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Thiolated chitosan microparticles: A vehicle for nasal peptide drug delivery

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Abstract

The goal of this study was to develop a microparticulate delivery system based on a thiolated chitosan conjugate for the nasal application of peptides. Insulin was used as model peptide. For thiolation of chitosan 2-iminothiolane was covalently linked to chitosan. The resulting chitosan–TBA (chitosan-4-thiobutylamidine) conjugate featured 304.89 ± 63.45 μmol thiol groups per gram polymer. 6.5% of these thiol groups were oxidised. A mixture of the chitosan–TBA conjugate, insulin and the permeation mediator reduced glutathione were formulated to microparticles. Control microparticles comprised unmodified chitosan and insulin. As second control served mannitol–insulin microparticles. All microparticulate systems were prepared via the emulsification solvent evaporation technique. In 100 mM phosphate buffer pH 6.8 chitosan–TBA–insulin microparticles swelled 4.39 ± 0.52-fold in size, whereas chitosan based microparticles did not swell at all. Chitosan–TBA microparticles showed a controlled release of fluorescein isothiocyanate (FITC)-labelled insulin over 6 h. Nasal administered chitosan–TBA–insulin microparticles led to an absolute bioavailability of 7.24 ± 0.76% (means ± S.D.; $n = 3$) in conscious rats. In contrast, chitosan–insulin microparticles and mannitol–insulin microparticles exhibited an absolute bioavailability of 2.04 ± 1.33% and 1.04 ± 0.27%, respectively (means ± S.D.; $n = 4$).

Because of these results microparticles comprising chitosan–TBA and reduced glutathione seem to represent a useful formulation for the nasal administration of peptides.

Keywords: Thiomer; Nasal drug delivery; Microparticles; Peptides; Glutathione; Bioavailability; Controlled release/delivery

1. Introduction

Besides inconvenient injections used to deliver peptides to the human body, the nasal route seems to be one of the most feasible alternative ways for peptide delivery. Because of the high vascularity and large surface area of the nasal cavity, nasally administered peptides are relatively rapid absorbed. After permeation into the nasal blood vessels, peptides are transported immediately to their site of action, avoiding a first-pass metabolism. The nasal delivery of most peptides representing polar and hydrophilic macromolecules, however, is even for this route of administration a great challenge. Consequently, only few nasal formulations containing peptides are on the market like nasal sprays for desmopressin and calcitonin. Generally most peptides show bioavailabilites of 1% or less when administered to the nasal cavity (Illum, 1996). This low bioavailability is the result of different barriers, namely the enzymatic barrier (I), the absorption barrier (II) and the mucociliary clearance (III). To overcome the enzymatic barrier, the coadministration of enzyme inhibitors like amastatin (O'Hagan et al., 1990) is possible, but most of these auxiliary agents are toxic if absorbed systemically. To overcome the absorption barrier, peptides can be chemically modified (Hashimoto et al., 1989) to enhance their absorption from nasal mucosa. The most common method to improve nasal peptide absorption is the use of permeation enhancers. Many permeation promoters cause unfortunately significant damage to the nasal mucosa when used in very effective concentrations (Hinchcliffe and Illum, 1999). The mucociliary clearance rate can be decreased by the use of mucoadhesive polymers. Chitosan has shown in different studies to prolong the residence time of nasal drug delivery systems at the site of drug absorption (Soane et al., 1999, 2001). Additionally, chitosan improves the absorption of peptides by opening transiently the tight junctions (Illum, 1998). Accordingly chitosan represents a promising polymer in nasal peptide delivery.

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In recent studies our research group demonstrated, that thiolation of chitosan results in a chitosan derivative with higher mucoadhesive (Bernkop-Schnürch et al., 2003a, 2004) and permeation enhancing properties (Bernkop-Schnürch et al., 2004) compared to unmodified chitosan. By the combination of thiolated chitosan in particular – chitosan–TBA (chitosan-4-thiobutylamidine conjugate) (Bernkop-Schnürch et al., 2003a) – with the permeation mediator reduced glutathione, the permeation enhancing effect of this chitosan derivative could be further improved (Bernkop-Schnürch et al., 2004). The usefulness of chitosan–TBA for the oral administration of peptide drugs has already been demonstrated in various in vivo studies (Guggi et al., 2003a, 2003b; Krauland et al., 2004), but its effectiveness in nasal peptide delivery was not yet evaluated.

