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Thiolated chitosan nanoparticles for the nasal administration of leuprolide: Bioavailability and pharmacokinetic characterization

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ABSTRACT

The purpose of this study was to develop thiolated nanoparticles to enhance the bioavailability for the nasal application of leuprolide. Thiolated chitosan–thioglycolic acid (chitosan–TGA) and unmodified chitosan nanoparticles (NPs) were developed via ionic gelation with tripolyphosphate (TPP). Leuprolide was incorporated during the formulation process of NPs. The thiolated (chitosan–TGA) NPs had a mean size of 252 ± 82 nm, a zeta potential of +10.9 ± 4 mV, and payload of leuprolide was 12 ± 2.8. Sustained release of leuprolide from thiolated NPs was demonstrated over 6 h, which might be attributed to inter- and/or intramolecular disulfide formation within the NPs network. Ciliary beat frequency (CBF) study demonstrated that thiolated NPs can be considered as suitable additives for nasal drug delivery systems. Compared to leuprolide solution, unmodified NPs and thiolated NPs provoked increased leuprolide transport through porcine nasal mucosa by 2.0 and 5.2 folds, respectively. The results of a pharmacokinetic study in male Sprague–Dawley rats showed improved transport of leuprolide from thiolated NPs as compared to leuprolide solution. Thiolated NPs had a 6.9-fold increase in area under the curve, more than 4-fold increase in elimination half-life, and a ~3.8-fold increase in maximum plasma concentration compared to nasal solution alone. The relative nasal bioavailability (versus s.c. injection) of leuprolide thiolated NPs calculated on the basis of $AUC_{(0-6)}$ was about 19.6% as compared to leuprolide solution 2.8%. The enhanced bioavailability of leuprolide is likely due to facilitated transport by thiolated NPs rather than improved release.

1. Introduction

Over the last six decades, the nasal route has revealed remarkable promise for systemic administration of peptides and proteins that are ineffective orally and need to be administered by injection. These peptides and proteins benefit of some major advantages of the nasal route like a rapid onset of therapeutic action, avoidance of first-pass effect and large surface area available for drug absorption due to microvilli present on epithelial surface (Bitko and Barik, 2007).

Leuprolide acetate is a synthetic nanopeptide and a potent agonist of the luteinizing hormone-releasing hormone (GnRH or LH–RH) receptor. Leuprolide has received considerable attention in the treatment of prostate cancer and hormone-dependent diseases such as endometriosis, uterine fibroids and central precocious puberty in children (Adjei et al., 1992, 1993). Nevertheless, chronic treatment with leuprolide has the shortcoming of requiring long-term, daily injections. The nasal route has frequently been described as a promising alternative to oral and parenteral administration of therapeutic peptides such as calcitonin, insulin, desmopressin, buserelin, and leuprolide. However, most peptides including leuprolide show bioavailabilities of 1% or less when administered to the nasal cavity (Krauland et al., 2006). In addition, the most important factor limiting the nasal bioavailabilities of peptides into the systemic circulation is low membrane permeability. Furthermore, bioavailabilities can be compromised by short residence time due to mucociliary clearance and high enzymatic turnover in the epithelium (O'Hagan et al., 1990). Numerous strategies are being used to facilitate the bioavailability of intranasally administered drugs. The four major advances that have been endeavoured are: (I) prolongation of drug residence time by using mucoadhesive polymers; (II) employ of permeation enhancers to improve absorption; (III) inclusion of enzyme inhibitors, and (IV) amalgamation of cilio-friendly excipients to minimize the effect of toxicological profile.

Thiolated polymers—or so-called thiomers—were established as a promising new class of polymeric excipients that combine these

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four strategies. First, thiomers have shown strongly improved residence time based on a thiol/disulfide exchange reactions between cysteine-rich subdomains of mucus glycoproteins and such polymers building up the mucus gel layer (Vetter et al., 2010). Second, thiomers have characteristics of permeation enhancement due to its ability to open tight junctions (Vetter and Bernkop-Schnürch, 2010). Third, thiomers had greater enzyme inhibitory activity by chelating essential metal ions from the enzyme structure (Vetter and Bernkop-Schnürch, 2010). Fourth, thiolated polymers exhibited no significant change in ciliary beat frequency (CBF) and thus nasal toxicological effects can be eliminated (Palmberger et al., 2011). It was therefore, the aim of this study to evaluate the potential of thiolated polymer to enhance the bioavailability and half life of leuprolide via nasal administration. Accordingly, nanoparticles based on thiolated chitosan–thioglycolic acid (chitosan–TGA) for non-invasive leuprolide delivery through the nasal route were developed. The resulting thiolated nanoparticles are characterised regarding their particle size and zeta potential. The permeation-enhancing properties on porcine nasal mucosa, viability of the porcine respiratory mucosa of the nasal cavity, ciliary beat frequency (CBF) profiles and the capability to provide a sustained release were evaluated in vitro. Prolongation of plasma half life time and improved bioavailability of leuprolide based on thiolated nanoparticles were investigated in vivo by using male Sprague–Dawley rats.

2. Materials and methods

2.1. Materials

Leuprolide acetate was purchased from Chemos, Germany. All other reagents were from commercial suppliers and of reagent grade.

2.2. Methods

2.2.1. Synthesis of thiolated chitosan (chitosan–TGA)

The synthesis of the thiolated chitosan was accomplished by the immobilization of amide bonds between primary amino groups of the polymer and the carboxylic acid groups of thioglycolic acid (TGA) mediated by EDAC as described by our research group previously (Martien et al., 2007).

