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Multilayer alginate/protamine microsized capsules: encapsulation of α-chymotrypsin and controlled release study

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Abstract

Stable polyelectrolyte microcapsules with size 6.5 μm were produced by means of the layer-by-layer adsorption of sodium alginate and protamine to surface of melamine formaldehyde microparticles. Core decomposition at low pH leads to formation of polyelectrolyte multilayered capsules filled with alginate gel. A proteolytic enzyme, α-chymotrypsin, was loaded into these microcapsules by embedding in alginate gel with high efficacy. The protein in the capsules was found to retain a high physiological activity of about 70% showed with fluorescent product. The protein was found to keep inside the microcapsules in water and acid (HCl solution, pH 1.7) during 24 and 4 h, respectively, while 75–85% of protein can be revealed in supernatant after 6 h incubation at pH 8.0 (0.05 M Tris buffer) in the presence of 2.5% w/v of sodium deoxycholate. The release rate of enzyme from multilayer alginate/protamine microcapsules can be regulated by additional adsorption of polyelectrolytes onto the microcapsules with encapsulated protein. Such protein-loaded capsules can be proposed as a drug delivery system with controllable release properties.

Keywords: Alginate/protamine microcapsules; α-Chymotrypsin; Controlled release; Drug delivery system

1. Introduction

A variety of both natural and synthetic polymeric systems have been investigated for controlled release of proteins and peptides. Recently, a new polymeric system based on layer-by-layer adsorption of oppositely charged macromolecules onto colloidal particles was proposed to encapsulate various materials synthetic polyelectrolytes, chitosan and its derivatives, proteins, DNA, lipids, multivalent dyes (Sukhorukov et al., 1998; Donath et al., 1998; Sukhorukov et al., 2000; Balabushevitch et al., 2001). Different templates with size ranging from 50 nm to tens of microns, such as organic and inorganic colloid particles, protein aggregates, biological cells and drug nanocrystals were coated with multilayer films (Sukhorukov, 2001). Some colloidal templates can be decomposed at conditions where the polymer shell is stable, which leads to the formation of hollow capsules with defined size, shape and shell thickness.

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In the present investigation sodium alginate and cationic protein protamine were chosen as biocompatible polymers.

Alginate, a naturally occurring biopolymer has several unique properties that have enabled it to be used as a matrix for the entrapment and/or delivery of a variety of biological agents (Pommersheim et al., 1994; Gombotz and Wee, 1998). The in situ gelation properties of alginate were reported as early as 1947 by Major George Blaine (Blaine, 1947). Usually, alginate can be ionically crosslinked by the addition of divalent cations in aqueous solution. On the other hand, alginate is well known to form strong complexes with polycations including proteins, polypeptides and synthetic polymers. For example, a poly-L-lysine coating can be performed for the formation of the alginate gel (Dupuy et al., 1994). One of the disadvantages of this system is the large size of the beads formed (large beads (>1 mm in diameter) and microbeads (0.2 mm in diameter)) (Martinsen et al., 1989; Ku et al., 1995). Moreover, numerous reports published on the encapsulation of proteins from alginate matrices claim that all loading techniques would require a lot of materials and time (Singh and Burgess, 1989; Ko et al., 1995; Vandenberg and De La Noue, 2001). The usage of the polyelectrolyte microcapsules described above allows fabrication of the formulation with reduced capsule size up to nanometric range and increasing the encapsulation efficacy.

Therefore, the objective of this work is to fabricate the polyelectrolyte microcapsules composed of alginate and protamine, encapsulate the protein and investigate the protein release from them. One of the serine proteinase—α-chymotrypsin that functions in intestinal tract has been chosen as a model protein.

2. Materials and methods

2.1. Materials

The sources of chemicals are as follows: protamine sulfate and alginic acid sodium salt (viscosity of 2%) and α-chymotrypsin (Sigma), deoxycholic acid sodium salt (Fluka), rhodamine 110, bis-(succinoyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl amide) (Molecular Probes), rhodamine isothiocyanate (Aldrich), melamine formaldehyde (MF)-particles (Microparticles GmbH, Berlin, Germany).