It was therefore, the aim of this study to determine the potential of chitosan–TBA as a nasal peptide delivery system in comparison to a formulation based on unmodified chitosan. As model peptide drug insulin was chosen. As insulin represents a polar, hydrophilic peptide of a comparatively high molecular mass, its nasal delivery is a challenging task. To achieve this goal, insulin loaded microparticles based on chitosan–TBA were generated. The resulting microparticles were characterised in vitro regarding their particle size, swelling behaviour and drug release. Furthermore, the efficacy of thiolated chitosan microparticles versus unmodified chitosan microparticles for nasal insulin delivery was evaluated in vivo.

2. Experimental

2.1. Synthesis and purification of the chitosan–TBA conjugate

Initially, 500 mg of chitosan (average molecular mass: 400 kDa; Fluka GmbH, Buchs, Switzerland) were dissolved in 50 mL of 1% acetic acid. After adjusting the pH to 6.5 with 1 M NaOH 200 mg of 2-iminothiolane HCl were added. The coupling reaction was allowed to proceed for 14 h at room temperature under continuous stirring. For purification the resulting polymer conjugate was dialysed (cellulose membrane dialysis tubing with molecular weight cutoff of 12 kDa, Sigma, St. Louis, MO, USA) against 5 mM HCl, two times against 5 mM HCl containing 1% NaCl, against 5 mM HCl and finally against 1 mM HCl to obtain a final pH of 3. Thereafter, the polymer was frozen at −30 °C until further use (Roldo et al., 2004). For the determination of oxidised and reduced thiol groups of the polymer, aliquots of 0.5 g were lyophilised at −30 °C and 0.01 mbar (Christ Beta 1–8 k; Osterode am Harz, Germany).

2.2. Determination of the thiol group and disulfide bond content

The amount of free thiol groups on the chitosan–TBA conjugate and within the resulting microparticles was determined via Ellman's reagent (5,5′-dithiobis(nitrobenzoic acid)) as described previously (Krauland et al., 2004). Disulfide content was measured after reduction with $NaBH_4$ and addition of 5,5′-dithiobis (nitrobenzoic acid) as described by Habeeb (1973).

2.3. FITC labeling of insulin

Fluorescein isothiocyanate (FITC; Sigma, St. Louis, MO, USA) was covalently linked to insulin in a slightly modified way as described previously (Clausen and Bernkop-Schnürch, 2000). In brief, a solution of FITC in dimethylsulfoxide (5 mg/mL) was slowly added in aliquot volumes of 25 μL to 40 mg of insulin (from bovine pancreas; Sigma; St. Louis, MO, USA) dissolved in 5 mL of 0.1 M Na_2CO_3. After an incubation period of 12 h at 4 °C, the coupling reaction was stopped by the addition of 50 mM NH_4Cl. The mixture was stirred for 2 h at 4 °C. Unbound FITC was separated on a Sephadex® G15 column (Pharmacia, Uppsala, Sweden). The FITC–insulin conjugate was frozen at −30 °C, lyophilised and stored at 4 °C in the dark until further use.

The amount of FITC being covalently linked to insulin was determined by measuring the fluorescence of 0.5 mg of FITC–insulin dissolved in 1 mL of $NaHCO_3$ (4% m/v) with a fluorimeter (SLT; Spectra Fluor; Tecan; Austria). The coupling rate was calculated using a standard curve obtained with a series of solutions of FITC dissolved in the same medium.

2.4. Preparation of the nasal dosage forms for in vivo studies

The compositions of chitosan–TBA–insulin microparticles and control microparticles are listed in Table 1.

2.4.1. Preparation of chitosan–TBA–insulin and chitosan–TBA–FITC–insulin microparticles

Microparticles were prepared by a water-in-oil (w/o) emulsification solvent evaporation technique. First, 3.0 mL

Table 1
Composition, insulin and glutathione load of the nasal dosage forms used for in vivo studies

Components	Chitosan–TBA–insulin microparticles (μg/mg microparticles)	Chitosan–insulin microparticles (μg/mg microparticles)	Mannitol–insulin microparticles (μg/mg microparticles)
Insulin	475	475	475
Chitosan–TBA conjugate	475	–	–
Chitosan	–	525	–
Mannitol	–	–	525
Reduced glutathione	50	–	–
Insulin load	181.58 ± 4.63 (38.23 ± 0.98%)	165.05 ± 2.58 (34.75 ± 0.54%)	165.89 ± 4.24 (34.92 ± 0.89%)
Glutathione load	4.76 ± 0.31 (9.52 ± 0.62%)	–	–

