Thiomers and thiomer-based nanoparticles in protein and DNA drug delivery

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Introduction: Thanks to advances in biotechnology, more and more highly efficient protein- and DNA-based drugs have been developed. Unfortunately, these kinds of drugs underlie poor non-parental bioavailability. To overcome hindrances like low mucosal permeability and enzymatic degradation polymeric excipients are utilized as drug carrier whereat thiolated excipients showed several promising qualities in comparison to the analogical unmodified polymer.

Areas covered: The article deals with the comparatively easy modification of well-established polymers like chitosan or poly(acrylates) to synthesize thiomers. Further, the recently developed "next generation" thiomers e.g. preactivated or S-protected thiomers are introduced. Designative properties like mucoadhesion, uptake and permeation enhancement, efflux pump inhibition and protection against enzymatic degradation will be discussed and differences between first and next generation thiomers will be pointed out. Additionally, nanoparticles prepared with thiomers will be dealt with regarding to protein and DNA drug delivery as thiomers seem to be a promising approach to avoid parenteral application.

Expert opinion: Properties of thiomers per se and results of in vivo studies carried out so far for peptide and DNA drugs demonstrate their potential as multifunctional excipients. However, further investigations and optimizations have to be done before establishing a carrier system ready for clinical approval.

Keywords: DNA drugs, nanoparticles, oral delivery, protein drugs, thiomers

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1. Introduction

At present, there are many highly efficient drugs that are only bioavailable when administrated parenterally. As parenteral application is associated with pain, fear and efforts, alternative routes are desirable. For example, oral, pulmonary or nasal application can help improve compliance and cut down side effects linked with parenteral application. Two important drug classes with growing demand in health care are protein- and DNA-based drugs. Non-parenteral application of these pharmaceutics leads to poor bioavailability [1,2]. Main problems are low mucosal permeability and instability due to enzymes and varying pH values in the gastrointestinal tract (GIT) [3]. To achieve therapeutically-efficient plasma levels, different approaches have been investigated. Besides derivatization of drugs, enzyme inhibitors, mucolytic compounds as well as stabilizing agents co-administrated to the drug and agents to facilitate permeation, different formulation techniques like micro- and nanoparticles are studied [3,4]. Nanoparticles have several advances due to their size and their properties in surface modification. Nanoparticulate formulations can enhance bioavailability, can be used in controlled/delayed release formulations and allow drug accumulation at specific

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Article highlights.

- Thiomers are multifunctional and biocompatible polymers for drug delivery.
- Development of preactivated thiomers improved mucoadhesion significantly.
- Drug-loaded thiomer nanoparticles can be prepared via simple ionic gelation method.
- *In vivo* studies showed improved bioavailability of protein-based drugs due to thiomer nanoparticulate formulations in comparison to formulations of non-thiolated polymers.
- Improved transfection rates and enhanced gene expression *in vivo* after application of thiomer-pDNA-nanocomplexes compared to non-thiolated polymer-pDNA nanocomplexes have been demonstrated.

This box summarizes key points contained in the article.

locations. Drug efficiency increases and both frequency and extent of side effects decrease [5-7]. Furthermore, nanoparticles are relatively stable in the gastrointestinal tract compared to other colloidal carriers like liposomes [8,9]. Nanoparticulate carrier systems consisting of multifunctional polymers showed considerable potential. Suitable excipients are demanded for protecting and targeting drugs. Multifunctional polymers show several useful properties: mucoadhesion, protective activity, controlled release as well as permeation and uptake enhancement.

One of the most promising groups of multifunctional polymers is thiolated polymers or so-called thiomers. Established functional polymers like chitosan and poly(acrylic acid) (PAA) are derivatized to improve properties for drug delivery. Immobilization of thiol-bearing ligands to the backbone of these polymers leads to strongly improved mucoadhesion, protective and permeation enhancing effects [10-14]. Additionally, efflux pump inhibition could be shown *in vitro* and *in vivo* [15,16]. Furthermore, nanoparticle preparation, stabilization and drug incorporation is possible employing simple methods [17]. In this paper, we will give an overview of different kinds, synthesis, and properties of thiomers. Furthermore, recent results of research in thiomer nanoparticles for drug delivery will be provided and discussed.

2. Thiomers

2.1 First-generation thiomers

In general, thiomers are synthesized by covalent attachment of thiol group-bearing ligands to the backbone of a polymeric substance under relatively simple and mild conditions. Due to the chemical structure of modified polymers, they can be subdivided into cationic and anionic thiomers. In case of the first-generation thiomers, all employed ligands used are of aliphatic structure. By now, cationic thiomers have mainly been based on chitosan. Thiolated chitosan derivatives are achieved by attaching a sulfhydryl-bearing ligand to chitosan's C2 amino groups. Chitosan-4-thiobutylamidine (Chitosan-TBA) can be synthesized by chemical reaction of 2-iminothiolane with chitosan leading to a cationic amidine conjugate in a one-step reaction [18]. Another thiolated chitosan derivative generated by amidine bond formation with isopropyl-*S*-acetylthioacetimidate is chitosan-thioethylamidine [19]. *N*-acteylcysteine, glutathione, thioglycolic acid, and 6-mercaptonicotinic acid are immobilized by amide bond formation between the ligands carboxyl group and chitosan's amino group mediated by carbodiimides [1,20,21].

Recently, a new cationic thiomer was developed based on hydroxyethylcelullose (HEC). Therefore, the polymer was oxidized to the aldehyde form. To thiolate the polymer, cysteamine was used. In a reductive amination reaction with sodium cyanoborohydride, the cationic HEC-cysteamine was generated [22].

So far, all anionic thiomers are based on carboxyl group-bearing polymers. Sulfhydryl-bearing ligands can be easily attached using the carboxyl group for amid bond formation. Therefore, ligands bearing both thiol and amino groups such as cysteine, cysteamine, and homocysteine are employed [23-26]. The amide bond formation is mediated by carbodiimides. Oxidation of thiol groups to disulfide bonds can be avoided as described below. Employing this synthesis pathway, poly(acrylic acid), polycarbophil, alginate, carboxymethylcellulose, and pectin are derivatized into thiomers [27-31]. Instead of forming an amino bond for thiolization, Sharma *et al.* induced an ester bond between the carboxyl group of pectin and thioglycolic acid in hydrochloric environment [32].

2.2 Next-generation thiomers

As a matter of fact, free thiol groups are comparatively unstable in solutions as they are oxidized at pH $\geq$ 5 leading to self-cross-linking of the polymer [33]. Different approaches have been made for both, using the affinity of disulfide bond formation and trying to delay the oxidation. Self-cross-linking properties of thiolated poly(acrylic acid) could be shown, resulting in a 10- to 12-fold increase in viscosity after incubation at 37 °C for 24 h (3 % m/v solutions in phosphate buffer pH 6.8), a quality that might be used in *in situ* gelling formulation [34]. Additionally, aromatic thiol-bearing ligands turned out to be extraordinary good electron donors, leading to a stronger notion to oxidation [35]. To benefit from this affinity, aromatic ligands have been attached to the polymer backbone instead of aliphatic ligands [36,37]. For example, Perera *et al.* linked the aromatic thiol group-bearing 4-aminothiophenol chemically to pectin to improve the *in situ* gelling properties and to be able to form pectin gel formulations with stronger cohesiveness. *In vitro* characterization of the new conjugate showed after oxidation *in situ* with hydrogen peroxide a 500-fold increased dynamic viscosity and disintegration time in comparison to unmodified pectin, further water-uptake and disintegration time was increased significantly whereas

