International Journal of Pharmaceutics 362 (2008) 88–92

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Improved adhesion to mucosal cells of water-soluble chitosan tetraalkylammonium salts

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ARTICLE INFO

Article history:
Received 22 April 2008
Received in revised form 13 June 2008
Accepted 17 June 2008
Available online 26 June 2008

Keywords:
Chitosan tetraalkylammonium salts
Ultrasound
Sonochemical reaction
Bioadhesivity
Buccal cells
In vitro test

ABSTRACT

Chitosan is a natural polymer whose bioadhesive properties make it a useful material for filming over and protecting damaged or sensitive mucosae. Much effort has been expended to develop this employ, and new applications are in the offing. The aim of the present study was to optimize the synthesis under sonochemical conditions of water-soluble chitosan tetraalkylammonium salts and to assess the mucoadhesive properties of the resulting water-soluble cationic polyelectrolytes. Aqueous solutions of several tetralkylammonium chitosan derivatives, viz. *N*-trimethyl- (1), *N*-diethylmethyl- (2), *N*-carboxymethyl- (3) and *N*-[*N*,*N*-diethylaminomethyl(diethyldimethylene ammonium)$_n$]methylchitosan (4) were tested along with the parent biopolymer and its citric acid salt (5), both at neutral and acidic pH. We used a published technique for evaluating *in vitro* bioadhesion to isolated buccal cells, a mucosal model that can predict bioadhesive behavior *in vivo*. Derivatives 1 and 4 gave the best results.

1. Introduction

In recent years much work has been aimed to improve mucosal and transmucosal drug delivery. This can follow different routes (e.g. ocular, buccal, nasal, rectal, vaginal), whether for topical or systemic therapy. The buccal route appears to offer several advantages compared with other ones: it leads to a rapid onset of action and can achieve high blood levels, avoiding first-pass inactivation of the drug and its exposure to the gastrointestinal contents (Ahn et al., 2001). Mucoadhesive materials can be used as therapeutic agents in their own right, to coat and protect damaged tissues (gastric ulcers or lesions of the oral mucosa) or as lubricating agents (in the oral cavity, eye and vagina) (Smart, 2005). Hence the currently active search for new, efficient protective agents, especially natural and polymeric ones, that are notable for various specific advantages (water solubility, prolonged effect, etc.). From this standpoint chitosan, a natural biopolymer, is of considerable interest as a basic matrix for making protective films.

Chitosan, a (1,4)-linked 2-amino-2-deoxy-β-D-glucan, is prepared by *N*-deacetylation of chitin, or poly-β-(1-4)-*N*-acetyl-D-glucosamine, which is (after cellulose) the most abundant natural polymer, being a structural component of shellfish, insects hexoskeleta and cell walls of bacteria and fungi. Because it is biocompatible and biodegradable (Hirano et al., 1988), devoid of toxicity and antigenic power, chitosan has been recommended for a variety of biomedical applications, including wound dressing and drug delivery systems. It has also been shown to be mucoadhesive and to enhance the penetration of macromolecules across intestinal, buccal and nasal mucosae (Illum et al., 1994). These potential applications are however severely limited by its insolubility at neutral pH, that is to say in most physiological environments (Singla and Chawla, 2001). Chitosan will dissolve in water only if pH is lowered below 6.0, when a substantial fraction of its amino groups becomes hence protonated; it is usually solubilised by adding 1% acetic acid, lactic acid or aqueous hydrochloric acid. In order to obtain chitosan derivatives that will dissolve in water at neutral or basic pH, many efforts have been made to covalently attach hydrophilic groups to reactive amino groups at the C2 position. Several synthetic strategies have been proposed. Many derivatives have been prepared by introducing in the macromolecule such polar groups as carboxymethyl, dihydroxyethyl, sulfate, phosphate, hydroxyalkylamino groups, etc. (Kurita, 2001). We tried