2.2.2. Synthesis of thiolated (chitosan–TGA) nanoparticles

The NPs were prepared by ionic interaction of thiolated (chitosan–TGA) and unmodified chitosan, respectively, with sodium tripolyphosphate (TPP) according to a method reported previously (Bernkop-Schnürch et al., 2006). Briefly, thiolated and unmodified low molecular mass chitosan were hydrated in 0.05 M acetic acid solution (pH 6.2) at a final concentration of 0.5% (m/v). Subsequently, 0.5% (m/v) TPP solution in demineralised water pH 5 containing 3 mg/ml leuprolide was gently added drop by drop to the solution to generate ionically crosslinked NPs. Afterwards, NPs were partially oxidized by the addition of 0.5% (v/v) H_2O_2 solution and the mixture was incubated for 1 h under continuous stirring at room temperature. NPs were collected by centrifugation for 10 min at 1340 rpm. In order to avoid aggregation of particles during the following centrifugation for 10 min at 13,400 rpm, trehalose was added to the suspension in a final concentration of 3% (w/v). To enhance the dispensability after the lyophilisation (Benchtop 2K, VirTis, NY, USA), particles were redispersed in a 3% (w/v) trehalose solution directly after the supernatant was discarded. For leuprolide content determination NPs were dispersed in 2 ml of acetonitrile and homogenized for 60 s using a vortex mixer (Vortex Mixer SA7, Stuart). To guarantee complete extraction of leuprolide the NPs were sonicated for 15 min and subsequently centrifuged at a speed of 1340 rpm for 10 min. The supernatant was collected and evaporated under shaking in a thermomixer (Thermomixer Comfort, Eppendorf, Hamburg, Germany) at 40 °C for 30 min. Then the residue was redispersed in 1 ml of mobile phase and 40 μl of the aliquot was injected into the HPLC system for analysis as previously described by our research group (Iqbal. et al., 2011).

2.2.3. Characterization of nanoparticles

The resulting control unmodified and modified leuprolide loaded nanoparticles were analyzed for mean particles size and zeta potential by PSS Nicomp 380 ZLS particles sizer as previously described by our research group (Martien et al., 2007).

2.2.4. Release behavior

For drug release evaluation thiolated (chitosan–TGA) and unmodified chitosan nanoparticles 1.6% (m/m) at leuprolide concentration of 1 mg/ml were suspended in 3 ml Krebs–Ringer buffer containing 40 mM HEPES at pH 6.5. NPs suspension was incubated in a water bath at 37 °C under continuous shaking. At predetermined time points, 200 μl samples were withdrawn from the solution for centrifugation and the supernatant collected. Subsequently, the supernatant was extracted with acetonitrile and analyzed via HPLC as described above (Iqbal. et al., 2011).

2.2.5. Permeation studies utilizing porcine respiratory mucosa of the nasal cavity

Tissue samples of 3–4 cm^2 of freshly excised porcine respiratory mucosa of the nasal cavity were inserted in Ussing-type chambers displaying a permeation area of 0.64 cm^2. The apical side of the tissue was thereby facing the donor compartment. One milliliter preheated (37 °C) KRB (Krebs–Ringer bicarbonate buffer) with 0.94% HEPES, pH 6.5, was added to the donor and acceptor chamber. The temperature within the chambers was maintained at 37 °C. After 30 min of pre-incubation with the artificial nasal fluid, the media of the donor compartment was substituted by either 0.8% (m/m) leuprolide loaded nanoparticles based on modified thiolated chitosan (chitosan–TGA) or the 0.8% (m/v) corresponding control leuprolide loaded nanoparticles (unmodified chitosan). Leuprolide was used as model drug in a final concentration of 0.05% (m/v). Over a time period of 180 min, samples of 200 μl were withdrawn from the acceptor compartment every 30 min and immediately replaced by 200 μl buffer at 37 °C to maintain sink conditions. After centrifugation, 150 μl of the supernatant was transferred into vials and the amount of permeated leuprolide was quantified by HPLC as described above with an injection volume of 30 μl. The apparent permeability coefficients (*P*app) for leuprolide were calculated according to the following equation:

$$P\text{app} = \frac{Q}{A \times c \times t} \quad (1)$$

where *P*app is the apparent permeability coefficient (cm/s), *Q* is the total amount of test substance permeated through the mucosa (mg), *A* is the diffusion area of the Ussing-type chamber (cm^2), *c* is the initial concentration of the drug in the donor compartment (mg/cm^3), and *t* is the total time of experiment (s).

2.2.6. Histological study

The viability of the porcine respiratory mucosa of the nasal cavity was evaluated by an embedding treatment as previously described by our research group (Vetter et al., 2010).

2.2.7. Preparation of a human nasal epithelial cell culture

Human nasal epithelial cells for cultures were harvested under the middle turbinate from healthy volunteer with nasal brushing (Toskala et al., 2005). Written informed and ethical

approved consent was obtained from volunteer before participation. Brushed cells were immediately immersed in DMEM-Ham's F12 1:1 medium supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. Then cells were incubated for 4–6 h at 37 °C in a 5% CO_2 atmosphere. The medium with brushed cells was centrifuged at 900 rpm for 5 min and the supernatant discarded. Cells were suspended in 1.0 ml of fresh medium consisting of DMEM-Ham's F12 1:1 medium with 2% Ultroser G, retinoic acid (Dimova, 2005), penicillin (50 IU/ml), streptomycin (50 μg/ml), amphotericin B (2.5 μg/ml) and 1% non-essential amino acids and plated in 0.2% rat tail collagen type I pre-coated 12-well plates in a final volume of 2 ml medium and incubated at 37 °C in atmosphere of 5% CO_2–95% O_2. The medium was changed 24 h after plating and subsequently every second day.