the modified pectin without induced oxidation showed a decreased water-uptake [38]. A similar approach has been taken by Millotti *et al.* from same group by attaching the aromatic heterocyclic compound 6-mercaptonicotinic acid. The molecule does have two tautomeric structures: thiol (S-H) and thione (C = N), whereat the thione form is the predominant structure in solution with polar solvents like water. As this structure can react as a nucleophile and a proton donor, disulfides can be formed without the presence of sulfhydryl groups meaning pH-independent reactivity of thiomers [20]. An improved mucoadhesion toward chitosan with aliphatic substituents was shown [36]. Following the idea of pH-independent and in general potentiated reactivity, preactivated thiomers have been developed recently by our group. Preactivated or designated *S*-protected thiomers are based on common thiomers at which free thiol groups are activated via pyridyl substructures. Resulting pyridyl disulfides are able to react rapidly with sulfhydryl groups by formation of disulfide bonds via disulfide exchange. These properties have already been employed by covalent chromatography [39,40]. The first preactivated anionic thiomer was poly(acrylic acid)-cysteine (PAA-Cys), as preactivating agent 2-mercaptonicotinic acid (2MNA) was employed. Therefore, 2MNA was oxidized to the dimer and coupled via disulfide exchange reaction to the cysteine-moiety [41]. 6-Mercaptonicotinamide (6MNA) was employed to preactivated chitosan-thioglycolic acid (Chitosan-TGA). 6MNA was synthesized out of 6-chloronicotinamide and thiourea according to the method developed by Forrest *et al.* [42]. After oxidation of the 6-MNA monomer into the 6,6-dithionicotiniamide (6-DTNA) dimer, it was coupled via disulfide exchange reaction to chitosan-TGA as well. The degree of preactivated thiol groups can be determined via spectrophotometrical detection of the aromatic substructure (**Figure 1**) [43].

3. Properties of thiomers

3.1 Mucoadhesive properties

For mucoadhesion, the possibility of interaction between participants must be provided. For most known mucoadhesive polymers like poly(acrylates), sodium cellulose derivatives, alginate and chitosan the attachment is achieved by chemical, non-covalent bonds such as ionic bonds, hydrogen bonds, and van der Waal's bonds [23,44]. Furthermore, physical interpenetration effects can be involved [17]. Covalent bonds with mucus components are possible with thiomers [24]. The reactive thiol groups are capable of forming disulfide bonds with proteins in the intestinal mucus layer leading to a strong adhesion effect [45]. Mucoadhesive properties of these thiolated polymers are improved in comparison to unmodified polymers. A positive correlation between thiol group content and mucoadhesive properties could be shown [23,45-47]. Dünnhaupt *et al.* investigated mucoadhesion of preactivated thiolated chitosan in comparison to thiolated and unmodified chitosan. **Figure 2B** illustrates the results of tensile studies [43]. **Figure 2A** shows the results of the same investigation for the anionic preactivated thiolated poly(acrylic acid) performed by Iqbal *et al.* [41]. Even if only about 50 – 70 % of attached thiol groups have been activated, significant improvement of mucoadhesion could be demonstrated. So far, there have been no mucoadhesion studies carried out for nanoparticles based on preactivated thiomers, but as for other types of thiomers mucoadhesion could be shown in nanoparticulate formulations, mucoadhesive properties can be assumed [48]. Dünnhaupt *et al.* described in a previous work the distribution of thiolated mucoadhesive nanoparticles on intestinal mucosa. Within this study, adhesion of thiol group-bearing nanoparticle formulations to the mucus layer could be shown via fluorescent labeling of the particles. It turned out that nanoparticles prepared using modified polymers showed a sixfold increase of mucoadhesion compared to nanoparticles consisting of unmodified polymers. Tests were performed with chitosan and poly(acrylic acid) [48]. The results are consistent with data published by Barthelmes *et al.* investigating mucoadhesion of thiomer nanoparticles on porcine urinary bladder. Therefore, chitosan and thiolated chitosan (TGA) nanoparticles have been labeled using the fluorescent marker FDA. For formulations based on thiolated chitosan, after 1 h around 80% of FDA was still adhered to the mucosa whereas for formulations based on unmodified chitosan only around 25% remained during the first hour [49].

Lately, Barthelmes *et al.* were able to confirm the *in vitro* mucoadhesive studies with an *in vivo* study carried out with female rats. Unmodified and thiolated chitosan nanoparticle suspension and pure fluorescent marker FDA suspension as control were delivered intravesical using a catheter. Remaining fluorescent marker was determined after sacrificing rats at predetermined time-points. Chitosan-TGA nanoparticles showed a significant fourfold increased mucoadhesive effect than unmodified chitosan nanoparticles and a 170-fold increased retention time compared to pure FDA suspension [50].

3.2 Permeation enhancement and efflux pump inhibition

The oral bioavailability of several drugs is reduced not only by enzymatic degradation but also by the absorption barrier consisting of epithelial cell membranes. The interconnecting tight junctions hinder paracellular uptake of hydrophilic drugs. Chitosan is one of the well-investigated permeation enhancers [51,52]. Several *in vitro* tests showed a further improvement of permeation enhancement by immobilizing thiol groups [19,53,54]. The underlying mechanism in uptake enhancement due to unmodified and thiolated chitosan is assumed to be the same: interactions with tight junctions [55-57,55]. Wang *et al.* investigated the permeation enhancer qualities of preactivated thiolated poly(acrylic acid) using sodium fluorescein as model drug in Ussing type chambers with freshly excised rat intestinal mucosa and

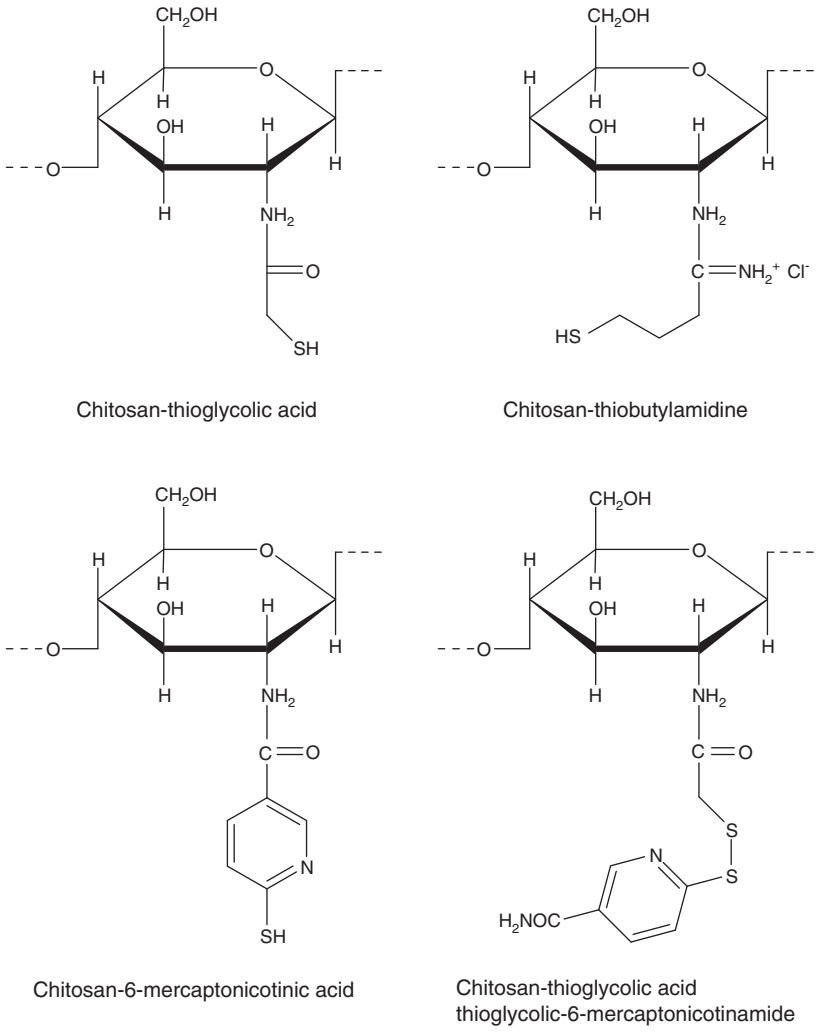

Figure 1. Substructure of selected thiolated chitosan derivatives.

Caco-2 cell monolayer as model barrier. It could be shown by both methods that the addition of PAA-Cys-MNA leads to a significant higher uptake of the model drug. The enhancement ratio was dependent on coupling rates of 2-MNA to the polymer [58].

