CELL BINDING AND PENETRATION OF QUATERNIZED CHITOSAN DERIVATIVES

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Abstract

Chitosan (Ch) is an attractive biopolymer with multiple reactive groups. However it is poorly soluble at neutral pH. Quaternization improves its solubility and permits the development of various positively charged drug delivery systems. The aim of this work was to study the solubility, toxicity, cell binding, and penetration of 20 kDa chitosan with 9, 40, 58 and 98% of quaternary ammonium group substitution (ChQ1 to ChQ4 accordingly). We showed that ChQ with substitution degree >40% was soluble in a wide pH range. Unexpectedly ChQ2 and ChQ3 were more toxic to cells than Ch, ChQ1 and ChQ4. Higher toxicity of ChQ was found against macrophage like cell line RAW264.7 than against epithelial cells MiaPaCa-2. All ChQ, in contrast to unmodified Ch, easily bound and penetrated the cells with the highest uptake by ChQ4. Thus, quaternized chitosan derivatives can be used for biomedical applications.

Key words: *chitosan, N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride, quaternized chitosan, cell uptake, trypan blue, cytotoxicity.*

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1. Introduction

Chitosan (Ch), a derivative of natural polysaccharide chitin, is a promising polymer for the development of drug delivery systems. Despite many advantages, the main limitation is its low solubility at pH≥6.5, which leads under physiological conditions to the formation of aggregates of micron size unable to penetrate the cells [1]. Introduction of some substitutes can increase Ch solubility at neutral pH [2]. One of the approaches to increase solubility is the replacement of the primary amino groups at C-2 position of Ch for quaternary ammonium groups [3,4]. Quaternization of Ch was shown to increase antibacterial [5] and antioxidant [6] activities, as well as the efficiency of Ch as a transfecting agent [7]. However, the information on the role degree of substitution (DS), cell toxicity, and the ability of ChQ to interact with the cells is poorly studied. M. Thanoua *et al*. showed that the increase in DS from 40 to 60% significantly increased the penetration of [14C]-mannitol into Caco-2 cells by opening tight junction, without causing cytotoxic effects [8]. These results were confirmed by B. Benediktsdóttir *et al*., which also found that co-administration of acyl and quaternary ammonium groups led to a better membrane permeability and 3-5 time increase in the transport of FITC-dextran into bronchial epithelium cells [9].

The aim of this work was to study the interaction of ChQ with different DS with epithelial (MiaPaCa-2) and macrophage (RAW 264.7) cell lines.

2. Materials and methods

2.1. Materials

Crab shell Ch with molecular weight (MW) 500 kDa and deacetylation degree (DD) 90% was purchased from ZAO Bioprogress (Moscow region, Russia). Ch with MW 20 kDa and DD 98% was obtained from 500 kDa chitosan by acidic hydrolysis in the presence of 6M HCl at heating (100ºC) during 3 h followed by precipitation with ethanol (yield 60%) [10]. Glycidyltrimethylammonium chloride (GTMAC) (Sigma, USA) was used to produce quaternized derivatives with different DS.

2.2. Methods

2.2.1. Synthesis and characterization of N-[(2-hydroxy-3-trimethylammonium)propyl]chitosan chloride

N-[(2-hydroxy-3-trimethylammonium)propyl]chitosan chloride (quaternized chitosan, ChQ) was prepared by a modified method. Ch (1 g, 6.2 mmol) was dispersed in distilled water (10 mL) at 85ºC. Three equal portions (3.55 ml each, 18.5 mmol) of GTMAC were added at 1 h intervals. The reaction mixture was stirred for 5 h at 85°C, and then it was gradually poured into cold acetone (30 ml) while stirring and kept at 4ºC overnight. The resulting precipitate was separated by decantation, dissolved in MeOH (15 ml), and precipitated in 4:1 acetone–ethanol. The product was separated by decantation and further purified by washing with hot EtOH. The final product was dried at 60°C [10]. The DS was measured by titrating the amount of Cl^- ions with HTCC and $AgNO_3$ solution according to [11]. As a result ChQ with DS 9, 40, 58 and 98% (ChQ1 to ChQ4) were obtained, The water solubility of ChQ in different pH was analyzed by solution absorbance at 600 nm [11].

