Alginate and chitosan gel nanoparticles for efficient protein entrapment

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Abstract

Alginate and chitosan nanoparticles were synthesized by ionic gelation of the polymers in the presence of stabilizers (PEG 1500, PEG 6000, TWEEN 80). The stability of 210-240 nm Ca-alginate colloids is affected by nanoparticles ageing and by the presence of a stabilizer. The diameter of chitosan nanoparticles is in the range of 180 to 260 nm and depends on polymer concentration in the reaction mixture, its molecular weight, and stabilizer type. The nanoparticles efficiently entrap a model protein, bovine serum albumin, in the amount up to 0.24 mg per 1 mg of polysaccharide.

Keywords: alginate, chitosan, nanoparticles, ionic gelation, BSA, PEG, TWEEN 80, ionic gelation, BSA, PEG, TWEEN 80

1. Introduction

Development of new nanosized vehicles that successfully entrap biologically active substances is one of the main directions in biomaterials science. Encasing in a polymeric matrix enhances chemical stability of the substances, improves their solubility in biological liquids, controls release of the active components, and alters drugs body distribution [1]. Such nanoparticles create a foundation for targeted delivery systems with improved blood circulation time and lower systemic toxicity [1-2].

Biocompatible and non-toxic biopolymers (chitosan, alginate, pectin, etc.) are often used as structural components for nano- and microcontainers [1-3]. Recently, polysaccharide nanoparticles have been reported as carriers for various classes of drugs: anti-inflammatory (ammonium glycyrrhizinate [4], prednisolone [5]), anticancer drugs (doxorubicine [6], taxol [7]), antiviral agents (lopinavir [8]), antibacterial drugs (rifampicin, isoniazid, pyrazinamide, ethanbutol [9]), proteins [10]. The common preparation technique for the nanoparticles is based on emulsification of diluted polysaccharide solutions in immiscible organic solvent followed by gelation of the polymer. The particles of 2-10 micron diameter
are easily produced by the method [11, 12], the preparation of nanoparticles of smaller diameter was rarely reported [9].

Here we propose a method that allows to obtain stable colloids of calcium alginate and chitosan gel particles with a diameter of 180-250 nm while swollen in water and 50-80 nm size after lyophilisation. The benefit of it is that only nontoxic compounds listed in the FDA Inactive Ingredients Database are used. It is based on crosslinking of corresponding polysaccharide with multivalent ions in an aqueous solution containing polyethylene glycol stabilizers. Incorporation of hydrophilic PEG tails on the surface of the nanoparticles allows to achieve high colloidal stability of the aqueous dispersion. A model protein, bovine serum albumin, was entrapped in the nanoparticles with high efficiency (ca. 0.24 mg per 1 mg of polysaccharide). The mixed protein/polysaccharide nanoparticles with hydrophobic patches of protein in the interior are considered perspective vehicles for hydrophobic drugs, such as anticancer drugs.

2. Materials and methods

Alginic acid (from brown algae, 240 kDa), chitosan (from crab shell, 800 kDa), polyethylenimine (PEI), poly(sodium-4-styrenesulfonate) (PSS), albumin from bovine serum (BSA), fluorescein isothiocyanate (FITC), polyethylene glycol (PEG) with molecular weight of 1500 (PEG 1500) were obtained from Sigma. TWEEN 80 and PEG with molecular weight of 6000 (PEG 6000) were from Panreac Quimica S.A.U. FITC-labeled albumin (BSA-FITC) was prepared according [13].

2.1. Ca-alginate nanoparticles preparation

A 2 M solution of calcium chloride (1.5 mL) was added dropwise to 30 mL of a 0.1 wt % sodium alginate solution (pH 5.0–5.5) under continuous ultrasonication (35 kHz). TWEEN 80 (0.5-5.0 wt %) or PEG 1500 (5-25 wt %) was added to the solution of sodium alginate prior crosslinking with Ca\(^{2+}\) ions. The obtained dispersion was ether aged in the supernatant at room temperature for 24 h or, on one occasion, separated immediately by centrifugation, rinsed twice with distilled water, and dispersed in a small volume of the solvent.

2.2. Chitosan nanoparticles preparation

Chitosan (0.025–0.75% w/v) was dissolved in aqueous acetic acid (2 % v/v, pH 3.0). TWEEN 80 (0.5-5.0 wt %), PEG 1500 (9-30 wt %) or PEG 6000 (9-30 wt %) was added to the solution of chitosan as a stabilizer. A sodium sulfate solution (20 wt %) was added dropwise to the chitosan solution under simultaneous stirring at 420 rpm and ultrasonication (35 kHz). After adding sodium sulfate the stirring and sonication were continued for 1 h. Chitosan nanoparticles were separated from supernatant by centrifugation at 4000 rpm and redispersed in water.