Thiomers have also been tested for their efficacy in improving uptake of efflux pump substrates. The bioavailability of some drugs is restricted because of being substrate of the multidrug efflux pump P-glycoprotein (P-gp). P-gp is located in the apical membrane of enterocytes and transports its substrates from the inner side of the membrane to the outer side [59]. To overcome this hindrance, excipients, which are able to inhibit the transporter, are on demand. It could be shown that using inhibitors improves intestinal absorption and tissue distribution and reduces elimination of drugs [15,60]. *In vivo* studies have been carried out by Föger *et al.* by applying a solid, enteric-coated chitosan-TBA/GSH oral formulation to rats and the P-gp substrate rhodamine 123 (rho-123) intravenously. The area under the plasma concentration–time curve of rho-123 increased by 58 % in comparison to unmodified chitosan and by 217 % in comparison to control buffer [16].

Dünnhaupt *et al.* studied permeation enhancing effect and efflux pump inhibition properties of thiolated and preactivated thiolated chitosan to achieve improved oral delivery of hydrophilic macromolecules. FD_4 was used as hydrophilic model drug, P-gp inhibition was investigated using Rhodamine-123 as P-gp substrate. Permeation studies using excised rat intestine showed within 3 h a P_{app} being 1.3-fold higher for preactivated thiolated chitosan in comparison to thiolated chitosan and more than twofold higher than the P_{app} of unmodified chitosan. For efflux pump inhibition, the increase was 1.4-fold for preactivated thiolated chitosan compared to thiolated chitosan [61].

4. Preparation of thiomer nanoparticles

The most common used method to prepare nanoparticles is "ionic gelation" by inducing ionic complex formation, a

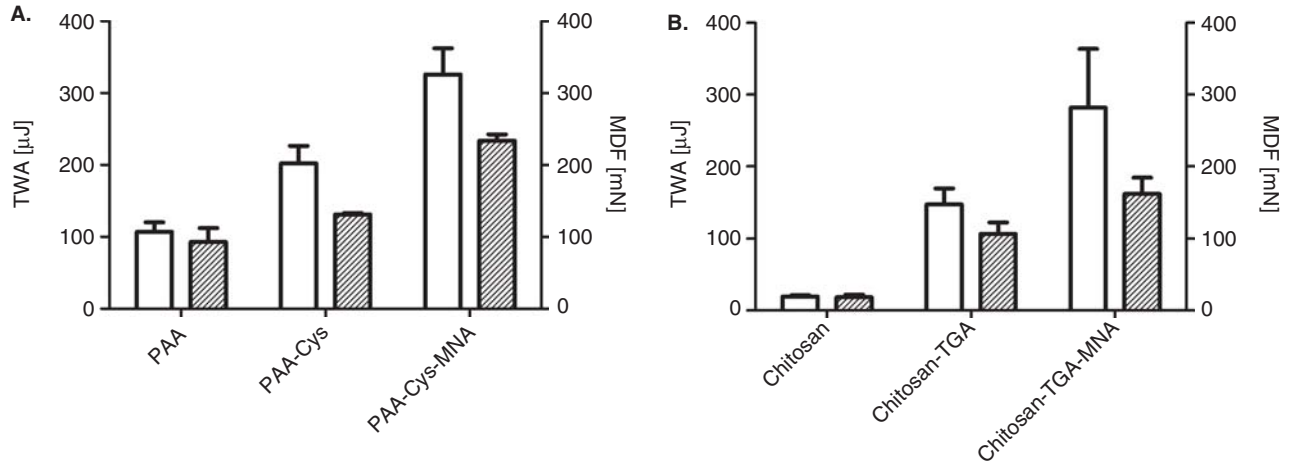

Figure 2. Bar charts show results of tensile studies, carried out on porcine intestinal mucosa.

Derived from data in [41] and [43].

MDF: Maximum detachment force, striped bars; TGA: Thioglycolic acid, TGA-MNA: Thioglycolic acid-6-mercaptonicotinamide, TWA: Total work of adhesion, representing the area under the force/distance curve, white bars.

simple and mild process. Electrostatic interactions between the positive charged amino group of chitosan and negative charged molecules like cyclodextrin, sodium sulfate and tripolyphosphate (TPP) lead to complex formation [62]. For nanoparticle formation, a TPP solution is dropped into an acidic solution of the thiomer under stirring. To stabilize the particles, the suspension can be stirred for several hours to induce disulfide bond formation within the particles. Size range of chitosan-6-mercaptonicotinic acid prepared according to this method was between 224 and 271 nm, for chitosan-TBA a mean diameter of 164 ± 6.9 nm was achieved. Particles can be collected by centrifugation and resuspended in water. After adding trehalose, particles can be lyophilized [63,64]. Bernkop-Schnürch *et al.* developed a method that allows formation of nanoparticles without ionically cross-linking. After inducing particle formation via TPP, thiol groups of resulting particles are partially oxidized. Using hydrogen peroxide or iodine solution leads to disulfide bond formation. Afterwards, the polyanion was removed via dialysis. Resulting chitosan-TBA particle mean size was 366 ± 30 nm and a zeta potential around + 11.3 ± 1.3 mV [65]. Barthelmes *et al.* employed this method for chitosan-TGA particles to develop stable thiomer nanoparticles. Resulting particles had an average size of 158 ± 8 nm showing a zeta potential of ~ + 16 mV. Investigating the stability of these covalently cross-linked nanoparticles showed that more than 99 % of these particles were stable over a 60-min period in simulated gastric fluid whereat only 10 % of the ionically cross-linked chitosan-TGA nanoparticles were stable over this time period [66].

The ionic gelation method can be used for anionic thiomers as well; the procedure is basically the same but using cationic cross-linking agents. Sharma *et al.* used magnesium chloride to form pectin-TGA nanoparticles whereas the polymer solution was added drop-wise to a magnesium chloride solution [32]. Thaurer *et al.* prepared PAA-Cys nanoparticles using calcium chloride as ionic cross-linker with an average size of 139 ± 34 nm [67]. Stabilizing via disulfide bond formation is also possible using hydrogen peroxide for partial oxidation. Dünnhaupt *et al.* prepared PAA-Cys nanoparticles using H_2O_2 for stabilization; resulting particle size was about 300 nm [48]. The ionic linker Ca^{2+} can be removed using ethylenediaminetetra-acetic acid and dialysis [17].

Furthermore, thiolated chitosan nanoparticles were prepared using a polymerization reaction. Radical polymerization used to prepare nanoparticles was first described by Chauvierre *et al.* in 2003. The polymerization process of isobutyl-cyanoacrylate was provoked by polysaccharides and cerium ammonium nitrate. Following this method, Bravo-Osuna *et al.* prepared nanoparticles based on thiolated chitosan/isobutyl-cyanoacrylate copolymer. Therefore, thiolated chitosan was dissolved in nitric acid, cerium ammonium nitrate and isobutylcyanate were added under stirring and argon gassing at 40°C for 40 min. After reaction time, the system was cooled down to room temperature and pH adjusted to 4.5 with NaOH solution. Nanoparticle size was about 229 nm and the resulting zeta-potential was determined to be 32.0 ± 0.2 mV [68]. Mazzaferro *et al.* also used isobutyl-cyanoacrylate to form nanocarrier with thiolated chitosan. Instead of a radical polymerization, an anionic polymerization was induced. To achieve the anionic polymerization in presence of chitosan-TBA, the polymer was solved in nitric acid, and then isobutyl-cyanoacrylate was added under stirring and argon. Reaction took place at 40°C, after reaction time and cooling down to room temperature; pH was adjusted to 6.5 using NaOH. Resulting particles had a size between 135 and 195 nm with a positive zeta-potential of about 40 mV [69].

