Protein-loaded microparticles prepared by layer-by-layer assembling of dextran sulfate and chitosan: characterization of composition

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Introduction

Protein encapsulation into polyelectrolyte micro- and nanoparticles is an actual task of applied biotechnology. One of the most preferable polyelectrolyte for protein microencapsulation is chitosan (Ch) as this biomaterial is biocompatible, biodegradable and possesses mucoadhesive properties. However, there are some difficulties with Ch quantification. The standard method for polysaccharides quantification based on the determination of corresponding monomers in their hydrolysates *(Bosworth T.R. et al., 1994)* appears to be unsuitable in case of Ch as the beta-glycosidic linkage between its monomers is difficult to hydrolyze *(Prochazkova S. et al., 1999)*. More efficient methods for the assay of Ch are colorimetric ones with various anionic dyes. These techniques are based on reactions between free amino groups of Ch and acting agents, that make them ineligible for determination of Ch in presence of substances bearing primary amino groups, including proteins *(Sekmokienė D. et al., 2005)*.

In consequence the Ch content in the protein loaded micro- and nanoparticles either could not be determined at all *(Chen Y. et al., 2003; Cui F. et al., 2004)* or is indirectly estimated with serious errors *(Balabushevich N.G. et al., 2006)*. To solve the problem, the possibility of application of technique early developed for amino acids determination with o-phthalaldehyde (o-PA) and *N*-acetylcysteine (NAC) *(Švedas V.-J.K. et al., 1980)* for Ch assay was investigated. The procedure conditions for chitosan determination in the presence of model compounds used for protein-loaded microparticles preparation were optimized. The method elaborated was used to characterize Ch content in microparticles prepared by polyelectrolyte assembling on insoluble protein-polyanion complex.

Materials and methods

Materials. Ch 22 kDa was kindly donated by Prof. Varlamov V.P. ("Bioengineering center" of Russian Academy of Sciences (RAS), Russia); Ch 100 kDa with a degree of deacetylation (DA) 84% was donaited by Dr. Yatluk Y.G., (Postovsky Institute of Organic Synthesis of RAS, Russia); Ch 150, 400, 600 kDa were purchased from Fluka (Switzerland); Ch 460 kDa with a DA 93% was purchased from Closed joint-stock company "Bioprogress" (Russia); dextran sulphate sodium salt (DS) 500 kDa, NAC were obtained from Sigma (USA); *o*-PA was purchased from Koch Light (UK); human recombinant insulin (Ins), 29 U/mg, was obtained from Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry of RAS (Russia).

Preparation of Ins-loaded microparticles. Ins-containing microparticles were obtained as described in *(Larionova N.I. et al.; 2005; Balabushevich N.G. et al., 2006)*. Insoluble complex between Ins and DS was prepared at pH 3.0. Then microaggregates were sequentially covered with Ch (M_w 400 kDa) and DS at pH 3.0 and 0.15 M NaCl. After a definite number of adsorption treatments microparticles were washed with 1 mM HCl and lyophilized.

Preparation of solutions. All Ch were dissolved in 0.01 M HCl and the pH value of the solutions was adjusted to 3.0. To prepare the model solutions Ins and DS were dissolved in 0.2 M borate buffer, pH 8.9. To determine Ch content in Ins-loaded microparticles, the lyophilized samples were dissolved in 0.01 M NaOH solution, and diluted by 0.2 M borate buffer, pH 8.9 to final Ins concentration 0.1-0.2 mg/mL. The reagent solution was freshly prepared before the assay by adding 0.2 mL of 0.11 M *o*-PA in ethanol and 0.2 mL of 0.071 M NAC in ethanol to 5 mL of 0.2 M borate buffer, pH 8.9. Buffer (0.5 mL) and reagent (0.5 mL) solutions containing 0.25 M NaCl were used to prepare the reference solution.

Characterization of microparticles. Protein concentration was determined by Lowry routine (*Lowry O.H. et al., 1951*). DS concentration was measured according to Dubois method *(Dubois M. et al., 1956)*. Ch content was determined as follows: 0.5 mL samples solutions were introduced into the tubes followed by addition of NaCl to final concentration 0.25 M and by addition of 0.5 mL of reagent solution. The reaction was allowed to proceed for 1 h, then the absorbance values were measured at 340 nm with a Shimadzu UV – 265 FW spectrophotometer (Japan) against the reference solution.

Results and discussion

Optimization of conditions of Ch determination with o-PA. The procedure conditions of colorimetric reaction between amino acids and o-PA proposed in *(Švedas V.-J.K. et al., 1980)* were taken as the basis. This method is based on derivatization of primary amino groups of an investigated compound with o-PA and a thiol resulting in formation of isoindoles which have a characteristic absorption maximum at 340 nm (Fig.1).

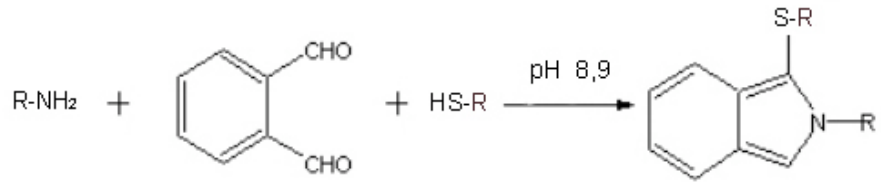

Fig.1. Derivatization of primary amino groups by o-PA and a thiol.