2.2.8. Ciliary beat frequency measurements

After three days, the medium was substituted with a suspension of control unmodified and modified leuprolide incorporated nanoparticles. CBF was measured in each sample before the treatment and after 20 min. After withdrawal of the formulation exposure, the CBF was also determined after rinsing the cells with Locke–Ringer buffer and incubating them for 60 min to see whether the effects on CBF are reversible. The negative controls consisted of the cells incubated with Locke–Ringer buffer only, and treated in the same way. Positive controls consisted of a 0.1% (m/v) solution of Triton X 100 and 0.005% (m/v) benzalkonium chloride. A high-speed digital camera (Redlake, Motion Scope M1, Eningen, Germany) and application software (Redlake Imaging Studio V.2.1.4) was used for image acquisition (Dimova, 2005). Furthermore, a computerized microscope photometry system consisting of a Laborvert FS light microscope and a Type MPV Combi photometer (Leitz, Jena, Germany) placed on a pneumatic vibration-absorbing table (Barry Controls, Ltd., Hersham, UK) were used. The images were captured at a frame rate of 512 fps with a sampling interval of 2 ms, measured at magnifications 500×, and the diameter of the photosensitive field on the sample was 5 μm. A sequence of 1000 images was recorded for each area for about 2 s. Each sequence of frame-by-frame images was stored in a file folder containing 1000 tiff format files for later retrieval and data analysis. Features of interest in the CBF or associated amplitude images were located in the original video images. The analysis method was implemented in Matlab 7.1 and a graphical user interface was used for CBF measurement and histogram analysis, which allows deriving the statistical data from the CBF of all beating cilia in the image.

The reversibility of the ciliary beat inhibition of the tested substance was calculated with the following equation (Ugwoke et al., 2000):

$$\%\ \text{reversibility} = \frac{\text{CBF after washing} - \text{CBF following treatment with test substance}}{\text{control CBF} - \text{CBF following treatment with test substance}} \times 100 \quad (3)$$

2.2.9. In vivo pharmacokinetic studies

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. Pharmacokinetic profiles of leuprolide dosage forms were determined in male Sprague–Dawley rats after nasal administration of a suspension of control unmodified and modified leuprolide incorporated nanoparticles in comparison to i.v. and s.c. injection and nasal leuprolide solution. A dose of 1 mg/kg was administered per rat for each formulation. Blood samples were collected from the tail vein at predetermined time points into a heparinized syringe. After centrifugation plasma was collected and stored at −80 °C till analysis of leuprolide concentration via LC–MS. Therefore, an internal standard was added to withdrawn plasma samples prior to stepwise addition of 400 μl of ice-cold acetonitrile in order to precipitate plasma proteins. After centrifugation for 8 min at 12,000 rpm the supernatant was collected and evaporated to dryness (SC210A SpeedVac® Plus, coupled with RVT400 Refrigerated Vapor Trap, Thermo Savant) for 60 min at 45 °C. The residue was reconstituted in 125 μl of mobile phase for LC-MS analysis as previously described by our research group (Iqbal. et al., 2011).

2.2.10. Pharmacokinetic analysis

Pharmacokinetic parameters were calculated using OriginPro. C_{max} and t_{max} were determined from the profiles generated by plotting the concentration of leuprolide against time. Area under the concentration time curves (AUC) was calculated according to the linear trapezoidal rule. Absolute bioavailability was calculated from the absolute dose and areas under curves (AUC) for nasal against intravenous administration.

2.2.11. Statistical analysis

For statistical analysis, analysis of variance and Student's *t*-test were used where appropriate. A probability of less than 0.05 ($P < 0.05$) was considered statistically significant. All results are presented as mean ± S.D.

3. Results and discussion

3.1. Characterization of thiolated chitosan

The amount of free thiol groups was quantified by Ellman's reagent according to the method described previously by our research group (Martien et al., 2007). The purified thiolated chitosan (chitosan–TGA) exhibited 1362 ± 64 μmol thiol groups per gram polymer (mean ± S.D. $n = 4$). Furthermore, by reducing all disulfide bonds present in the polymer network, 284 ± 30 μmol disulfide bonds per gram polymer could be quantified (mean ± S.D. $n = 4$).

3.2. Characterization of nanoparticles

Unmodified chitosan and thiolated (chitosan–TGA) nanoparticles were prepared via ionic gelation with TPP followed by formation of intra- and intermolecular disulfide bonds within the thiolated particles. According to the outcome of numerous experiments, pH 6.2 was investigated as most suitable for the polymer solution whereas pH 5 appears to be most favorable for the TPP solution. The ideal polymer:crosslinker ratio was 7.5:1 with a positive zeta potential as demonstrated in Table 1. The surface charge is likely to be pivotal factor on the stability of colloidal formulations and adhesion of particle formulations onto biological surfaces. Therefore, investigation of zeta potential is a significant part of nanoparticle characterization. Positive zeta potential of these particles can give rise to a strong electrostatic interaction with negatively charged mucus layer. Furthermore, nanoparticles developed under standard conditions exhibit a narrow size distribution as shown in Fig. 1. In order to enhance the stability of thiolated (chitosan–TGA) nanoparticles, inter- and intramolecular disulfide bonds were formed due to the addition of H_2O_2. Subsequently, decrease in thiol groups within the particles was determined by Ellman's reagent, to characterize the oxidation process. Results of this study are shown in Table 1. In addition, leuprolide loaded unmodified chitosan or thiolated nanoparticles were prepared by a

Table 1
Characterization of particle size, payload, zeta potential, thiol groups and disulfide bonds on ionically cross-linked as well as covalently cross-linked (oxidized; ox) nanoparticles. Indicated values are means ± SD of at least 3 experiments.

