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Research paper

In vitro evaluation of the viscoelastic properties of chitosan–thioglycolic acid conjugates

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Abstract

The aim of this study was to evaluate the viscoelastic properties of chitosan–thioglycolic acid conjugates with different amounts of thiol groups immobilized on the polymer. The modification of chitosan was achieved via the covalent attachment of thioglycolic acid mediated by a carbodiimide. Chitosan–thioglycolic acid conjugates displaying 120, 209 and 439 μM thiol groups per gram of polymer were synthesized. The rheological properties of the three different conjugates were investigated. The elastic properties of the gels were found to increase significantly at pH 5.5. After 6 h the elastic modulus G' of chitosan–TGA 120, chitosan–TGA 209 and chitosan–TGA 439 gels increased 7-, 32- and 168-fold, respectively. In parallel the formation of disulfide bonds was observed. Accordingly, proof of principle that chitosan modified by the introduction of thiol groups has in situ gelling properties due to the formation of inter- and intramolecular disulfide bonds at physiological pH values is provided. Based on their in situ gelling properties, chitosan–thioglycolic acid conjugates seem to be very promising new excipients for liquid or semisolid formulations which should stabilize themselves once applied on the site of drug delivery.

Keywords: In situ gelling; Chitosan, Chitosan–thioglycolic acid conjugate; Thiomer

1. Introduction

Rapid clearance from the site of drug action is one important factor that limits the efficacy of drugs administered to the ocular, nasal and vaginal mucosa, either for local treatment or systemic effect. These routes have effective protective mechanisms that often lead to a poor bioavailability of applied drugs.

It is widely accepted that limiting the clearance by increasing the viscosity of a drug formulation and also prolonging the contact time through mucoadhesive interactions will result in an increased bioavailability of the drug. A very promising strategy to obtain drug formulations of sufficient viscosity is based on in situ gel formation. The formation of a gel at the site of drug delivery combines the advantages of a solution, being easy to administer for the patient, with the favorable properties of a gel. The sol–gel transition occurs as a result of physicochemical changes induced by the physiological environment.

So far, several concepts for in situ gelling systems have been investigated. Polymers can undergo sol–gel transition because of changes in pH (i) as described by Gurny et al. [1], in temperature (ii) as described by Edsman et al. [2] and Bromberg [3], or in electrolyte concentration (iii) as described by Deasy and Quigley [4] and Paulsson et al. [5]. A new concept for in situ gelling polymers has been introduced recently [6,7]. Polymers with thiol groups – so-called thiomers – display in situ gelling properties due to the formation of inter- and intramolecular disulfide bonds at physiological pH values. Thiomers have been reported to have excellent mucoadhesive properties as well [8–10]. The enhancement of mucoadhesion can be explained by the formation of covalent bonds between the thiol groups of the polymer and of the mucus layer.

Thiolated chitosan was investigated in this study. Chitosan, a biopolymer obtained by alkaline deacetylation of chitin, was used, since it exhibits several favorable properties such as biodegradability, non-toxicity and biocompatibility [11]. Furthermore, it was shown that the mucoadhesive properties of thiolated chitosan are strongly improved in comparison to unmodified chitosan [10]. Within this study chitosan was modified by the introduction of thioglycolic acid via amide bond formation.

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The aim of this study was to provide a proof of principle that chitosan–thioglycolic acid conjugates display in situ gelling properties due to the formation of inter- and intramolecular disulfide bonds at physiological pH values. The rheological behavior of chitosan–thioglycolic acid gels was investigated and in parallel the formation of disulfide bonds was monitored.

2. Materials and methods

2.1. Materials

Chitosan (molecular mass: ~150 kDa; degree of deacetylation: 83–85%; as specified by the supplier) was purchased from Fluka Chemie (Buchs, Switzerland). Thioglycolic acid (TGA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Synthesis of chitosan–thioglycolic acid conjugates

The immobilization of thiol groups on the surface of the cationic polymer chitosan was achieved by the covalent attachment of thioglycolic acid to the primary amino groups of chitosan via an amide bond. The coupling reaction was mediated by a carbodiimide. Chitosan–thioglycolic acid conjugates were synthesized by a method described previously [10].

