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Research paper

Development of a novel method for the preparation of submicron particles based on thiolated chitosan

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Abstract

It was the aim of this study to develop a simple method for the production of thiolated chitosan particles without being ionically crosslinked. In the first step, thiolated chitosan was ionically gelated with tripolyphosphate (TPP) and sulphate in aqueous solution forming submicron particles and microparticles, respectively. In the next step, thiol groups in and on the particles were partially oxidized forming stabilizing inter- and intramolecular disulfide bonds. As the degree of oxidation can be controlled during the production process, the share of thiol and disulfide groups can be adjusted on demand. Thereafter the polyanions were removed. Utilizing this novel preparation method stable particles of a mean size of 366 ± 30 nm and a zeta potential of around + 11.3 ± 1.3 mV can be produced using TPP as ionic crosslinker. On average 83% of all thiol groups were oxidized. In contrast, particles did not remain stable after removing sulphate as temporary auxiliary ionic crosslinker. Neither ionically nor covalently crosslinked particles were degraded by lysozyme under physiological conditions.

Utilizing the novel method described here allows a simple production of thiolated chitosan particles without losing the cationic charge of chitosan.

Keywords: Chitosan; Submicron particles; Microparticles; Thiomers; Crosslinking

1. Introduction

Due to its polycationic character chitosan has been increasingly used in the design of drug delivery systems. The positive charges of chitosan are believed to be responsible for high mucoadhesive properties because of ionic interactions with negatively charged sialic acid and sulphonic acid substructures of the mucus [1]. In addition, the positive charges of chitosan are likely essential for the permeation enhancing effect of this polymeric excipient. These mucoadhesive and permeation enhancing properties of chitosan were even significantly further improved by the immobilization of thiol groups on the polymer. Roldo et al. [2], for instance, could show that the mucoadhesive properties are even 140-fold improved due to the covalent attachment of thiol groups on the polymer. In another study, a 3-fold higher permeation enhancing effect of thiolated chitosan was achieved in comparison to unmodified chitosan [3].

As micro- and nanoparticles offer the advantage of a prolonged residence time on mucosal membranes [4], of a comparatively higher drug uptake [5] and the possibility to reach greater mucosal surface areas, consequently numerous types of chitosan micro- and nanoparticles have been developed and evaluated in vitro and in vivo [6]. Stability of such particles is in most cases provided by the addition of polyanionic excipients such as tripolyphosphate [7], sulphate [8] or hyaluronic acid [9] leading to an ionic crosslinking of chitosan. Due to the addition of polyanions the positive charges of chitosan, however, are neutralized resulting in a loss of the mucoadhesive and permeation enhancing properties of chitosan [10,11].

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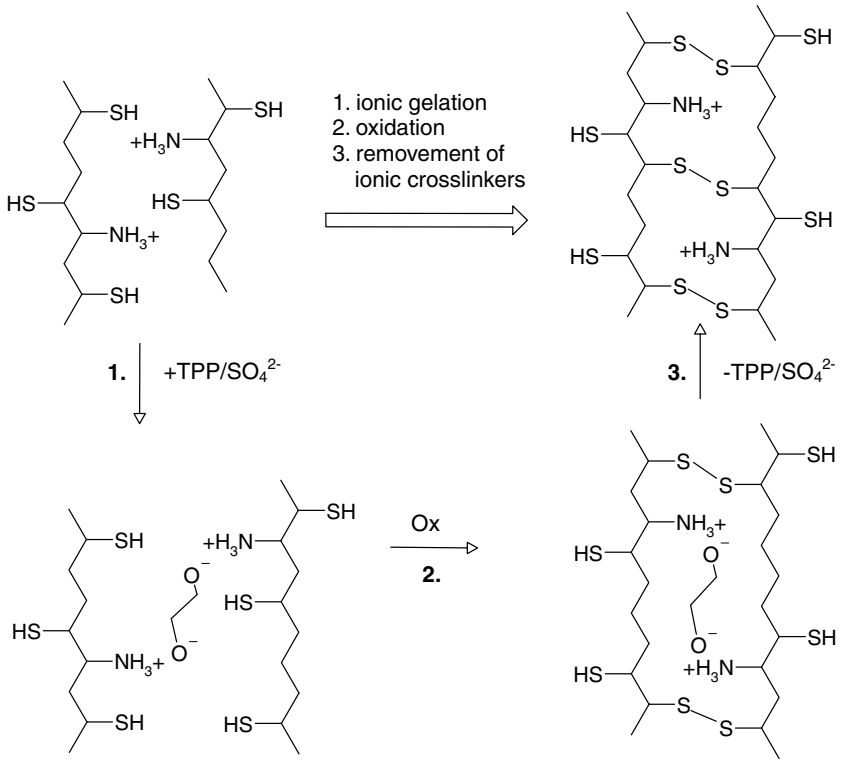