Preparation	–SH [μmol/g]	–S–S– [μmol/g]	Size (nm)	Zeta potential (mV)	Payload (%)
Unmodified nanoparticles	–	–	546 ± 134	+8.9 ± 3	13 ± 3.5
Thiolated nanoparticles	1240 ± 124	268 ± 73	367 ± 69	+7.7 ± 1	15 ± 4.2
Thiolated nanoparticles (ox)	946 ± 105	470 ± 192	272 ± 82	+10.9 ± 4	12 ± 2.8

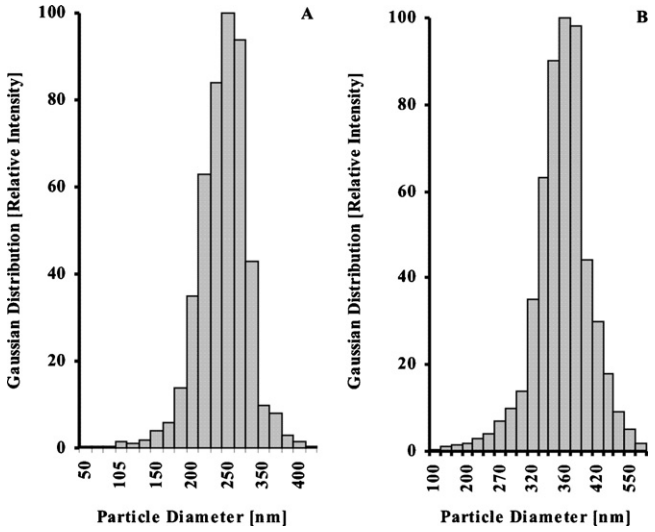

Fig. 1. Size distribution of ionically crosslinked nanoparticles based on thiolated chitosan–thioglycolic acid (chitosan–TGA) with 268 μmol/g disulfide bonds (A), ionically as well as covalently crosslinked nanoparticles based on thiolated chitosan–thioglycolic acid (chitosan–TGA) 470 μmol/g disulfide bonds (B). The relative intensity of particles depicts the outcome achieved by the analysis based on the theory of the Gaussian distribution analysis. Indicated values are means ± SD of last three experiments.

combination of embedding and diffusion. The payload of leuprolide loaded NP is illustrated in Table 1.

3.3. Release profile studies

The percentage of leuprolide released from unmodified chitosan or thiolated (chitosan–TGA) nanoparticles was plotted as a function of time in Fig. 2. An initial burst release is achieved for the unmodified chitosan NP, which is approximately 50% within 30 min. In contrast, the release of leuprolide from thiolated nanoparticles was relatively slower and more sustained with 43% of liberated drug within 2 h. Due to the swelling of the thiolated polymer (Roldo et al., 2004) immobilized on the surface of the nanoparticles, a slight improvement of the initial burst could be attained. Therefore, it is important that the leuprolide is not released to a large extent before the leuprolide-loaded nanoparticles pass the nasal mucosal barrier during their residence in the nasal cavity (Table 2).

Table 2
Apparent permeability coefficients and transport enhancement ratio of leuprolide based on chitosan and thiolated chitosan–thioglycolic acid (chitosan–TGA) nanoparticles by using a porcine respiratory mucosa of the nasal cavity (indicated values are means ± SD of at least 3 experiments).

Formulations	Apparent permeability coefficient (*P*app $\times 10^{-6}$ (cm/s))	Enhancement ratio (*P*app enhancer system/*P*app control)
Control	2.58 ± 0.3	–
Unmodified nanoparticles	5.30 ± 1.4	2.0
Thiolated nanoparticles	13.1 ± 2.7	5.2

3.4. In vitro transport studies

The influence of thiolated (chitosan–TGA) nanoparticles on the transport of leuprolide was evaluated on freshly excised porcine nasal mucosa mounted in Ussing-type chambers. As control, unmodified chitosan nanoparticles and leuprolide alone were used. Results of permeation studies with leuprolide on porcine nasal mucosa are represented in Fig. 3 and *P*app values with enhancement ratios are summarized in Table 3. Within this study it could be demonstrated that due to thiolation of chitosan improved transport of leuprolide was achieved in comparison to the buffer control. The *P*app enhancement ratio was 5.2-fold higher for the thiolated formulation and 2.0-fold higher for the unmodified formulation as compared to the buffer control. Concerning the tissue damage, staining of porcine nasal mucosa with trypan blue after permeation studies with thiolated nanoparticles did not show any nonviable cells (data not shown). The TEER of the tissue was determined before and after permeation to be 82.7 ± 6 and 80.1 ± 5 Ω cm^2, respectively. Previous studies reported that the principal mechanism of permeation enhancement by thiomers based on the inhibition of protein tyrosine phosphatase (PTP). The inhibition of PTP can be achieved by a disulfide bond formation of the active site cysteine of the protein. Subsequently, a higher extent of phosphorylated tyrosine groups on the extracellular loops of the membrane protein occludin, leading to the opening of the tight junctions. GSH released by intestinal cells oxidises the cysteine groups on PTP (Barrett et al., 1999). It is believed that reduced thiol functions on the thiolated polymer reduce the oxidised glutathione, thereby raising the amount of GSH at the absorption area for PTP

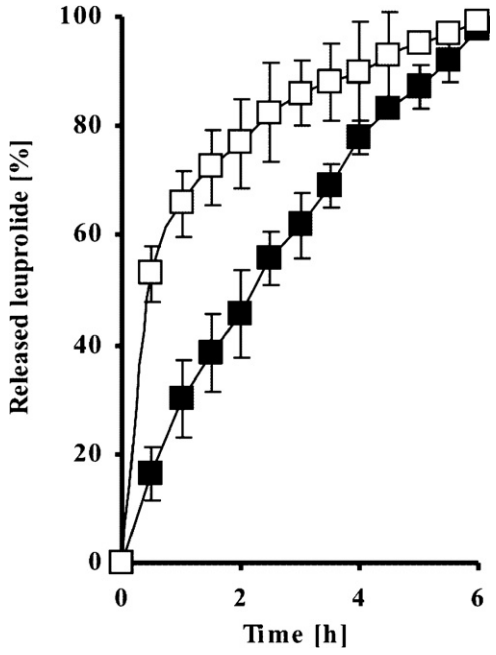

Fig. 2. Release behavior of leuprolide nanoparticles based on chitosan (□) and thiolated chitosan–thioglycolic acid (chitosan–TGA) (■) in Krebs–Ringer buffer containing 40 mM HEPES. Indicated values are means ± SD ($n \geq 3$).