of the frozen chitosan–TBA solution (containing 7.83 mg chitosan–TBA per millilitre) were thawed and 2.5 mg of reduced glutathione (GSH; Sigma, St. Louis, MO, USA) and 2.5 mL of a 0.95% insulin solution (from bovine pancreas; Sigma; St. Louis, MO, USA) or 2.5 mL of a 0.95% FITC–insulin solution were added. The mixture was stirred for 20 min before it was added dropwise to 90 g of paraffin oil (viscosity 11–230 mPa s) containing 0.25% Span 20 as emulsifying agent. Once the emulsion was formed by utilising an ultraturax (Omni 5000; Omni International), the dispersed aqueous phase was completely evaporated by maintaining the temperature at 25 °C. Additionally the emulsion was bubbled with air (5 L/min) and stirred with a paddle at 300 rpm for 12 h. In this time period the aqueous phase was totally evaporated. Petroleum ether (20 mL) was added and mixed with the oil phase for 10 min. The microparticles were separated from the oil phase by centrifugation (Sorvall RC; 3000 rpm; 5 min), washed several times with petroleum ether to remove remaining traces of paraffin oil, dried at air temperature and stored at 4 °C until nasal administration.

2.4.2. Preparation of chitosan–insulin microparticles

Chitosan–insulin microparticles were obtained by thawing 2.26 mL of a frozen unmodified chitosan pH 3 solution (containing 11.62 mg unmodified chitosan per millilitre (average molecular mass: 400 kDa; Fluka GmbH, Buchs, Switzerland)). After addition of 2.5 mL of a 0.95% insulin solution, the mixture was stirred for 20 min and microparticles were prepared as described above. Chitosan–insulin microparticles were stored at 4 °C until nasal administration.

2.4.3. Preparation of mannitol–insulin microparticles

Mannitol–insulin microparticles, representing a second control, were obtained by dissolving 26.25 mg mannitol in 3 mL demineralised water. After adjusting the pH to 3, 2.5 mL of a 0.95% insulin solution were added. The mixture was stirred for 20 min and microparticles were prepared as described above. Mannitol–insulin microparticles were stored at 4 °C until nasal administration.

2.5. Insulin load determination

The drug load of microparticles was determined as follows: 1 mg of microparticles was hydrated in 2 mL of a mixture of 70% 0.1 M HCl and 30% dimethyl sulfoxide (DMSO) and incubated in an oscillating waterbath (GFL 1092; 60 rpm) at 37 ± 0.5 °C. After 24 h the suspension was centrifuged 10 min (24,000 × *g*, Hermle Z 323 K) to remove the microparticles. Insulin in the supernatant was evaluated by HPLC analysis (series 200 LC; Perkin-Elmer) according to a method described previously by our research group (Marschütz and Bernkop-Schnürch, 2000). The insulin concentration was determined by interpolation from a standard curve obtained from increasing insulin concentrations.

2.6. Determination of reduced glutathione loading

The amount of the permeation mediator reduced glutathione comprised in the microparticles was determined by hydrating 1 mg of microparticles in 0.5 mL demineralised water following by incubation in an oscillating waterbath (GFL 1092; 60 rpm) at 37 ± 0.5 °C. After 24 h the suspension was centrifuged 10 min in order to remove the microparticles. Reduced glutathione in the supernatant was evaluated by HPLC analysis. Reduced glutathione was separated from insulin on a Nucleosil 100-5 C18 column (250 mm × 4 mm) at 40 °C. Gradient elution was performed as follows: flow rate 1.0 mL/min, 0–17 min; linear gradient from 100% A/0% B to 93% A/7% B (eluent A: 0.1% trifluoroacetic acid in water; eluent B: 0.1% trifluoroacetic acid in 90% acetonitrile/9.9% water). Reduced glutathione was detected by absorbance at 220 nm with a diode array absorbance detector (Perkin-Elmer 235 C). The reduced glutathione concentration was determined by interpolation from a standard curve obtained from increasing reduced glutathione concentrations.

2.7. Particle size and swelling behaviour

Particle size and the water absorbing capacity of the chitosan–TBA–insulin microparticles and chitosan–insulin microparticles were determined by using a laser diffraction particles size analyser (Shimadzu SALD 1100). The size of the microparticles was analysed in paraffin oil (viscosity: 5 mPa s) as a non-dissolving dispersion medium. Particles were suspended by sonification during the measurement.

For determination of the swelling behaviour microparticles were incubated in 100 mM phosphate buffer pH 6.8 preequilibrated to 37 °C. The increasing size of the microparticles was measured immediately after addition of the buffer and after 30 min, 1 and 2 h of incubation in the same buffer medium under continuous shaking on an oscillating water bath (GFL 1092; 100 rpm) at 37 °C. All particle size distributions were calculated by the number of microparticles.