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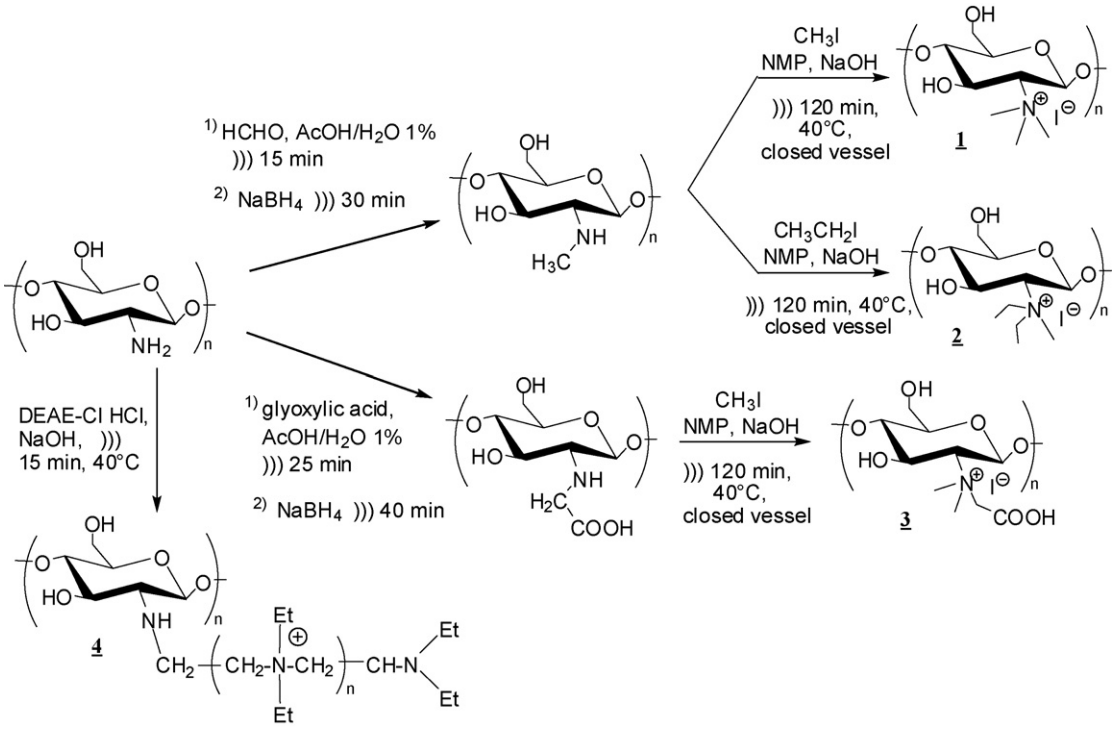
doi:10.1016/j.ijpharm.2008.06.017



Scheme 1.

the grafting of chitosan with cyclodextrins (Binello et al., 2004; Aime et al., 2006); however, chitosan-βCD adducts proved less mucoadhesive than the parent polymer (Venter et al., 2006). Other authors grafted chitosan with sugars (Park et al., 2003), polypeptides such as polylisine (Chi et al., 2008) and betaine (Stepnova et al., 2007).

The present work aimed: (1) to quaternize amino groups of chitosan or to attach to it extra tetraalkylammonium groups using optimized sonochemical synthetic protocols and (2) to assess the mucoadhesive properties of the resulting water-soluble cationic polyelectrolytes. In fact one of the known strategies to increase the positive charge density and the solubility of chitosan is to introduce quaternary ammonium groups (Muzzarelli and Tanfani, 1985) by reacting the polymer with an alkyl halide. This modification improves its properties as a drug carrier across epithelial barriers (Thanou et al., 2001), also increases its antimicrobial activity (Rabea et al., 2003). Numerous quaternization studies have been carried out with an alkyl iodide or glycidyltrimethylammonium chloride (Lim and Hudson, 2004). Unfortunately, these reactions usually proceed at basic pH and relatively high temperatures (70–90 °C), under which conditions chitosan is in the form of a fine, heterogeneous dispersion. This method led to a non-random distribution of quaternary groups and the formation of highly 3-*O*-alkylated and 6-*O*-alkylated side-products of lesser solubility (Polnok et al., 2004) as well as other by-products, *N*-methyl- and *N*,*N*-dimethylchitosans (Snyman et al., 2002).