Table 3
Pharmacokinetic parameters of leuprolide based on thiolated (chitosan–TGA) NPs, unmodified chitosan NPs and saline solution after nasal administration at 1 mg/kg dose to male Sprague–Dawley rats. Indicated values are means ± S.D. of at least five experiments: thiolated (chitosan–TGA) NPs differs significantly from unmodified chitosan NPs and saline solution with $P < 0.05$. *Abbreviations:* AUC = areas under the concentration time curves; AUMC = area under the first-moment curve; MRT = mean residence time; T_{max} = time for maximum plasma concentration; C_{max} = maximum plasma concentration; $T_{1/2}$ = half life; V_d = volume of distribution; K_e = elimination rate constant; C_L = total plasma clearance.

Pharmacokinetic parameters	Results after nasal administration		
	Thiolated nanoparticles	Unmodified nanoparticles	Control (saline solution)
$AUC_{(0-6)}$ (ng h/ml)	88.9 ± 11.2	20.6 ± 3.2	12.9 ± 1.6
$AUC_{(0-\infty)}$ (ng h/ml)	113 ± 21	21.0 ± 1.7	13.0 ± 1.2
$AUMC_{(0-6)}$ (ng h/ml)	215 ± 47	18.4 ± 0.4	10.2 ± 1.5
$AUMC_{(0-\infty)}$ (ng h/ml)	408 ± 53	20.7 ± 1.3	10.6 ± 0.3
MRT (h)	3.60 ± 0.4	0.90 ± 0.06	0.80 ± 0.01
T_{max} (h)	1.51 ± 0.01	0.50 ± 0.01	0.50 ± 0.02
C_{max} (ng/ml)	58.3 ± 12	23.5 ± 3.1	16.6 ± 0.87
$T_{1/2}$ (h)	2.53 ± 0.12	0.87 ± 0.07	0.56 ± 0.02
K_e (h^{-1})	0.27 ± 0.03	0.81 ± 0.02	1.24 ± 0.02
V_d (l)	12.2 ± 1.6	14.5 ± 2.1	19.1 ± 2.4
C_L (l/h)	3.37 ± 0.5	14.3 ± 0.8	23.2 ± 3.7

inhibition. Accordingly, a significantly improved permeability through tight junctions was observed (Clausen et al., 2002).

3.5. Influence of thiolated chitosan–TGA nanoparticles on CBF

Mucociliary clearance (MCC) is one of the most significant defense mechanisms of the respiratory tract (Toskala et al., 2005). Damage of the ciliary beating might consequence poor mucociliary clearance, with subsequent upper and lower respiratory infection. The ciliary beat frequency (CBF) is often believed as an indicator for toxicity. CBF and mucociliary clearance (MCC) are interconnected and a formulation which is capable to change CBF has consequently also an impact on MCC. The nasal cell culture model employed for this study demonstrated stable ciliary beating around 7–10 Hz at room temperature which compares with other studies. The preliminary investigations showed that Locke–Ringer solution pH 7.4 do not significantly affect the CBF in this cell culture system within the study periods. CBF of the thiolated (chitosan–TGA) NPs and unmodified chitosan NPs (0.5% (m/v), 1% (m/v) and 2.0% (m/v)) solution were determined in Locke–Ringer solution pH 7.4. The effect of thiolated nanoparticles and control nanoparticles on CBF was both concentration- and time-dependent as shown in Fig. 4a–c. The reductions in CBF after thiolated NPs exposure was not statistically significant different to the unmodified NPs. The effect on CBF obtained with 2.0% (m/v) solution was moderate after 25 min and reversibility was not ascertained because of the solution viscosity. With the 1% (m/v) concentration, mild inhibitory effect was observed after 25 min. While 0.5% (m/v) solution showed no inhibition after 10 min and mild inhibition after 25 min. The cilio-inhibition that was observed with the 0.5% (m/v) solution was absolutely reversible, while washing had partially reversible effect on the cilioinhibition caused by the 1% (m/v) solution. In contrast, Triton X and benzalkonium chloride, known to be cilio-toxic when applied in a concentration of 0.1% (m/v) irreversibly inhibited the ciliary beating. Thiolated NPs caused decreased CBF probably by mechanical interference with the cilia. Thiolated chitosan when used as permeation enhancers, are mostly used in concentrations of 0.5% (m/v) and at this concentration they appeared as safe excipients from a cilio-inhibiting point of view. A compulsion for nasally applied formulation is that drugs and excipients in the dosage forms do not hamper with normal nasal functioning such as the nasal mucociliary systems (Dimova, 2005). From this study it can be inferred that thiolated NPs formulations can be considered as suitable excipients for nasal drug delivery systems.