2.8. In vitro release studies from chitosan–TBA–insulin microparticles

The FITC–insulin release rate from chitosan–TBA–FITC–insulin microparticles was analysed in vitro. First, 0.5 mg of microparticles were placed in an Eppendorf vial containing 1.0 mL of release medium (100 mM phosphate buffer pH 6.8 preequilibrated to 37 °C). The vial was closed, placed on an oscillating water bath (GFL 1092; 100 rpm) and incubated at 37 °C. At predetermined time points aliquots of 100 μL were withdrawn carefully in order to prevent withdrawal of microparticles and replaced by an equal volume of release medium preequilibrated to temperature. Released FITC–insulin was determined by measuring the fluorescence of the aliquots with a fluorimeter (SLT; Spectra Fluor; Tecan; Austria). Concentrations were calculated by interpolation from a standard curve. Sink conditions were maintained throughout the whole study.

2.9. Preparation of an insulin solution for intravenous injection

Intravenous injection of an insulin solution served as positive control. For i.v. injection 3.48 μg of insulin was dissolved in

0.1 mL of a sterile 154 mM phosphate-buffered saline pH 7.5, then filtered through a cellulose acetate filter unit (pore size: 0.22 μm, Millipore S.A., Molsheim, France) and subsequently injected.

2.10. In vivo evaluation of the delivery systems

The protocol for the studies on animals was approved by the Animal Ethical Committee of Vienna, Austria and adhered to the Principles of Laboratory Animal Care. For in vivo studies male Wistar rats SPF (200–300 g body weight) obtained from the Institut für Labortierkunde und Genetik, University of Vienna were used. All rats were kept in restraining cages with free access to water. Before dosing the fasted animals, 200 μL of blood were taken from the tail vein. This initial insulin level was used as reference level (time point zero). Rats were divided in four groups and treated separately with the different dosage forms. On the one hand, 1 mg of chitosan–TBA–insulin microparticles containing 726.31 μg insulin/kg (three rats) or chitosan–insulin microparticles containing 660.2 μg insulin/kg (four rats) or mannitol–insulin microparticles containing 663.55 μg insulin/kg (four rats) was administered into each nostril (Table 2). The application device comprised a 200 μl gel-load pipette tip (Greiner, Kremsmünster, Austria), filled with the formulations connected via polyethylene tubing to a 5 mL syringe to aerosolise the formulation. A cotton filter served to prevent the formulation from entering the polyethylene tubing. The dry particles were delivered without anaesthesia by holding the rats in an upright position and blowing air through the device. Another group of rats was dosed with 13.87 μg of insulin/kg by i.v. injection (four rats) (Table 2). The dosed rats were fasted for 6 h and kept in restraining cages with free access to water. Two hundred microlitre of blood were taken 15, 30 min, 1, 2, 4 and 6 h after drug application, immediately centrifuged (4000 × *g*, 5 min, 4 °C) and plasma samples were collected. The plasma was kept frozen until the concentration of insulin was determined via ELISA (Biosource, Nivelles, Belgium).

For i.v. injections 200 μL of blood were taken additionally 2.5, 5 and 10 min postadministration, as a decrease of the injected insulin was expected already in this time period.

2.11. Statistical data analysis

Statistical data analysis was 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).

3. Results

3.1. Characterisation of the chitosan–TBA conjugate

TBA was attached to chitosan via an amidine bond between the carboxylic group of the reagent and a free primary amino group of the polymer. The purified chitosan–TBA conjugate exhibited 304.89 ± 63.45 μmol thiol groups per gram polymer (mean ± S.D.; $n = 4$). Thereof 6.5% were oxidised thiol groups. The obtained polymer was white, odourless and showed a fibrous structure.

3.2. Characterisation of FITC labelled insulin

On average, 1.91 ± 0.28 mol FITC were bound to 1 mol insulin (means ± S.D.; $n = 4$) as determined by fluorimetry.

3.3. Characterisation of microparticles

The microparticles were spherical and showed a rough surface. Size measurements in paraffin oil as non-dissolving dispersion medium showed that more than 99.8% of chitosan–TBA–insulin microparticles and 100% of the chitosan–insulin microparticles were in the range of 1–59 μm. The average particle diameter was 18.7 ± 0.3 μm for chitosan–TBA–insulin microparticles and 18.5 ± 0.3 μm for the chitosan based control particles. These results could be confirmed by measuring the particle size in a transmission light microscope. Due to the size of chitosan–TBA–insulin microparticles as well as of chitosan–insulin microparticles with a fraction smaller than 10 μm, the deposition of this fraction of microparticles after nasal administration in the lower respiratory tract could not be excluded.