Fig. 1. Schematic presentation of the preparation method of submicron particles as well as microparticles based on thiolated chitosan (Chitosan-TBA).

In order to make use on the one hand of the properties of thiolated chitosan and micro- and nanoparticles on the other hand without losing the positive charges of the polymer due to the addition of polyanions, it was the aim of this study to develop according particulate systems. The strategy pursued to achieve that goal is outlined in Fig. 1. Micron and submicron particles shall in a first step be produced via well-established ionic gelation with thiolated chitosan and polyanions. In a second step a certain share of thiol groups within these particles shall be oxidized forming stabilizing intra- and intermolecular disulfide bonds. In a third step, the polyanions shall be removed. In addition, certain parameters such as the used polyanions influencing the properties of resulting particles will be evaluated within this study.

2. Materials and methods

2.1. Preparation of low molecular weight chitosan

First, 2 g of chitosan (medium molecular mass: 400 kDa; Fluka GmbH, Buchs, Switzerland) was dissolved in 100 ml of acidic acid (6% (v/v)). To this solution, 80 mg of sodium nitrite dissolved in 10 ml of demineralized water were added. After 1 h of incubation under continuous stirring, chitosan was precipitated by the addition of a 4 M solution of sodium hydroxide until pH 9 was reached. The resulting precipitate was filtered and washed with cold acetone. The residue was resolubilized in 15 ml of 0.1 M acidic acid and exhaustively dialysed against demineralized water. The dialysed product was concentrated partially under vaccum followed by lyophilization at −30 °C and 0.01 mbar (Benchtop 2K, VirTis, NY, USA).

2.2. Synthesis of thiolated chitosan

Thiolated chitosan was synthesized according to a method described previously [2]. In brief, 500 mg of low molecular weight chitosan prepared as described above was dissolved in 50 ml of 1% acetic acid. After adjusting the pH to 6.5 with 1 M NaOH, 200 mg of 2-iminothiolane HCl (Traut's reagent; Sigma, Vienna, Austria) was added. The reaction mixture was incubated for 12 h at room temperature under stirring. The resulting polymer conjugate was dialysed against 5 mM HCl, then two times against 5 mM HCl containing 1% NaCI, against 5 mM HCl and finally against 0.4 mM HCl. Thereafter, the polymer was freeze-dried at −30 °C and 0.01 mbar (Benchtop 2K, VirTis, NY, USA) and stored at 4 °C until further use.

2.3. Determination of thiol/disulfide groups

The amount of thiol groups on thiolated chitosan was evaluated via iodometric titration as described previously [12]. Disulfide content was measured after reduction with $NaBH_4$ and iodometric titration as described above.

2.4. Preparation of micro- and submicron particles

Submicron particles were spontaneously obtained upon addition of a tripolyphosphate (TPP) or sulphate in

aqueous solution to the chitosan solution according to methods described previously [7,8].