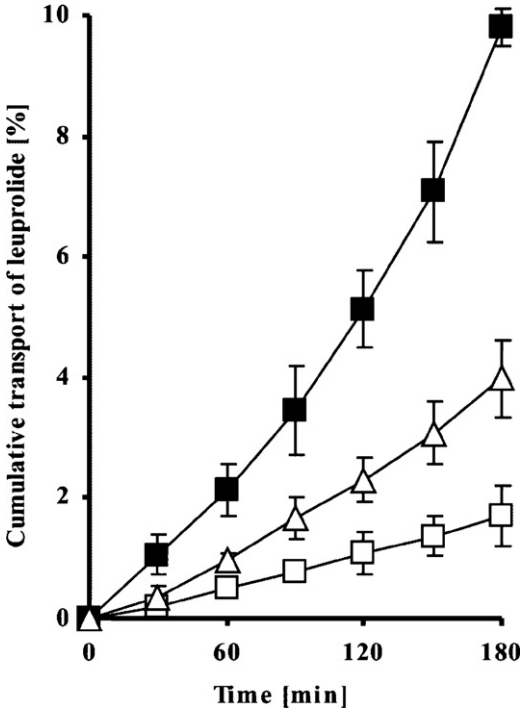

Fig. 3. Permeation of leuprolide across porcine respiratory mucosa of the nasal cavity in the presence of 0.8% (m/m) leuprolide loaded nanoparticles based on modified thiolated chitosan (■), 0.8% (m/v) corresponding control leuprolide loaded nanoparticles (△), and versus medium only (□). Transport data (means ± SD, $n = 4$) are expressed as percentage of total leuprolide.

3.6. In vivo evaluation of the delivery systems

Bioavailability of nasally administered drugs is particularly restricted by short residence time due to mucociliary clearance and rapid enzymatic degradation in nasal cavity as well as poor membrane penetration. The incorporation of drugs into nanoparticles might be a promising strategy, since colloidal formulations have been revealed to shield them from the degrading milieu in the nasal cavity and facilitate their transport across the mucosal barriers. Furthermore, the most common approach to improve nasal bioavailability is the use of permeation enhancers, enzyme inhibitors and use of mucoadhesive polymers. The potential of thiolated polymers has been demonstrated for the nasal route (Leitner et al., 2004a). The nasal administration of human growth hormone (hGH) incorporated in a polycarbophil-cysteine gel formulation resulted in considerably higher plasma concentrations compared to the gel formulation of unmodified polymer (Leitner

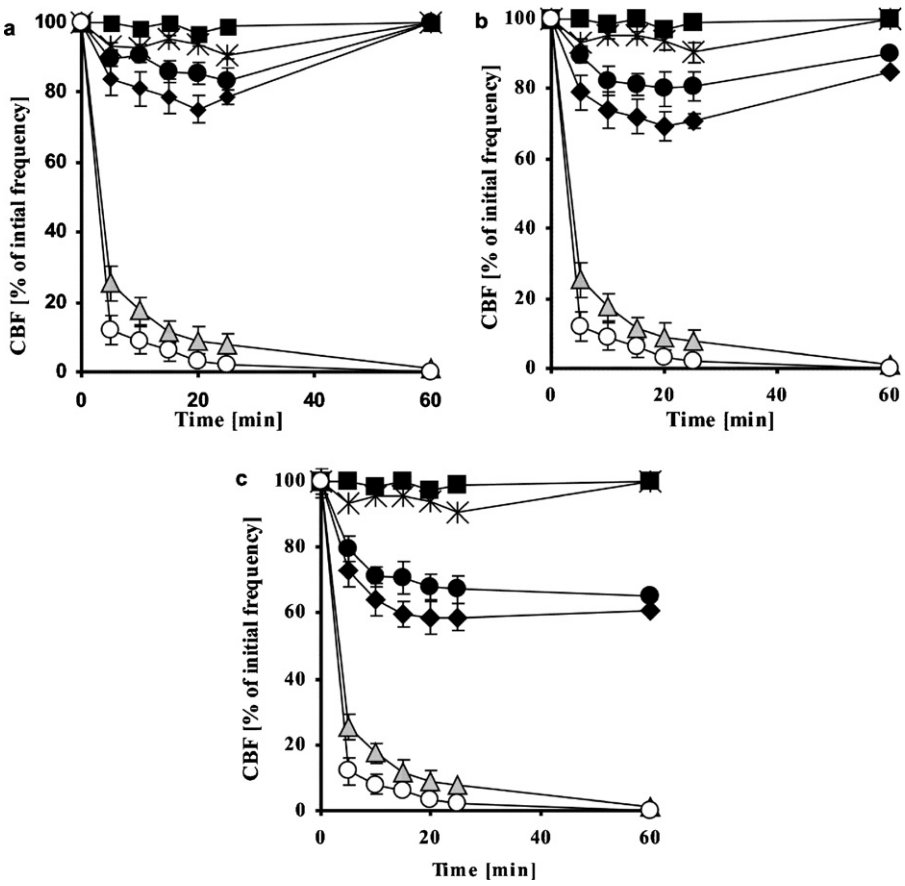

Fig. 4. The effects of thiolated (chitosan–TGA) NPs with different concentration [a: 0.5% (m/v), b: 1% (m/v), c: 2% (m/v)] on the ciliary beat frequency (CBF) of human respiratory mucosa of the nasal cavity in vitro. Control solutions: Locke–Ringer pH 7.4 (■), saline solution (*), unmodified chitosan (●), chitosan–TGA (♦), positive control benzalkonium chloride (0.005%, w/v) (△) and positive control Triton X-100 (0.1%, v/v) (○). Data are expressed as the mean ± SD of 3–5 experiments.