In brief, 500 mg of chitosan were hydrated in 4 ml of 1 M HCl and dissolved by the addition of demineralized water to obtain a 1% solution of chitosan hydrochloride. Thioglycolic acid 500 mg was added to this solution. Thereafter EDAC dissolved in 1 ml demineralized water was added to a final concentration of 125 mM. During the coupling reaction the pH was kept constant at either 3, 4 or 5, by adding 1 M HCl. The reaction was modified with respect to the pH of the reaction mixture in order to obtain chitosan–TGA conjugates with different amounts of thiol groups immobilized on the polymer. The reaction mixtures were incubated for 3 h at room temperature under permanent stirring. In order to isolate the chitosan–TGA conjugates, the polymer solutions were dialyzed in tubings (molecular weight cut-off 12 kDa; dialysis tubings, cellulose membrane; Sigma, St. Louis, MO) for 3 days at 10 °C in the dark against 5 mM HCl, then twice against the same medium but containing 1% NaCl to reduce ionic interactions between the cationic polymer and the anionic sulfhydryl compound. Then the samples were dialyzed twice against 1 mM HCl to adjust the pH of the polymer to 4. Thereafter samples and controls were lyophilized by drying frozen aqueous polymer solutions at −30 °C and 0.01 mbar (Christ Beta 1-8K; Germany) and stored at 4 °C until further use. Samples prepared in the same way but omitting EDAC served as controls.

2.2.2. Determination of thiol group content

The degree of modification, i.e. the amount of thiol groups immobilized on chitosan, was determined spectrophotometrically with Ellman's reagent. First, 0.50 mg conjugate were hydrated in 250 μl of demineralized water. Then 250 μl 0.5 M phosphate buffer (pH 8.0) and 500 μl Ellman's reagent (3 mg of 5,5′-dithiobis(2-nitrobenzoic acid) in 10 ml of 0.5 M phosphate buffer, pH 8.0) were added. The samples were incubated for 3 h at room temperature. The supernatant was separated from the precipitated polymer by centrifugation (24 000 × *g*, 5 min). Thereafter 300 μl of the supernatant were transferred to a microtitration plate and the absorbance was measured at a wavelength of 450 nm with a microtitration plate reader (Anthos reader, 2001; Salzburg, Austria). Thioglycolic acid standards were used to calculate the amount of thiol groups immobilized on the polymer.

2.2.3. Formation of disulfide bonds

Chitosan–TGA conjugates were hydrated in demineralized water. The pH of the resulting solution was 4. After a hydration time of 1 h, 0.5 M acetate buffer, pH 5.5 was added. The final concentration of the polymer was 1.50% (m/v). Samples were incubated at 37 °C under permanent shaking. At predetermined time points aliquots of 50 mg were withdrawn and 50 μl of 1 M HCl was added in order to quench any further reaction. The amount of remaining thiol groups was determined spectrophotometrically with Ellman's reagent as described above.

2.2.4. Rheological studies

The viscoelastic properties of chitosan–TGA conjugates were determined with a cone-plate (C35/2°) rheometer (RotoVisco RT20, Haake GmbH, Karlsruhe, Germany). Chitosan–TGA conjugates and controls were hydrated in demineralized water. The pH of the resulting solution was 4. After a hydration time of 1 h, 0.5 M acetate buffer, pH 5.5 was added. The final concentration of the polymer was 1.50% (m/v). The samples were incubated at 37 °C. Preliminary strain sweep measurements were made to determine the linear viscoelasticity region for all samples. Dynamic oscillatory tests within the linear viscoelasticity region were performed on 1-ml aliquots of the samples at 2-h intervals over a time period of 6 h. The frequency was varied from 0.1 to 10 Hz.

The measurements were used to determine the following parameters: the phase angle δ, the elastic modulus (G'), and the viscous modulus (G''). Loss tangent (tan δ), a parameter that represents the ratio between the viscous and elastic properties of the polymer, was also calculated ($tan(\delta) = G''/G'$).

In parallel, the formation of disulfide bonds was monitored by determining the decrease in free thiol groups with Ellman's reagent as described above.