In a previous paper we had described several efficient protocols for the chemical modifications of chitosan under power ultrasound (US) (Cravotto et al., 2005a,b). Besides cutting down reaction times, our experimental procedure did not lead to blockwise modification. Following this start, here we describe the preparation under US of several tetraalkylammonium salts of chitosan (Scheme 1).

These derivatives, along with the parent chitosan and its citric acid salt, were then studied by a technique for evaluating *in vitro* polymer bioadhesion to buccal cells, a mucosal model that can predict bioadhesive behavior *in vivo* (Patel et al., 1999).

2. Materials and methods

2.1. Materials

Chitosan of medium molecular weight, Streptavidin peroxidase, biotinylated concanavalin A from *Canavalia ensiformis* (Con-A), *o*-phenylenediamine dihydrochloride (*o*-pd), trypan blue, hydrogen peroxide, and other reagents of analytical or high-purity grade were purchased from Sigma–Aldrich (Italy); all solvents from Carlo Erba (Italy). NMR and IR spectra were recorded on a Bruker Avance 300 spectrometer and a Shimadzu FT-IR 8001 spectrophotometer, respectively.

2.2. General procedure for reductive amination

The procedure first described by Kim et al. (1997) and adopted by Jia et al. (2001) was more efficiently carried out under power ultrasound using an improved sonochemical reactor (18.6 kHz) (Cravotto et al., 2005a,b). Chitosan powder (5 g) was suspended in 110 mL hot deionized water (60 °C), then 1.5 mL of glacial acetic acid were added and the mixture was sonicated 15 min at 60W with a titanium horn inserted in the reaction vessel, a 250 mL tube made of PTFE (Teflon®). An excess of aldehyde (formalin 35%, 60 mL, or glyoxylic acid, 5 g) was added and the solution was sonicated 15–25 min (150 W) in a closed reactor whose top was joined to the horn by an elastomeric sleeve fastened to it with a tight seal. Then $NaBH_4$ (2.5 g) was added portionwise to the mixture under sonication (40 W for 10 min, then 100 W for 20–30 min) in the same vessel. The reacted mixture was brought to pH 9 with 0.5N NaOH and the product, after precipitation with acetone (30 mL), was collected on a paper filter in a Buckner funnel. The crude precipitate of monoalkylamino derivative was washed twice with 70 mL acetone.

2.3. General procedure for N-alkylation to prepare quaternized chitosan derivatives **1**–**3**

Each chitosan monoalkylamino derivative (2.5 g) was dispersed in NMP (100 mL) under nitrogen atmosphere in the above-mentioned, closed sonochemical reactor (5 min, 80 W). Then NaOH and the alkyl iodide (MeI or EtI, in a four-fold excess to chitosan amino groups) and NaI (1 eq) were added. The mixture was sonicated 120 min at 50 °C in the closed reactor. The product, precipitated with acetone (80 mL), was collected on a paper filter in a Buckner funnel and dried under vacuum.

2.4. Preparation of N-[N,N-diethylaminomethyl(diethyldimethylene ammonium)$_n$]methyl chitosan (**4**)

We modified the procedure described by Zambito et al. (2006) to work under ultrasound. Our sonochemical method avoided the preliminary preparation of chitosan microparticles. Aqueous 15% NaOH was slowly dropped at 40 °C under sonication (15 min, 120 W) into a mixture of chitosan (5 g) and 2-diethylaminoethyl chloride (9 g, 50.2 mmol) until pH rose to 8. Work-up and purification followed the original procedure.

2.5. Solutions of chitosan derivatives

Each chitosan derivative was dissolved in 0.9% saline to a concentration of 0.05% (w/v). The pH of the solutions was adjusted to 7.6 or 5.5 with concentrated aqueous sodium hydroxide or hydrochloric acid; chitosan however was used at pH 5.5 exclusively in all experiments. The solutions of derivatives were stored at 4 °C for at least 48 h to allow the polymer chains to become fully hydrated prior to biological testing.

