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Research Article

The Impact of Vehicles on the Mucoadhesive Properties of Orally Administrated Nanoparticles: a Case Study with Chitosan-4-Thiobutylamidine Conjugate

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Abstract. The aim of this study was to evaluate the impact of various vehicles on mucoadhesive properties of thiolated chitosan nanoparticles both *in vitro* and *in vivo*. Nanoparticles (NPs) were prepared by *in situ* gelation technique followed by labeling with fluorescein diacetate. Comparative studies on mucoadhesion were done with these thiolated chitosan NPs and unmodified chitosan NPs (control). The obtained nanoparticles displayed a mean diameter of 164.2±6.9 nm and a zeta potential of 21.5±5 mV. In an *in vitro* adhesion study, unhydrated thiolated NPs adhered strongly to freshly excised porcine small intestine, which was more than threefold increase compared to the control. In contrast, in the presence of various vehicles (PEG 300, miglyol 840, PEG 6000, cremophor EL, and caprylic triglyceride), the mucoadhesive properties of thiolated NPs were comparatively weak. Thiolated NPs suspended in caprylic triglyceride, for example, had a percent mucoadhesion of 22.50±5.35% on the mucosa. Furthermore, results from *in vivo* mucoadhesion studies revealed that the dry form of nanoparticles exhibits the strongest mucoadhesion, followed by nanoparticles suspended in PEG 300, miglyol, and 100 mM phosphate buffer, in that order. Three hours after administration, the gastrointestinal residence time of the dry form of thiolated NPs was up to 3.6-fold prolonged. These findings should contribute to the design of highly effective oral mucoadhesive nanoparticulate drug delivery systems.

KEY WORDS: chitosan-TBA; mucoadhesion; nanoparticles; thiomer; vehicles.

INTRODUCTION

Since the last decade, the use of mucoadhesive particulate delivery systems for oral administration has been extensively investigated in order to retain drugs at a site of action such as the gastrointestinal tract and consequently to achieve a comparatively higher drug uptake (1,2). Adhesive particles, for example, could prolong the residence time of a drug in the gastrointestinal lumen of volunteers due to cohesive forces such as hydrogen bonds or van der Waal forces between the particles and the membrane (3,4). However, the full potential of oral mucoadhesive particulate delivery systems has so far not been reached. A number of natural and synthetic polymers are currently being employed to improve the residence time of drugs. These approaches are mainly based on various polymers, for instance, polyacrylates (5), alginate (6), or chitosan (2).

Unlike other polymers, chitosan provides high mucoadhesive properties because of ionic interactions between the positive charges of chitosan and the negative charges of mucus (7). Indeed, these mucoadhesive features of chitosan could be further improved by the immobilization of thiol groups. The thiolation of chitosan with 2-iminothiolane HCl, for example, yielded chitosan-4-thiobutylamidine conjugate (chitosan-TBA), which exhibited an even more than 100-fold higher mucoadhesion than a corresponding unmodified chitosan (8). Recently, thiolated chitosans were chosen for nanoparticles, owing to their recognized mucoadhesivity and ability to enhance the bioavailability of drugs (9). The surface modification of poly(lactide-co-glycolide) nanoparticles with chitosan-TBA nanoparticles showed a 3.3-fold improvement in mucoadhesive properties (10). The prolonged residence time of a thiolated chitosan has been confirmed in a previous study (9). The mucoadhesive features of chitosan-TBA nanoparticles comprising a fluorescence marker were up to twofold improvement in comparison to unmodified nanoparticles (9). Regarding the different types of polymers and particles sizes, a further study to prolong the residence time of drug delivery systems is to formulate a nanoparticulate preparation comprising a polymer and a vehicle, which could enhance mucoadhesive properties of the dosage form.

So far, however, to our knowledge, the impact of a formulation approach on the mucoadhesiveness of particles has not been investigated. It was therefore the aim of the present study to evaluate the effect of various vehicles on mucoadhesive properties of nanoparticulate drug delivery systems using thiolated chitosan *in vitro*. PEG 300, miglyol 840, PEG 6000, cremophor EL, and caprylic triglyceride being widely used in pharmaceutical formulations were

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chosen as liquid vehicle for particle administration. The most promising vehicles were subsequently selected and used for an *in vivo* study. The mucoadhesiveness of the particles was quantified via a visual and simple method by determining the amount of remaining fluorescein diacetate (FDA) on a target site. In addition, characterization of particles and FDA loading were determined.