Both Ins and Ch should be involved in this reaction as they contain primary amino groups. We suggested that we may calculate Ch concentration in presence of Ins by determination of summary protein and Ch content and by determination of protein concentration using Lowry routine.

In preliminary experiments we have varied the concentration of reagents (o-PA, NAC), pH, derivatization time. The obtained results demonstrated that the pH 8.9 is optimal for analysis of Ch in presence of Ins. At pH>9 the contribution of Ins significantly increase, while at pH<8.0 the reaction rates with both Ch and Ins dramatically decrease (A_{340} < 0.1 during 60 min).

Ch determination was shown to be possible in these conditions in model solutions composed of known quantities of Ins and Ch. However, in real systems when Ch is used for protein microencapsulation, tested solutions often contain other components, such as polysaccharides, polyelectrolytes (*Cui F. et al., 2004; Balabushevich N.G. et al., 2006)* etc. For example, when DS (0.04 mg/mL) was added to the mixture of Ch and Ins calculated Ch concentration turned to be 3 times lower than the real one. We suggested that the underestimation of Ch may be a consequence of interactions between DS and the reagent, Ch and Ins.

Thus the influence of DS on all reaction components was investigated. DS didn't almost change the absorbance value of the reference solution. When added to Ins solution, DS caused the increase in absorbance at 340 nm due to the turbidity caused by insoluble complexes formation between polycation Ins and strong polyanion DS. When added to Ch solution, DS caused a significant decrease in absorbance at 340 nm. It is well known *(Petrov A.I. et al., 2003)* that strong polyelectrolyte can cause a shift of a potentiometric titration curve of weak oppositely charged polyelectrolyte on 2-3 pH units. It means in our case that at pH 8.9 in the DS presence Ch amino groups would be protonated and capable of interpolyelectrolyte complex formation. Thus we may conclude that DS influenced on Ins and Ch determination.

To prevent DS interference NaCl was added to all samples to a final concentration 0.25 M. NaCl presence didn't influence the slope of calibration lines of Ins and Ch. As the maximum of absorbance growth was not reached during 2 h, standard calibration lines of Ins and Ch were constructed according to the absorbance values at 340 nm after 1 h from reaction beginning using the stock solutions. The correlation (R^2) of all calibration lines was greater than 0.99.

Ch concentration in a model mixture of Ins, Ch and DS was calculated according to the formula: $[Ch] = \frac{A_{340} - [Ins] \times tg_{Ins}}{tg_{Ch}}$, where tg_{Ch} and tg_{Ins} are the slopes of calibration lines for Ch and Ins, correspondingly, [Ins] – Ins concentration in the model mixture, A_{340} – the absorbance value of the model mixture against the reference solution.

Table 1 shows the results of Ch concentration determination in the presence of protein and DS. It can be seen that Ch concentrations differed from the real ones in the model mixtures on 3-7 %.

A possibility of Ch assay of various molecular weights by the technique elaborated was also investigated. Six different Ch were used for assay (Table 2). Ch concentrations were from 0.01 to 0.13 mg/mL. The correlation (R^2) of calibration curves was greater than 0.99 for all Ch tested. The results of Ch determination are presented in Table 2.

[Ch], mg/mL	Measured [Ch], mg/mL	Recovery, %
0.020	0.019±0.001	95±5
0.040	0.041±0.002	103±5
0.060	0.056±0.002	93±4
0.080	0.086±0.003	107±3

Table 1. Assay of Ch in the model mixtures containing 0.125 mg/mL Ins and 0.050 mg/mL DS.

Ch, M_W, kDa	22	100	150	400	460	600
Slope of calibration line	11.3	8.38	8.11	7.80	9.38	7.81
R^2	0.9982	0.9925	0.9958	0.9969	0.9948	0.9916

Table 2. Assay of Ch of various molecular weights.

Characterization of microparticles composition. The share of all components was determined in Ins-loaded microparticles (Table 3). It should be noted that even after 4th stage of adsorption treatment Ins content in microparticles was higher than 50 %. Ch content was determined by the technique elaborated and using difference method. The data obtained by 2 methods were in good coincidence.

Sample	Content, w %			
	Ins	DS	Ch	
			By difference*	By the method elaborated
(Ins-DS)	80±4	22±5	0	0
(Ins-DS)-Ch	55±3	16±3	19±5	19±2
(Ins-DS)-Ch-DS	48±2	28±5	14±6	15±2
(Ins-DS)-Ch-DS-Ch	53±4	20±3	17±6	17±2

Table 3. Composition of Ins-loaded microparticles prepared from DS and Ch.
* - taking into account the mean water content in lyophilized microparticles (10 %)

Conclusions

Thus we developed a novel colorimetric technique of Ch determination. The method has proven to be convenient and reproducible assay for quantification of Ch of various molecular weights. The assay is both precise and accurate within the Ch concentration ranged from 0.01 to 0.15 mg/mL. The method elaborated is suitable for Ch determination in the presence of proteins and strong polyanion DS. The o-phthalaldehyde assay may be considered as an essential aid in characterization of protein-loaded microparticles composed of chitosan and polyanions.

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