In case of TPP gelation thiolated and unmodified low molecular weight chitosan were dissolved in 0.05% (m/v) acetic acid solution at a final concentration of 0.25% (m/v) and the pH was adjusted to 5.5 by the addition of a 0.5% (m/v) NaOH solution. Sodium tripolyphosphate was dissolved in demineralized water in a final concentration of 0.2% (m/v). Following this 1 ml of the TPP solution was added to 3 ml of the low molecular weight chitosan solution leading to the formation of chitosan submicron particles.

In case of sulphate gelation 300 μl of a 10% (m/v) Na_2SO_4 solution were added to 10 ml of a 0.25% low molecular weight chitosan solution in aqueous 0.25% acetic acid solution containing 1% Tween 80 under stirring. In the following step, 2 ml of each particle suspension was partially oxidized in three different ways: (1) due to the addition of hydrogen peroxide (3%; 5 μl); (2) due to the addition of 5–10 μl of a 1 mM iodine solution; (3) by adjusting the pH of the nanoparticle suspension to 5.8 leading to air-oxidation as a function of time. Thereafter the anions and oxidants were removed by dialysis against 0.1 M HCl over 12 h.

2.5. Particle characterization

The amount of thiol groups on particles was determined via iodometric titration (1 mM iodine solution; indicator: starch) at pH 1–2. Remaining traces of tripolyphosphate were determined spectrophotometrically after the addition of ammonium molybdate in sulphate solution and subsequent reduction with ascorbic acid by measuring the absorbance of the resulting molybdene blue at 830 nm (DU Series 600 spectrophotometer, Beckman Instruments, Fullerton, USA) according to [13]. Size distribution and zeta potential of particles were determined with a particle sizer (Zeta Potential/Particle Sizer, Nicomp™ 380 ZLS, PSS, Santa Barbara, CA, USA). Measurements were performed via dynamic light scattering analyses of particle suspensions in demineralized water at 22 °C. Shape of particles was monitored with an in-column energy filter transmission electron microscope (ZEISS 902; Zeiss AG; Oberkochen; Germany). Particles were photographed using global imaging and inelastic imaging with a selected energy loss of 50 eV.

2.6. Biodegradation studies

First, 2 ml of both suspensions containing either ionically crosslinked or via disulfide bonds crosslinked particles as described above was added to 0.5 ml of demineralized water. To this suspension 1 ml of a hen egg white lysozyme solution was added to each sample. The final concentration of lysozyme was 1.5 mg/ml. The pH was adjusted to 5.0 by addition of 0.5% NaOH and samples were incubated at 37 °C. At predetermined time points the particle size and quantity of remaining particles was evaluated with a particle sizer (Zeta Potential/Particle Sizer, Nicomp™ 380 ZLS, PSS, Santa Barbara, CA, USA). Particle suspensions without lysozyme were evaluated under the same conditions and served as controls.

2.7. Statistical data analyses

Statistical data analyses were performed using the Student t test with $p < 0.05$ as the minimal level of significance. Calculations were done using the software Xlstat version 5.0 (b8.3).

3. Results and discussion

3.1. Polymer preparation and characterization

The lower molecular mass of chitosan, the smaller particles can be generated. In order to be able to produce even thiolated chitosan submicron particles, with a mean size below 200 nm, low molecular weight chitosan with a mean molecular mass of 10 kDa has been prepared according to a method described previously [14]. To this low molecular weight chitosan, thiol groups were immobilized. In total, 194 ± 26 μmol thiol groups per gram polymer were covalently attached. During the preparation process 46% of these thiol groups were already oxidized forming inter- and intrachain disulfide bonds. The lyophilized thiolated polymer was of fibrous structure, light yellow and odourless.

3.2. Preparation and characterization of ionically crosslinked particles

In order to evaluate the influence of polyanions on the particle size, two different ionic crosslinkers namely TPP and sulphate were utilized. Results of these studies demonstrated a significant impact of the polyanions used on the particle size. As shown in Fig. 2 particles being crosslinked with TPP displayed a mean particle size in the range of 240 nm, whereas particles crosslinked with sulphate showed a mean particle size of 1500 nm. The particle size determined via dynamic light scattering technique could be confirmed by electron microscopic images as shown in Fig. 3. Particles were of spherical shape and displayed a smooth surface.