2.2.2. Preparation of FITC-labeled chitosan and its quaternized derivatives

To prepare FITC-labeled samples, 25 nmoles of FITC dissolved in DMSO was added to 5 mg of Ch dissolved in 50 mM acetic buffer (pH 4.5) (for samples Ch, ChQ1, ChQ2, ChQ3) or in 50 mM phosphate-buffered saline (pH 7.4) for ChQ4. The reaction mixtures were incubated for 1.5 hrs at room temperature protected from light. The FITC-labeled Ch was purified from unbound dye by dialysis against bi-distilled water for 24 hours. Efficacy of FITC-labeling was measured by microfluorimetry assay using FITC-standard curve (λ ex/em=494/518 nm).

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2.2.3. MTT-assay

Cytotoxic effect of Ch and ChQ samples was estimated by a standard 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) test as was described earlier [12].

2.2.4. Flow cytometry

Ch binding to cells was estimated in MiaPaCa-2 and RAW 264.7 cells. For this, cells were trypsinized, washed and pre-plated overnight in complete RPMI-1640 culture medium with 10% fetal calf serum, L-glutamine, and antibiotics. Ch of ChQ (10 μg/ml) were added to the cells and incubated for different periods of time. For the flow cytometry analysis cells were again trypsinized, washed and studied using FACScan device (BD, USA). Dead cells were excluded using propidium iodide. To differentiate between extracellular and internalized samples, trypsinized cells were incubated for 10 min with 0.1% trypan blue (TB) solution according to [13]. The results were analyzed using WinMDI 2.8 software.

2.2.5. Confocal analysis

For confocal analysis cells were grown overnight on the sterile cover slips in 150 μl of complete culture medium in 6-well plates (Costar). Before the analysis extracellular Ch was quenched by 0.1% trypan blue solution as above. LysoTrackRed was added for 3 hrs before the analysis. Finally cells were fixed with 1% paraformaldehyde, washed, and polymerized with Mowiol 4.88 medium (Calbiochem, Germany). Slides were analyzed using Eclipse TE2000 confocal microscope (Nikon, Japan).

3. Results and discussion

3.1. Synthesis and characterization of quaternized chitosan derivatives

The reaction between Ch and GTMAC was conducted in heterophase conditions, providing a more selective N-substitution. General scheme of synthesis is presented in ***Figure 1 a***. ChQ with DS 9, 40, 58 and 98% designated as ChQ1 to ChQ4 accordingly were obtained. DS was determined by conductometric titration. The solubility in a wide pH range of the synthesized derivatives is shown in *Figure 1 b.* It was found that unlike unmodified Ch which has pKa ≤ 6.5, modified polymers dissolved at a pH from 3 to 7.5 in the case of ChQ1 and from 3 to 11 for ChQ2-ChQ4. These results correlate with the data obtained earlier [11].

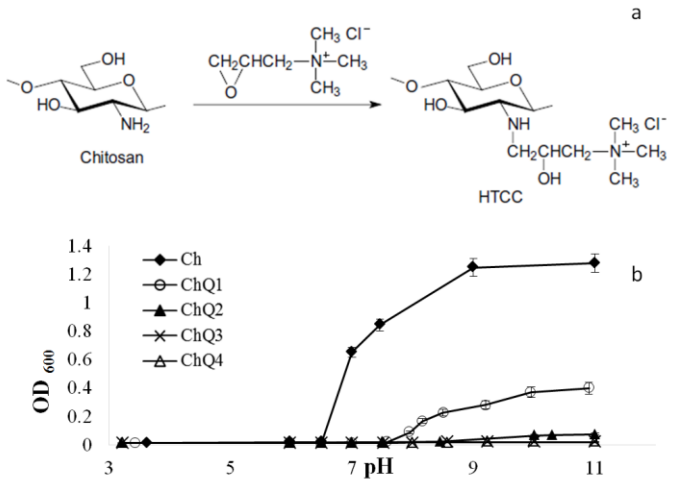

Figure 1. Preparation and characterization of quaternized chitosan. a) Scheme of synthesis; b) pH-dependence solubility of modified chitosan with different DS.

3.2. Effect of substitution degree on cell viability

The cytotoxicity of Ch and ChQ was studied *in vitro* by MTT test in macrophage and epithelial cells. Rather unexpectedly it was found that the cytotoxicity significantly depended on DS and was significantly higher for ChQ2 and ChQ3 in both cell lines used *(Figure 2 a, b)*. The dependence of toxicity from a combination of different substituents in the polymer chain was shown previously [14]. The second rather interesting result was the difference in the toxicity of ChQ against macrophages and epithelial cells, the former were more sensitive to ChQ *(Figure 2 a, b)*. Probably, the key parameters that determine the cytotoxicity of these polysaccharides are the solubility and positive charge at a neutral pH. Earlier we demonstrated the relationship of toxicity exhibited by charged polymer chains [15].