Another preparation method for nanoparticles is the complex coacervation method. Similarly to the ionic gelation method described above, particles are stabilized by Coulombic interactions. Intermolecular complexes are formed due to electrostatic interactions for example of cationic amino groups of chitosan and negatively charged groups like carboxyl groups of anionic polymers such as alginate or sulfate groups of dextran sulfate [62,70]. Saboktakin *et al.* formed insulin-loaded dextran sulfate-chitosan-TGA particles by complex coacervation. Complexation occurred when chitosan solution was dropped in dextran sulfate-insulin solution under stirring. Resulting particles had a size range from 64 to 169 nm [71].

In general, loading of the particles with an active pharmaceutical ingredient (API) can be done during the particle formation process or afterwards – the latter happens while incubating the particle suspension with the drug. The achieved drug load rate is higher when the API is added to the solution before inducing particle formation but a drawback for this method is that the API has to undergo the complete process and may be affected [72]. In the case of DNA drugs, the drug itself can be used to precipitate nanocomplexes: Chitosan-TGA/pDNA complexes prepared by Lee *et al.* had an average size about 100 nm and a positive zeta-potential of about 15 mV [73]. This polyelectrolyte complexation method was also employed by Yin *et al.* who prepared different trimethyl-chitosan-cysteine nanoparticles in a size range between approximately 100 and 200 nm with a positive zeta-potential between 10 and 20 mV through self-assembly of positively charged polymer and negatively charged insulin [74].

5. Thiomer nanoparticles – *in vitro* and *in vivo* studies

5.1 Protein delivery

Progress in biotechnology has made production of peptide drugs at a commercial scale possible, making their use as highly efficient drugs more interesting and drawing the attention of researchers to finding ways to improve their oral bioavailability [4].

Perera *et al.* could demonstrate that insulin encapsulated in thiomer nanoparticles could be protected from enzymatic degradation. Insulin-loaded PAA-Cys/insulin particles (122 – 136 nm) were prepared using poly(vinyl pyrrolidon) as linking agent. Particles were stabilized via disulfide formation followed by incorporation into a triglyceride. Around 45 % of encapsulated insulin could be protected from trypsin and elastase and approximately 21 % from chymotrypsin degradation in *in vitro* studies [75]. The influence of the degree of thiol group content on release from core-shell nanoparticles has been investigated. Therefore, chitosan-TBA has been employed for encapsulation of drug Leu-Enkephalin in core-shell isobutylcyanoacrylate/chitosan-TBA nanoparticles. Particles were obtained by anionic emulsion polymerization [69,76,77]. To associate the protein with the resulting particles, two different methods have been employed, either during the nanoparticle formation process (inclusion method, "loading") or via adsorption on preformed nanoparticles. The higher content of drug was obtained by the inclusion method. Investigators discovered that, with increasing degree of thiolation, a decrease in both loading and adsorption efficiency occurred and was linked to an increased initial release. It is assumed that the protein drug interacts with the positively charged, free amino groups of chitosan shell of the formulation [69]. As increasing TBA attachment leads to reduction in free amino groups, the strength of linkage between excipient and active pharmaceutical ingredient declined. However, Millotti *et al.* found a reverse effect using the protein drug insulin. Chitosan-6-mercaptonicotinic acid (6MNAcid) particles were prepared using insulin-containing TPP solution as linking agent, average size of resulting particles was 271 nm. *In vitro* release studies showed that within the first 30 min, 50% of the incorporated insulin was released, whereas 70% of incorporated insulin was released from unmodified chitosan nanoparticles prepared for comparison. Both formulations released 100% of insulin whereas a more sustained release was observed from 6MNAcid-modified chitosan within the first 2 h. *In vivo* evaluation of this carrier system was carried out using non-diabetic rats and determining the human insulin content in the plasma via ELISA after oral administration. Maximal insulin level was achieved at 120 min for unmodified chitosan particles and after 180 min for particles with thiolated chitosan. There was a significant 7.8-fold higher maximal plasma level of insulin detected after administration of thiolated particles compared to non-thiolated particles [63]. The highest concentration of human insulin determined in plasma after oral application (C_{max}) was for thiolated chitosan-based (chitosan 6MNAcid) nanoparticles 76.6 mU/l whereas chitosan nanocarrier led to C_{max} = 9.75 mU/l. The AUC calculated for insulin after administration with chitosan 6MNAcid particles was with 1810 mU four times higher than the AUC after administration of insulin-loaded chitosan nanoparticles.

To combine the positive qualities of trimethyl chitosan (TMC) and thiolated chitosan, Yin *et al.* synthesized thiolated trimethyl chitosan (TMC-Cys). To this end, TMC was synthesized by using CH_3I in NaOH solution. After reaction time I^- was exchanged to Cl^- with anion-exchange resin and purified via dialysis. TMC was thiolated using L-cysteine HCl. Insulin-loaded nanoparticles were prepared via ionic interactions due to positively charged polymers and negatively charged insulin. Resulting particles size range was between 100 and 200 nm with insulin encapsulation efficiency about 90%. *In vitro* insulin release was investigated in phosphate buffer 0.2 M, pH 7.4 at 37°C. TMC-Cys-insulin particles (chitosan 500 and 200 kDa) showed peptide release of 80 – 90% after 4 h, TMC-Cys-insulin particles (chitosan 30 kDa) showed faster release with almost 100% peptide release after 1 h. Furthermore, an increased permeation enhancing effect for insulin from TMC-Cys-nanocarrier

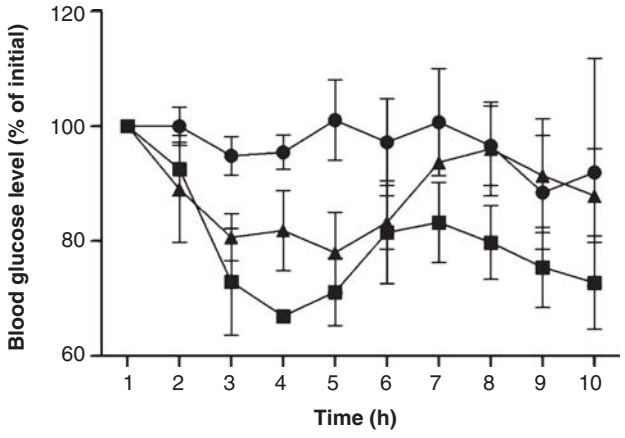

Figure 3. Serum glucose levels in normal rats after oral administration of trimethyl-chitosan nanoparticles (-▲-), trimethyl-chitosan-cysteine nanoparticles (-■-) with insulin dose of 50 IU/kg, insulin solution 50 IU/kg (-●-).
Adapted from [74] with permission of Elsevier.

compared to TMC was found (in rat intestine). Pharmacological *in vivo* effect of insulin administrated orally with TMC-Cys nanoparticles was determined in rats. For comparison, oral insulin solution and TMC-insulin nanoparticles were employed. In addition, insulin suspension, TMC-Cys- and TMC-insulin nanoparticles were administrated into the ileum. Both oral and ileal application led to considerable decrease of blood glucose level compared to insulin solution. The effect lasted for 7 – 8 h after administration and a maximal blood glucose depression of 35% for oral administration and 70% for ileal application was measured. Compared to TMC-insulin nanoparticles TMC-Cys-insulin nanoparticles showed a prolonged and higher hypoglycemic effect (**Figure 3**) [74].

Recently, Shahnaz *et al.* published their work on nasal administration of leuprolide in rats. The size of the chitosan-TGA nanoparticles was about 252 nm, zeta-potential 10.9 ± 4 mV and sustained leuprolide release was determined over 6 h. Particles were prepared by ionic gelation and leuprolide was incorporated during particle formation process. It was observed that unmodified chitosan nanoparticle and chitosan-TGA nanoparticles enhanced leuprolide transport through nasal mucosa compared to leuprolide solution 2.0- and 5.2-folds, respectively. The relative bioavailability of leuprolide administered nasally via thiomer nanoparticle (versus subcutaneous injection) was about 19.6% compared to leuprolide solution with 2.8%. The maximum plasma concentration of leuprolide administered nasally via chitosan-TGA nanoparticles was about 3.8-fold improved compared to leuprolide solution administered nasally [78].