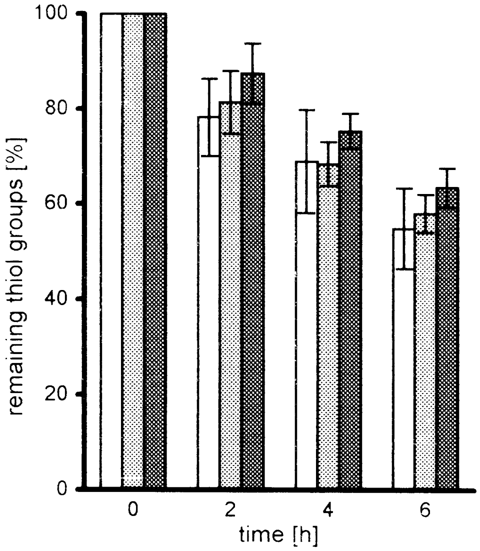

Fig. 1. Decrease of the thiol group content within a gel of 1.50% (m/v) chitosan–TGA 120 (white bars), chitosan–TGA 209 (light gray bars) and chitosan–TGA 439 (dark gray bars), respectively, at pH 5.5 and 37 °C. Indicated values are means (± SD, $n = 3$).

2.2.5. Statistical data analysis

Statistical data analysis was performed using the Mann–Whitney test with $P < 0.05$ as the minimal level of significance. Calculations were performed with the software Xlstat version 5.1 v1 (Addinsoft).

3. Results and discussion

3.1. Synthesis of chitosan–thioglycolic acid conjugates

Chitosan–TGA conjugates with increasing amounts of immobilized thiol groups were synthesized. Different degrees of modification with thioglycolic acid were achieved, dependent on the pH during the coupling reaction. The lyophilized polymers appeared as white, odorless powder of fibrous structure. For the conjugates, the resulting amount of thiol groups immobilized on 1 g of chitosan was determined to be 119.65 ± 3.59 μM (for chitosan–TGA 120), 208.56 ± 8.33 μM (for chitosan–TGA 209), and 438.98 ± 13. 22 μM (for chitosan–TGA 439). Less than 0.1% (m/m) of thioglycolic acid was detected in the controls.

3.2. Decrease of thiol group content within the polymer

The free thiol groups of thiolated polymers are oxidized depending on their pK_a value and the pH value of the thiomer solution. The pK_a value of the thiol group of thioglycolic acid covalently attached to chitosan was calculated by the software ChemSketch (Advanced Chemistry Development) to be 8.52. At a higher pH the decreasing H^+-concentration leads to a higher amount of negative thiolate anions, representing the active form for oxidation. This results in the formation of inter- and intramolecular disulfide bonds.

A significant decrease in thiol group content of chitosan–TGA conjugates could be observed at pH 5.5. Over a time period of 6 h the thiol groups were nevertheless not completely oxidized; more than 50% of the thiol groups remained stable. The results are illustrated in Fig. 1. They are in good agreement with results published previously [10], investigating the decrease of thiol groups in chitosan–TGA conjugate solutions at pH 5 and 6.5. At pH 4, thiol groups of chitosan–TGA conjugates remained stable towards oxidation.

As shown in Fig. 1, the total amount of thiol groups in the conjugate had no statistically significant influence on the rate at which disulfide bonds were formed, whereas a higher polymer concentration resulted in an increased oxidation process [12].

3.3. Rheological studies

Oscillatory measurements provide information about the dynamic properties, the elastic modulus G' and the viscous modulus G'' of a gel. The internal three-dimensional structure of the gel usually remains intact during oscillatory measurements, whereas it may be destroyed during a classical rotational viscometry test. Oscillatory measurements in this study were performed within the linear viscoelasticity region and therefore describe the properties of the intact gel.

The rheological investigation of chitosan–TGA solutions demonstrated a clear correlation between the total