During the microparticle preparation process the chitosan–TBA conjugate formed inter- and intra-molecular disulfide bonds. As the chitosan–TBA conjugate displayed 6.5% oxidised thiol groups, resulting microparticles exhibited 78.4% oxidised thiol groups indicating inter- and intra-molecular crosslinking via disulfide bonds. Therefore, an increased stability of the resulting microparticles was obtained during the preparation process.

Table 2
Main pharmacokinetic parameters after nasal administration of chitosan–TBA–insulin microparticles, chitosan–insulin microparticles and mannitol–insulin microparticles, as well as after intravenous injections of insulin to rats (means ± S.D.; $n = 3$–4)

Formulation	Nasally given chitosan–TBA–insulin microparticles	Nasally given chitosan–insulin microparticles	Nasally given mannitol–insulin microparticles	Intravenous injection
Insulin dose (μg/kg)	1452.64	1320.4	1327.12	13.87
C_{max} (ng/mL)	91.03 ± 10.98	26.95 ± 40.09	20.03 ± 10.47	–
t_{max} (min)	30	15	15	–
$AUC_{0 \rightarrow 6}$/rat	69.23 ± 7.25	16.71 ± 9.66	9.96 ± 2.68	9.12 ± 3.8
Absolute bioavailability (%)	7.24 ± 0.76	2.04 ± 1.33	1.04 ± 0.27	–

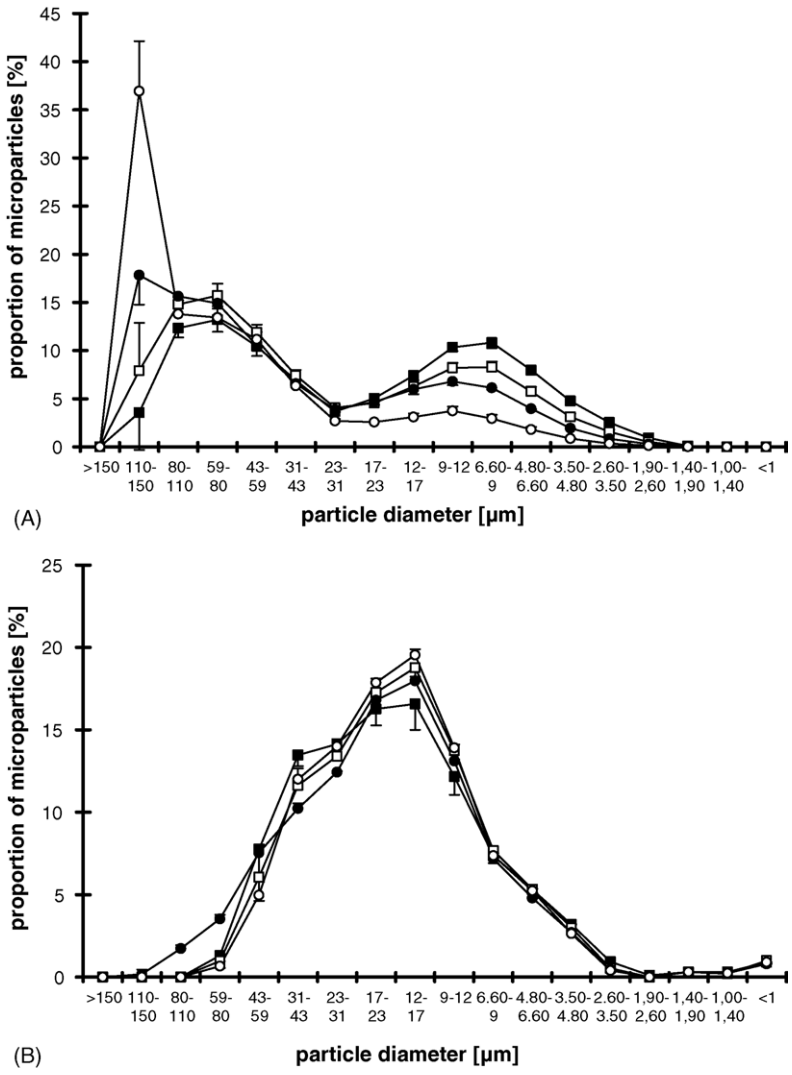

Fig. 1. Size distribution of chitosan–TBA–insulin microparticels (A) and chitosan–insulin microparticles (B), determined after dispersion in 100 mM phosphate buffer pH 6.8 at 37 °C (■) and after keeping the particles in the same buffer medium for 30 min (□), 1 (●) and 2 h (○). The means ($n = 3$) and standard deviation bars are shown.