Makhlof and coworkers achieved an improved pulmonary delivery of calcitonin after encapsulation in glycol-chitosan-thioglycolic acid nanoparticles compared to non-thiolated glycol-chitosan nanoparticles (size range of 230 – 330 nm). Mucoadhesion of nanoparticle bearing free thiol groups was increased twofold compared to non-thiolated ones in rats (intra tracheal application). For *in vivo* studies, calcitonin solution, calcitonin-loaded glycol-chitosan (GCS) or thiolated glycol-chitosan nanoparticles (GCS-TGA) were administrated to rats via a liquid Micros-Sprayer technique. Blood calcium level was investigated over a 24-h time period. All formulations were able to reduce blood calcemia, whereas free calcitonin showed a short duration time that may be caused by the rapid elimination due to mucociliary clearance and/or drug degradation by peptidases. GCS and GCS-TGA formulations were able to prolong the hypocalcemic effect of the peptide with a pharmacological availability of 27 and 40%, respectively. Makhlof *et al.* assume that this improvement is due to mucoadhesive properties and permeation enhancing effects of glycol-chitosan; further, the higher efficacy and longer lasting effect of thiolated glycol-chitosan could be the result of its higher mucoadhesive qualities. **Table 1** presents the area above blood calcium curve after pulmonary application [79].

5.2 Gene delivery

More and more attention is being given to therapeutic nucleic acids for vaccination and therapeutic gene expression. Carlisle *et al.* demonstrated that plasmid DNA conjugated with thiolated polyethylenamine to nanoparticles coated with thiol-reactive poly[*N*-(2-hydroxypropyl)methacrylamide] bearing 2-pyridyldisulfanyl groups, leading to reducible disulfide-linked coating, could improve transfection activity 40- to 100-fold compared to thioether-linked particles [80]. Furthermore, it was shown that thiolation of gelatin used as DNA drug carrier brought benefits compared to investigated non-thiolated gelatin-based formulations. It was observed that transfection was more effective in NIH-3T3 murine fibroblast cells using the nanoparticles of thiolated gelatin [81]. Based on these results, investigating thiomers as non-viral carriers for DNA seems promising. Nanoparticles can be prepared readily using thiolated chitosan: Coacervation between positively charged amino groups of chitosan and negatively charged phosphate groups of nucleic acids leads to chitosan-DNA complexes in the nano-range [82]. For their evaluation of thiolated chitosan nanocarrier in antisense therapy, Talaei *et al.* employed *N*-acetylcysteine-chitosan (chitosan-NAC) and *N*-acetyl penicillamine (chitosan-NAP). Nanoparticles were prepared by ionic gelation containing antisense oligonucleotide (ASOND) complementary to positions 3811 – 3825 of the human EGFR (epidermal growth factor receptor) cDNA, resulting particle size range was from 200 to 300 nm. *In vitro* release studies were carried out in phosphate buffered saline pH 7.4 (PBS) as well as under stimulated reducing cytosol conditions (dithiothreitol-containing environment). During 15 h in PBS there was a lower (22% instead of complete) but more sustained release to detect compared to unmodified chitosan-ASOND particles. In reducing cytosol-stimulating medium particles dissociated leading to release of about 50%. The

Table 1. Area above blood calcium curve over 24 h after administration. Pharmacological bioavailability in % relative to subcutaneous administration.

	Calcitonin (μg/kg)	Area above blood calcium curve	Pharmacological bioavailability (%)
Solution (subcutaneous)	5	447.4 ± 110.3	-
Solution (pulmonary)	25	215.5 ± 145.2	9.63 ± 6.49
Glycol chitosan nanoparticles (pulmonary)	25	609.9 ± 144.1	27.27 ± 6.44
Glycol chitosan-TGA nanoparticles (pulmonary)	25	909.6 ± 26.2	40.67 ± 1.17

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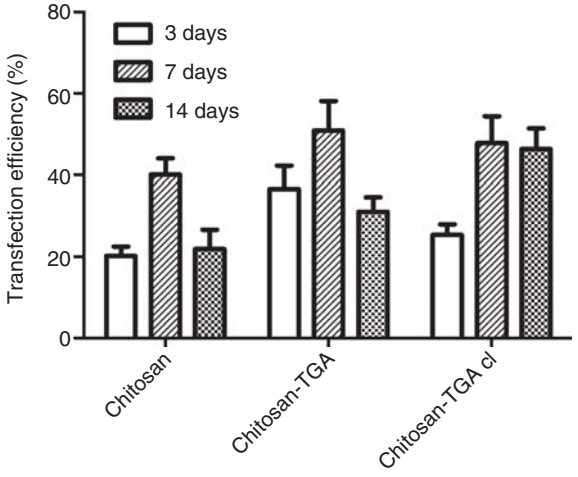

Figure 4. Application of green fluorescent protein pDNA/chitosan nanoparticles intranasally in mice. Chart shows level of gene expression in BAL cells for 14 days after administration. n=4. cl: cross-linked nanoparticles, TGA: 4-thiobutylamidine. Figure adapted from [74] with permission of Springer.

Adapted from [73].

cl: Cross-linked nanoparticles; TGA: 4-thiobutylamidine.

impact on EGFR expression was investigated *in vitro* using T47D breast cancer cells. The expression of EGFR protein was down-regulated by ASOND-containing particles (NAC-chitosan, NAP-chitosan, unmodified chitosan) about twofold compared to free ASOND [83].

Martien *et al.* achieved protection of DNA against nucleases via encapsulation into chitosan-TGA carrier, particles were stabilized via oxidative cross-linking. Chitosan-TGA particles resisted DNase I for 30 min whereat unprotected DNA and chitosan/DNA particle showed substantial degradation [84]. It is assumed that the enzyme is inhibited via complexation of divalent cations, essential for enzyme activity, by the modified polymer [84,85]. Furthermore, a fivefold increase in transfection rate (expression of green fluorescence protein) of chitosan-TGA/pDNA nanoparticles (average size of 100 – 200 nm) compared to unmodified chitosan/pDNA nanoparticles in Caco-2 cells was detected [84]. Lee *et al.* found enhanced and sustained gene delivery while investigating chitosan-TGA/DNA nanoparticles (113 – 220 nm): For the investigation, pDNA encoding the reporter gene for the green fluorescent protein (GFP) was employed. A study to determine the effect of thiolation of chitosan and thiolation followed by cross-linking on protection of pDNA from DNAse showed that unmodified chitosan and cross-linked thiolated chitosan protected pDNA from digestion whereas particles with thiolated non-cross-linked chitosan turned out to provide less protection. Additionally, Lee *et al.* compared transfection efficiency of thiolated chitosan and thiolated chitosan with Lipofectin (a liposomal transfection reagent) in HEK 293 cells. Results showed higher transfection efficiency for thiolated chitosan nanocomplexes compared to Lipofectin (approximately 30% after 60 h compared to about 10% for Lipofectin system) and unmodified chitosan. Results from Lee *et al.* were in accordance with data published by Martien *et al. In vitro* transfection study using HEK 293 cells showed that a higher degree of free thiol groups led to a higher efficiency in transfection whereas oxidation of free thiol groups entailed a reduction in transfection efficiency. The *in vivo* gene transfer potential was determined using intranasal application to mice. Particles prepared with thiolated chitosan led to increased gene expression of green fluorescent protein after 3 days, particles containing cross-linked thiolated chitosan after 7 days compared to particles prepared with unmodified chitosan (**Figure 4**). Additionally, mucoadhesion study showed that mucoadhesion-enhancing effect of thiolation is not lost in particle formulation [73].