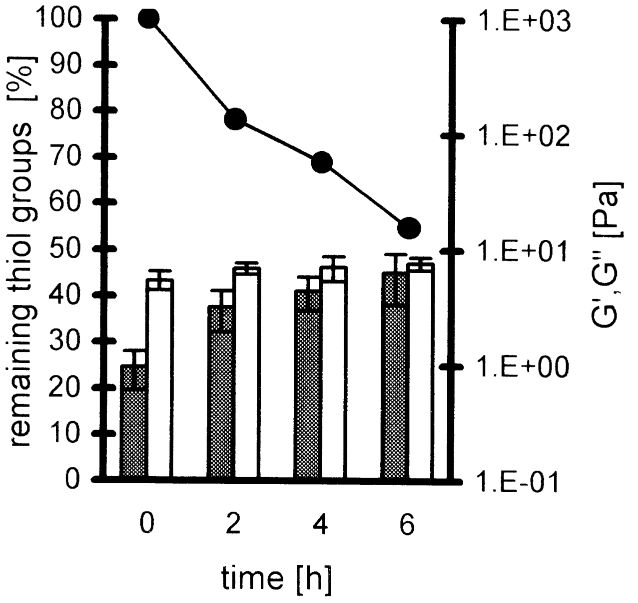

Fig. 2. Correlation between the increase of G' and G'' and the decrease of free thiol groups of chitosan–TGA 120 at pH 5.5. Indicated values are means (± SD, $n = 3$) of thiol content as percent of the total amount (-●-), the elastic modulus G' (gray bars) and the viscous modulus G'' (white bars).

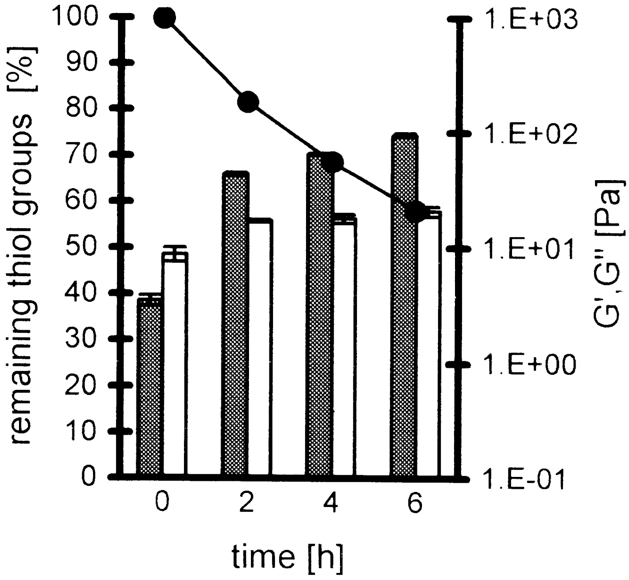

Fig. 3. Correlation between the increase of G' and G'' and the decrease of free thiol groups of chitosan–TGA 209 at pH 5.5. Indicated values are means (± SD, $n = 3$) of thiol content as percent of the total amount (-●-), the elastic modulus G' (gray bars) and the viscous modulus G'' (white bars).

amount of polymer-linked TGA and the in situ gelling properties due to the formation of intra- and intermolecular disulfide bonds at pH 5.5. The controls did not display any changes in their viscoelastic behavior over a time period of 6 h (data not shown), whereas the elastic modulus of chitosan–TGA 120, chitosan–TGA 209 and chitosan–TGA 439 was significantly increased after 6 h compared to the same solutions at $t = 0$. Results of rheological studies in comparison with the formation of disulfide bonds are shown in Figs. 2–4. In order to simplify the comparison of the rheological behavior of different chitosan–TGA conjugates the indicated values of G' and G'' at a single representative frequency (1 Hz) are plotted as a function of time, although this approach does not characterize the complete viscoelastic behavior of the samples.

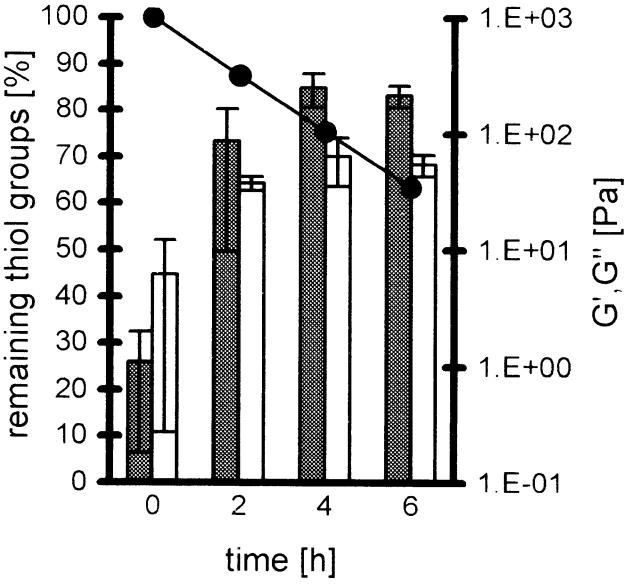

Fig. 4. Correlation between the increase of G' and G'' and the decrease of free thiol groups of chitosan–TGA 439 at pH 5.5. Indicated values are means (± SD, $n = 3$) of thiol content as percent of the total amount (-●-), the elastic modulus G' (gray bars) and the viscous modulus G'' (white bars).