MATERIALS AND METHODS

Materials

Chitosan (medium viscous, molecular mass of 400 kDa, and degree of deacetylation of 70–85% as described by a supplier), sodium nitrite, acetic acid, PEG 300, FDA, sodium tripolyphosphate (TPP), trehalose, sodium borohydride ($NaBH_4$), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and 2-iminothiolane were purchased from Sigma-Aldrich, Austria. Miglyol 840 was obtained from Sasol, Germany. Cremophor EL was purchased from BASF, Germany. Acetonitrile and PEG 6000 were obtained from Merck, Germany. Caprylic triglyceride was gifted from Abitec Corporation, Germany. Dulbecco's minimum essential (DMEM) was purchased from PAA, Germany. Lactate dehydrogenase assay (LDH)-based CytoTox 96 kit was obtained from Roche.

Methods

Preparation of Low Molecular Mass Chitosan

Medium molecular mass chitosan was chemically treated with sodium nitrite in acetic acid to produce low molecular mass chitosan (9,400 g/mol) (11). Thereafter, resulting chitosan was precipitated with 4 M NaOH, filtered, and washed with cold acetone. Subsequently, chitosan was resolubilized in 100 ml of 0.1 M acetic acid and dialyzed three times against distilled water. Finally, the resulting solution was lyophilized at −70°C and 0.01 mbar (Benchtop 2 K, VirTis, NY, USA) and kept at −20°C for further use.

Synthesis of Chitosan-TBA

Thiolated chitosan was synthesized as described previously (9). In brief, 500 mg of low molecular mass chitosan was dissolved in 80 ml of 1% acetic acid. After adjusting the pH of the mixture to 7.0 with 1 M NaOH, 200 mg of 2-imminothiolane HCl was added and continuously stirred at room temperature for 18 h. The obtained mixture was then dialyzed and dried under vacuum by lyophilization at −70°C and 0.01 mbar.

Fourier-Transform Infrared Spectroscopy Determination

The spectra of unmodified chitosan and chitosan-TBA were characterized by using a Perkin Elmer Spectrum 100 Fourier-Transform Infrared (FT-IR) Spectrometer in the range of 4,000–650 cm^{-1}.

Preparation of Fluorescent Nanoparticulate Chitosan-TBA

In situ gelation method was used to accomplish nanoparticles in this study (12). Modified low molecular mass chitosan was hydrated in 100 ml of 0.05% acetic acid to achieve a final concentration of 0.15%, 0.2%, and 0.25% (*m*/*v*). Subsequently, 0.5% (*m*/*v*) of NaOH was added to the mixtures to reach a pH of 4.5, 5.5, and 6.0, respectively. A 0.1% and 0.2% (*m*/*v*) TPP solution, respectively, was then added dropwise to the mixtures at a volume ratio of 1:7. Opalescent suspension was formed spontaneously under vigorous stirring at room temperature. Nanoparticles were purified by centrifugation at 4,300 rpm for 45 min. Supernatant was discarded and the precipitate was resuspended in 50 ml of distilled water. The 0.1% (*m*/*v*) FDA solution in acetonitrile was incorporated to the nanoparticle suspension in a ratio of 1:1. Then, the suspension was incubated in a thermomixer (Thermomixer Comfort, Eppendorf, Hamburg, Germany) at 25°C for 1 h. In order to avoid an aggregation of nanoparticles during the process of centrifugation, trehalose was added to the samples to obtain a final concentration of 3% (*m*/*v*). After that, the samples were centrifuged at a speed of 4,300 rpm for 20 min. The supernatant was removed and particles were resuspended in 100 ml of 0.05% acetic acid.

To determine the FDA load of particles, the chitosan-TBA nanoparticles were resuspended in 5 M NaOH and incubated at 37°C for 30 min under shaking in order to quantitatively hydrolyze FDA to sodium fluorescein (13). Thereafter, the formed sodium fluorescein was quantified by using microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Increasing amounts of sodium fluorescein served as standard curve.

Determination of the Content of Thiol Groups and Disulfide Bonds

The amount of thiol groups immobilized on the polymers was quantified by Ellman's method using a spectrophotometer (14). Disulfide contents were evaluated after reduction with $NaBH_4$ and determined by Ellman's reagent. The total amount of thiol moieties is represented by the summation of reduced thiol groups and oxidized thiol groups in the form of disulfide bonds (15).

Characterization of Chitosan-TBA Nanoparticles

The quantity of thiol groups on nanoparticles surface was determined with Ellman's reagent as described previously. Particle size and zeta potential of chitosan-TBA nanoparticles were evaluated by a particle determination equipment (Zeta Potential/Particle Sizer, Nicomp™ 380 ZLS, Tokyo, Japan).

Cytotoxicity Studies

Cytotoxicity studies of chitosan-TBA nanoparticles were determined by the MTT test and LDH on Caco-2 cells (16). Five milligrams of chitosan-TBA nanoparticles was added to the cell lines. The cells were incubated for 3 h, and viability of cells was then determined. In addition, toxicity of nanoparticles suspended in various vehicles was quantified as well.