3.4. Insulin and glutathione loading

Insulin and glutathione loading of the different microparticulate systems are displayed in Table 1. The percentage insulin loading of chitosan–TBA–insulin microparticles was $38.23 \pm 0.98\%$ (w/w) and the load of reduced glutathione was $9.52 \pm 0.62\%$ (w/w). No oxidised glutathione was detected in the particles. Possible thiol/disulfide exchange reactions between the thiolated polymer and insulin likely leading to ineffective protein fragments can be obviated as no additional peaks arose in HPLC-analyses. The percentage insulin loading for chitosan–insulin microparticles and mannitol–insulin microparticles was determined to be $34.75 \pm 0.54\%$ (w/w) and $34.92 \pm 0.89\%$ (w/w; means ± S.D.; $n = 3$–4), respectively.

3.5. Swelling behaviour of microparticles

The swelling behaviour of chitosan–TBA–insulin microparticles and chitosan–insulin microparticles was determined in 100 mM phosphate buffer pH 6.8. As depicted in Figs. 1 and 2, the mean particle size of the chitosan–TBA–insulin microparticulate formulation increased from 23.5 ± 3.1 μm measured immediately after contact with the buffer to 82.1 ± 8.7 μm after

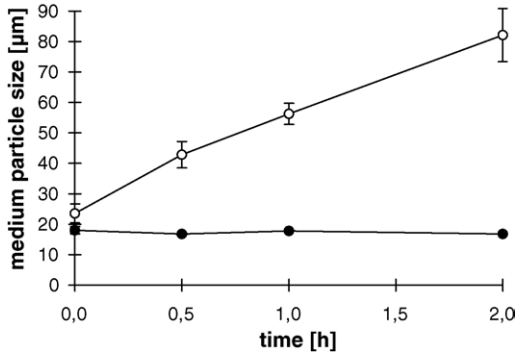

Fig. 2. Mean particle size of chitosan–TBA–insulin microparticles (○) and chitosan–insulin microparticles (●), determined in 100 mM phosphate buffer pH 6.8 at 37 °C. The means ($n = 3$) and standard deviation bars are shown.

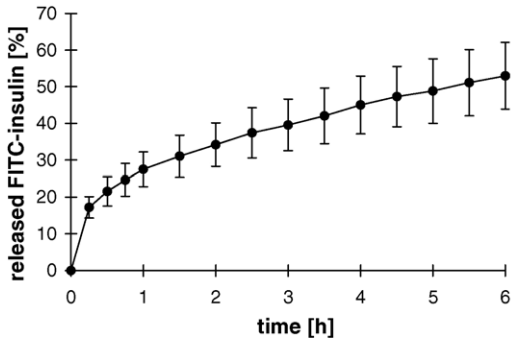

Fig. 3. Release profile of FITC–insulin from chitosan–TBA–FITC–insulin microparticles. The release study was performed in 100 mM phosphate buffer pH 6.8 at 37 °C. The means ($n = 3$) and standard deviation bars are shown.

2 h. The size of the microparticles at the end of the experiment was 4.39 ± 0.52 times higher than the size of the same microparticles measured in paraffin oil. Chitosan–insulin microparticles, in contrast, showed no size enlargement during the swelling experiment.

3.6. In vitro release of FITC–insulin

The release rate of FITC–insulin from chitosan–TBA–FITC–insulin microparticles is displayed in Fig. 3. Within 6 h $53.0 \pm 9.1\%$ (mean ± S.D.; $n = 3$) FITC–insulin were released from the particles. The release profile indicates a fast release of FITC–insulin within 15 min following by a controlled release over the remaining time period. Due to this controlled and sustained release profile, objectionable interactions between the thiolated polymer matrix and FITC–insulin can be excluded.

3.7. In vivo study—proof of principle

To evaluate the efficacy of chitosan–TBA in comparison with unmodified chitosan for the nasal administration of insulin, both microparticle systems were tested in vivo. As control served on the one hand unmodified chitosan microparticles comprising insulin and on the other hand a mixture of mannitol and insulin also prepared by the emulsification solvent evaporation technique. Additionally, insulin was injected intravenously as positive control. The absolute bioavailability was calculated referred to the i.v. injection results. The resulting insulin concentration–time curves after i.v. injections and after nasal administration of chitosan–TBA–insulin microparticles, chitosan–insulin microparticles and mannitol–insulin microparticles, respectively, are shown in Fig. 4. The main biofeedback parameters are listed in Table 2. As all nasal applied formulations contained a different amount of insulin, calculative corrections have been made regarding the bioavailabilities, in order to obtain comparable results.