3.3. Preparation of covalently crosslinked particles

Substituting the ionic crosslinking with TPP in the next step by disulfide crosslinking leads to 1.54 greater particles as shown in Fig. 4, which might be explained by the lower number of disulfide crosslinkages compared to ionic crosslinkages. Based on the assumption that each polyphosphate provides just one ionic crosslinking 723 μmol polyphosphate crosslinkages per gram chitosan are feasible, whereas in maximum only 98 μmol disulfide

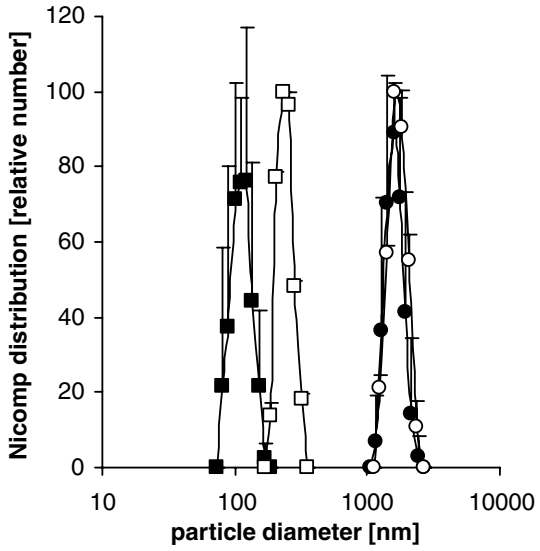

Fig. 2. Size distribution of ionically crosslinked particles being determined via dynamic light scattering analyses of particle suspension in demineralized water at 22 °C. Particles, based on chitosan (■,●) or chitosan-TBA (□,○), were obtained by ionic gelation with TPP (■,□) and sodium sulphate (●,○), respectively. The relative number of particles describes the result gained by the measurement based on the principle of the Nicomp Distribution Analysis. Indicated values are means of at least three experiments (±SD).

crosslinkages per gram chitosan can be formed. In case of particles being ionically crosslinked with sulphate, no covalent crosslinking was feasible at all. An explanation for this observation might be given by the comparatively much greater particle size making an oxidation of thiol groups inside the particles more difficult. Analysis of thiolated chitosan/sulphate particles demonstrated that by applying the oxidation methods described here no significant oxidation of thiol groups within these particles can be achieved. Evidence for the covalent crosslinking in TPP particles but not in sulphate particles was provided by incubation of particles in hydrochloride solution. Whereas chitosan/TPP, chitosan/sulphate, thiolated chitosan/TPP, thiolated chitosan/sulphate and oxidized thiolated chitosan/sulphate particles dissolved in this medium, oxidized thiolated chitosan/TPP particles remained stable. In contrast to ionically crosslinked chitosan particles, disulfide crosslinked chitosan particles display therefore a comparatively greater stability, which might be advantageous for various types of delivery systems. In case of oral drug delivery, for instance, disulfide crosslinked chitosan particles do not need to be additionally enteric coated, as stability in gastric fluids is obviously provided.

On the one hand, disulfide bonds are responsible for the stability of chitosan micro- and submicron particles and on the other hand free thiol groups are essential for improved mucoadhesive and permeation enhancing properties [2,3]. Thiolated chitosan particles should therefore exhibit both disulfide bonds and free thiol groups. In order to achieve that goal, the oxidation of thiol groups has to be well controlled during the preparation process. In orientating studies three different ways to control thiol group oxidation were evaluated for suitability. The first method was based on the oxidation of thiol groups by the addition of hydrogen