2.2. Preparation of polyelectrolyte microcapsules

Polyelectrolyte multilayer assembly was fabricated on 5.2 ± 0.2 μm MF-particles at pH 6.7, 0.5 M NaCl by alternating adsorption of 8 alginate/protamine layers as described in Sukhorukov et al. (1998). The cores were dissolved at pH 1.6 by 0.1 M HCl solution and after dissolution the hollow capsules swelled up to 6.5 ± 0.5 μm. Confocal images were taken by means of a confocal laser scanning microscope (TCS Leica). The concentration of microcapsule suspension was calculated directly from the numerous confocal images by counting the amount of microcapsules in defined volume and was equal $2 \pm 1 \times 10^{10}$ particles/l.

2.3. Encapsulation of α-chymotrypsin into the alginate/protamine microcapsules

0.2 ml capsule suspension in water (concentration $2 \pm 1 \times 10^{10}$ particles/l) was centrifuged (3000 rpm, 10 min, Sigma, Bioblock Scientific) and supernatant was removed. Then 0.2 ml of protein solution (1 mg/ml) in Tris–HCl buffer pH 8.0 were mixed in the Eppendorf tube with 8-layers alginate/protamine capsules. This mixture was incubated for 15 min at room temperature. Afterwards, it was centrifuged (3000 rpm, 10 min) and washed three times with 0.2 ml of water. Additionally, the part of the capsules loaded with protein was covered by 8 layer alginate/protamine polyelectrolytes to produce 16 layer microcapsules. The supernatant and capsule suspensions were used for determination of the protein amount and its activity. The protein concentration in solutions and in suspensions of microcapsules was determined according to Lowry et al. method (Lowry et al., 1951). The enzymatic activity of free and encapsulated α-chymotrypsin was determined by fluorescence microscopy using rhodamine 110, bis-(succinoyl-L-alanyl-L-alanyl-L-

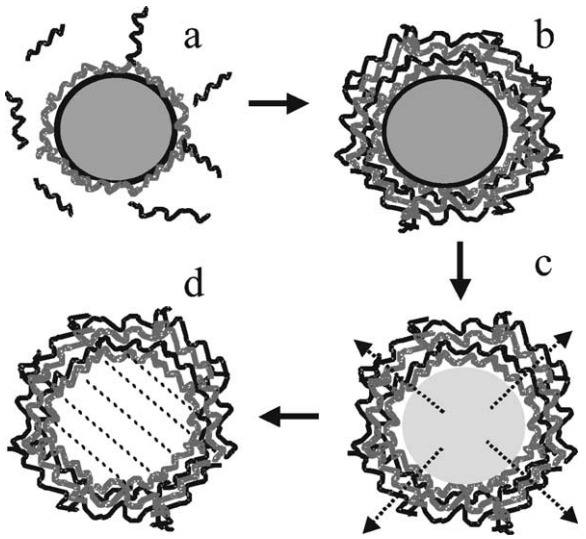

Fig. 1. Consecutive adsorption of negatively (black) and positively (gray) charged polyelectrolytes—protamine and sodium alginate onto negatively charged colloidal particles (a, b), dissolution of the colloidal core at pH 1.6, HCl solution (c, d) and the formation of the polyelectrolyte capsules filled with alginate matrix.

prolyl-L-phenylalanyl amide) as a substrate in 0.05 M Tris–HCl, pH 8.0 at room temperature as described in Leytus et al. (1983). The activity of encapsulated α-chymotrypsin was calculated from the activity of the native enzyme measured at standard conditions (at pH 8.0 (Tris–HCl buffer), at 25 °C).

2.4. Confocal microscopy

The micrographs were obtained by means of a Leica confocal laser scanning microscope mounted to a Leica Aristoplan. A 100× oil immersion objective and standard filter settings for rhodamine excitation and emission were used. Chymotrypsin was labeled by rhodamine isothiocyanate at pH 9.0, 0.1 M carbonate buffer, over night, at molar ratio [chymotrypsin]/[rhodamine B isothiocyanate] = 1/3.