et al., 2004a). The results of another study emphasize the effectiveness of thiomers as a permeation enhancer for nasal delivery of peptide drugs. With a polycarbophil-cysteine–glutathione–hGH microparticle formulation, a three-fold bioavailability compared to polycarbophil–hGH microparticle formulation was investigated (Leitner et al., 2004b). In recent studies our research group has proven that thiolation of chitosan results in the improved bioavailability of nasally administered peptide due to a combination of mucoadhesion and a permeation enhancement (Krauland et al., 2006). The objective of the present work was to evaluate the potential of thiolated (chitosan–TGA) NPs as nasal leuprolide delivery system in comparison to a formulation based on unmodified NPs and nasal saline solution. Pharmacokinetic parameters (AUC, MRT, K_e, $T_{1/2}$, V_d, C_{max}, T_{max} and C_L) of leuprolide based on thiolated (chitosan–TGA) NPs and unmodified chitosan NPs were calculated and are provided in Table 3. Plasma concentration–time curves of leuprolide given nasally as solution or with the test formulations based on either thiolated NPs and unmodified NPs are presented in Fig. 4. The areas under plasma concentration of leuprolide administered as subcutaneous injection or intravenously are shown in Table 4. The peptide drug leuprolide was poorly absorbed after administration in the form of a nasal solution with absolute bioavailability of 2.6%. The relative bioavailability of the nasal solution of leuprolide was 2.8% as shown in Table 4. As illustrated in Fig. 4, the nasal administration of leuprolide incorporated in the thiolated NPs resulted in a significantly enhanced absorption of leuprolide compared with both controls. Thiolated NPs had a 6.9-fold increase in area under the curve, 4.5-fold increase in mean residence time, more than 4-fold increase in elimination half-life, ~3.8-fold increase in maximum plasma concentration, and a 7-fold decrease in plasma clearance rate compared to nasal solution alone. Leuprolide loaded in thiolated NPs could be transported across the nasal epithelium via the paracellular pathway. From this point of view, the supremacy of thiolated NPs nanoparticles for facilitating higher drug concentration gradient of leuprolide as compared to unmodified NPs (Fig. 4) could be attributed to the following

Table 4
Bioavailability assessment of leuprolide based on thiolated (chitosan–TGA) NPs, unmodified chitosan NPs and saline solution after nasal administration at 1 mg/kg dose to male Sprague–Dawley rats. Indicated values are means ± S.D. of at least five experiments.

Formulation	$AUC_{(0-6)}$ (ng h/ml)	Bioavailability [%]	
		Relative (F_{rel})	Absolute (F_{abs})
Nasal solution	12.9 ± 1.6	2.8	2.6
Nasal unmodified nanoparticles	20.6 ± 3.2	4.4	4.3
Nasal thiolated nanoparticles	88.9 ± 11.2	19.6	18.5
Subcutaneous solution	454 ± 67	94	–
Intravenous solution	479 ± 83	–	100

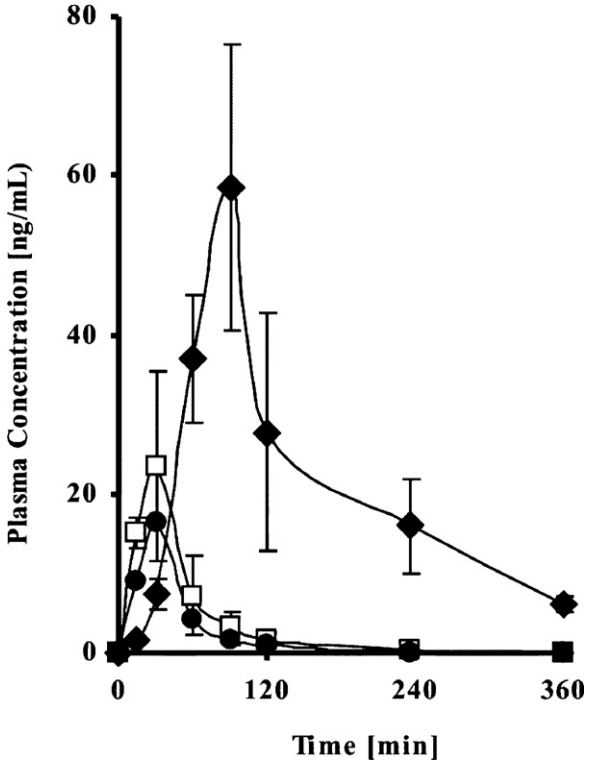

Fig. 5. Plasma concentration–time curves of leuprolide given nasally as solution (●), with the test formulations based on thiolated NPs (■) and unmodified NPs (□) to male Sprague–Dawley rats: Indicated values are mean ± S.D. of at least five experiments. Bars represent standard deviations.

aspects. Firstly, positively charged nanoparticles persuaded transient loosening of tight junctions through electrostatic interaction with negatively charged constituents in them (Yin et al., 2009), and thiolation could additionally promote opening of tight junctions through inhibition of protein tyrosine phosphatase (Marschütz and Bernkop-Schnürch, 2002). Consequently, drug transport via the paracellular route could be improved. Secondly, resistance of leuprolide towards enzymatic degradation could be enhanced, which might be due to shielding of the enzymatic cutting sites (Bernkop-Schnürch et al., 2004). Thirdly, thiomers display excellent mucoadhesive properties because they were shown to interact with cysteine-rich residues of mucus glycoproteins thereby forming disulfide bridges. Therefore, thiolated nanoparticles can provide an intimate contact of the polymer with the nasal mucosa and a prolonged residence time on it. Moreover, a higher drug concentration gradient was offered at the absorption sites, which facilitated drug transport (Fig. 5).

4. Conclusion

In the present work the potential of thiolated (chitosan–TGA) NPs as nasal delivery system for leuprolide in comparison to unmodified chitosan NPs was evaluated. The thiolated (chitosan–TGA) NPs were obtained via ionic gelation, which possessed a narrow size distribution (252 ± 82 nm) and a positive zeta potential (+10.9 ± 4 mV). Sustained released and permeation enhancing effect of thiolated (chitosan–TGA) NPs were significantly higher than those of unmodified chitosan NPs. Ciliary beat frequency (CBF) evaluation demonstrated lack of toxicity of thiolated NPs from a cilio-inhibiting point of view. The nasal administration of leuprolide incorporated in the thiolated (chitosan–TGA) NPs resulted in a significantly greater AUC and higher bioavailability in comparison to unmodified chitosan NPs. Therefore, according to the achieved results it is suggested that thiolated (chitosan–TGA) NPs is a valuable tool for improving the nasal bioavailability of the peptide drug leuprolide.