Chitosan–insulin microparticles and mannitol–insulin microparticles led to a peak in the insulin concentration–time curve 15 min after nasal application with 26.95 ± 40.09 ng/mL and 20.03 ± 10.47 ng/mL, respectively. In contrast, after nasal administration of chitosan–TBA–insulin microparticles to rats, the insulin maximum in plasma was determined after 30 min

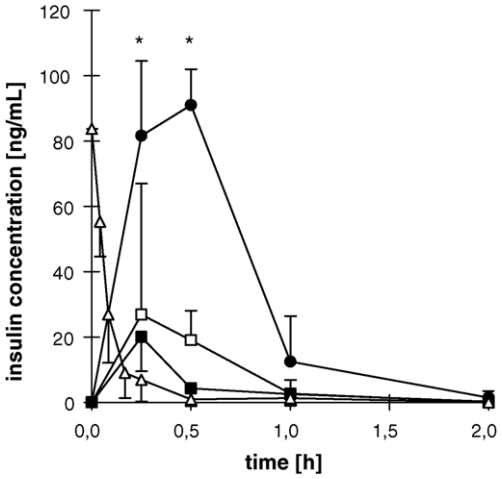

Fig. 4. Concentration–time profiles of insulin in rat plasma obtained after nasal application of chitosan–TBA–insulin microparticles (containing 1452.64 μg insulin/kg) (●), of chitosan–insulin microparticles (containing 1320.4 μg insulin/kg) (□) and of mannitol–insulin microparticles (containing 1327.12 μg insulin/kg) (■), respectively, and after intravenous injection of 13.87 μg insulin/kg (△). Indicated values are the mean results of 3–4 rats ± S.D. (*) differs from control $p < 0.05$.

with 91.03 ± 10.98 ng/mL. The absolute bioavailability for these microparticles was $7.24 \pm 0.76\%$ and therefore, more than 3.5-fold higher than the absolute bioavailability obtained for the chitosan–insulin microparticles. Mannitol–insulin microparticles led to an almost seven-fold lower effect than chitosan–TBA–insulin microparticles.

4. Discussion

Within this study microparticles with chitosan–TBA as carrier matrix containing the model peptide insulin and reduced glutathione as permeation mediator were prepared via the emulsification solvent evaporation method. Several studies could demonstrate that insulin microparticles and insulin powder formulations lead to a significantly higher bioavailability than insulin solutions administered nasally. Nagai et al. (1984) described a better absorption of insulin in powder form than in liquid form using dogs as experimental animals. Schipper et al. (1993) achieved an almost 13-fold higher bioavailability after nasal application of a lyophilised insulin/dimethyl-β-cyclodextrin powder dosage form to rabbits as after application of the same insulin/cyclodextrin mixture in solution. After getting in contact with the nasal mucosa, microparticles or powder formulations are believed to form a viscous gel by withdrawing water from the nasal mucus. The gel formation decreases the ciliary clearance rate and as a consequence the residence time of the formulation at the nasal mucosa is prolonged (Pereswetoff-Morath, 1998). The same effect on the mucociliary transit time was also shown for the mucoadhesive polymer chitosan. Soane et al. determined in sheep a half-time of clearence for chitosan microspheres of 115 min, whereas a chitosan solution was half cleared from nasal mucosa in 43 min (Soane et al., 2001). In human volunteers the half-time clearance of chitosan microspheres was two-fold higher than that of a chitosan solution

(Soane et al., 1999). These mucoadhesive properties of chitosan microspheres were attributed to the previous mentioned spontaneous gel formation on nasal mucosa. Additionally, chitosan in general has mucoadhesive properties because of its positive charge. The electrostatic attraction between the positively charged chitosan and the negatively charged mucus was demonstrated in vitro (Schipper et al., 1997; He et al., 1998). According to this, starch microspheres lacking a positive charge displayed a significant shorter half-time of nasal clearance than chitosan microspheres in human volunteers (Soane et al., 1999). These great mucoadhesive properties of chitosan could be further improved by the covalent attachment of 2-iminothiolane to chitosan, as chitosan–TBA–conjugates were shown to be in vitro approximately 130-fold more mucoadhesive than unmodified chitosan (Bernkop-Schnürch et al., 2003a, 2004). This additional improvement in the mucoadhesive properties of chitosan is believed to base on the formation of disulfide bonds between thiol groups of the thiomer and cysteine-rich subdomains of mucus glycoproteins (Leitner et al., 2003). Due to the covalent attachment of the gel-formed microparticles to the nasal mucus, the residence time of chitosan–TBA based microparticles should be higher compared to chitosan microparticles. Furthermore, our research group demonstrated that the mucoadhesive properties of thiomers do not decrease because of microparticle preparation using the emulsification solvent evaporation method (Bernkop-Schnürch et al., 2003b; Krauland and Bernkop-Schnürch, 2004).