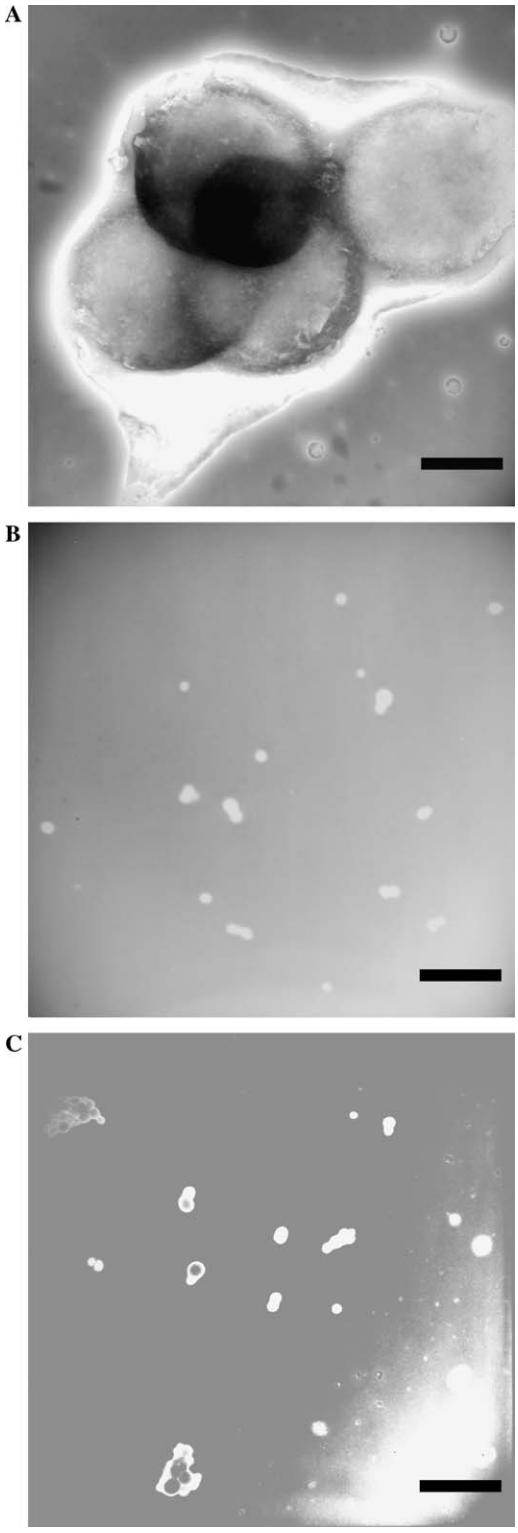

Fig. 3. TEM micrographs of micro- and submicron particles based on chitosan-TBA. (A) Ionically crosslinked with Na_2SO_4; (B) ionically crosslinked with TPP; (C) covalently crosslinked after ionic gelation with TPP. Displayed squares represent an area of 6×6 μm and black bar represents 1.0 μm.

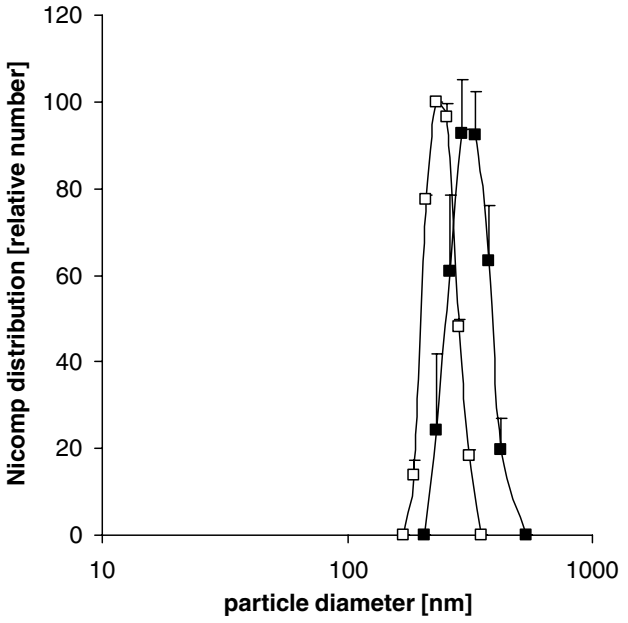

Fig. 4. Comparison of size distribution of ionically crosslinked (□) and covalently crosslinked (■) submicron particles based on chitosan-TBA obtained by ionic gelation with TPP. Measurement was performed via dynamic light scattering analyses of particle suspension in demineralized water at 22 °C. The relative number of particles describes the result gained by the measurement based on the principle of the Nicomp Distribution Analysis. Indicated values are means of at least three experiments (±SD).