In Vitro Mucoadhesion Studies

Freshly excised porcine intestinal mucosa was used for the evaluation of mucoadhesive properties. An analytical

method to determine the amount of remaining fluorescent marker was described by Albrecht *et al.* (17). Briefly, the intestinal mucosa was mounted on a plastic platform, placed in an angle of 45° in an incubation chamber where 100% relative humidity and 37°C was maintained. Thereafter, the mucosa was continuously rinsed with 100 mM phosphate buffer pH 6.5, which served as an artificial intestinal fluid. To humidify the mucosa, an equilibration period of 5 min was maintained before administering the nanoparticles. A constant flow rate of 1 ml/min was provided using a peristaltic pump. After the equilibration period, 10 mg of unhydrated thiolated NPs, the particles coated with PEG 6000, and the particles suspended in vehicles (PEG 300), miglyol 840, cremophor EL, and caprylic triglyceride in a final concentration of 10 mg/100 µl were transferred onto the mucosa. After 30, 60, 120, and 180 min, the treated mucosal membranes were incubated in 25 ml of 5 M NaOH under shaking at 37°C for 30 min. The samples were then centrifuged at a speed of 13,400 rpm for 5 min, and 200 µl of the supernatants was transferred to microplate reader. For a calibration curve, increasing amounts of chitosan-TBA nanoparticles served as standard samples. All experiments were performed in triplicate.

In Vivo Study

The animal study complied with the regulations of the Animal Ethical committee of Vienna, Austria, and the protocol adhered to the Principles of Laboratory Animal care. Male Wistar rats weighing 280–350 g were used in the study. They were fasted and housed at room temperature to maintain an alimentary deficiency for overnight. Chitosan-TBA nanoparticles were orally administered to the rats in form of either dry powder in a fast-release capsule described by our research group (17) or in form of suspensions. For the suspensions, the treated nanoparticles were suspended in 100 mM phosphate buffer pH 6.5, PEG 300, and miglyol 840 in a final concentration of 10% (*m*/*v*). The nanoparticles were placed deeply into the throat to initiate the swallow-reflex and immediately fed with 1 ml of water. Dosed rats were kept in restraining cages and allowed free access to water. After 3 h of administration, rats were sacrificed by an injection of an overdose of a 5:1 mixture of ketamine/xylazine hydrochloride (250 mg/kg). Thereafter, the stomach, small intestine (divided into three segments), and colon were removed for evaluation. Each experiment was performed at least three times.

Statistical Data Analysis

Data were expressed as mean±SD. The differences between groups were tested by ANOVA assay with p value <0.05 as the minimal level of significance.

RESULTS AND DISCUSSION

Synthesis and Characterization of Chitosan-TBA

The depolymerization method used to produce a low molecular weight chitosan was originally established by Mao and coworkers (18) and this method has been carried out and adapted by our research group (11). Schmitz *et al.* examined the molecular weight of low molecular weight chitosan using capillary viscosity measurements. It was found that the obtained chitosan had 9,400 g/mol and the production yield was 70% (11).

The pathway for the synthesis of chitosan-TBA is a simple one-step reaction. By the covalent attachment of 2-iminothiolane HCl to the amine groups of chitosan, chitosan-TBA was obtained (19). The lyophilized chitosan-TBA was a white and odorless powder of fibrous structure. The total amount of thiol groups immobilized on chitosan was 643.7±71.28 µmol/g chitosan and approximately 8% of thiol groups were oxidized.

The FT-IR spectra of unmodified chitosan and chitosan-TBA are shown in Fig. 1. A characteristic band at 3,363 cm^{-1} is pointed out to $-NH_2$ and –OH groups stretching vibration, and at 1,621 cm^{-1} the band for amide groups can be seen in the infrared spectrum of chitosan. In case of FT-IR spectra of chitosan-TBA, the characteristic band from 4,000 to 1,600 cm^{-1} is similar to chitosan. However, at 1,520 cm^{-1}, a new peak appears. This peak might be attributed to –C=N group stretching vibration, which belongs to amidine groups