As the swelling behaviour of microparticles plays an important role in the in situ gel formation on nasal mucosa, the swelling behaviour of the microparticles was determined. Chitosan–TBA–insulin microparticles had a size of $18.7 \pm 0.3\,\mu m$ in paraffin oil, but seconds after contact with phosphate buffer, their size increased to $23.5 \pm 3.1\,\mu m$ to reach after 2 h a more than four-fold increased size. Particles based on unmodified chitosan showed no swelling behaviour in phosphate buffer. Besides the absence of thiol groups, the incapability of swelling following by lower mucoadhesion could be another factor for the low insulin bioavailability of unmodified chitosan microparticles in this study.

Chitosan–TBA–insulin microparticles investigated in our study displayed a 3.5-fold higher bioavailability than chitosan–insulin microparticles based on unmodified chitosan. This difference is attributed not only to the stronger mucoadhesive properties of the chitosan–TBA conjugate as mentioned above, but also to the permeation enhancing properties of this conjugate itself (Bernkop-Schnürch et al., 2004). By the combination of chitosan–TBA with the permeation mediator reduced glutathione the amount of permeated drug could be even further improved (Bernkop-Schnürch et al., 2004). The permeation enhancing effect of thiomer/glutathione mixtures has already been demonstrated in vitro on bovine nasal mucosa for human growth hormone (Leitner et al., 2004). Furthermore, insulin tablets based on chitosan–TBA and glutathione led to a significantly higher pharmacological efficacy after oral application to rats compared to control tablets (Krauland et al., 2004).

The slow in vitro insulin release however, affects the efficacy of the chitosan–TBA–insulin microparticulate system. As particles in general deposited in the nasal cavity are cleared with a half-life of 15–30 min from nasal mucosa (Hinchcliffe and Illum, 1999) and chitosan microparticles showed a half-time of clearence of 115 min in sheeps (Soane et al., 2001), the slow FITC–insulin release of 21.57% within 30 min and 34.23% of the total amount within 120 min, respectively, represents a limitation for achieving higher insulin plasma levels in vivo with chitosan–TBA microparticles. Considering this rapid mucociliary clearance in the nasal cavity, the excellent mucoadhesive properties of chitosan–TBA might be reduced and the permeation enhancing effect might be therefore, the more crucial component in vivo. This hypothesis seems to be confirmed by the quick absorption of insulin with high insulin levels after 15 and 30 min indicating a strong permeation enhancement, whereas rapid mucociliary clearance of the microparticles might cause the short duration of insulin absorption of about 1 h.

According to the results of the present study chitosan–TBA leads not only as oral, but also as nasal peptide delivery system to a significantly higher effect than unmodified chitosan representing a well-established polymer in nasal peptide delivery (Illum et al., 1994; Dyer et al., 2002). Although the present study represents only a ‘proof of concept’ for a new nasal peptide delivery system, the bioavailability of chitosan–TBA based microparticles with $7.24 \pm 0.76\%$ in rats has to be seen with caution as Merkus et al. observed species differences between rat, rabbit and man. Whereas a dimethyl-β-cyclodextrin insulin solution exhibited bioavailabilities of 100% in rats, no insulin could be detected in rabbit and man plasma using the same formulation (Merkus et al., 1999). Furthermore, the insulin bioavailability of $7.24 \pm 0.76\%$ achieved in the present study seems to be low compared with the results of other research groups. Callens and Remon (2000), for instance, achieved for drum-dried waxy maize starch/Carbopol® 974 P powder formulations an absolute nasal insulin bioavailability of 14.4% in rabbits. Starch microspheres with the permeation enhancer glycodeoxycholate led to a relative bioavailability of 31.9% in sheep (Illum et al., 2001). Dyer et al. (2002) determined for a chitosan powder insulin delivery system a relative bioavailability of 17.0% in sheep. Results of different studies, however, are difficult to compare, because different animal models are used, experimental conditions and nasal formulations are varied, results are referred either to relative or absolute bioavailabilities and variances exist between different ELISA-kits. This study should therefore, be regarded as a ‘proof of concept’ for the potential of thiolated chitosan for nasal insulin delivery.

5. Conclusion

In the present study a ‘proof of concept’ for the capability of a thiolated chitosan derivative as nasal delivery system for peptides in comparison to a delivery system based on unmodified chitosan was presented. Administered nasally as microparticulate delivery system for insulin thiolated chitosan resulted in a significantly higher bioavailability than microparticles comprising unmodified chitosan. The difference in the efficacy of the two delivery systems could be seen in the higher mucoadhesive and permeation enhancing properties of the chitosan–TBA/glutathione system. This drug delivery system

might represent a useful vehicle for the nasal administration of therapeutic peptide drugs.

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