superoxide. Results of this study, however, showed that thiol groups are too rapidly oxidized and that the process is too difficult to be controlled with regard to a standardized large-scale production. In contrast, oxidation of thiol groups on particles was much better controllable by oxidation with iodine. In the same way as done by an iodometric titration, thiol groups can be oxidized to a demanded degree by the addition of an exact quantity of iodine. The resulting iodide is then removed with the polyanion during the purification process. The third method is based on a simple oxidation process of thiol groups in aqueous solution at pH values above 5.5. The higher pH is adjusted and the longer the reaction is allowed to continue, the more thiol groups are oxidized. For this method no additional oxidants are needed and a good control is provided.

3.4. Characterization of covalently crosslinked particles

3.4.1. Thiol/disulfide content

The degree of oxidation as listed in Table 1 has been achieved via the pH-controlled method. In case of covalently crosslinked particles having been generated by utilizing TPP during the preparation, 17% of all thiol groups remained unoxidized. By none of the methods described here, however, it was possible to guarantee a selective oxidation of thiol groups only inside the particles, where disulfide bonds are needed for stabilization leaving free thiol groups on the surface of the particles unoxidized. Such a selective oxidation might be feasible in a two-step process.

Table 1
Amount of thiol groups and disulfide bonds immobilized on the basic polymer and particles after ionic gelation and oxidation, respectively

	–SH (μmol/g)	–S–S– (μmol/g)	Σ–SH (μmol/g)
Chitosan-TBA (polymer)	106	44	194 ± 26
Chitosan-TBA (+TPP)	70	61	192 ± 7
Chitosan-TBA (ox)	33	82	197 ± 21
Chitosan-TBA $+SO_4^{2-}$	103	43	189 ± 13
Chitosan-TBA (ox)	100	47	194 ± 17

Thiol groups, for instance, can be entirely oxidized followed by a selective reduction on the surface via a reducing agent, which cannot penetrate into the particles.

3.4.2. Zeta potential

The zeta potential of ionically and covalently crosslinking particles is listed in Table 2. Due to the removal of the ionic crosslinkers the positive zeta potential of particles increased. Removing the ionically crosslinker TPP from chitosan-TBA particles led to a more than 2-fold increase in the zeta potential. As the positive charges of chitosan are responsible for its mucoadhesive properties, particles of a more positive zeta potential should display comparatively more pronounced mucoadhesive properties. Moreover, as the positive charges of chitosan seem to be essential for its permeation enhancing properties [15], a more positive zeta potential might contribute to a more pronounced permeation enhancing effect of such particles. Quantifying the amount of remaining traces of disturbing polyphosphate revealed that approximately 5–10% of the polyanion are still present within the particles. An improved purification process of polyanions will therefore contribute to an even more positive zeta potential of this new type of particles.

3.4.3. Biodegradability

Chitosan is extensively degraded by lysozyme, which is ubiquitous in a variety of tissues and secretions [16]. In order to evaluate whether this enzyme is also capable of

Table 2
Mean particle diameter and zeta potential of various particles; indicated values are the means of at least three experiments (±SD)

	Mean particle diameter (nm)	Zeta potential (mV)
Ionically crossslinked		
TPP		
Chitosan	150 ± 22	7.7 ± 2.2
Chitosan-TBA	239 ± 34	5.1 ± 0.9
SO_4^{2-}		
Chitosan	1608 ± 81	6.7 ± 1.5
Chitosan-TBA	1543 ± 92	4.8 ± 2.3
Covalently crosslinked		
TPP	366 ± 30	11.3 ± 1.3
SO_4^{2-}	–	–

degrading ionically and/or covalently crosslinked particles, particles were incubated with this enzyme in physiological concentration. Results as shown in Fig. 5, however, revealed no degradation of both types of particles. These results are in good agreement with previous studies demonstrating that chitosan particles are not degraded by lysozyme [17]. Apart from these orientating studies, however, further more detailed studies will be necessary to determine the extent of biodegradability of such particles.

