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Nanoparticles of glycol chitosan and its thiolated derivative significantly improved the pulmonary delivery of calcitonin

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ABSTRACT

A novel thiomer derivative of glycol chitosan (GCS) was synthesized by coupling with thioglycolic acid (TGA) and evaluated for the pulmonary delivery of peptides. Nanoparticles (NPs) based on GCS and GCS–TGA were obtained by the ionic gelation method and demonstrated a particle size in the range of 0.23–0.33 μm with positive surface charge and high calcitonin entrapment. Fluorescent GCS–TGA NPs resulted in a 2-fold increase in mucoadhesion to lung tissue after intra-tracheal administration to rats as compared to non-thiolated NPs. Evaluation of pulmonary toxicity revealed the biocompatibility of the two nanoparticulate formulations with lung tissue. The efficacy of the prepared NPs to enhance the pulmonary absorption of peptides was evaluated after pulmonary administration to rats using a liquid micro-sprayer technique. Calcitonin-loaded GCS and GCS–TGA NPs resulted in a pronounced hypocalcemic effect for at least 12 and 24 h, and a corresponding pharmacological availability of 27 and 40%, respectively. These findings suggest that both GCS and its thiomer derivative are promising and safe carriers for pulmonary peptide delivery.

Calcitonin is a polypeptide hormone that inhibits calcium resorption from bones by an action on osteoclasts. It is indicated for the treatment of postmenopausal osteoporosis, Paget's disease, and bone metastasis. Currently, calcitonin from salmon species is commercially available in the form of injection and intranasal spray (Miacalcin®). Data on the bioavailability of Miacalcin® shows great variability with an average bioavailability of 3% as compared to injection (Hinchcliffe et al., 2005). The systemic delivery of calcitonin via the lungs is as a promising alternative (Patton, 2000). In contrast to other mucosal barriers, the pulmonary epithelium is more permeable to macromolecules and has lower enzymatic activity (Patton, 1996). Yet, certain barriers still compromise the systemic absorption of protein drugs. These barriers include pulmonary mucus, mucociliary clearance, alveolar epithelium, pulmonary enzymes, basement membrane and macrophages (Agu et al., 2001). Over the last two decades, various approaches were evaluated for enhancing the absorption of proteins after pulmonary administration (Agu et al., 2001; Siekmeier and Scheuch, 2009). Table 1 summarizes the outcomes of these investigations with regard to calcitonin.

Recently, there is an increased interest in nanocarriers for pulmonary delivery. Compared to microparticles, nanoparticles have the potential to diffuse through the mucus layer and translocate through the alveolar epithelium by endocytosis (Yang et al., 2008). In addition, it is reported that particles smaller than 0.26 μm can escape from phagocytosis in the alveoli (Lauweryns and Baert, 1977). To enhance the retention of the nanocarrier in the lung and to prevent rapid elimination by ciliary movement, much concern is also given to mucoadhesive formulations.

Chitosans (CS) are cationic polysaccharides composed of D-glucosamine and N-acetyl-D-glucosamine units. In addition to safety, biological compatibility, and bioadhesive properties, CS can enhance the paracellular absorption of drugs by reversible opening of the tight junctions between the cells (Yamamoto et al., 2005). The mucoadhesive and permeation enhancing properties of CS are ascribed to ionic interaction between the positive amino groups of CS and anionic substructures in mucous layer or epithelial cells. However, the amino groups of CS have pK_a values of 5.5–6.5. Therefore, CS is subjected to aggregation and loss of surface charge at physiological pH values, which limits its usefulness as a carrier for pulmonary drug delivery. To optimize the physico-chemical and biological properties of CS, various chemical modifications have been introduced. Glycol chitosan, CS conjugated with ethylene glycol, is water soluble at the entire pH ranges and, at the same time, it retains its positive charge at physiological pH values (Trapani et al., 2009). Thiolated polymers, or thiomers, have been developed to further improve the mucoadhesive properties of well-established mucoadhesive polymers such as CS. Via chemical synthesis, a thiol group bearing small molecule is covalently bonded to the polymeric backbone. The reason for this kind of modification is based

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Table 1
Calcitonin pulmonary formulations.

