher than that from the colon, release study of the microparticles was determined for this time period. In dependence on the drug and therapeutic strategy, however, an even more sustained release might be of interest. As shown in Fig. 6, the initial burst release of FITC-dextran embedded in chitosan-TBA microspheres was 25% from the total FITC-dextran load. Thereafter, a controlled release with a release rate of approximately 0.2% per min was provided for the next 2.5 h. The release of FITC-dextran from unmodified chitosan microspheres reached the maximum peak level of the model compound release after 1 h, whereas the maximum release of the drug load in case of chitosan-TBA microspheres was not obtained even after 3 h of incubation. As FITC-dextran represents an uncharged model compound, it can be assumed that the release from microparticles is mainly dissolution and diffusion controlled. In case of peptide and protein drugs, an even more sustained release can be achieved by making use of ionic interactions. Peptides exhibiting an anionic net charge will interact with the cationic moieties of chitosan leading to a comparatively slower release. In addition, as ionic polymers exhibit a high buffer capacity, the pH within the polymeric carrier matrix and, consequently, the extent of ionic interactions between the polymer and the peptide, can be adjusted on demand.

CONCLUSION

In the present study, chitosan-TBA microspheres prepared by emulsification solvent evaporation technique were shown to have controlled drug release characteristics. Taking into consideration the previous results of our research group which proved that chitosan-TBA exhibits absorption enhancing, enzyme inhibiting, in situ gelling, and improved mucoadhesive properties, the use of this thiomer for particulate delivery systems could be a promising concept. Chitosan-TBA provides a good candidate for carrier systems which prolong the residence time of the drug at mucosal membrane and favor the epithelial drug uptake. Therefore, chitosan-TBA could be a useful tool in improving the bioavailability of poorly absorbed drugs which in turn assist reduction of dosing frequency, decrease drug side effects, and thereby increase patient compliance.

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