

**PROTEIN DELIVERY BY NANOPARTICLES FORMED
BY CHITOSAN-N-ACYL DERIVATIVES**

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Abstract

Biodegradable nanoparticulated carriers have important potential applications for administration of therapeutic proteins and peptides. Chitosan based nanoparticles have attracted attention due to their biological properties such as biodegradability and biocompatibility. However, chitosan nanoparticles are not stable in colloid form. Therefore, to increase the stability of these particles is an important problem. The aim of this work was to obtain stable nanoparticles formed by N-acyl derivatives of chitosan by ionic gelation and to investigate their ability to interact with acidic, neutral and basic proteins.

Key words: *chitosan nanoparticles, protein delivery, N-acyl derivatives of chitosan, ionic gelation.*

1. Introduction

Chitosan is a polycationic biopolymer with remarkable physicochemical and biological properties [1 - 3]. Chitosan nanoparticles (Chi-NPs) have found many applications in *in vivo* systems since they are biocompatible with live tissues; do not induce allergic reactions and necrotic rejections; and, in general, do not induce any side effects. Chi-NPs are nontoxic and biodegradable by tissue enzymes producing aminosaccharides which are completely assimilated within the body. While the efficacy of most bioactive substances is limited by their ability to reach target organs without the loss of activity, Chi-NPs can efficiently interact with cell membranes due to a high positive charge density on Chi-NPs.

The use of Chi-NPs as a vehicle for bioactive substances would provide the targeted delivery as well as a decrease in the drug dosage, prolong the efficient time of interaction and facilitate noninvasive oral, intranasal, or sublingual administration for various medicines [4 - 6].

Since the primary amino groups of chitosan in acidic aqueous media are positively charged, the cationic background of this biopolymer permits the ionic binding of multivalent anions and gelation. The ionotropic gelation method is widely used for Chi-NPs formation [7]. The aim of this work was to obtain stable nanoparticles formed by N-acyl derivatives of chitosan by ionic gelation and to investigate their ability to interact with acidic, neutral and basic proteins.

2. Materials and methods

2.1. Chitosan

Crab shell chitosan with molecular weight (MW) 700 kDa and deacetylation degree (DD) 85% was purchased from ZAO "Bio-progress" (Moscow region, Russia). Chitosan with MW 340 kDa and DD 85 % was obtained from 700 kDa chitosan by acidic hydrolysis as described in [8]. Chitosan with MW 7 kDa and DD 98% was obtained from 700 kDa chitosan by acidic hydrolysis in the presence of 6M HCl at heating (100 °C) during 3 h followed by precipitation with ethanol (yield 60%). In order to decrease the polydispersity of chitosan 340 kDa chitosan was precipitated by addition of aqueous 12 wt% aqueous ammonium solution to 2% chitosan solution in acetic acid. Collection of chitosan precipitation was carried out in range of pH values from 5.9 to 7.4. Chitosan precipitate was collected and dried in vacuum.

MW and polydispersity of chitosan samples were determined by HPLC method as described earlier [9].

2.2. N-Acylchitosan derivatives

N-acylchitosan derivatives were synthesized in homogenous conditions using acetic or hexanoic acid anhydrides as described in [10]. The yields of N-acylchitosan derivatives after dialysis and freeze-drying ranged from 80 to 85%.

The structures of the synthesized chitosan derivatives, their degrees of N-substitutions were determined by $^1\text{H-NMR}$ method [11]. Signals of the corresponding N-acyl groups were observed at 0.52 - 2.5 ppm.

2.3. Formation and characterization of chitosan nanoparticles

Chi-NPs were formed using 340 kDa or 7 kDa chitosans by ionotropic gelation method [7,12]. Briefly: PEG-2000 was added to 0.25% solution of N-acetyl-chitosan derivative in 0.4% aqueous acetic acid to final concentration of 20 mg/ml. After that 0.1% TPP (pH 3) was added to N-acetyl-chitosan solution at vigorous stirring at 30 rpm. The dispersion formed was additionally stirred at 30 rpm during 20 min at 22 °C. The optical density of the solution at 580 nm was 0.030 - 0.032. Chi-NPs were separated by centrifugation (14000 g, 20 min) and then resuspended in deionized water.

Hydrodynamic diameters and zeta-potentials of Chi-NPs were determined by dynamic light scattering in 10 mM phosphate buffer (pH 7.5) at 22 °C. Dynamic stability of the particles stored at 22 °C for 14 days was evaluated by the same method.