Formulation	Dose[a], method of administration, animal model	Finding	Refs.
hCT and sCT solutions	50 μg/rat, intra-tracheal, rat	17% BA compared to i.v.	Patton et al. (1994)
eCT dry powder	20 IU/kg (5 μg/kg), intra-tracheal, rat	11.5% BA compared to i.v.	Komada et al. (1994)
sCT dry powder	320 U/subject, dry powder inhaler, human	28% relative bioavailability compared to i.m.	Deftos et al. (1997)
eCT/sodium glycocholate solution (10 mM)	6 IU/rat (1 μg/rat), intra-tracheal, rat	11.4% PA compared to i.v.	Yamamoto et al. (2001)
eCT/chitosan modified PLGA nanosphere suspension	100 IU/kg, pulmonary nebulizer, Guinea pig	Significant and prolonged reduction of blood calcemia	Yamamoto et al. (2005)
PEGylated sCT solution	80 μg/rat, intra-tracheal, rat	Improved stability and pharmacological response	Youn et al. (2008)
sCT powder or aqueous aerosol	670–790 IU/subject, pulmonary nebulizer, human	10–18% relative bioavailability compared to s.c.	Clark et al. (2008)

BA, absolute bioavailability; eCT, eel-calcitonin; hCT, human calcitonin; i.m., intramuscular; i.v., intravenous; s.c., subcutaneous; sCT, salmon calcitonin.
[a] Unit system and drug potency vary from one study to another depending on the supplier.

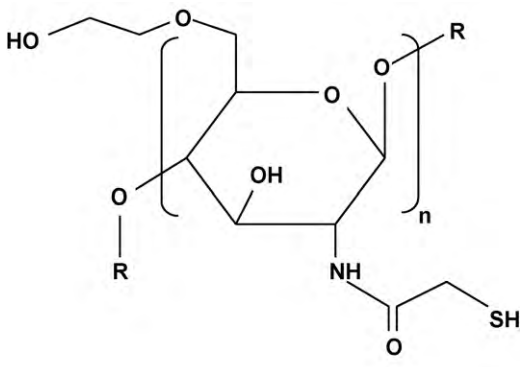

Fig. 1. Structural illustration of GCS–TGA polymer conjugate.

on the assumption that thiol groups of the polymer can form covalent disulfide bonds with thiol groups of cysteine-rich sub-domains of the mucus (Leitner et al., 2003).

It was the aim of this study to (1) synthesize a novel glycol chitosan–thioglycolic acid (GCS–TGA) polymer conjugate, (2) prepare GCS and GCS–TGA NPs by ionic gelation with tripolyphosphate (TPP), (3) evaluate the in vivo pulmonary mucoadhesive properties and toxic effects of the two nanoparticulate formulations, and finally (4) evaluate their role in enhancing the pulmonary absorption of incorporated peptide.

Chemical synthesis was performed by coupling amine groups of GCS with carboxylic groups of TGA using the carbodiimide method. Initially, one gram of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDAC, Sigma) was dissolved in 10% TGA (Sigma) solution with or without 10% N-hydroxysuccinimide (NHS, Sigma). The mixture was slowly added to 50 ml of 1% GCS (250 kDa, Sigma) aqueous solution, and the pH was adjusted to 6.0. The coupling reaction was allowed to proceed for 3 h at room temperature and the polymer was purified by repeated dialysis (3 days) followed by freeze drying. The amount of free sulphhydryl groups on the modified polymer was determined photometrically by Ellman's test (Werle and Hoffer, 2006), using free TGA as a calibrating standard (R^2 = 0.999). Schematic presentation of GCS–TGA polymer conjugate is depicted in Fig. 1. The density of sulphhydryl groups on the modified polymers was 20 ± 5 and 112 ± 12 μmol free thiol groups/g polymer using EDAC and EDAC/NHS for activation of TGA, respectively. NHS increases the reaction yield by forming a more stable amine-reactive intermediate ester with the carboxylic acid moiety (Weissenböck et al., 2004).