2.6. Harvesting of buccal cells

Young healthy volunteers, students at the University of Catania (from 10 to 12, male and female in equal numbers) provided the buccal cells. Donors were required not eat or drink for 30–60 min prior to harvesting of the cells. These were removed by gently scraping the inner aspect of the cheeks with a wooden spatula. The cells were pooled and suspended in 10 mL isotonic 0.05 M Tris buffer saline (TBS), pH 7.6 (Patel et al., 1999). A 0.9 mL sample of the suspension was then added to 0.1 mL 0.5% (w/v) trypan blue. The total number of cells was determined using a haemocytometer. For each test 48×10^4 cells were used immediately after harvesting.

2.7. Con A/o-pd assay

Our technique was based on that described by Patel et al. (1999). Polymer solutions were tested at concentrations of 0.5% (as reported by Patel) and 0.05%. Because our derivatives were equally active at the lower concentration, only the results obtained with the latter are reported here.

The buccal cell suspension was centrifuged at 2000 rpm for 5 min. Each polymer solution (5 mL) was added to the cell suspension (2 mL) and incubated for 15 min at 30 °C under gentle shaking. A control contained saline in place of the solution of chitosan derivative.

The cells were then washed twice by adding 5 mL isotonic 0.05 M TBS (pH 7.6 or 5.5) followed by centrifugation at 2000 rpm for 5 min. After the second wash the cells were transferred to a clean tube and given a final wash. This step was necessary, that any material bound to the walls of the tube might not be carried over and interfere with the assay. The cells were sedimented by centrifuging at 2000 rpm for 5 min, after which all but 2 mL of the supernatant was removed. The residue was then vigorously stirred with a vortex mixer and washed by adding 12 mL isotonic 0.05 M TBS (pH 7.6) followed by centrifugation at 2000 rpm for 5 min. Chitosan was assayed at a lower pH by adjusting its suspension in 0.9% w/v saline to pH 5.5, then washing with isotonic phosphate buffer saline, pH 5.5, in place of TBS. The washing step was repeated twice, after which the cells were transferred to a clean tube and given a final wash prior to the addition of the next reagent. 5 mL of 0.05 M TBS, pH 7.6, or phosphate buffer saline pH 5.5 (for chitosan), containing 1 mM calcium chloride and 10 mg/L biotinylated Con-A were added to the cells and the mixture was incubated at 30 °C for 30 min under gentle shaking. It was then centrifuged at 2000 rpm for 5 min and the supernatant removed leaving 2 mL buffer. The cells were washed twice with TBS, and their suspension transferred to a clean tube. 5 mL 0.125 M TBS containing 5 mg/L streptavidin peroxidase were added and the tube was incubated at 30 °C for 60 min under gentle shaking. The cells were then washed twice, transferred to a clean tube, and washed again. To each pellet 1 mL of *o*-pd solution (containing 0.4 mg *o*-pd and 0.4 μL 30% H_2O_2 in 1 mL 0.05 M citrate phosphate buffer) was added, and the suspension constantly stirred. The oxidation of *o*-pd to produce a yellow color was stopped after 2 min with 1 mL of 1 M H_2SO_4 and the optical density measured at 492 nm (spectrophotometer from Genesis, Sigma–Aldrich, Italy).

A control was run in parallel which was identical to the above except that cells were exposed to plain saline in place of chitosan or a chitosan derivative. Results were expressed as percentage reduction relative to the control. The whole experiment was repeated 5 times for the control, chitosan and each derivative.

2.8. Statistical analysis

Statistical analysis of results was performed by means of one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons with control. All the statistical analyses were performed using the statistical software package SYSTAT, Version 9 (Systat Inc., Evanston, IL, USA).