Morphological characteristics of nanoparticles were studied by electron microscopy JEM-100CX (JEOL) at 80 kV. A drop of Chi-NPs (5 - 10 μl) was deposited onto a carbon-collodium film grid and left for 3 min till complete water evaporation. Then, the grid was stained with 2% uranyl acetate solution for 2 min, and the excess of solution was removed by a filter paper. Finally, the grid was dried at room temperature.

2.4. Complex formation protocol

Protein binding ability of Chi-NPs was estimated as follows: Protein solution (protein amount 10 μg , concentration 1 mg/ml) in 7 mM Tris-HCl (pH 7.4) was mixed with 30 μl of Chi-NPs (0.5 mg/ml) in water. Different buffers (see **Table 3**, page ...) were added to the mixtures to estimate the efficacy of Chi-NP-protein complex formation. The mixture was thoroughly shaken and incubated at room temperature for 10 min and then at 4 °C for 20 min without shaking. After the incubation the mixture was centrifuged at 10000 rpm for 15 min to separate Chi-NPs and residual proteins. Supernatant was transferred to a new tube, and protein content before (blank) and after incubation with Chi-NPs was estimated by BioRad Protein Assay Kit.

2.5. In vitro study

In some experiments the sediments were dissolved in culture medium and added to cell cultures. Human keratinocyte cells (line HaCaT) were grown to confluence on cover slips in DMEM, 10% fetal calf serum, antibiotics and L-glutamine. Cells were incubated at 5% CO_2 at 37 °C with Chi-NPs loaded with various proteins for 4 hrs. Then nuclei were stained with Hoechst dye, and the cells were fixed by PFA, washed three times and polymerized by Mowiol 4.88 (Calbiochem, Germany). For confocal experiments chitosan was labeled with rhodamine using sulforhodamine B acid chloride (Sigma) before Chi-NPs formation [13]. Proteins were labeled with FITC (Sigma) as described [14]. HaCaT cells were grown to confluence on cover slips. Chi-NPs labeled with Rhodamine were incubated with Asp f 2-FITC, BSA-FITC, SOD-FITC or SWM-FITC at 3:2 ratio for 30 min as described in

Experimental. Chi-NPs were washed out from unbound proteins and added to HaCaT for 4 hrs. Nuclei were stained with Hoechst. After incubation cells were fixed and polymerized. Slides were analyzed by confocal microscopy at 3000x magnitude.

3. Result and discussion

3.1. Production and characterization of Chi-NPs formed by chitosan-N-acyl derivatives

For production of N-acylchitosan derivatives we used partially hydrolyzed chitosan samples obtained by acid hydrolysis of a high MW chitosan. It was presumed that chitosan samples with smaller polydispersity and MW could produce nanoparticles of smaller sizes. In order to decrease polydispersity and, consequently, to decrease particle sizes the 340 kDa chitosan was partially fractionated. We showed that Chi-NPs produced from: A) fractionated chitosans of almost equal MWs but differing in polydispersity in 50% (**Table 1**, samples 1 and 2); or B) chitosans with equal polydispersity but 10-fold differing in MW (**Table 1**, samples 2 and 3) demonstrated the equal physicochemical characteristics. Therefore, all further investigations were carried out with Chi-NPs formed from non-fractionated chitosan samples.

Table 1. Physicochemical characteristics of N-acetylchitosan derivatives (DD was 85%) and Chi-NPs.

Sample №	MW, kDa	Polydispersity index	Average particle size, nm	Zeta-potential, mV
1	6.2	1.3	70 ± 30	+ 32 ± 0.5
2	7.3	2.0	70 ± 30	+ 34 ± 0.4
3	86.0	2.0	85 ± 20	+ 33 ± 0.8

The ionotropic gelation method was used for Chi-NPs preparation. The choice of this method was determined both the chemical structure of chitosan and the simplicity of the method. The mechanism of particle formation was based on the electrostatic interaction between positively charged chitosan amino groups and negatively charged TPP phosphoric groups. For Chi-NPs formation 0.1-0.3% chitosan solutions in acetic acid were used. The concentration of acetic acid was about 1.5 times higher than that of chitosan solution.

Earlier it had been shown that Chi-NPs obtained in a colloidal form were unstable at storage due to their self-aggregation [15]. Our studies demonstrated that the nanoparticles formed by N-acetylchitosan having MW 340 kDa and DD 85% had the initial size 60 - 200 nm and zeta-potential 30 mV. These nanoparticles aggregated spontaneously and enlarged their sizes to 250 - 800 nm at storage at 22 °C during 5 days. Also we observed 30% reduction in zeta-potential during the storage time. The freeze-drying process followed by re-suspension in 10 mM phosphatic buffer (pH 7.2) did not lead to the formation of stable Chi-NP dispersion supporting the data presented in (Lopez-Leon et al, 2005).