These results confirm studies from Schmitz *et al.* and Loretz *et al.* who investigated chitosan-TBA and chitosan-NAC, respectively. Improved stability, enhanced pDNA release under reducing conditions and higher transfection rates in Caco-2 cell could be shown for these chitosan derivatives [86,87]. Loretz *et al.* showed that chitosan/DNA particles prepared with chitosan-NAC followed by cross-linking via oxidation were more stable in environments with an excess of the polyanion heparin or with pH 10 then non-oxidized chitosan-NAC particles but not more stable than non-thiolated chitosan/DNA particles. It turned out in the *in vitro* transfection study that oxidized chitosan-NAC/DNA particles could not improve transfection efficacy, apparently the vehicle is to stable to release DNA, whereat chitosan-TBA/DNA particles raised

Table 2. Main features of thiomer nanoparticles in drug delivery.

Thiomer	Drug/model drug	Outcome	Ref.
PAA-Cys	Insulin	*In vitro* degradation studies, nanoparticles protected 44.47% of the initial insulin amount from trypsin, 21.33% from chymotrypsin, 45.01% from elastase compared to insulin solutions	[75]
Chitosan-6MNAcid	Insulin	AUC *in vivo* after oral administration to rats fourfold improved compared to unmodified chitosan nanoparticles	[63]
TMC-Cys	Insulin	Oral and ileal application in rats blood glucose depression of 35% for oral administration and 70% for ileal application *in vivo*, hypoglycemic effect higher and longer-lasting compared to TMC-insulin nanoparticles	[74]
Glycol chitosan-TGA	Calcitonin	Pulmonary application to rats, pharmacological availability of 40% compared to 27% after administrating non-thiolated glycol chitosan/calcitonin particles	[79]
Chitosan-NAC/Chitosan-NAP	ASOND	*In vitro* release after 15 h showed a more sustained release of 22% whereas ASOND was completely released from non-thiolated chitosan particles	[83]
Chitosan-TGA	pDNA	Fivefold higher transfection rate (Caco-2) due to protection from nucleases	[84]
Chitosan-TGA	pDNA	Intranasal application in mice lead to gene expression over 14 days	[73]
Chitosan-NAC	pDNA	2.5-fold increase of transgene expression (Caco-2) in comparison to non-thiolated chitosan carrier	[87]
Chitosan-TBA	pDNA	Increased transfection efficiency in Caco-2 cells compared to naked plasmid	[86]

6MNAcid: 6-mercaptonicotinic acid; ASOND: Antisense oligonucleotide; NAC: Chitosan-N-acteylcysteine; NAP: N-acetyl penicillamine; PAA-Cys: Poly(acrylate)-cysteine; TBA: 4-thiobutylamidine; TGA: Thioglycolic acid; TMC-Cys: Trimethylchitosan-cysteine.

transfection 2.5-fold in comparison to chitosan/DNA particles (**Table 2**) [87].

6. Expert opinion

This paper reviews thiomers and thiomer-based nanoparticulate delivery systems. Thiomers are a class of different polymeric excipients characterized by bearing free thiol groups. In many studies, common properties have been demonstrated. First of all, remarkable improvements in mucoadhesion came along with thiolation independent of the underlain polymer. With the introduction of preactivated thiomers, mucoadhesion could be further improved, although the currently used synthesis pathway does not allow one to preactivate the total amount of attached thiol groups. To investigate the influence of preactivation in more detail, it would be desirable to modify up to 100 %. Additionally, permeation enhancing and efflux pump inhibition could be shown in several studies for different kind of thiomers. However, until recently, it was only possible to demonstrate these properties *in vitro*. It is not certain that these effects of widely biodegradable polymers under physiological conditions including enzymes and chyme as well as gastric and intestinal peristalsis still be notable. As a matter of fact, qualities of thiomers are depending on the polymeric backbone, the chosen thiol group-bearing ligand and the degree of modification, making general conclusions about thiomers difficult but expand application area. For instance, modification with hydrophilic aromatic ligands led to increased viscosity being an advantage in gel or *in situ* gelling formulations. Further, viscosity can have an impact on drug release. As the viscosity and solubility can be adjusted by varying ligands and degree of modification, tailor-made thiomers-based release medium may be possible someday. Sustained release could already be detected for thiomer nanoparticulate formulations being a first step in controlled-release area. It turned out that thiol groups attached to the excipients could be employed for preparation of relatively stable drug-loaded nanoparticles. Therefore, particles can be cross-linked via oxidation of thiol groups into disulfides. Cross-linking of particles does besides having an impact on stability have an influence on drug release. It seems to be a balancing act in introducing enough disulfide bonds for stabilizing particles and granting release. Further, drug release does not only depend on carrier system, individual interactions between the active pharmaceutical ingredient and excipients are important. Several *in vivo* studies with thiomer-drug formulations have been carried out showing that these formulations can succeed in protecting the DNA- or peptide-based drug and showed increased bioavailability in comparison to the unmodified polymer. These results seem promising for future investigations. It has to be considered that there are, by now, no systematic *in vivo* studies available that would make a general prediction possible. It is hardly possible to compare given results from different designed studies using different polymers

with different kind and degree of modification and to draw general conclusion. As far as there are no clinical studies available evaluation and forward-looking statements are difficult. Nevertheless, encouraging results in drug formulation of protein- and DNA-based drugs have been achieved, even though for DNA drugs mainly model drugs are used and by now there is no possibility to predict a therapeutic relevance of obtained bioavailability. Another advantage of thiomers-based nanoparticles, besides permeation enhancing, mucoadhesion, biocompatibility, uptake and transfection enhancement is the cheap and simple synthesis of thiomers and the mild conditions during nanoparticle formulation being essential for sensitive drugs like proteins.

A further aspect to keep in mind is that most formulations are only produced in laboratory scale. Therefrom, before any clinical trials are to debate, future research should concentrate on optimizing production and upscaling of the carrier system as well as providing more and better comparable *in vivo* studies. *In vitro* toxicological investigations have been carried out using different kind of cell lines showing all no noteworthy toxic effect. It has to be mentioned that these kinds of investigation only have been performed with an incubation time up to 24 h [63,73,84]. Therefrom, no long-term data are provided making it even more difficult to transpose conclusion to human application.

In conclusion, various thiomers have been developed and investigated in terms of nanoparticulate carrier systems. Considering the low bioavailability of peptide- and DNA-based drugs when administrated non-parenterally, very encouraging results have been reached. As the individual thiomers differ in their properties, the excipient fitting best for a certain formulation can be evaluated, leading to tailored carrier system for each drug and each application route. With promising qualities of thiomers per se and results of *in vivo* studies carried out so far, we are dealing with a substance class which will not be ruled out easily in the field of drug delivery development. It is also obvious that further optimizations are necessary for establishing a carrier system ready for clinical approval.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