3. Results and discussion

In this study we used 5 different chitosan salts that have been previously described and characterized by others. We introduce here new improved synthetic protocols carried out under US. The most widely reported quaternized chitosan is the *N*-trimethyl derivative (**1**) (Amidi et al., 2006), while *N*-diethylmethyl chitosan (**2**) was first described by Avadi et al. (2004), whose NMR spectra have been recently discussed by Sadeghi et al. (2008). As depicted in Scheme 1, tetraalkylammonium salts **1**–**3** were obtained in two steps: reductive amination with formaldehyde (**1**, **2**) or glyoxylic acid (**3**) followed by quaternization with methyl iodide (**1**, **3**) or ethyl iodide (**2**). Reductive amination with glyoxylic acid is an efficient way to selectively prepare *N*-carboxymethyl chitosan (CMC), an improvement over the classic reaction with monochloroacetic acid that affords mixtures of *N*- and *O*-carboxymethyl derivatives (Chen et al., 2002, 2003). It is well known that CMC can be dissolved in water at acidic, neutral or basic pH provided the carboxymethylation degree is more than 50–60%. In product **4** the cationic nitrogen is not that of glucosamine but lies on the DEAE side chain (Sadeghi et al., 2008). We propose here a sonochemical version of the procedure described by Zambito et al. (2006). Compound **5** was a simple salt prepared by adding a stoichiometric amount of citric acid to an aqueous solution of chitosan and freeze-drying the product.

Table 1
Percentage reduction of lectin binding after treatment with chitosan derivatives

Entry	Mean percentage reduction in lectin binding relative to control at pH 7.6	Mean percentage reduction in lectin binding relative to control at pH 5.5
Chitosan	65 ± 3	66 ± 4
1	76 ± 5[a]	74 ± 6[a]
2	68 ± 2	72 ± 2[a]
3	None	60 ± 3
4	78 ± 4[a]	75 ± 2[a]
5	15 ± 4	None

[a] Significant compared to chitosan.

All spectroscopical data of derivatives **1**–**4** were in good agreement with published ones.

The basis of our assay is the following: if chitosan or one of its derivatives binds to buccal cells, it will mask cell–surface glycoconjugates, hence proportionally suppress Con-A lectin binding.

Our results indicate that chitosan significantly inhibited lectin binding (65 ± 3% inhibition compared to the control), confirming it to be an effective mucoadhesive (Table 1). **2** also caused a large reduction of binding (68 ± 2% compared to the control). Among our five derivatives, **1** and **4** showed the greatest reduction (76 ± 5 and 78 ± 4, respectively); **3** showed no significant effect (*t*-test >5), and **5** but a modest one (15 ± 4%).

The same results were found when our chitosan derivatives were tested at acidic pH (5.5) except for compound **2**, for which the reduction value was 72 ± 3, demonstrating an improved mucoadhesion compared to chitosan. This result was probably due to the higher solubility of **2** at pH 5.5.

Bioadhesion is said to take place when two materials, at least one of which is biological in nature, are held together for extended periods of time by interfacial forces (Smart, 2005). In the pharmaceutical sciences, when the adhesive attachment is to mucus or a mucous membrane, the phenomenon is called mucoadhesion (Gu et al., 1988). The most widely investigated group of mucoadhesives, the so-called "first-generation" ones, consists of hydrophilic macromolecules containing numerous hydrogen-bond-forming groups (Harding et al., 1999; Lee et al., 1993). The presence of hydroxyl, carboxyl or amino groups on the macromolecule favours adhesion. Typical examples are carbomers, chitosan, sodium alginate and some cellulose derivatives (e.g. sodium carboxymethylcellulose and hydroxypropylmethylcellulose). These were initially used because they were available "off-the shelf" and had regulatory approval, but in last few years new, more effective materials have been developed. In fact the formulation of first-generation mucoadhesive polymers presents no mean hurdles, as they are poorly soluble in non-aqueous solvents; in water even at low concentrations they form highly viscous, often pH-sensitive solutions. In the case of cationic polymers like chitosan, the positive charge will favour binding to a negatively charged surface although *in vivo* binding to soluble luminal mucins may inhibit this effect (Fiebrig et al., 1995).

At low pH values the fraction of protonated basic groups, depending on the degree of deacetylation of chitosan, can be relatively high, so as to provide a large number of potential interaction sites for the cells. It was in order to increase this further, and ensure that it is maintained even at pH 7.6, we prepared some water-soluble chitosan tetraalkylammonium salts.