Fluorescence labeling of polymers was performed to facilitate quantitative detection of the NPs. Briefly, 1% GCS or GCS–TGA solution was prepared in 0.1% acetic acid, and fluorescein isothiocyanate (FITC, Sigma) in methanol was slowly added to the polymer solution and allowed to react in the dark for 3 h. The mixture was dialyzed in the dark against deionized water till no fluorescence was detected in the dialysis medium (Ex; 490 nm, Em; 520 nm), freeze-dried, and stored at 4 °C till further use.

The NPs were prepared by the method of ionic gelation using TPP (Sigma) as a cross linking polyanion. Briefly, 4 ml of TPP solution (1 mg/ml) in deionized water was slowly added to 5 ml of the polymer solution (3 mg/ml in acetate buffer pH 5.5) under magnetic stirring. For drug loading, calcitonin (5000 U/mg, Asahi, Japan) was pre-mixed with TPP solution to give a final concentration of 12.5 μg/ml of the NPs dispersion. The obtained dispersions were ultra-centrifuged (75,000 rpm, 45 min), and supernatant content of calcitonin was determined by Micro-BCA protein assay (Pierce, USA). The size and the surface charge of the prepared particles were determined using Zetasizer Nano (Malvern, UK). As indicated by Table 2, NPs in the size range of 0.23–0.33 μm with low polydispersity and positive surface charge were obtained. The lower surface potential of GCS–TGA NPs may be attributed to the partial decrease in the surface free amine groups, while larger particle size and higher drug entrapment efficiency may the result of inter- and intra-particular disulfide bonding (Sakloetsakun et al., 2009).

The pulmonary mucoadhesion and toxic effects of the NPs were evaluated after intra-tracheal administration to male Wistar rats (10 weeks). All animal experiments were approved by the animal welfare commission of Gifu Pharmaceutical University. The rats were fasted 24 h prior to I.P. injection of phenobarbital, trachea was exposed by a midline incision in the neck, and 5 cm long PE tubing was inserted. The rats were administered 1 ml of the fluorescent NPs through the intra-tracheal tube and maintained in a supine position. Two hours later, the rats were killed, the bronchoalveolar lavage fluid (BALF) was collected, and the whole lung tissue was separated for quantitative fluorescence analysis. The pulmonary toxicity was estimated by measuring the amount of proteins leached out from the epithelial tissue into the BALF (BCA

Table 2
Physico-chemical properties of GCS and GCS–TGA NPs.

Formulation	Particle size (μm)	Polydispersity	Zeta potential (mV)	Calcitonin entrapment (%)
FITC GCS NPs	0.232	0.213	25.7 ± 1.6	–
FITC GCS–TGA NPs	0.301	0.195	21.1 ± 0.3	–
GCS NPs	0.245	0.264	27.4 ± 4.1	54.2 ± 2.6
GCS–TGA NPs	0.332	0.337	22.3 ± 1.9	63.6 ± 5.9

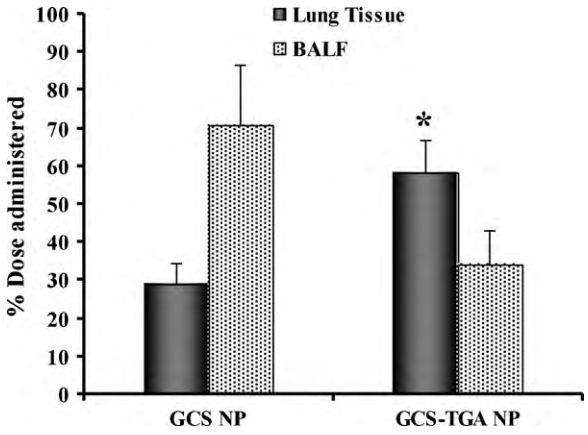

Fig. 2. Dose % of FITC-labeled GCS and GCS–TGA NPs in the lung tissue and BALF, 2 h after intra-tracheal administration to rats ($n = 3$, $^*P < 0.05$).