2.5. Protein release from polyelectrolyte microcapsules

Microcapsules were resuspended in 1 ml of 0.05 M Tris–HCl buffer (pH 8.0) containing 2.5% w/v of sodium deoxycholate or in 1 ml of HCl solution (pH 1.7) obtaining the final concentration 0.2 mg protein/ml. After 15, 35, 75, 120, 180, 300, 360 and 720 min aliquots of suspension were with-

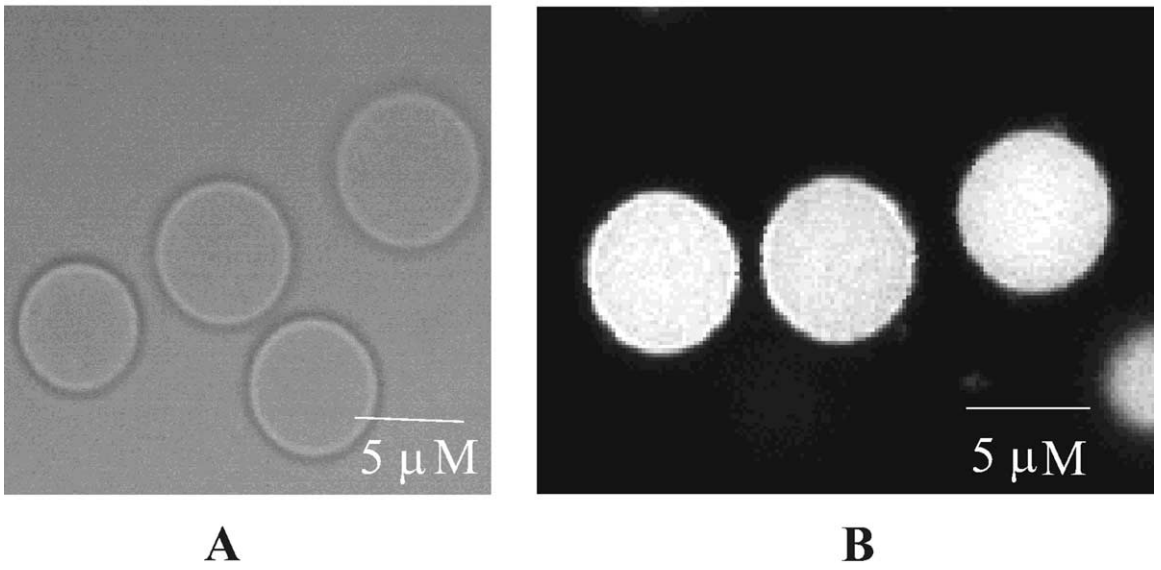

Fig. 2. Confocal microscope image of the alginate/protamine microcapsules in water (A) and alginate microcapsules loaded with rhodamine labeled α-chymotrypsin (B).

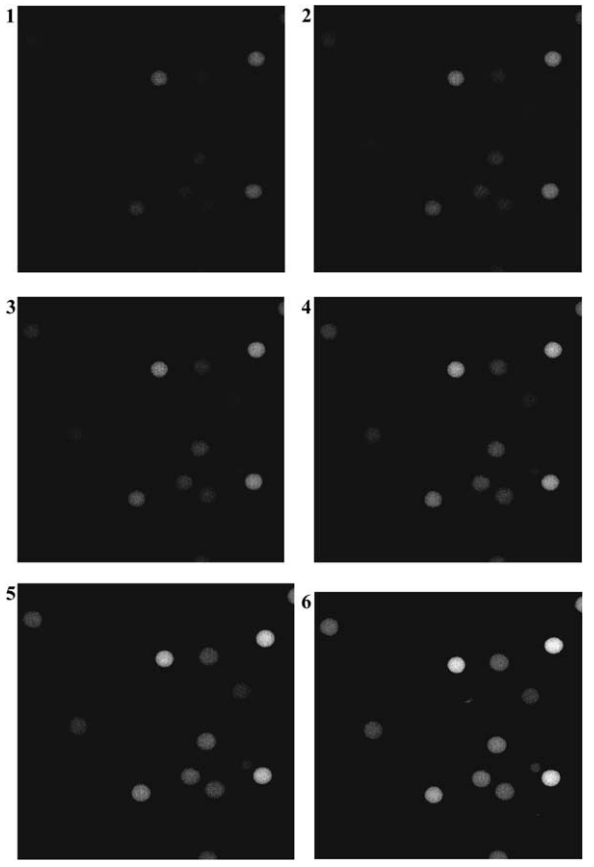

Fig. 3. Simultaneous imaging of chymotrypsin function inside the alginate/protamine microcapsules: fluorescence images of rhodamine 110, bis-(succinoyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl amide) (10 μM) in 0.05 M Tris buffer, pH 8.0. The images were obtained at 10-s intervals.

drawn and centrifuged. Protein content in the supernatants was determined as described earlier.