4. Conclusion

In this paper we present a number of sonochemical reactions carried out on chitosan to yield several water-soluble tetraalkylammonium salts that showed improved adhesion to mucosal cells. The possibility of employing these compounds at neutral, not just acidic pH opens the road for their wide application to mucosal treatments, in particular for filming over aphtae or other painful mucosal lesions of the oral cavity whose treatment with acidic formulae is objectionable because it is bound to cause irritation.

Acknowledgements

This work was financially supported by Regione Piemonte—CIPE 2004 and the University of Catania.

References

Ahn, J.S., Choi, H.K., Cho, C.S., 2001. A novel mucoadhesive polymer prepared by template polymerization of acrylic acid in the presence of chitosan. Biomaterials 22, 923–928.
Aime, S., Gianolio, E., Barge, A., Tagliapietra, S., Cravotto, G., 2006. New paramagnetic supramolecular adducts for MRI applications based on non-covalent interactions between Gd(III)-complexes and beta- or gamma-cyclodextrin units anchored to chitosan. J. Inorg. Biochem. 100, 931–938.
Amidi, M., Romeijn, S.G., Borchard, G., Junginger, H.E., Hennink, W.E., Jiskoot, W., 2006. Preparation and characterization of protein-loaded *N*-trimethyl chitosan nanoparticles as nasal delivery system. J. Control. Release 111, 107–116.
Avadi, M.R., Mahdavinia, G., Sadeghi, A.M., Erfan, M., Amini, M., Tehrani, M.R., Shafiee, A., 2004. Synthesis and characterization of *N*-diethyl methyl chitosan. Iran. Polym. J. 13, 431–436.
Binello, A., Cravotto, G., Nano, G.M., Spagliardi, P., 2004. Synthesis of chitosan–cyclodextrin adducts and evaluation of their bitter-masking properties. Flavour Fragr. J. 19, 394–400.
Chen, L.Y., Du, Y.M., Wu, H.Q., Xiao, L., 2002. Relationship between molecular structure and moisture-retention ability of carboxymethyl chitin and chitosan. J. Appl. Polym. Sci. 83, 1233–1241.
Chen, L., Du, Y., Huang, R., 2003. Novel pH, ion sensitive polyampholyte gels based on carboxymethyl chitosan and gelatin. Polym. Int. 52, 56–61.
Chi, P., Wang, J., Liu, C., 2008. Synthesis and characterization of polycationic chitosan-graft-poly(L-lysine). Mater. Lett. 62, 147–150.
Cravotto, G., Omiccioli, G., Stevanato, L., 2005a. An improbe sonochemical reactor. Ultrason. Sonochem. 12, 213–217.
Cravotto, G., Tagliapietra, S., Trotta, M., Robaldo, B., 2005b. Chemical modification of chitosan under high-intensity ultrasound. Ultrason. Sonochem. 12, 95–98.
Fiebrig, I., Harding, S.E., Rowe, A.J., Hyman, S.C., Davis, S.S., 1995. Transmission electron microscope studies on pig gastric mucin and its interactions with chitosan. Carbohydr. Polym. 28, 239–244.
Gu, J.M., Robinson, J.R., Leung, S.H.S., 1988. Binding of acrylic polymers to mucin/epithelial surfaces: structure property relationships. Crit. Ther. Drug Carr. Syst. 5, 21–67.
Harding, S.E., Davis, S.S., Deacon, M.P., Fiebrig, I., 1999. Biopolymer mucoadhesives. Biotechnol. Genet. Eng. Rev. 16, 41–85.
Hirano, S., Seino, H., Akiyama, Y., Nonaka, I., 1988. Biocompatibility of chitosan by oral and intravenous administration. Polym. Eng. Sci. 59, 897–901.
Illum, C., Farraj, N.F., Davis, S.S., 1994. Chitosan as a novel nasal delivery system for peptide drugs. Pharm. Res. 11, 1186–1189.
Jia, Z., Shen, D., Xu, W., 2001. Synthesis and antibacterial activities of quaternary ammonium salt of chitosan. Carbohydr. Res. 333, 1–6.
Kim, C.H., Choi, J.W., Chun, H.J., Choi, K.S., 1997. Synthesis of chitosan derivatives with quaternary ammonium salt and their antibacterial activity. Polym. Bull. 38, 387–393.
Kurita, K., 2001. Controlled functionalization of the polysaccharide chitin. Progress in Polymer Science 26, 1921–1971.
Lee, J.W., Park, J.H., Robinson, J.R., 1993. Bioadhesive-based dosage forms: the next generation. J. Pharm. Sci. 89, 253–270.
Lim, S.H., Hudson, S.M., 2004. Synthesis and antimicrobial activity of a water-soluble chitosan derivative with a fiber-reactive group. Carbohydr. Res. 339, 313–319.
Muzzarelli, R.A., Tanfani, F., 1985. The *N*-permethylation of chitosan and the preparation of *N*-trimethyl chitosan iodide. Carbohydr. Polym. 5, 297–307.
Park, J.H., Cho, Y.W., Chung, H., Kwon, I.C., Jeong, S.Y., 2003. Synthesis and characterization of sugar-bearing chitosan derivatives: aqueous solubility and biodegradability. Biomacromolecules 4, 1087–1091.
Patel, D., Smith, A.W., Grist, N., Barnett, P., Smart, J.D., 1999. An in vitro mucosal model predictive of bioadhesive agents in the oral cavity. J. Control. Release 61, 175–183.
Polnok, A., Borchard, G., Verhoef, J.C., Sarisuta, N., Junginger, H.E., 2004. Influence of methylation process on the degree of quarternisation of *N*-trimethyl chitosan chloride. Eur. J. Pharm. Biopharm. 57, 77–83.
Rabea, E.I., Badawy, M.E.-T., Stevens, C.V., Smagghe, G., Steurbaut, W., 2003. Chitosan as antimicrobial agent: applications and mode of action. Biomacromolecules 4, 1457–1465.
Sadeghi, A.M.M., Dorkoosh, F.A., Avadi, M.R., Saadat, P., Rafiee-Tehrani, M., Junginger, H.E., 2008. Preparation, characterization and antibacterial activities of chitosan, *N*-trimethyl chitosan (TMC) and *N*-diethylmethyl chitosan (DEMC) nanoparti-