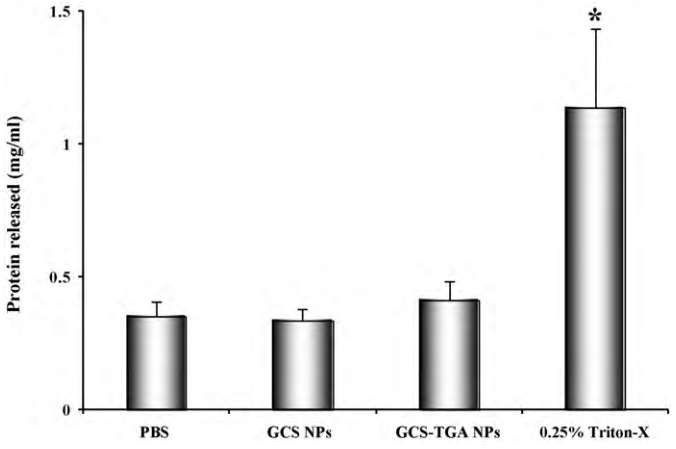

Fig. 3. Protein concentration in BALF 2 h after intra-tracheal administration of GCS and GCS–TGA NPs to rats ($n = 3$, $^*P < 0.05$ as compared to PBS).

protein assay), using PBS and 0.25% Triton-X as negative and positive controls, respectively. Fig. 2 shows the dose fraction of the administered fluorescent NPs in the lung tissue and BALF. The association of the NPs to the lung tissue was significantly improved about 2-fold by the attachment of thiol groups. We have previously shown that CS coating of PLGA NPs prolong their residence time in the lungs of guinea pigs (Yamamoto et al. 2005). Moreover, thiolated CS derivatives display strong mucoadhesive properties and can improve the epithelial uptake of drugs both in vitro and in vivo (Bernkop-Schnürch et al., 2006). The improved mucoadhesion is probably mediated by the formation of disulfide bonds between the surface thiol groups and mucin (Leitner et al., 2003). To investigate the potential toxic effects of the NPs, protein concentration in the BALF was measured as a biomarker for epithelial tissue damage (Fig. 3). The effect of GCS and GCS–TGA NPs was comparable to that of the control buffer, whereas 0.25% Triton-X resulted in significant tissue damage. These results indicate that mucoadhesion of GCS

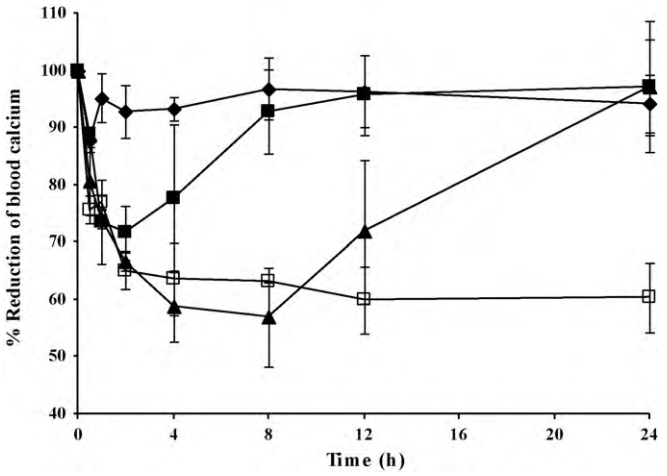

Fig. 4. Percent reduction of blood calcemia after pulmonary administration of PBS (♦), calcitonin solution (■), calcitonin-loaded GCS NPs (▲), or calcitonin-loaded GCS–TGA NPS (□), ($n = 3$, calcitonin dose = 25 μg/kg).

and its thiolated derivative NPs is not associated with epithelial damage or toxicity.