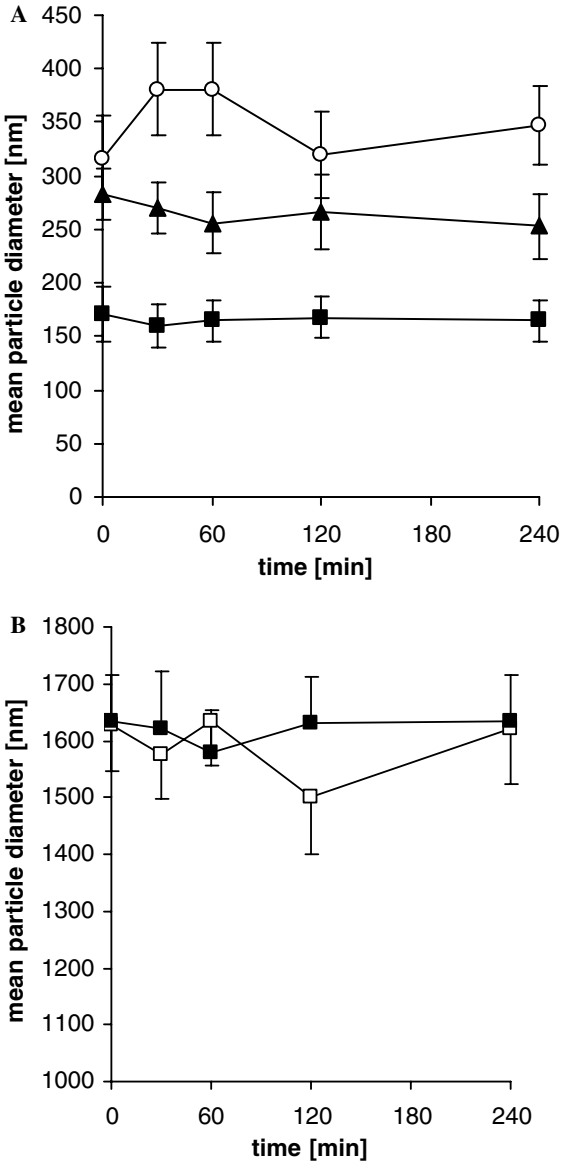

Fig. 5. (A) Stability of submicron particles obtained by ionic gelation with TPP. Biodegradability was studied in the presence of lysozyme. Submicron particles based on chitosan (■) and chitosan-TBA (ionically crosslinked ▲; covalently crosslinked ○). (B) Stability of microparticles obtained by ionic gelation with sodium sulphate. Biodegradability of ionically crosslinked microparticles based on chitosan (■) and chitosan-TBA (□), respectively, was studied in the presence of lysozyme. Indicated values are the means of at least three experiments (±SD).

4. Conclusion

Due to the addition of polyanions ionically crosslinked chitosan particles lose at least to some extent their positive charge being essential for high mucoadhesive and permeation enhancing properties. Utilizing the novel technique described here, this drawback can be excluded as the ionically crosslinking is substituted by a covalent crosslinking based on disulfide bonds. According to their high stability being based on covalent bonds (I), a more pronounced positive charge (II) and remaining free thiol groups (III), having been shown to be responsible for improved mucoadhesive and permeation enhancing properties, these particles might be a promising novel vehicle for the delivery of various drugs via mucosal membranes.