3. Results and discussion

The first step of this work was to prepare the multilayer polyelectrolyte microcapsules made of biocompatible polymers as negatively charged sodium alginate and positively charged protamine. Scheme (Fig. 1) shows the steps of the preparation. The final size of the microcapsules obtained was equal about 6.5 μm (Fig. 2A). The effect of accumulation of the positively charged dyes such as rhodamine 6G, rhodamine B inside the microcapsules was observed by confocal microscopy. This indicates the presence of negatively charged matrix inside the microcapsules formed by alginate. Apparently, in our system cationic protein—protamine can play the role of crosslinking agent by formation the complex with alginate matrix similar to poly-L-lysine as mentioned above in Section 1. The rhodamine-labeled protein was used to demonstrate the encapsulation of chymotrypsin ($pI_{\alpha\text{-chymotrypsin}}$ 8.5 (Kunitz and Northrop, 1936)) into the alginate/protamine microcapsules in water. Fig. 2B demonstrates the confocal image of the microcapsules loaded with chymotrypsin that was entrapped successfully inside and did not eliminate after two times of washing. Moreover, α-chymotrypsin was found to be retained inside the microcapsules in aqua media during incubation upto 24 h. Therefore, the encapsulation of positively charged macromolecules can be performed due to the catching the macromolecules by alginate gel formed (Fig. 1).

Taking into account the concentration of microcapsules and protein encapsulated, the loading capacity was evaluated according the following equation:

$$L = ([c]N_A)/(\mathrm{Mw}[N]),$$

where $[c]$—concentration of encapsulated protein, mg/ml, N_A—Avogadro constant, Mw–molecular weight of chymotrypsin, g/mol, $[N]$—microcapsule concentration, number of microcapsules per liter, and was found to be equal 10^9 protein molecules/microcapsule.

The functioning the serine proteinase was measured using the substrate rhodamine 110, bis-(succinoyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl amide). The enzyme encapsulated was found to retain about 70% of activity. The substrate used was developed in Molecular probes for revealing chymotrypsin activity by indication of fluorescent product and possesses the high degree of sensitivity and selectivity. Fig. 3 shows the time-resolving appearance of the fluorescent product obtained by confocal microscope. Moreover, this method can be proposed for measuring directly the kinetic parameters of enzyme function revealed by fluorescent substrate or product. This approach

allows reduced the sample amount up to 1 μl and has very high sensitivity.

To study the protein release from alginate/protamine microcapsules the following pH values were chosen—pH 1.7 (HCl solution) and pH 8.0 (0.05 M Tris buffer, 2.5% sodium deoxycholate) that simulates the physiological microenvironment in the stomach and intestinal tract, respectively. Proteins encapsulated in alginate matrices are known to release by two mechanisms: (i) diffusion of the protein through the pores of the polymer network because of the high porosity of alginate gels (pore size ranges from 5 to 200 nm in diameter (Andresen et al., 1977) and (ii) degradation of the polymer network (Gombotz and Wee, 1998). The results showed that the protein was not released from the alginate/protamine microcapsules when incubated in HCl solution, pH 1.7 during 4 h. No changes in capsule morphology were observed by confocal microscope. However, when the microcapsule suspensions were transferred to Tris buffer, pH 8.0 (2.5% of sodium deoxycholate), 85% of protein was released within 6 h and practically all of protein (95%) was released in 12 h (Fig. 4). On the other hand, practically the same release profile was obtained without preincubation at acidic conditions. It means that the alginate/protamine microcapsules were stable during 4 h at pH 1.7. The similar data were obtained with the alginate beads loaded with TGF-β_1 protein, which was encapsulated inside the beads at pH 1.0 during 1 day and released at pH 7.4 during 2 h. Authors explained the fast release by the degradation of the alginate in the low pH solution (Mumper et al., 1994). Apparently, in our case the protein–alginate interaction stabilized the gel structure and protected the alginate from degradation at acidic pH values. Moreover, the increase of the alginate/protamine layers from 8 to 16 by further adsorption of 8 additional layers resulted in decreasing the release rate ($\approx$ by 10–15%) (Fig. 4). It is necessary to take into account that the enzyme restored its complete activity. Therefore, variation of the capsule prepa-