2.3. Preparation of BSA-loaded nanoparticles

BSA was admixed to a polysaccharide solution (0.25% for chitosan, 0.1 % for alginate) to a final ratio varying from 1:10 to 2:1 by weight. The BSA-loaded alginate and chitosan nanoparticles were formed using the same procedures as those for unloaded nanoparticles. The concentration of BSA in chitosan nanoparticles was determined by the Lowry method [14]. BSA-FITC was used for encapsulation in Ca-alginate nanoparticles and its concentration was evaluated spectrofluorometrically (\(\lambda_{ex} = 495\) nm, \(\lambda_{em} = 520\) nm, a Solar CM 2203, Belarus spectrofluorometer). The BSA encapsulation efficiency (EE) of nanoparticles was calculated as: EE(%) = (1 – C\(_1\)/C\(_0\))·100, where C\(_0\) and C\(_1\) are the total and supernatant concentrations of BSA.
2.4. Chitosan hydrolysis

Chitosan (1 g) was dissolved in 100 mL of a 1% lactic acid solution and kept at 20±1 °C. This hydrolyzing agent has been chosen due to the fact that no additional deacetylation of chitosan is observed under these conditions [15]. Aliquots were withdrawn after certain time periods and their intrinsic viscosity was measured until no further decrease was observed. The hydrolyzed for 90 days chitosan was precipitated by 0.1 M NaOH (pH 9.0), rinsed with distilled water to pH 7.0, and lyophilized. Molecular weights of unhydrolyzed (800 kDa) and hydrolyzed (310 kDa) chitosan were determined by viscosimetry in a 0.2 M sodium acetate–2% acetic acid using the following constants in the Mark-Houwink equation (25°C): a = 0.85, K_m = 1.38·10^-4 [15].

2.5. Nanoparticles characterization

The morphology and size of nanoparticles were studied by atomic force microscopy (Multimoda III, Veeco, USA) and transmission electron microscopy (JEM-100 CX, Japan). For TEM, a drop of colloid was placed on a copper grid with polyvinyl formal film. The samples for AFM were prepared by adsorbing the polysaccharide nanoparticles on a silicon substrate modified with a PEI and PEI/PSS sublayer for Ca-alginate and chitosan nanoparticles, accordingly. The mean hydrodynamic diameter of swollen polysaccharide nanoparticles was estimated by light scattering [16].

3. Results and discussion

3.1. Calcium alginate nanoparticles

Calcium cations have been chosen as a gelating agent for alginate because of their biocompatibility, non-toxicity, and high gelating ability towards such polysaccharides, as alginates and pectins [3]. Besides, they promote formation of smaller particles in comparison with other cations [17].

The synthesized calcium alginate nanoparticles are negatively charged and readily adsorb on the surface coated with a layer of positively charged polyethylenimine (fig. 1a).

![AFM image of calcium alginate nanoparticles on a PEI-coated surface](image1a.png)

![Apparent diameter of Ca-alginate nanoparticles](image1b.png)

Fig. 1. a) AFM image of calcium alginate nanoparticles on a PEI-coated surface; b) apparent diameter of Ca-alginate nanoparticles (black – dried, grey – swollen) prepared in the presence of different stabilizers
Dried particles are flat and have a round form (fig. 1a). As determined from AFM images analysis, in all synthesized samples the mean nanoparticles diameter does not exceed 280 nm, and in some cases, it is less than 130 nm. The particles height is below 20 nm. At the same time, the mean hydrodynamic diameter of particles in water varies from 210 to 540 nm (fig. 1b). It indicates that initially swollen in water gel particles collapse as drying occurs. The size distribution and stability of the Ca-alginate colloids are affected by ageing in the supernatant and the presence of the stabilizers. However, the stabilizer type and its concentration do not essentially influence the size of lyophilized nanoparticles (fig. 1b, black).

At the same time, the apparent diameter of swollen prepared nanoparticles obtained with TWEEN 80 is somewhat smaller than in the presence of PEG. TWEEN 80 is an nonionogenic surfactant with critical micelle concentration of 0.01 mM and HLB value of 15 [18]. Being adsorbed on the surface of calcium alginate nanoparticles, TWEEN 80 apparently forms a shell that structure reminds that of a micelle. The interaction between neighboring hydrophobic tails prevents extreme swelling of nanoparticles in water.

We assume that the main role of PEG during the synthesis consists in increasing viscosity of the reaction mixture, thus decreasing the rate of nanoparticles aggregation. PEG macromolecules apparently adsorb on the surface of already formed nanoparticles through hydrogen binding [19]; their presence results in less sticking of nanoparticles in concentrated colloids. Stable for more than 30 days calcium alginate colloids of 200-300 nm particles are formed in the presence of 15 wt % PEG 1500 and 0.5 – 5 wt % TWEEN 80. However, the utilization of PEG solutions with a concentration higher than 15 wt.% results in highly dispersed nanoparticles due to uneven distribution of the crosslinking agent CaCl₂ in the viscous solution.