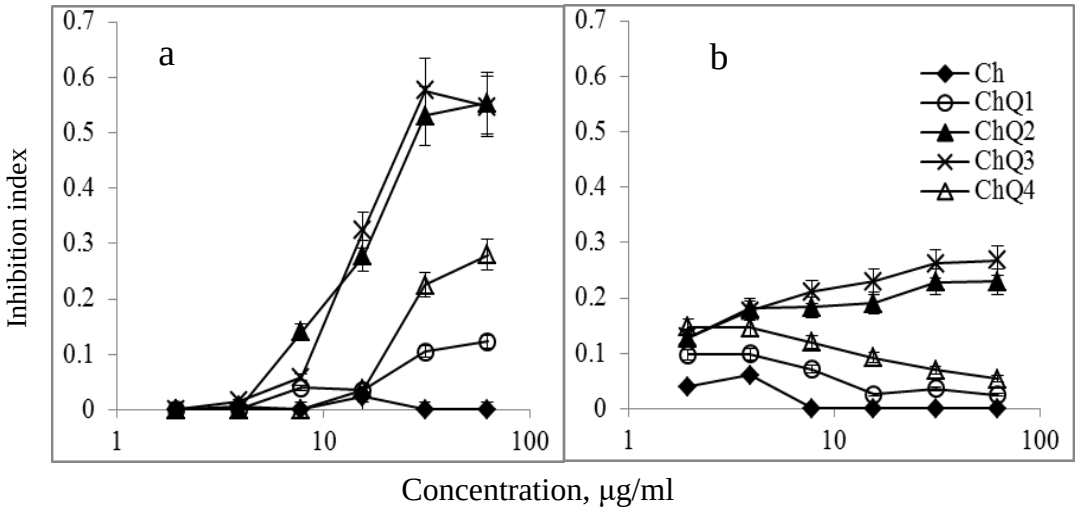

Figure 2. Toxicity of chitosan and its quaternized derivatives against RAW 264.7 (a) and MiaPaCa-2 (b) cells in the 72h MTT-test.

3.3. Cellular uptake of chitosan and its quaternized derivatives

Cell binding and penetration was studied by flow and confocal cytometry using trypan blue quenching of non-internalized Ch [16]. Cells were incubated with Ch samples for 80 hrs. Histograms in Figure 3 a-c and g-i show the results of flow cytometry analysis where green and blue curves correspond to Ch and quenched Ch samples accordingly. The results are shown only for Ch, ChQ1 and ChQ4. The results for ChQ2 and ChQ3 are alike ChQ1 and ChQ4 and are not shown. Unmodified Ch poorly bound the cells and was significantly quenched by trypan blue (Figure 3 a, g) while ChQ1 and ChQ4 much better bound the cells and did not respond to trypan blue quenching (Figure 3 b, h and c, i).

Confocal analysis demonstrated that unmodified Ch was found in large aggregates outside the cells both for macrophages (Figure 3 d) and epithelial cells (Figure 3 j). The introduction into the polymer chain even 9% of quaternary ammonium groups substantially increased the efficiency of penetration into the cell regardless of the type of cell line (Figure 3 e, k). A similar effect was found for all ChQ ((Figure 3 f, l).