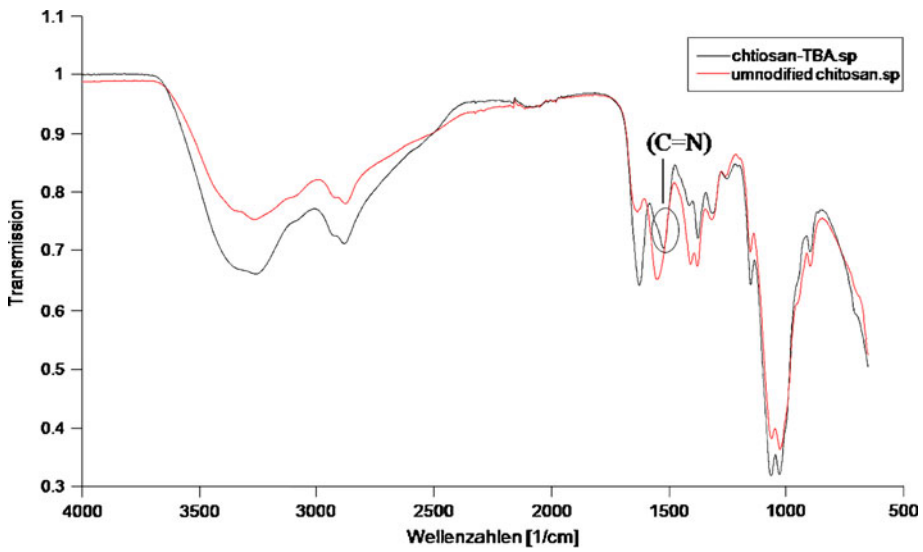

Fig. 1. FT-IR of chitosan and chitosan-TBA

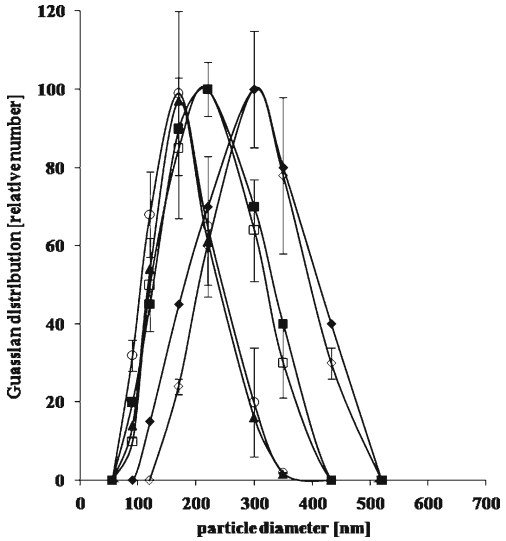

Fig. 2. Size distribution of chitosan-TBA nanoparticles obtained by ionic gelation with TPP based on chitosan (0.15%, *filled diamonds*; 0.2%, *filled squares*; 0.25%, *filled triangles* chitosan to 0.1% TPP and 0.15%, *empty diamonds*; 0.2%, *empty squares*; 0.25%, *empty circles* chitosan to 0.2% TPP). Indicated values are means ± SD of at least three experiments

confirming the immobilization of 2-imminothiolane on the polymer backbone.

In the present study, nuclear magnetic resonance (NMR) was not tested due to a solubility problem of chitosan-TBA. The lyophilized chitosan-TBA was easily soluble in distilled water but insoluble in $CDCl_3$ and partially soluble in DMSO. Guggi *et al.* (20) demonstrated that ^{1}H- and ^{13}C-NMR analysis of thiomers-TBA conjugate in DMSO exhibited a much higher tendency of hydrolysis of the product. In addition, Bravo-Osuna *et al.* (21) reported the use of ^{1}H-NMR analysis for the determination of the degree of deacetylation of chitosan. Results displayed that, after depolymerization, no structural change of chitosan and chitosan-TBA was observed due to the fact that both the unmodified chitosan and chitosan-TBA showed the same degree of deacetylation (21). Within the study, however, the obtained chitosan-TBA was indirectly identified via Ellman test. Results showed an intensive colorimetric reaction with Ellman's reagent, confirming the presence of high quantities of recued thiol groups (20).

Preparation and Characterization of Chitosan-TBA Nanoparticles

The formation of chitosan-TBA nanoparticles can be explained by the interaction between the positively charged chitosan and the negatively charged TPP solution. The obtained particles were in the nanosize range and showed a positive zeta potential. The ratio of chitosan-TBA to TPP solution was a crucial factor for the preparation. When 0.1% and 0.2% (*m*/*v*) TPP solution was added to 0.15% (*m*/*v*) chitosan-TBA solution, aggregates of large diameter were formed. By an addition of TPP solution to 0.2% and 0.25% (*m*/*v*) chitosan solution, however, the smaller sizes of particles were obtained. A certain volume ratio and a specific concentration between chitosan and TPP solution play a key factor for a formation of nanoparticles. In this study, a volume ratio of 1:7 of chitosan to TPP solution was used, which resulted in a particle size in the range of 160–305 nm (as depicted in Fig. 2). The zeta potential was determined to be +8.0 to +21.5 mV (data not shown). The highest zeta potential was achieved when the concentration of chitosan and TPP solution was 0.25% (*m*/*v*) and 0.2% (*m*/*v*), respectively. Goto *et al*. have reported on the effect of microparticles in prolonging the residence time of a drug adhering on the intestinal mucosa of rats (22). Microparticles of diameters of <53 μm (SS size) composed of a 1:1 molar ratio of methacrylic acid/poly(ethylene glycol) remained in the intestinal lumen to a higher extent than particles having a larger size. Moreover, when these small microparticles comprising insulin were orally administered to rats, the oral bioavailability of insulin was 9.5% compared to subcutaneous insulin injection. These results imply that the mucoadhesive capacities of polymers depend on the particles size.