Bibliography

1. Werle M, Bernkop-Schnurch A. Thiolated chitosans: useful excipients for oral drug delivery. J Pharm Pharmacol 2008;60(3):273-81
2. Khafagy E-S, Morishita M, Onuki Y, Takayama K. Current challenges in non-invasive insulin delivery systems: a comparative review. Adv Drug Deliv Rev 2007;59(15):1521-46
3. Antosova Z, Mackova M, Kral V, Macek T. Therapeutic application of peptides and proteins: parenteral forever? Trends Biotechnol 2009;27(11):628-35
4. Frokjaer S, Otzen DE. Protein drug stability: a formulation challenge. Nat Rev Drug Discov 2005;4(4):298-306
5. Emerich DF, Thanos CG. The pinpoint promise of nanoparticle-based drug delivery and molecular diagnosis. Biomol Eng 2006;23(4):171-84
6. Hobbs SK, Monsky WL, Yuan F, et al. Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. Proc Natl Acad Sci USA 1998;95(8):4607-12
7. Yih TC, Al-Fandi M. Engineered nanoparticles as precise drug delivery systems. J Cell Biochem 2006;97(6):1184-90
8. des Rieux A, Fievez V, Garinot M, et al. Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. J Control Release 2006;116(1):1-27
9. Chaudhury A, Das S. Recent advancement of chitosan-based nanoparticles for oral controlled delivery of insulin and other therapeutic agents. AAPS PharmSciTech 2011;12(1):10-20
10. Makhlof A, Tozuka Y, Takeuchi H. Design and evaluation of novel pH-sensitive chitosan nanoparticles for oral insulin delivery. Eur J Pharm Sci 2011;42(5):445-51
11. Grabovac V, Guggi D, Bernkop-Schnurch A. Comparison of the mucoadhesive properties of various polymers. Adv Drug Deliv Rev 2005;57(11):1713-23
12. Hornof MD, Bernkop-Schnurch A. In vitro evaluation of the permeation enhancing effect of polycarbophil-cysteine conjugates on the cornea of rabbits. J Pharm Sci 2002;91(12):2588-92
13. Kast CE, Guggi D, Langoth N, Bernkop-Schnurch A. Development and in vivo evaluation of an oral delivery system for low molecular weight heparin based on thiolated polycarbophil. Pharm Res 2003;20(6):931-6
14. Takeuchi H, Yamamoto H, Kawashima Y. Mucoadhesive nanoparticulate systems for peptide drug delivery. Adv Drug Deliv Rev 2001;47(1):39-54
15. Werle M, Hoffer M. Glutathione and thiolated chitosan inhibit multidrug resistance P-glycoprotein activity in excised small intestine. J Control Release 2006;111(1-2):41-6
16. Foger F, Schmitz T, Bernkop-Schnurch A. In vivo evaluation of an oral delivery system for P-gp substrates based on thiolated chitosan. Biomaterials 2006;27(23):4250-5
17. Albrecht K, Bernkop-Schnurch A. Thiomers: forms, functions and applications to nanomedicine. Nanomedicine (Lond) 2007;2(1):41-50
18. Bernkop-Schnurch A, Hornof M, Zoidl T. Thiolated polymers–thiomers: synthesis and in vitro evaluation of chitosan-2-iminothiolane conjugates. Int J Pharm 2003;260(2):229-37
19. Kafedjiiski K, Krauland AH, Hoffer MH, Bernkop-Schnurch A. Synthesis and in vitro evaluation of a

novel thiolated chitosan. Biomaterials 2005;26(7):819-26

20. Millotti G, Samberger C, Frohlich E, Bernkop-Schnurch A. Chitosan-graft-6-mercaptonicotinic acid: synthesis, characterization, and biocompatibility. Biomacromolecules 2009;10(11):3023-7
21. Bernkop-Schnurch A, Hoffer MH, Kafedjiiski K. Thiomers for oral delivery of hydrophilic macromolecular drugs. Expert Opin Drug Deliv 2004;1(1):87-98
22. Rahmat D, Sakloetsakun D, Shahnaz G, et al. Design and synthesis of a novel cationic thiolated polymer. Int J Pharm 2011;411(1-2):10-17
23. Bernkop-Schnurch A. Thiomers: a new generation of mucoadhesive polymers. Adv Drug Deliv Rev 2005;57(11):1569-82
24. Bernkop-Schnurch A, Schwarz V, Steininger S. Polymers with thiol groups: a new generation of mucoadhesive polymers? Pharm Res 1999;16(6):876-81
25. Bernkop-Schnurch A, Clausen AE, Hnatyszyn M. Thiolated polymers: synthesis and in vitro evaluation of polymer-cysteamine conjugates. Int J Pharm 2001;226(1-2):185-94
26. Bernkop-Schnurch A, Leitner V, Moser V. Synthesis and in vitro characterization of a poly(acrylic acid)-homocysteine conjugate. Drug Dev Ind Pharm 2004;30(1):1-8
27. Bernkop-Schnurch A, Kast CE, Richter MF. Improvement in the mucoadhesive properties of alginate by the covalent attachment of cysteine. J Control Release 2001;71(3):277-85
28. Bernkop-Schnurch A, Steininger S. Synthesis and characterisation of mucoadhesive thiolated polymers. Int J Pharm 2000;194(2):239-47
29. Majzoob S, Atyabi F, Dorkoosh F, et al. Pectin-cysteine conjugate: synthesis and in-vitro evaluation of its potential for drug delivery. J Pharm Pharmacol 2006;58(12):1601-10
30. Jindal AB, Wasnik MN, Nair HA. Synthesis of thiolated alginate and evaluation of mucoadhesiveness, cytotoxicity and release retardant properties. Indian J Pharm Sci 2010;72(6):766-74
31. Davidovich-Pinhas M, Harari O, Bianco-Peled H. Evaluating the mucoadhesive properties of drug delivery systems based on hydrated thiolated alginate. J Control Release 2009;136(1):38-44
32. Sharma R, Ahuja M, Kaur H. Thiolated pectin nanoparticles: preparation, characterization and ex vivo corneal permeation study. Carbohydr Polym 2011;87:1606-10
33. Brandt J, Svenson A, Carlsson J, Drevin H. Covalent coupling of unsaturated compounds to thiol agrarose using gamma-Radiation. A new method for preparation of adsorbents for affinity chromatography. Appl Biochem Biotechnol 1977(2):2:109
34. Marschutz MK, Bernkop-Schnurch A. Thiolated polymers: self-crosslinking properties of thiolated 450 kDa poly(acrylic acid) and their influence on mucoadhesion. Eur J Pharm Sci 2002;15(4):387-94
35. Burner U, Jantschko W, Obinger C. Kinetics of oxidation of aliphatic and aromatic thiols by myeloperoxidase compounds I and II. FEBS Lett 1999;443(3):290-6
36. Mueller Christiane, Verroken Andrea, Iqbal Javed, Bernkop-Schnuerch Andreas. Journal of Applied Polymer Science, 2012; 124(6):5046?5055
37. Millotti G, Samberger C, Frohlich E, et al. Chitosan-4-mercaptobenzoic acid: synthesis and characterization of a novel thiolated chitosan. J Mater Chem 2010;20(12):2432-40
38. Perera G, Hombach J, Bernkop-Schnurch A. Hydrophobic thiolation of pectin with 4-aminothiophenol: synthesis and in vitro characterization. AAPS PharmSciTech 2010;11(1):174-80
39. Carlsson J, Drevin H, Axen R. Protein thiolation and reversible protein-protein conjugation. N-Succinimidyl 3-(2-pyridyldithio)propionate, a new heterobifunctional reagent. Biochem J 1978;173(3):723-37
40. Norris R, Brocklehurst K. A convenient method of preparation of high-activity urease from Canavalia ensiformis by covalent chromatography and an investigation of its thiol groups with 2,2′-dipyridyl disulphide as a thiol titrant and reactivity probe. Biochem J 1976;159(2):245-57
41. Iqbal J, Shahnaz G, Dunnhaupt S, et al. Preactivated thiomers as mucoadhesive polymers for drug delivery. Biomaterials 2012;33(5):1528-35
42. Forrest HS, Fuller AT, Walker J. Chemotherapeutic agents of the sulphone type; effect of homologation on the antibacterial activity of p-methylsulphonyl-benzamidine and p-methylsulphonyl-benzylamine hydrochlorides. J Chem Soc 1948;3:1501-6
43. Dunnhaupt S, Barthelmes J, Rahmat D, et al. S-protected thiolated chitosan for oral delivery of hydrophilic macromolecules: evaluation of permeation enhancing and efflux pump inhibitory properties. Mol Pharm 2012;9(5):1331-41
44. Smart JD. The basics and underlying mechanisms of mucoadhesion. Adv Drug Deliv Rev 2005;57(11):1556-68
45. Bernkop-Schnurch A, Hornof M, Guggi D. Thiolated chitosans. Eur J Pharm Biopharm 2004;57(1):9-17
46. Kast CE, Bernkop-Schnurch A. Thiolated polymers–thiomers: development and in vitro evaluation of chitosan-thioglycolic acid conjugates. Biomaterials 2001;22(17):2345-52
47. Hombach J, Bernkop-Schnurch A. Mucoadhesive drug delivery systems. Handb Exp Pharmacol 2010(197):251-66
48. Dunnhaupt S, Barthelmes J, Hombach J, et al. Distribution of thiolated mucoadhesive nanoparticles on intestinal mucosa. Int J Pharm 2011;408(1-2):191-9
49. Barthelmes J, Perera G, Hombach J, et al. Development of a mucoadhesive nanoparticulate drug delivery system for a targeted drug release in the bladder. Int J Pharm 2011;416(1):339-45
50. Barthelmes J, Dunnhaupt S, Unterhofer S, et al. Thiolated particles as effective intravestical drug delivery system for treatment of bladder related diseases. 2012;Under revision