The efficiency of the prepared NPs in enhancing the pulmonary absorption of calcitonin was evaluated in vivo. Fasted rats were anesthetized by inhalation of isoflurane for a brief period (10–20 s). Calcitonin solution or NPs suspension (dose = 25 μg/kg, 0.4 ml/rat) was directly delivered to the lungs of anesthetized rats using a liquid Micro-Sprayer technique that generates an aerosol by an atomizer-attached syringe (PennCentury, USA). This method avoids surgical manipulation with animals and allows for a much deep and uniform deposition of the spray droplets in the lung. Following pulmonary administration, the rats recovered from anesthesia within 30 s. Blood was collected from jugular vein over an entire period of 24 h, and blood calcium level was determined using a commercially available calcium kit (Wako, Japan). Fig. 4 shows the blood calcium profiles of rats after pulmonary administration of different samples and Table 3 presents the calculated area above the blood calcium curves (AAC) and the pharmacological bioavailability (PA) values relative to s.c. injection. All calcitonin formulations immediately reduced blood calcemia of rats, confirming the higher permeability of lung epithelium to macromolecules as compared to the intestinal barrier (Makhlof et al., 2010). The relative short duration of action of free calcitonin may be the result of rapid elimination of drug solution by mucociliary clearance and/or drug degradation by pulmonary peptidases (Morita et al., 1994). On the other hand, GCS and GCS–TGA NPs significantly prolonged the hypocalcemic effect of calcitonin with approximate PA values of 27 and 40%, respectively. This improvement of drug absorption may be attributed to the mucoadhesive and permeation enhancing effects of GCS. Moreover, partial protection against enzymatic degradation is expected due to the steric hindrance effect of the NPs. The higher efficacy and longer duration of GCS–TGA NPs could be the result of its higher mucoadhesive property as aforementioned. The ability of thiolated polymers to form rigid and cohesive NPs by inter- and

Table 3
Pharmacodynamics of calcitonin absorption after s.c. and pulmonary administration to rats.

Formulation	Dose (μg/kg)	$AAC_{(0-24)}$	% PA
Calcitonin solution (s.c.)	5	447.4 ± 110.3	–
Calcitonin solution (pulmonary)	25	215.5 ± 145.2	9.63 ± 6.49
Calcitonin/GCS NPs (pulmonary)	25	609.9 ± 144.1	27.27 ± 6.44
Calcitonin/GCS–TGA NPs (pulmonary)	25	909.6 ± 26.2	40.67 ± 1.17

PA, pharmacological availability relative to s.c. administration.

intra-molecular disulfide bonding could allow for better control of drug release (Sakloetsakun et al., 2009).

Comparison of the results of the current study with previous findings should be made with caution because of the differences in the in vivo model, dose, calcitonin type, administration method, and evaluation procedure. However, GCS and its thiolated derivative should be considered as potentially safe and effective polymers for enhancing the pulmonary delivery of peptide drugs. Chitosan is generally regarded as biologically compatible and safe material as compared to the use of conventional protease inhibitors or surfactant enhancers (Siekmeier and Scheuch, 2008, Kean and Thanou, 2010). Its action is mainly mediated by transit opening of the tight junction between epithelial cells without inducing cell injury. Additionally, formulation of CS as NPs offers an appropriate size for avoiding alveolar macrophage clearance and promoting transepithelial transport in the alveolar region.

The pulmonary delivery of NPs may be challenged by a number of physico-chemical and physiological parameters that influence the inhalation deposition in the lungs. Small particles in the size range of 0.1–1.0 μm are inspired in the alveoli, but also exhaled without being deposited significantly (Scheuch and Siekmeier, 2007). Nebulization of NPs suspension as aerosol droplets (Dailey et al., 2003) or microencapsulation of NPs as dry powders (Grenha et al., 2005) that are appropriate for pulmonary administration are reported to enhance the handling and delivery of nanocarriers.

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