3.2. Chitosan nanoparticles

Chitosan nanoparticles were obtained by crosslinking polysaccharide macromolecules with sulfate anions. A higher than 0.75 % concentrations of the polymer is not practical because viscosity of the solution becomes too high. As a consequence, a homogeneous distribution of the added sodium sulfate is not possible; it would have led to the formation of agglomerates. The addition of a stabilizer (TWEEN 80, PEG 1500, PEG 6000) in a chitosan solution during the synthesis results in a reduction of the nanoparticles hydrodynamic diameter by approximately 12-30 % (fig. 2) but the type and concentration of the stabilizer insignificantly affect the nanoparticles size.
However, prepared without stabilizers colloids are collapsed in one day. The utilization of any stabilizer on the preparation stage increases later colloidal stability of the nanoparticles in water. No separation of colloids prepared in the presence of TWEEN 80 was observed after 30 days. We assume that the forces behind the interaction of TWEEN 80 and PEG with chitosan nanoparticles that support the high stability of the colloids are similar to the discussed above for alginate nanoparticles.

By changing the polymer concentration in the reaction mixture the size of formed gel nanoparticles can be slightly altered (fig. 2b). A decrease in chitosan concentration from 0.5 to 0.025 wt.% leads to a 1.3 fold reduction of the average hydrodynamic diameter of the nanoparticles. The data are in good agreement with Ref.20 on the influence of chitosan concentration on the size of nanoparticles prepared by sodium tripolyphosphate mediated gelation.

The chitosan nanoparticles have positively charged surface and form a uniform layer on a negatively charged PEI/PSS precursor film (fig. 3a). They are monodisperse and have a spherical shape with the diameter of about 80 nm (fig. 3b). The size of these nanoparticles is 2.8 times smaller than that determined by light scattering method (~ 220 nm) in water; the difference is caused by gel nanoparticles swelling like that for calcium alginate particles.

The size of particles formed by ionic gelation of a biopolymer strongly depends on its molecular weight [21]. Chitosans of two different molecular weights were analyzed in order to develop a reliable method of formation of chitosan nanoparticles with a given size. Under similar conditions, 800 kDa chitosan produces nanoparticles of a 220 nm diameter, while the hydrodynamic diameter of nanoparticles prepared from the sample with Mw 310 kDa was ca.190 nm, and those calculated from TEM data – 80 and 50 nm, accordingly. This trend of decreasing particle size with reducing chitosan molecular weight may be explained by larger initial globule size of higher molecular weight chitosan that result in nanoparticles with increasing diameter. The similar results have been previously reported for chitosan-tripolyphosphate nanoparticles [22, 23].

3.3. Encapsulation of BSA in alginate and chitosan nanoparticles

BSA was incorporated into both alginate and chitosan nanoparticles by admixing the protein to a polysaccharide solution before the corresponding nanoparticles were formed. The BSA encapsulation
efficiency (EE) was significantly affected by its initial concentration in the reaction mixture. For example, the encapsulation efficiency by alginate nanoparticles from the reaction mixture with a 0.25 mg/mL BSA concentration is more than 99%. An increase in protein concentration (from 0.25 to 1.0 mg/mL) leads to a decrease in encapsulation efficiency to 15%.

In the case of chitosan nanoparticles, a fourfold increase of initial protein concentration (from 0.25 to 1.0 mg/mL) enhances the BSA encapsulation efficiency approximately two times (table 1). However, any further increase in protein concentration does not change its amount included per 1 mg of chitosan. The maximum capacity of chitosan nanoparticles for the protein of ca. 0.24 mg/mg was observed in the range of BSA concentrations from 1.0 to 5.0 mg/mL. The amount is comparable previously reported data on BSA entrapment in chitosan nanoparticles [24].

### Table 1. BSA entrapment efficiency in chitosan nanoparticles

<table>
<thead>
<tr>
<th>Initial BSA concentration, mg/ml</th>
<th>Chitosan:BSA ratio in initial solution</th>
<th>EE, %</th>
<th>Amount BSA in the nanoparticles, mg per mg of chitosan</th>
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<tr>
<td>0.25</td>
<td>10:1</td>
<td>25.0±1.0</td>
<td>0.02</td>
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<td>5:1</td>
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<tr>
<td>5.0</td>
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<td>13.0±1.0</td>
<td>0.24</td>
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</tbody>
</table>

### 4. Conclusion

A non-emulsion method of preparation of stable aqueous colloids of alginate and chitosan gel-like nanoparticles is developed. By varying synthesis conditions (type of stabilizer and its concentration, molecular weight and concentration of biopolymer) calcium alginate nanoparticles with a diameter from 210 to 240 nm and chitosan nanoparticles with a diameter from 180 to 260 nm in water are obtained. The size of lyophilized chitosan nanoparticles does not exceed 80 nm. By admixing protein to polysaccharide solution prior the nanoparticles formation, its efficient encapsulation is achieved. The formed polysaccharides nanoparticles are promising carriers for biologically active compounds.

### References