In order to characterize the oxidation process during nanoparticle production, the decrease in thiol groups on the surface of nanoparticles was quantified by Ellman's reagent. Thiol moieties on nanoparticles were decreased in each step of the production as shown in Table I. Disulfide bond formations within the particles, however, increased from 8% to 29% due to air oxidation (23). These covalently cross-linked particles offer adequate stability to nanoparticles even at low pH levels. The particles will therefore not disintegrate in acidic conditions such as in the stomach. The mean diameter of nanoparticles in various vehicles is shown in Table II. The particle size of chitosan-TBA nanoparticles suspended in 100 mM phosphate buffer pH 6.5, PEG 300, miglyol 840, cremophor EL, and caprylic triglyceride was determined to be 332.2, 146.3, 125.1, 150.7, and 135.2 nm, respectively. The various sizes of the particles may be explained by the swelling behavior of the particles. The more prehydrated chitosan nanoparticles are, the greater the size is.

Cytotoxicity Studies

Caco-2 cell lines were used to determine cytotoxicity of thiolated NPs. The cells were cultured in 24-well plates, supplemented with DMEM with fetal calf serum, and

Table I. Amount of Thiol Groups and Disulfide Bonds Covalently Attached to Chitosan-TBA and Chitosan-TBA Nanoparticles After Ionic Gelation with TPP Solution (the Concentration Ratio Between Chitosan-TBA and TPP Solution Was 0.25:0.2)

Compounds	–SH (μmol/g)	–S–S– (μmol/g)	Σ–SH (μmol/g)
Chitosan-TBA	534.3	54.5	643.7
Chitosan-TBA nanoparticles	381.8	138.5	638.7
Chitosan-TBA nanoparticles after removing TPP by centrifugation	69.6	36.7	142.5
Lyophilized chitosan-TBA NPs	24.2	16.6	57.3

Table II. Mean Particle Diameter of Chitosan-TBA Nanoparticles in Various Vehicles: Chitosan-TBA Nanoparticles, Chitosan-TBA Nanoparticles Suspended in 100 mM Phosphate Buffer pH 6.5, PEG 300, Miglyol 840, Cremophor EL, and Caprylic Triglyceride, Respectively

Formulations	Mean particle diameter [nm] (means ± SD)
Chitosan-TBA nanoparticles	164.2±6.9
Chitosan-TBA nanoparticles suspended in 100 mM phosphate buffer pH 6.5	332.2±52.1
Chitosan-TBA nanoparticles suspended in PEG 300	146.3±2.3
Chitosan-TBA nanoparticles suspended in miglyol 840	125.1±4.4
Chitosan-TBA nanoparticles suspended in cremophor EL	150.7±8.4
Chitosan-TBA nanoparticles suspended in caprylic triglyceride	135.2±10.8

incubated for 24 h. Cytotoxicity (%) of chitosan-TBA nanoparticles quantified by LDH assay showed no significant difference in comparison to untreated cells (control). In contrary, nanoparticles suspended in PEG 300 slightly induced a damage of cell membrane after 3 h of incubation (data not shown). This finding is in good correlation with the result obtained from MTT assay. After 3 h of incubation with the particles suspended in PEG 300, the viability of cells was approximately 80%, whereas those of particles suspended in other vehicles were about 90%. In addition, the cytotoxicity (%) of the excipients used in the study was determined. Results showed that the concentration of all excipients was in the safety range. The decrease in the viability of cells was not dramatically changed. More than 70% of the cells incubating with all media were viable after 3 h of the experiment. One hundred micromolars of phosphate buffer pH 6.5 was the most harmless followed by caprylic triglyceride, miglyol 840, PEG 6000, cremophor EL, and PEG 300, respectively. According to the cytotoxicity results, chitosan-TBA nanoparticles are considered relatively less toxic for the cells.

In Vitro Mucoadhesion Studies

Mucoadhesion Studies of Chitosan-TBA Nanoparticles

Mucoadhesive properties of thiolated chitosan nanoparticles on excised porcine intestinal mucosa were evaluated indirectly using FDA as fluorescence marker. FDA was embedded in thiolated NPs by a simple diffusion technique. The reason for using this marker is its insolubility in aqueous media such as artificial intestinal fluid; thereby, it can remain on the mucosa (13). By utilizing this method, an FDA load of 2–3% was achieved. The percentage of FDA-loaded particles remaining on freshly excised porcine intestine after administration of the formulations is shown in Fig. 3. After 30 min of incubation, an average of 95% chitosan-TBA nanoparticles stuck on the mucosa, whereas only 44% of unmodified chitosan nanoparticles were still adhering. The modified chitosan nanoparticles theoretically bind to the intestinal mucosa via covalent bonds with cysteine-rich subdomains of glycoproteins in the mucus based on the mechanism of thiol/disulfide exchange reactions and on an oxidation process (7).