It is well known that introduction of hydrophobic groups into chitosan molecule increases the stability of a hydrophobically modified chitosan colloid system [16]. Taking into

account this effect we synthesized several N-acylchitosan derivatives shown in **Figure 1**. Chitosan N-acylation was carried out using anhydrides of the corresponding acids [10].

We showed that Chi-NPs based on N-hexanoylchitosan were stable during 14 days. Depending on the degree of N-hexanoyl group content (DS) and MW of N-hexanoyl-chitosan derivatives the sizes of Chi-NPs differed. The sizes decreased from 120 to 80 nm for 340 kDa chitosan (DS 10%) and from 50 to 30 nm for 7 kDa chitosan (DS 5%). Vice versa, the size of Chi-NPs produced from 7 kDa chitosan (DS 27%) increased from 60 to 80 nm. Chi-NPs of 340 kDa (DS 21%) and of 7 kDa (DS 17%) were found stable at storage.

The ionotropic gelation method used for the formation of Chi-NPs usually presumes the usage of PEG in order to maintain the hydrophilic-hydrophobic balance and provide an increase in particle stability. The introduction of N-hexanoyl groups into chitosan molecule allowed the formation of the Chi-NPs stable at storage without PEG addition (**Table 2**, samples Ib,c and IIa,b,c). Physicochemical characteristics of nanoparticles based on N-acyl-chitosan derivatives are presented in **Table 2**.

Table 2. Degrees of N-acetyl group (DA), amino group (DD) and N-hexanoyl group (DS) contents, particle sizes and zeta-potentials of N-acylchitosan derivatives; * Sample description is given in **Figure 1**.

N-acylchitosan*	MW, kDa	DA,%	DD,%	DS,%	Average particle size, nm	Zeta-potential, mV
I*	340	15	85	0	90 ± 5	+30 ± 0.5
I a	—	43	42	0	350 ± 15	+22 ± 0.8
I b	—	15	65	5	158 ± 10	+25 ± 0.3
I c	—	15	49	21	533 ± 25	+22 ± 0.6
II	7	2	98	0	50 ± 3	+34 ± 0.5
II a	—	2	88	10	237 ± 12	+41 ± 0.4
II b	—	2	81	17	257 ± 14	+33 ± 0.7
II c	—	2	71	27	219 ± 9	+27 ± 0.6

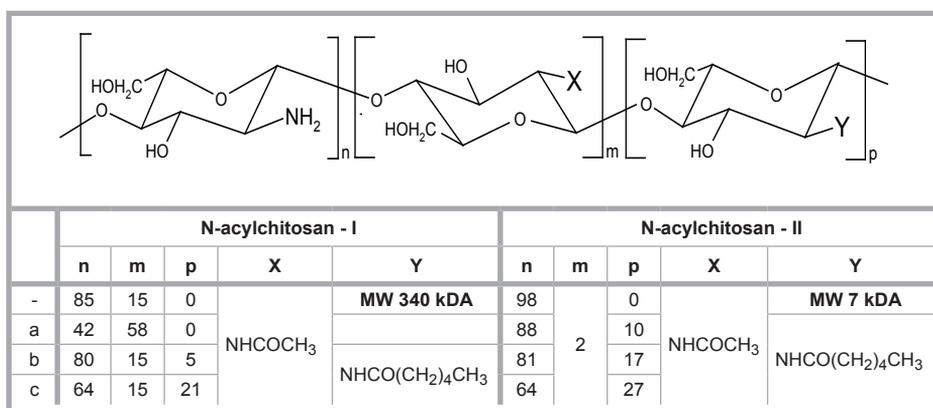


Figure 1. The structure of chitosan (I and II) and its N-acyl derivatives (I a,b,c u II a,b,c) used for Chi-NPs formation.

3.2. Complex formation and protein delivery by Chi-NPs formed from chitosan-N-acyl derivatives

Several publications demonstrated that chitosan and its derivatives form stable complexes with proteins and peptides [7, 17, 18]. Ionic, hydrophilic-hydrophobic interaction or Van der Waals forces are responsible for chitosan-protein interaction. In order to study complex formation between chitosan and proteins in more detail we chose eight proteins having different physicochemical characteristics (**Table 3**).