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References

[1] E.E. Hassan, J.M. Gallo, A simple rheological method for the in vitro assessment of mucin-polymer bioadhesive bond strength, Pharm. Res. 7 (1990) 491–495.
[2] M. Roldo, M. Hornof, P. Caliceti, A. Bernkop-Schnürch, Mucoadhesive thiolated chitosans as platforms for oral controlled drug delivery: Synthesis and in vitro evaluation, Eur. J. Pharm. Biopharm. 57 (2004) 115–121.
[3] A. Bernkop-Schnürch, D. Guggi, Y. Pinter, Thiolated chitosans: development and in vivo evaluation of a mucoadhesive permeation enhancing oral drug delivery system, J. Control. Release 94 (2004) 177–186.
[4] R.B. Umamaheshwari, S. Ramteke, N.K. Jain, Anti-Helicobacter pylori effect of mucoadhesive nanoparticles bearing amoxicillin in experimental gerbils model, AAPS Pharm. Sci. Technol. 5 (2004) 32.
[5] V.M. Leitner, D. Guggi, A.H. Krauland, A. Bernkop-Schnürch, Nasal delivery of human growth hormone: in vitro and in vivo evaluation of a thiomer/glutathione microparticulate delivery system, J. Control. Release 100 (2004) 87–95.
[6] Y. Kato, H. Onishi, Y. Machida, Application of chitin and chitosan derivatives in the pharmaceutical field, Curr. Pharm. Biotechnol. 4 (2003) 303–309.
[7] P. Calvo, C. Remunan-Lopez, J.L. Vila-Jato, M.J. Alonso, Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers, J. Appl. Polym. Sci. 63 (1997) 125–132.
[8] I.M. van der Lubben, J.C. Verhoef, A.C. van Aelst, G. Borchard, H.E. Junginger, Chitosan microparticles for oral vaccination: preparation, characterization and preliminary in vivo uptake studies in murine Peyer's patches, Biomaterials 22 (2001) 687–694.
[9] S.T. Lim, B. Forbes, G.P. Martin, M.B. Brown, In vivo and in vitro characterization of novel microparticulates based on hyaluronan and chitosan hydroglutamate, AAPS Pharm. Sci. Technol. 2 (2001) 20.
[10] H.L. Luessen, B.J. de Leeuw, M.W. Langemeyer, A.B. de Boer, J.C. Verhoef, H.E. Junginger, Mucoadhesive polymers in peroral peptide drug delivery. VI. Carbomer and chitosan improve the intestinal absorption of the peptide drug buserelin in vivo, Pharm. Res. 13 (1996) 1668–1672.

[11] A. Bernkop-Schnürch, M.E. Krajicek, Mucoadhesive polymers as platforms for peroral peptide delivery and absorption: synthesis and evaluation of different chitosan–EDTA conjugates, J. Control. Release 50 (1998) 215–223.
[12] C.E. Kast, A. Bernkop-Schnürch, Thiolated polymers: development and in vitro evaluation of chitosan-thioglycolic acid conjugates, Biomaterials 22 (2001) 2345–2352.
[13] B. Kakac, Z.J. Vejdelek, Handbuch der photometrischen Analyse organischer Verbindungen, Verlag Chemie (1977) 416.
[14] C. Vauthier, Personal communication.
[15] P. Younessi, M.R. Avadi, K. Shamimi, A.M. Sadeghi, L. Moezi, E. Nahid, K. Bayati, A.R. Dehpour, M. Rafiee-Tehrani, Preparation and ex vivo evaluation of TEC as an absorption enhancer for poorly absorbable compounds in colon specific drug delivery, Acta Pharm. 54 (2004) 339–345.
[16] H.A. McKenzie, Alpha-lactalbumins and lysozymes, EXS 75 (1996) 365–409.
[17] A. Vila, A. Sanchez, M. Tobio, P. Calvo, M.J. Alonso, Design of biodegradable particles for protein delivery, J. Control. Release 78 (2002) 15–24.