Table 1

Loss tangent (tan δ) measured at a frequency of 1 Hz for 1.50% (m/v) solutions of chitosan–TGA at pH 5.5 over a time period of 6 h[a]

Time (h)	Loss tangent		
	Chitosan–TGA 120	Chitosan–TGA 209	Chitosan–TGA 439
0	6.06 ± 1.57	2.65 ± 0.66	9.67 ± 7.32
2	2.17 ± 0.14	0.40 ± 0.06	1.75 ± 2.28
4	1.67 ± 0.24	0.28 ± 0.02	0.35 ± 0.36
6	1.26 ± 0.26	0.22 ± 0.02	0.27 ± 0.12

[a] Indicated values are means ($n = 3$, ± SD).

The greater the number of thiol groups immobilized on chitosan, the higher was the increase in elastic modulus G' in chitosan–TGA solutions over a time period of 6 h. The elastic modulus of chitosan–TGA 120, chitosan–TGA 209 and chitosan–TGA 439 gels increased 7-, 32- and 168-fold, respectively. The ratio between the elastic modulus and the viscous modulus is described by tan δ. The significant increase in the elastic properties of chitosan–TGA gels is evidenced by a decrease in tan δ (Table 1). The sol–gel transition of chitosan with the lowest amount of thiol groups available for cross-linking seems to be incomplete after 6 h, whereas tan δ is smaller than 1 for chitosan 209 and 439 in accordance with their elastic nature. After a gelation time of 2 h G' and G'' were not influenced by the frequency of oscillation (Fig. 5). This indicates the formation of a cross-linked gel. At $t = 0$ h, i.e. before the formation of disulfide bonds could take place, G' shows a decline at low frequencies. This is typical for a system that is only physically entangled [13].

A pH of 5.5 was chosen for this study as a compromise with regard to possible application on vaginal, nasal or ocular mucosa. Since the formation of disulfide bonds in chitosan–TGA conjugates could be observed within a pH range of 5–6.8, in situ gel formation should be possible within this pH range as well. Due to the faster formation of disulfide bonds at pH 6.8, the gel formation should also take place more rapidly in comparison to chitosan–TGA solutions gelling at pH 5.5.

Long-term stability of chitosan–TGA solutions at pH values between 5 and 7.4 can be achieved by storage under inert conditions in single dose units. The absence of oxygen prevents the formation of disulfide bonds during storage, so that gelation occurs only after administration. Solutions or gels for vaginal drug delivery could be filled at pH 4 under inert conditions in multi-dose units where the stability of free thiol groups can be guaranteed for a duration of therapy