Table 3. Sorption efficiency and physical-chemical properties of proteins; ^a B1 – buffer containing 1 mM Tris-HCl, pH 7.4, ^b pI values are taken from Righetti P.R. and Tudor G. (1981) Isoelectric points and molecular weights of proteins. A new table. *J Chrom*, 220, 115-194, ^c Protein charge was calculated by accounting of K, R, E and D charges in protein sequences.

	Asp f2	BSA	SOD	Insulin	IFN	SWM	IgG	Lys
H ₂ O	37	40	0	-	50	0	0	0
B1 ^a	93	64	9	81	70	0	0	0
B1+0.9% NaCl	90	0	0	0	32	0	0	0
B1+1xPBS	68	0	0	0	0	0	0	
pI ^b		4.8 - 5.8	4.2 - 5.9	5.4	5.5 - 7.0	7.7 - 8.5	5.5 - 7.9	>11
Charge ^c	-16	-14	-6	-4	-2	+2	+7	+14
MW, kDa	37	66	16	5	20	17	150	14

Among them Asp f2, a major allergen from *Aspergillus fumigatus* fungi, was the most acidic protein (total charge -16), and lysozyme was the most basic one (total charge +14). Also the proteins varied significantly in their MWs so that it was possible to register the effect of MW on complex formation of the proteins with Chi-NPs. For this work we used Chi-NPs made of 340 kDa chitosan derivative (**Figure 1, Table 2**, sample Ic). The proteins were dissolved in 7 mM Tris-HCl pH 7.4 while Chi-NPs were in water. Complexes between Chi-NPs and proteins were formed at 3:2 ratios as described in the experimental part. Protein bound to Chi-NPs was removed by centrifugation and a residual protein concentration in supernatant was compared with that of the parent solution.

In solution at low ionic strength Chi-NPs formed complexes with the most acidic proteins: Asp f2, BSA, insulin and IFN but not with SOD showing that the ionic interactions play a role in complex formation. On the contrary, Chi-NPs did not form complexes with all basic proteins studied: SWM, IgG and Lysozyme (**Table 3**). MWs of acidic proteins were in the range from 16 to 66 kDa and MWs of basic proteins ranged from 14 to 150 kDa. So that we could suppose that MW did not affect the complex formation while ionic interaction played a major role in the interaction of proteins with Chi-NPs. In order to estimate the role of ionic interaction in chitosan-protein complexes we increased the ionic strength by the addition of 0.9% NaCl. This completely abrogated the interaction of Chi-NPs with BSA and insulin and significantly decreased IFN binding while had no effect on Asp f2. These results showed that the stability of Chi-NPs complexes with different proteins depended on a spatial distribution of ionic groups in proteins. Small counterions Na⁺ and Cl⁻ could mask the ionic groups of proteins preventing them from the interaction with Chi-NPs. Moreover, the ionic groups could be hidden inside protein molecule. This explained why SOD with the total distributed charge of -6 and pI 4.2 - 5.9 did not form complex with Chi-NPs.