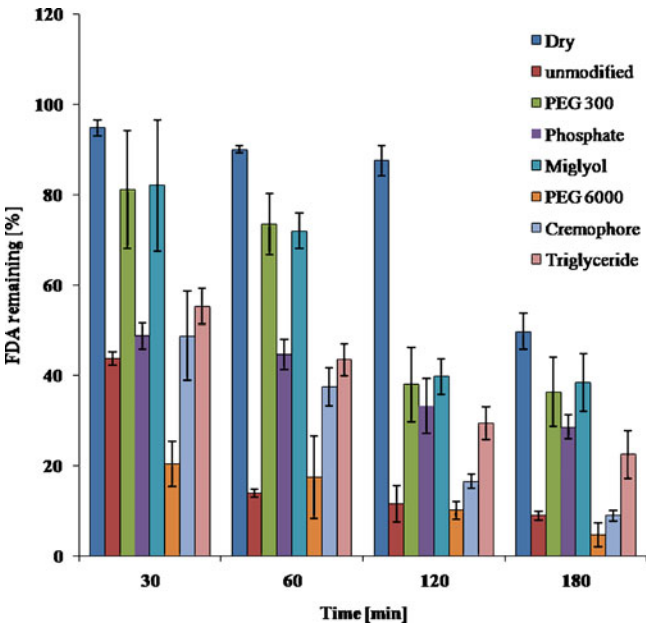

Fig. 3. Percentage of FDA remaining on excised porcine small intestinal mucosa over 180 min of incubation. Chitosan-TBA nanoparticles were applied in dry form, suspended in PEG 300, in miglyol 840, in cremophor EL, in caprylic triglyceride, in PEG 6000, or in 100 mM phosphate buffer pH 6.5 and compared to unmodified chitosan nanoparticles applied in dry form. Indicated values are means ± SD of at least three experiments

The amount of FDA remaining was decreased as a function of time, for example, after 180 min of the measurement the marker remained to a lower extent on the mucosa than at $t=0$. This finding was in good agreement with the result obtained from Bernkop-Schnürch *et al.* (9). The results revealed that at the end of the experiment the percentage of the remaining marker being incorporated in thiolated chitosan nanoparticles was approximately 43% where the improvement ratio between the remaining marker within modified chitosan nanoparticles and unmodified chitosan nanoparticles was 2.38-fold (9). The comparatively low mucoadhesive properties of 10 kDa thiolated NPs might be explained by the amount of free thiol groups. The more free the thiol moieties' availability are for the disulfide bond formation with the mucus layers, the higher are the mucoadhesive features of the nanoparticles.

Effect of Various Vehicles on Mucoadhesion Studies

The amount of FDA remaining on nanoparticles suspended in various vehicles is provided in Fig. 3 The total amount of FDA remaining on the intestinal mucosa after 180 min was significantly (p value<0.05) higher (49.71±3.94%) when applied in dry form. Nevertheless, when applied in the form of chitosan-TBA nanoparticles suspended in cremophor EL, only 8.95±1.12% of FDA adhered on the mucosa. The mucoadhesive properties were maximal for lyophilized chitosan-TBA nanoparticles followed by the nanoparticles suspended in miglyol 840, PEG 300, 100 mM phosphate buffer pH 6.5, caprylic triglyceride, cremophor EL, and PEG 6000, respectively. Actually, all prepared thiolated chitosan particles had the average size of 164.2±6.9 nm. When they dispersed in various media, particles swelled. Consequently, the particles had different sizes. Therefore, mucoadhesion of the particles on the one hand might be a result of the particle sizes, and on the other hand, it could be due to the properties of the excipients used in the study. Basically, the mechanism of mucoadhesion of particles involves two steps of adhesive process: contact stage and consolidation stage. Mucoadhesive substances and the mucus membrane have initial contact to form an intimate contact. Thereafter, moisture will allow the mucoadhesive substances to conform to the shape of the surface and to adhere predominantly by weak van der Waals and hydrogen bonding (1). In case of the dry form, the strongest mucoadhesion is a consequence of covalent bond formations between the polymer and the mucosal layer. Once dry particles contact to the mucus layer, the gastrointestinal fluids will hydrate particles. Subsequently, the particles will swell and form strong bonds with the mucosal membrane (13). The more unhydrated nanoparticles are, the more particles adhere to the mucosa after a certain time. Chitosan-TBA nanoparticles coated with PEG 6000, however, exhibited the lowest mucoadhesive properties. It is likely that, after administration of the formulation, the coated particles detach from the mucosa because of rapid swelling of PEG 6000. To clarify this observation, swelling behavior of particles suspended in various vehicles once in contact with an artificial intestinal fluid was determined. As depicted in Fig. 4, the size of particles increased as a function of time in case of particles suspended in PEG 300, PEG 6000, and cremophor EL. These results support that PEGs allow nanoparticles to hydrate in the artificial fluid. Furthermore, it is likely that cremophor EL can form an emulsifying/microemulsifying system when exposed to aqueous media, resulting in a bigger size of particles. In contrast,