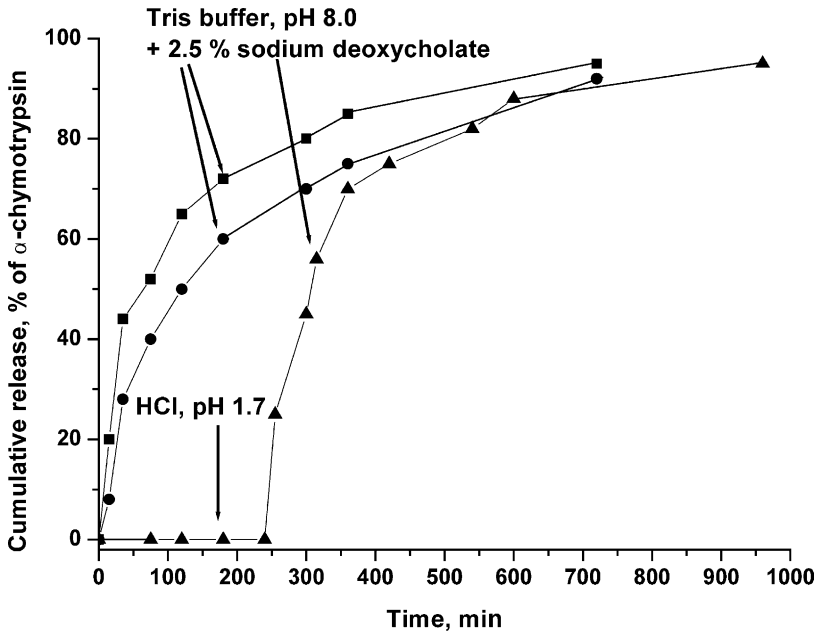

Fig. 4. Release profile of chymotrypsin in 0.05 M Tris buffer + 2.5% sodium deoxycholate, pH 8.0 from alginate/protamine microcapsules with 8 polyelectrolyte layers (■), 16 polyelectrolyte layers (●) and in HCl solution, pH 1.7 transferred to 0.05 M Tris buffer + 2.5% sodium deoxycholate for 8 polyelectrolyte layer capsule (▲).

ration conditions and the number of polyelectrolyte layers allows preparing the delivery system with controlled release properties.

4. Conclusions

A new delivery system—multilayer alginate/protamine microcapsules with size 6.5 μm were obtained by layer-by-layer technique. Changing the core size and the positively charged polymer is a good tool to vary the alginate matrix properties. The encapsulation procedure of α-chymotrypsin is based on the electrostatic entrapment of the protein inside the capsules filled with alginate gel. In comparison with known techniques of protein loading into the alginate beads, the procedure proposed in this work is quite simple and fast. The microcapsules possessed the high loading capacity—10^9 protein molecules per microcapsule while for PSS/PAH microcapsules prepared by the same technique (α-chymotrypsin was encapsulated due to the concentration gradient through the 'opened' wall of PSS/PAH layers at acidic pH value) this parameter was less by 100 times (Tiourina et al., 2001).

The protein release can be controlled by two ways: (i) by the alginate matrix properties (pore size, water content, stability etc.); (ii) by the number of further assembled polyelectrolyte layers. Furthermore, this work established that the alginate/protamine capsules are capable to protect the entrapped protein from harsh environment of the stomach and later slowly release it at its potential site of action in the small intestine. Taking into account the mucoadhesive properties of alginate this system could be successfully used as a mucosal drug delivery vehicle for the delivery of drugs to the intestinal tract.

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