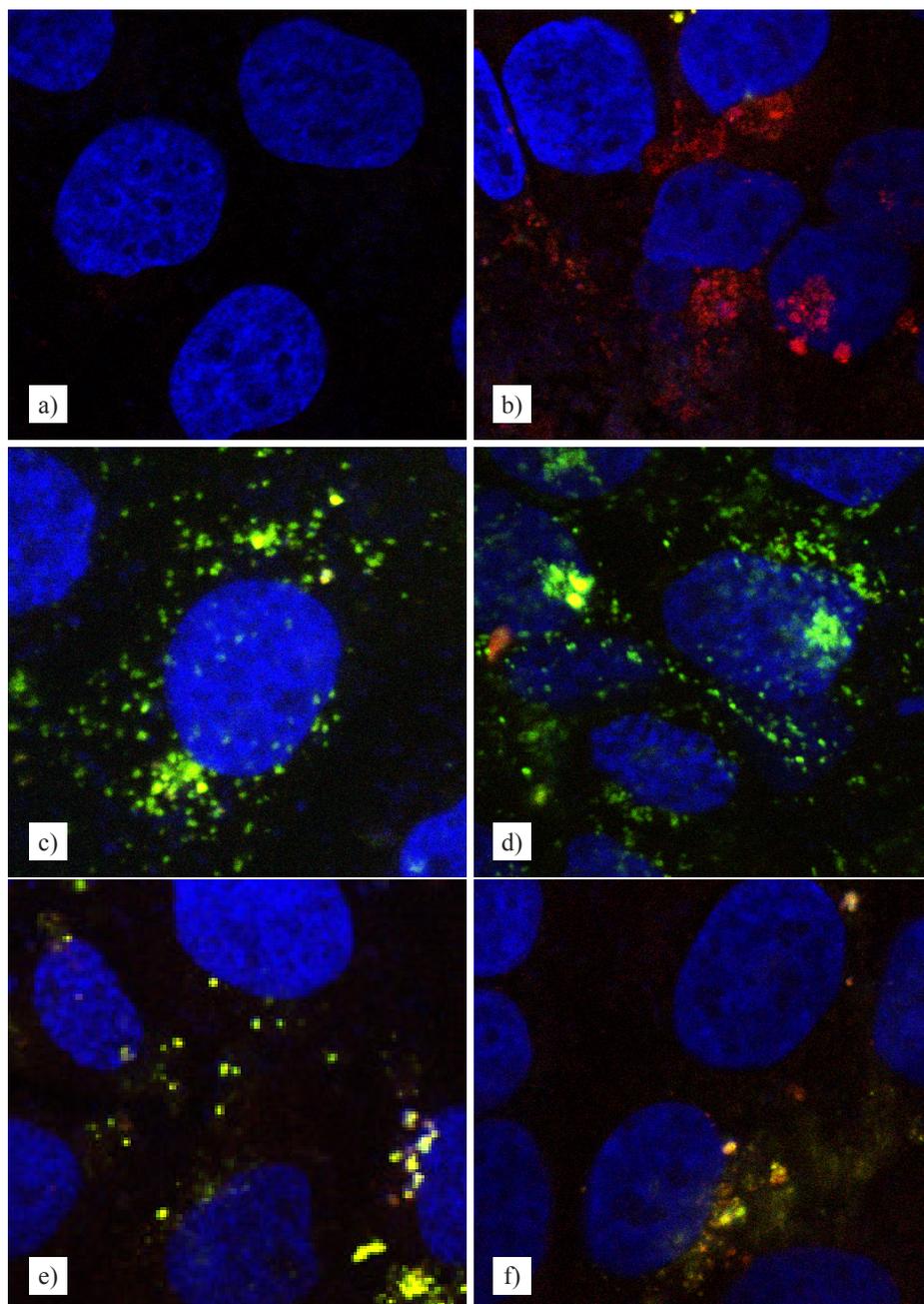


Figure 2. Confocal fluorescent microscopic images. a) Control, b) Chi-NP-Rh, c) Chi-NP-Rh+Asp j2-FITC, d) Chi-NP-Rh+BSA-FITC, Chi-NP-Rh+SOD-FITC, f) Chi-NP-Rh+SWM-FITC

Many proteins contain sites capable of interacting with phosphates. Since Chi-NPs contained triphosphate ions which could interact with proteins, it was interesting to estimate the effect of phosphate ions on Chi-NPs-protein complex formation. Incubation of Chi-NPs with proteins in the presence of 10 mM phosphates and 0.9% NaCl (saline phosphate buffer, PBS) abrogated the complex formation between Chi-NPs and IFN and decreased Asp f2 binding. These results confirmed that both chitosan and TPP took part in the formation of complexes with proteins. As a result we could conclude that Chi-NPs demonstrated considerable selectivity in the formation of complexes with proteins. This selectivity mostly depended on the steric availability of protein ionic groups for the interaction with Chi-NPs. In future the observation can provide a possibility of separation of target proteins from protein mixtures by means of Chi-NPs. Indeed, it was recently shown that the low MW chitosan was able to precipitate β -lactoglobulin but not the other proteins in cow whey [19].

Confocal fluorescent microscopy experiments demonstrated that the rhodamine-labeled Chi-NPs loaded with proteins were first absorbed by cell membranes. Then, the particles steadily penetrated into the intercellular cavities and, finally, they were found in the intracellular or peri-nuclear locations (**Figure 2.c, 2.d**), while free FITC-labeled proteins did not penetrate the cells and were found as the patches on cell membranes (**Figure 2.e, 2.f**).

Control without (A) and with Chi-NP (B). Cell incubated with Chi-NPs complexed with acidic proteins Asp f 2 (C) and BSA (D). Membrane locations of complexes are shown by white arrows. Intracellular locations of complexes are shown by white asters. Chi-NPs do not form complexes with SOD (E) or SWM (F). Proteins are bound to cell membranes.

4. Conclusions

Thus, it was shown that the colloidal stable Chi-NPs based on by N-hexanoylchitosan and TPP which and produced by ionotropic gelation could form complex with acidic proteins. Stability of these complexes depended on the presence of counterions ions and phosphates in medium. Chi-NPs can be used for targeted protein delivery into epithelial cells or protein purification from complex mixtures.

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