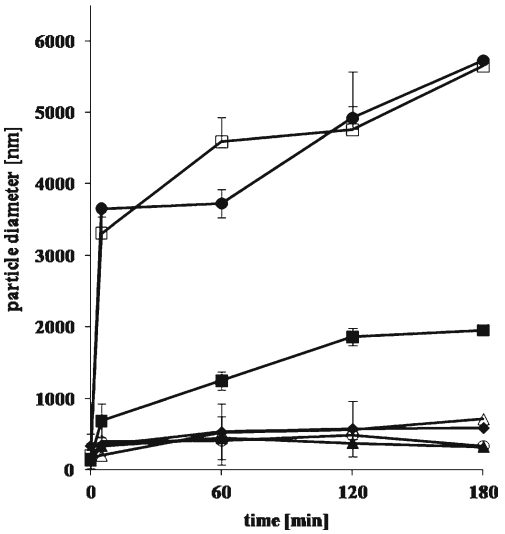

Fig. 4. Swelling behaviors of chitosan-TBA nanoparticles suspended in various vehicles: PEG 300 (*filled diamonds*), miglyol 840 (*filled squares*), PEG 6000 (*empty squares*), caprylic triglyceride (*empty circles*), cremophor EL (*filled circles*), and phosphate buffer pH 6.5 once in contact with an artificial gastrointestinal fluid (100 mM phosphate buffer pH 6.5; *filled triangles*) compared to unmodified chitosan nanoparticles (*empty triangles*). Indicated values are means ± SD of at least three experiments

unmodified chitosan nanoparticles showed no significant difference in swelling behaviors when the particles size was less than 500 nm. Mucoadhesion of unmodified nanoparticles, however, was comparatively weak because of hydrogen bond formations between the polymer and mucus layers (p value<0.05, compared to $t=0$ min). Once particles were suspended in nonaqueous phase such as in miglyol 840 and caprylic triglyceride, the particles might be entrapped and small droplets are formed. The entrapped particles were therefore not swollen while aqueous phase was introduced to the formulations. According to this phenomenon, the mean diameter of these particles was smaller than that of particles suspended in cremophor EL and PEG 6000.

The effect of the zeta potential in chitosan-TBA nanoparticles mucoadhesion has been studied by Bravo-Osuna *et al.* (24). The zeta potential seemed not to be important enough to explain the differences in mucoadhesion. When the formulations were orally administered, part of the soluble mucin fraction located in the mucus could interact with the positively charged nanoparticles and partially mask the charges (24).

***In Vivo* Studies**

Distribution of Chitosan-TBA Nanoparticles in the Gastrointestinal Tract of Rats

According to the *in vitro* study, the most promising formulations were taken forward for an *in vivo* study. The four formulations include dry chitosan-TBA nanoparticles in a fast-release capsule, particles suspended in 100 mM phosphate buffer pH 6.5, PEG 300, and miglyol 840, respectively. The capsule was prepared as described previously (17). The distribution of particles having been administered in the form of these formulations in the digestive tract of rats is shown in Fig. 5. In the stomach, more than 70% of dry nanoparticulate thiolated chitosan remained adhering. It showed a signifi-

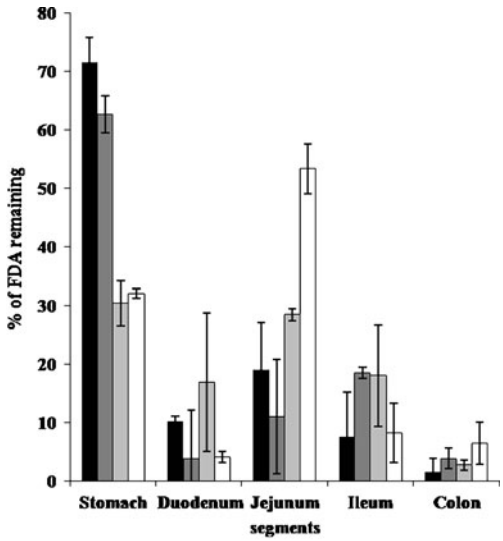

Fig. 5. Percentage of FDA remaining on five different segments: stomach, duodenum, jejunum, ileum, and colon of rats. Chitosan-TBA nanoparticles were administered to rats a form of a fast capsule release (*black bar*) or the nanoparticles suspended in PEG 300 (*dark gray bar*), in 100 mM phosphate buffer pH 6.5 (*light gray bar*) and in miglyol 840 (white bar). Indicated values are means ± SD of at least three experiments