51. Aungst BJ. Absorption enhancers: applications and advances. The AAPS Journal 2012;14(1):10-18

52. Luessen HL, de Leeuw BJ, Langemeyer MW, et al. Mucoadhesive polymers in peroral peptide drug delivery. VI. Carbomer and chitosan improve the intestinal absorption of the peptide drug buserelin in vivo. Pharm Res 1996;13(11):1668-72

53. Werle M, Kafedjiiski K, Kolmar H, Bernkop-Schnurch A. Evaluation and improvement of the properties of the novel cystine-knot microprotein McoEeTI for oral administration. Int J Pharm 2007;332(1-2):72-9

54. Bernkop-Schnurch A, Guggi D, Pinter Y. Thiolated chitosans: development and in vitro evaluation of a mucoadhesive, permeation enhancing oral drug delivery system. J Control Release 2004;94(1):177-86

55. Clausen AE, Kast CE, Bernkop-Schnurch A. The role of glutathione in the permeation enhancing effect of thiolated polymers. Pharm Res 2002;19(5):602-8

56. Grafstrom R, Stead AH, Orrenius S. Metabolism of extracellular glutathione in rat small-intestinal mucosa. Eur J Biochem 1980;106(2):571-7

57. Staddon JM, Herrenknecht K, Smales C, Rubin LL. Evidence that tyrosine phosphorylation may increase tight junction permeability. J Cell Sci 1995;108(Pt 2):609-19

58. Wang X, Iqbal J, Rahmat D, Bernkop-Schnurch A. Preaktivated thiomers: permeation enhancing properties. In preparation

59. Silverman JA. Multidrug-resistance transporters. Pharm Biotechnol 1999;12:353-86

60. Varma MV, Ashokraj Y, Dey CS, Panchagnula R. P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement. Pharmacol Res 2003;48(4):347-59

61. Dunnhaupt S, Barthelmes J, Rahmat D, et al. S-protected thiolated chitosan for oral delivery of hydrophilic macromolecules: evaluation of permeation enhancing and efflux pump inhibitory properties. Mol Pharmaceutics 2012;9(5):pp 1331-1341

62. Amidi M, Mastrobattista E, Jiskoot W, Hennink WE. Chitosan-based delivery systems for protein therapeutics and antigens. Adv Drug Deliv Rev 2010;62(1):59-82

63. Millotti G, Perera G, Vigl C, et al. The use of chitosan-6-mercaptonicotinic acid nanoparticles for oral peptide drug delivery. Drug Deliv 2011;18(3):190-7

64. Sakloetsakun D, Perera G, Hombach J, et al. The impact of vehicles on the mucoadhesive properties of orally administrated nanoparticles: a case study with chitosan-4-thiobutylamidine conjugate. AAPS PharmSciTech 2010;11(3):1185-92

65. Bernkop-Schnurch A, Heinrich A, Greimel A. Development of a novel method for the preparation of submicron particles based on thiolated chitosan. Eur J Pharm Biopharm 2006;63(2):166-72

66. Barthelmes J, Dunnhaupt S, Hombach J, Bernkop-Schnurch A. Thiomer nanoparticles: stabilization via covalent cross-linking. Drug Deliv 2011;18(8):613-19

67. Thaurer MH, Deutel B, Schlocker W, Bernkop-Schnurch A. Development of nanoparticulate drug delivery systems based on thiolated poly(acrylic acid). J Microencapsul 2009;26(3):187-94

68. Bravo-Osuna I, Schmitz T, Bernkop-Schnurch A, et al. Elaboration and characterization of thiolated chitosan-coated acrylic nanoparticles. Int J Pharm 2006;316(1-2):170-5

69. Mazzaferro S, Bouchemal K, Vauthier C, et al. What are parameters affecting Leu-enkephalin loading and release from poly(isobutylcyanoacrylate) nanoparticles coated with thiolated chitosan? J Drug Deliv Sci Technol 2011;21(5):385-93

70. Nagpal K, Singh SK, Mishra DN. Chitosan nanoparticles: a promising system in novel drug delivery. Chem Pharm Bull (Tokyo) 2010;58(11):1423-30

71. Saboktakin MR, Tabatabaie RM, Maharramov A, Ramazanov MA. Synthesis and in vitro evaluation of thiolated chitosan-dextran sulfate nanoparticles for the delivery of letrozole. 2010;1(2):62-7

72. Agnihotri SA, Mallikarjuna NN, Aminabhavi TM. Recent advances on chitosan-based micro- and nanoparticles in drug delivery. J Control Release 2004;100(1):5-28

73. Lee D, Zhang W, Shirley SA, et al. Thiolated chitosan/DNA nanocomplexes exhibit enhanced and sustained gene delivery. Pharm Res 2007;24(1):157-67

74. Yin L, Ding J, He C, et al. Drug permeability and mucoadhesion properties of thiolated trimethyl chitosan nanoparticles in oral insulin delivery. Biomaterials 2009;30(29):5691-700

75. Perera G, Greindl M, Palmberger TF, Bernkop-Schnurch A. Insulin-loaded poly (acrylic acid)-cysteine nanoparticles: stability studies towards digestive enzymes of the intestine. Drug Deliv 2009;16(5):254-60

76. Bouchemal K, Ponchell G, Mazzaferro S, et al. A new approach to determine loading efficiency of Leu-enkephalin in poly(isobutylcyanoacrylate) nanoparticles coated with thiolated chitosan. J Drug Deliv Sci Technol 2008;18(6):392-7

77. Requena V, Bouchemal K, Vauthier C, Ponchel G. Encapsulation of leu-enkephalin in core-shell isobutylcyanoacrylate - thiolated chitosan nanoparticles for oral administratton. J Chilean Chem Soc 2008;53(4):1677-81

78. Shahnaz G, Vetter A, Barthelmes J, et al. Thiolated chitosan nanoparticles for the nasal administration of leuprolide: bioavailability and pharmacokinetic characterization. Int J Pharm 2012;428(1-2):164-70

79. Makhlof A, Werle M, Tozuka Y, Takeuchi H. Nanoparticles of glycol chitosan and its thiolated derivative significantly improved the pulmonary delivery of calcitonin. Int J Pharm 2010;397(1-2):92-5

80. Carlisle RC, Etrych T, Briggs SS, et al. Polymer-coated polyethylenimine/ DNA complexes designed for triggered activation by intracellular reduction. J Gene Med 2004;6(3):337-44

81. Kommareddy S, Amiji M. Preparation and evaluation of thiol-modified gelatin nanoparticles for intracellular DNA delivery in response to glutathione. Bioconjug Chem 2005;16(6):1423-32

82. Mao HQ, Roy K, Troung-Le VL, et al. Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. J Control Release 2001;70(3):399-421

83. Talaei F, Azizi E, Dinarvand R, Atyabi F. Thiolated chitosan nanoparticles as a delivery system for

antisense therapy: evaluation against EGFR in T47D breast cancer cells. Int J Nanomedicine 2011;6:1963-75

84. Martien R, Loretz B, Thaler M, et al. Chitosan-thioglycolic acid conjugate: an alternative carrier for oral nonviral gene delivery? J Biomed Mater Res A 2007;82(1):1-9

85. Counis MF, Torriglia A. DNases and apoptosis. Biochem Cell Biol 2000;78(4):405-14

86. Schmitz T, Bravo-Osuna I, Vauthier C, et al. Development and in vitro evaluation of a thiomer-based nanoparticulate gene delivery system. Biomaterials 2007;28(3):524-31

87. Loretz B, Thaler M, Bernkop-Schnurch A. Role of sulfhydryl groups in transfection? A case study with chitosan-NAC nanoparticles. Bioconjug Chem 2007;18(4):1028-35

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