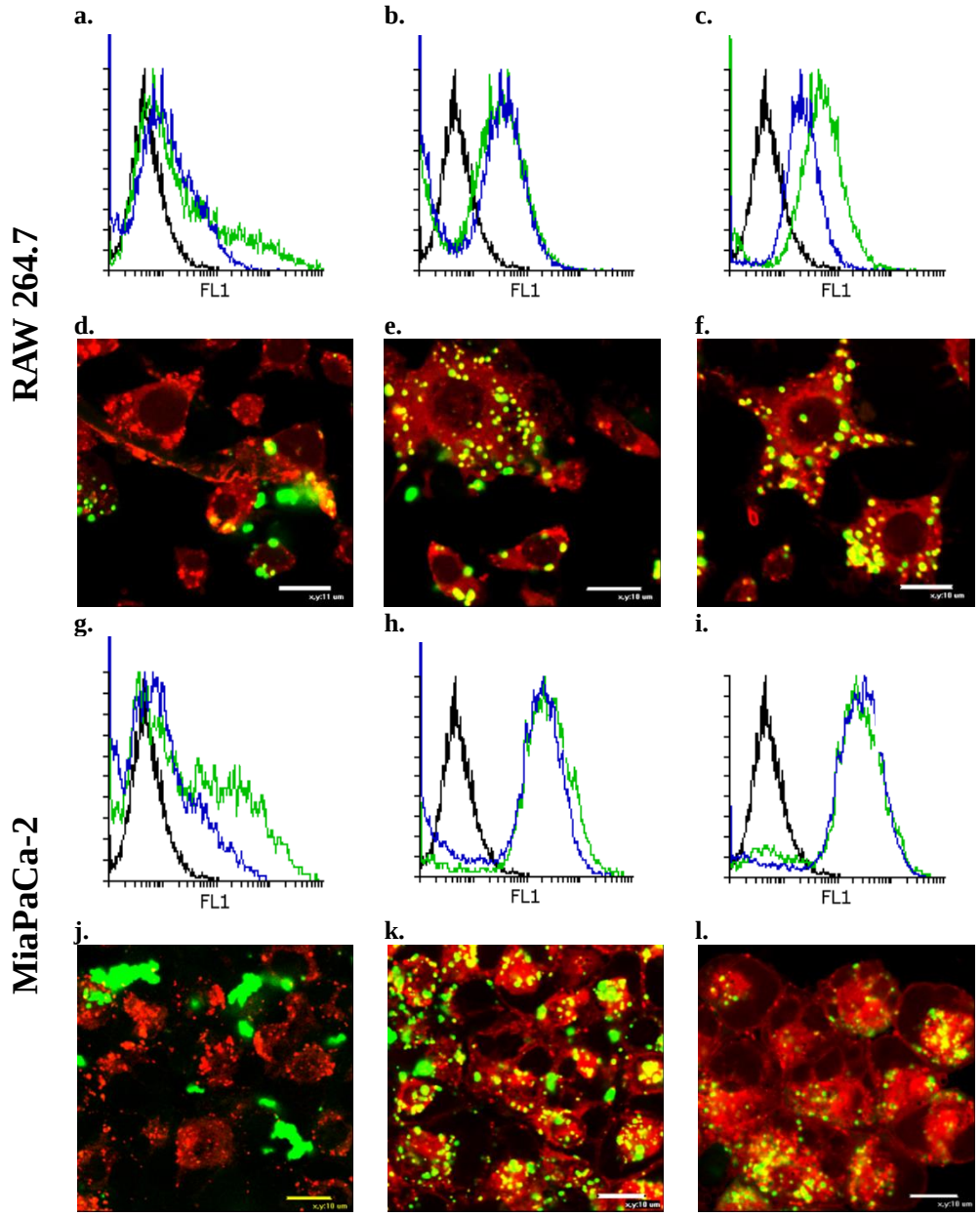

Figure 3. Cellular uptake of chitosan and its quaternized derivatives. a-c and g-i Flow-cytometry profile of RAW 264.7 (a-c) or MiaPaCa-2 cells (g-i) after 80 h of incubation with 10 μg/ml of FITC-labeled Ch (a, g); ChQ1 (b, h), or ChQ4 (c, i) (green line). Blue lines correspond to quenched FITC after trypan blue treatment. d-f and j-l Confocal images of cellular uptake by RAW 264.7 (d-f) or MiaPaCa-2 (j-l) of Ch (d, j), ChQ1 (e, k), or ChQ4 (f, l). Lysosomes were stained with LysoTrackerRed. Scale bar 15 nm.

Note that some amount of ChQ1 was still present in extracellular space in contrast to ChQ4 which was found exclusively inside the cells. Co-localization with lysosomal tracker (red) demonstrated that ChQ predominantly traffic to lysosomes.

4. Conclusions

The results of this study has showed that low molecular weight unmodified chitosan is nontoxic to cells, it forms large aggregates at neutral pH due to the charge loss, poorly adheres to cell membranes and is not able to penetrate cells. Contrary to it, quaternized derivatives with 40% or more substitution degree are soluble in a wide range of pH, do not aggregate, adhere to cells and penetrate cells efficiently. Quaternized chitosan derivatives demonstrate toxicity against macrophage cells and, to a lesser extent, to epithelial cells, possibly due to high positive charge which interfere with cell membrane conductance. On average, quaternized derivatives even with low substitution degree were able to penetrate cells which make them good candidates as polymers for the development of drug delivery systems.

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