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References

Bitko, V., Barik, S., 2007. Intranasal antisense therapy: preclinical models with a clinical future? Curr. Opin. Mol. Ther. 9, 119–125.
Adjei, A., Love, S., Johnson, E., Diaz, U., Greer, J., Haviv, F., Bush, E., 1993. Effect of formulation adjuvants on gastrointestinal absorption of leuprolide acetate. J. Drug Target. 1, 251–258.
Adjei, A., Sundberg, D., Miller, J., Chun, A., 1992. Bioavailability of leuprolide acetate following nasal and inhalation delivery to rats and healthy humans. Pharm. Res. 9, 244–249.
Krauland, A.H., Guggi, D., Bernkop-Schnürch, A., 2006. Thiolated chitosan microparticles: a vehicle for nasal peptide drug delivery. I. J. Pharm. 307, 270–277.
O'Hagan, D.T., Critchley, H., Farraj, N.F., Fisher, A.N., Johansen, B.R., Davis, S.S., Illum, L., 1990. Nasal absorption enhancers for biosynthetic human growth hormone in rats. Pharm. Res. 7, 772–776.
Vetter, A., Martien, R., Bernkop-Schnürch, A., 2010. Thiolated polycarbophil as an adjuvant for permeation enhancement in nasal delivery of antisense oligonucleotides. J. Pharm. Sci. 99, 1427–1439.
Vetter, A., Bernkop-Schnürch, A., 2010. Nasal delivery of antisense oligonucleotides: in vitro evaluation of a thiomer/glutathione microparticulate delivery system. J. Drug Target. 18, 303–312.
Palmberger, T.F., Augustijns, P., Vetter, A., Bernkop-Schnürch, A., 2011. Safety assessment of thiolated polymers: effect on ciliary beat frequency in human nasal epithelial cells. Drug Dev. Ind. Pharm. 37, 1455–1462.
Martien, R., Loretz, B., Thaler, M., Majzoob, S., Bernkop-Schnürch, A., 2007. Chitosan–thioglycolic acid conjugate: an alternative carrier for oral nonviral gene delivery? J. Biomed. Mater. Res. A 82, 1–9.
Bernkop-Schnürch, A., Weithaler, A., Albrecht, K., Greimel, A., 2006. Thiomers: preparation and in vitro evaluation of a mucoadhesive nanoparticulate drug delivery system. Int. J. Pharm. 317, 76–81.
Iqbal., J., Vigl, C., Moser, G., Gasteiger, M., Perera, G., Bernkop-Schnürch, A., 2011. Development and in vivo evaluation of a new oral nanoparticulate dosage form for leuprolide based on polyacrylic acid. Drug Deliv. 18, 432–440.
Toskala, E., Haataja, J., Shirasaki, H., Rautiainen, M., 2005. Culture of cells harvested with nasal brushing: a method for evaluating ciliary function. Rhinology 43, 121–124.
Dimova, S., 2005. High-speed digital imaging method for ciliary beat frequency measurement. J. Pharm. Pharmacol. 57, 1–6.
Ugwoke, M.I., Agu, R.U., Jorissen, M., Augustijns, P., Sciot, R., Verbeke, N., Kinget, R., 2000. Nasal toxicological investigations of Carbopol® 971P formulation of apomorphine: effects on ciliary beat frequency of human nasal primary cell culture and in vivo on rabbit nasal mucosa. Eur. J. Pharm. Sci. 9, 387–396.
Roldo, M., Hornof, M., Caliceti, P., Bernkop-Schnürch, A., 2004. Mucoadhesive thiolated chitosans as platforms for oral controlled drug delivery: synthesis and in vitro evaluation. Eur. J. Pharm. Biopharm. 57, 115–121.
Barrett, W.C., DeGnore, J.P., König, S., Fales, H.M., Keng, Y.F., Zhang, Z.Y., Yim, M.B., Chock, P.B., 1999. Regulation of PTP1B via glutathionylation of the active site cysteine 215. Biochemistry 38, 6699–6705.
Clausen, A.E., Kast, C.E., Bernkop-Schnürch, A., 2002. The role of glutathione in the permeation-enhancing effect of thiolated polymers. Pharm. Res. 19, 602–608.
Leitner, V.M., Guggi, D., Bernkop-Schnürch, A., 2004a. Thiomers in noninvasive polypeptide delivery: in vitro and in vivo characterization of a polycarbophil-cysteine/glutathione gel formulation for human growth hormone. J. Pharm. Sci. 93, 1682–1691.
Leitner, V.M., Guggi, D., Krauland, A.H., Bernkop-Schnürch, A., 2004b. Nasal delivery of human growth hormone: in vitro and in vivo evaluation of a thiomer/glutathione microparticulate delivery system. J. Control Release 100, 87–95.
Yin, L., Ding, J., He, C., Cui, L., Tang, C., Yin, C., 2009. Drug permeability and mucoadhesion properties of thiolated trimethyl chitosan nanoparticles in oral insulin delivery. Biomaterials 30, 5691–5700.
Marschütz, M.K., Bernkop-Schnürch, A., 2002. Thiolated conjugates: self-crosslinking properties of thiolated 450 kDa poly (acrylic acid) and their influence on mucoadhesion. Eur. J. Pharm. Sci. 15, 387–394.
Bernkop-Schnürch, A., Hornof, M., Guggi, D., 2004. Thiolated chitosans. Eur. J. Pharm. Biopharm. 57, 9–17.