cantly 3.6-fold higher mucoadhesion compared to the unmodified chitosan NPs (p value <0.05). The obtained results, however, were not consistent with a previous study (17). Albrecht *et al*. showed that, after 3 h of administration, microparticles retained to a much higher extent in the small intestinal segments, whereas within this study particles were mainly accumulated in the stomach. Generally, the gastric fate of dosage forms primarily depends on the state of the stomach (fed or fasted) and the size of the formulations (4). Liquids and small particles are emptied quickly from the stomach, whereas large particles (bigger than 2 mm in size) are retained until reduction to a smaller size. Two theories have been postulated to explain the retention of particles on the stomach mucosa. First, large indigestible solids such as a capsule may be cleared from the stomach to the small intestine by the migrating myoelectric complex (MMC) (25). The capsule was retained in the stomach until it was emptied by waves of MMC, but MMC may not be strong enough to move the whole capsule to the small intestine. Second explanation for this finding is that the folds of the stomach wall may lodge the particles upon dispersion in the stomach; thereby, the residence time was prolonged. Apparently, the twine of the capsule would be expected to be dissolved in a medium with a pH value exceeding 6.8. However, results showed that the capsule abruptly released its content in the stomach. The explanation for this result is that the stomach pH of fasted rats is higher than fed rats (26). Hence, the capsule could be erupted and followed by a release of the particles from the capsule. Once the nanoparticles were released, they might be trapped in the folds of the stomach wall, resulting in high accumulation in the stomach.

Effect of Various Vehicles on Mucoadhesion Studies

In comparison to unmodified chitosan nanoparticles, thiolated chitosan nanoparticles suspended in various vehicles showed significantly higher residence time (p value <0.05; data not shown). The corresponding mean mucoadhesion (%) for thiolated NPs in the capsule, the nanoparticles suspended in 100 mM phosphate buffer pH 6.5, PEG 300, and miglyol 840 in the stomach were 71.57%, 30.44%, 62.68%, and 32.09%, respectively. In addition, approximately 60% of thiolated chitosan nanoparticles suspended in PEG 300 were adhering in the stomach after 3 h of administration. The result of the present study seems to correlate with earlier findings on the transit effect of PEG 400 (27). In the presence of PEG 400, an increase of ranitidine uptake was achieved. The bioavailability of the model drug ranitidine was accelerated based on a stimulation effect on gastrointestinal mobility and transit time (27). In addition, PEG chains can enhance adhesion to the membrane due to interdiffusion and entanglement with the mucus layers, thereby the prolongation of the retention to the GI tract was observed (22).

When chitosan-TBA nanoparticles were dispersed in 100 mM phosphate buffer, pH 6.5, particles remained on the intestinal mucosa rather than the stomach, indicating that the particles could not stick on the gastric mucosa. Generally, solutions of drugs of a size less than 2 mm would be emptied from the stomach quite rapidly by the intragastric pressure, which is produced by the contractions of muscles in the upper body of the stomach (28,29). It can be assumed that aqueous suspensions of nanoparticles are less adhesive in the stomach due to previous swelling. Thus, they are cleared faster to the intestine. However, dry particles from capsules or particles suspended in nonaqueous media displayed higher adhesion to membrane of the stomach due to less extensive swelling. The time for small molecules to traverse the small intestine of rats is approximately 3–4 h, and the velocity of transport is faster in the proximal segments than in the ileum (30). Hence, particles were mainly accumulated in the jejunum segments. In the presence of miglyol 840, particles remained by 55% on the jejunum mucosa after 3 h of oral administration. This phenomenon might be explained by the fact that disulfide bond formations between thiol groups of thiolated nanoparticles and the mucus gel layer of the digestive tract is favored at higher pH values. During a fasted state, the pH value in the jejunum–ileum segments is comparatively high (pH value >7) compared to a fed state (26). When the pH value of the fluid content in the digestive system is greater than the p*K*a value of the thiol groups attached to chitosan, higher amounts of thiol anions (S^-) are available, leading to thiol/disulfide exchange reactions and/or an oxidation process. Subsequently, chitosan-TBA nanoparticles could adhere in the jejunum.

CONCLUSION

In this study, the mucoadhesive properties of polymers both *in vitro* and *in vivo* were quantified using fluorescence analysis. Moreover, the impact of various vehicles on the mucoadhesion of thiolated nanoparticles was reported for the first time as a case study. Results from *in vitro* and *in vitro* mucoadhesion studies showed that chitosan-TBA nanoparticles being applied in dry form exhibit the strongest improvement in mucoadhesion. In the presence of PEG 300 and miglyol 840, the mucoadhesiveness of the polymer could be enhanced due to the properties of vehicles. It can therefore

be concluded that vehicles have a tremendous influence on the mucoadhesive properties of orally administered nanoparticles. In addition, the strong mucoadhesive features of these formulations could be helpful for target-specific drug delivery with a prolongation in the residence time of drugs.

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