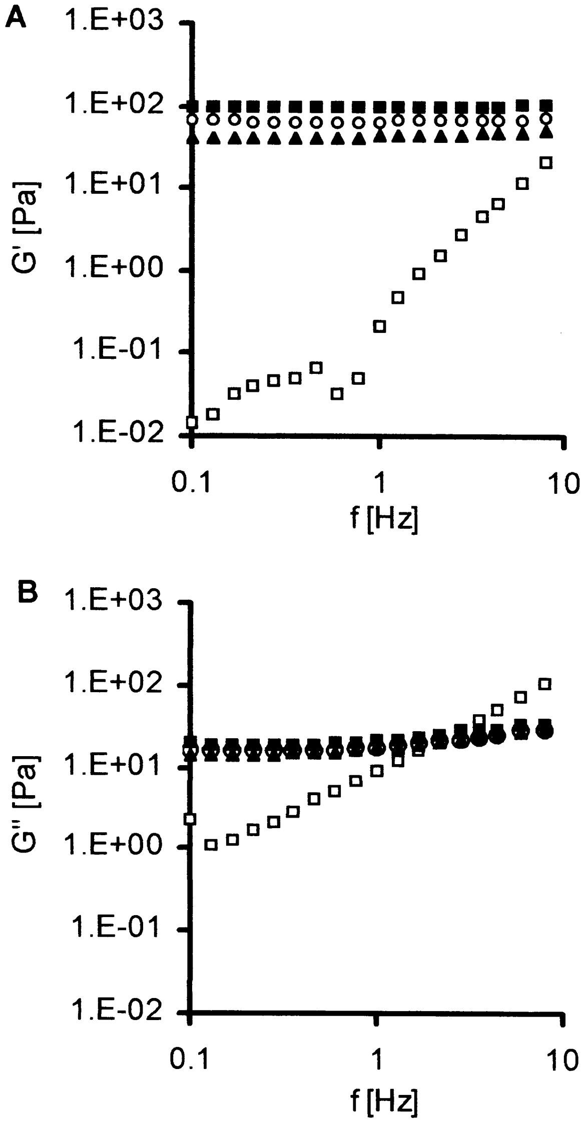

Fig. 5. Example of 'typical' mechanical spectra of chitosan–TGA gels. (A) The elastic modulus G' of chitosan–TGA 209 after 0 (□), 2 (▲), 4 (○) and 6 (■) h of gelation at pH 5.5. G' is plotted against oscillating frequency. (B) The viscous modulus G'' of chitosan–TGA 209 after 0 (□), 2 (▲), 4 (○) and 6 (■) h of gelation at pH 5.5. G'' is plotted against oscillating frequency.

of about 4 weeks. Gelation of the polymer takes place on the vaginal mucosa after a pH shift to pH 5.

3.4. Views on chitosan–thioglycolic acid conjugates

The features of chitosan–thioglycolic acid conjugates are very advantageous from the drug delivery point of view. In this study they were shown to form gels of high elasticity at physiological pH values. In combination with the strongly improved mucoadhesive properties [10], chitosan–thioglycolic acid conjugates could be used to provide a longer contact time of dosage forms with mucosal tissues. Recently, the efficacy of thiolated chitosan was verified in vivo. In contrast to unmodified chitosan, tablets based on a thiolated chitosan matrix could significantly increase the oral bioavailability of calcitonin [14]. Chitosan–thioglycolic acid conjugates also seem to be very promising new excipients for liquid or semisolid formulations which should stabilize themselves once applied on the site of drug delivery.

Since one problem in vaginal drug delivery is the rapid removal of inserted systems [15,16], the mucoadhesive and in situ gelling properties of chitosan–TGA might be advantageous in vaginal drug delivery systems. Chitosan–TGA was already evaluated in vitro as a potential carrier matrix for vaginal tablets for the local delivery of the antimycotic drug clotrimazole. With chitosan–TGA it was possible to significantly improve the cohesive and mucoadhesive properties of the delivery system in comparison to unmodified chitosan [17]. Based on another thiomer – thiolated poly(acrylic acid) – a novel vaginal delivery system with a mucoadhesive gel was developed for the systemic delivery of progesterone [6].

One strategy to increase the poor bioavailability of ocularly applied drugs is the prolongation of the ocular residence time with mucoadhesive and/or viscoelastic polymeric excipients. In situ gel formation is a new and promising strategy to obtain polymer solutions of sufficient viscosity on the surface of the eye [18,19]. Chitosan was also shown to significantly prolong the residence time of ophthalmic formulations [20]. For the use of chitosan–TGA in ocular drug delivery systems the polymer still has to be optimized in order to shorten its gelation time. The formation of disulfide bonds can be accelerated for example by decreasing the pK_a of the thiol groups via electrostatic interactions or by addition of traces of Cu^{2+} [21]. This will be the subject of further studies.

Another possible use for chitosan–TGA conjugates might be as excipients in nasal drug delivery. With in situ gelling and mucoadhesive polymeric excipients the residence time of the drug on the nasal mucosa can be prolonged since the major disadvantage is the removal of substances by mucociliary activity [22]. It was demonstrated that unmodified chitosan is able to improve the transnasal absorption of polar drugs such as peptides [23]. The mechanism of action was suggested to be a combination of mucoadhesion and a transient opening of the tight junctions in the mucosal membrane [24]. According to this, formulations for nasal drug delivery containing chitosan–TGA conjugates with improved mucoadhesive and viscoelastic properties might increase the bioavailability of polar drugs much more than unmodified chitosan.

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