cles loaded with insulin using both the ionotropic gelation and polyelectrolyte complexation methods. Int. J. Pharm. 355, 299–306.
Singla, A.K., Chawla, M., 2001. Chitosan: some pharmaceutical and biological aspects—an update. J. Pharm. Pharmacol. 53, 1047–1067.
Smart, J.D., 2005. The basics and underlying mechanisms of mucoadhesion. Adv. Drug Deliv. Rev. 57, 1556–1568.
Snyman, D., Hamman, J.H., Kotse, J.S., Rollings, J.E., Kotze, A.F., 2002. The relationship between the absolute molecular weight and the degree of quaternization of *N*-trimethyl chitosan chloride. Carbohydr. Polym. 50, 145–150.
Stepnova, E.A., Tikhonov, V.E., Babushkina, T.A., 2007. New approach to the quaternization of chitosan and its amphiphilic derivatives. Eur. Polym. J. 43, 2414–3242.
Thanou, M., Verhoef, J.C., Verheijden, J.H.M., Junginger, H.E., 2001. Intestinal absorption of octreotide using trimethyl chitosan chloride: studies in pigs. Pharm. Res. 18, 823–828.
Venter, J.P., Kotze, A.F., Auzely-Velty, R., Rinaudo, M., 2006. Synthesis and evaluation of the mucoadhesivity of a CD-chitosan derivative. Int. J. Pharmacol. 313, 36–42.
Zambito, Y., Uccello-Barretta, G., Zaino, C., Balzano, F., Di Colo, G., 2006. Novel transmucosal absorption enhancers obtained by aminoalkylation of chitosan. Eur. J. Pharm. Sci. 29, 460–469.