Biochemistry (Moscow), Vol. 69, No. 7, 2004, pp. 763-769. Translated from Biokhimiya, Vol. 69, No. 7, 2004, pp. 937-944.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM03-276, March 28, 2004.

Encapsulation of Catalase in Polyelectrolyte Microspheres Composed of Melamine Formaldehyde, Dextran Sulfate, and Protamine

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Received December 8, 2003
Revision received December 29, 2003

Abstract—Immobilization of catalase (molecular weight 240,000 daltons) in polyelectrolyte microspheres was studied. The microspheres were obtained by alternating adsorption of dextran sulfate and protamine on commercially available melamine formaldehyde cores followed by the core hydrolysis at pH 1.7. As the interior of the microspheres was filled with homogeneous matrix, the catalase distribution inside the microspheres was uniform. The quantity of entrapped catalase was dependent on the initial concentration of the enzyme and pH of solution, and the peak value was 10^8-10^9 molecules per microsphere. It was demonstrated that catalase was entrapped in the microspheres via electrostatic and hydrophobic interactions. The catalase activity inside the microspheres increased as the quantity of enzyme decreased, which was due to the switch between diffusion and kinetic regimes of the enzymatic reaction. The microspheres could be applied for separation and concentration of high molecular weight proteins.

Key words: microencapsulation, microspheres, polyelectrolytes, catalase, dextran sulfate, protamine, melamine formaldehyde

Immobilization of proteins in polymeric microcapsules is of great scientific and practical interest [1]. Special attention should be given to microencapsulation of proteins into prefabricated polyelectrolyte microparticles under mild conditions: hollow microcapsules [2-4] and microspheres [5-7] with internal composition of homogeneous, weakly cross-linked, gelatinous matrix. These microparticles were obtained by the alternating adsorption of a certain number of oppositely charged polyelectrolyte layers on commercially available melamine formaldehyde (MF) micron sized carrier [8-10] (Fig. 1). After that, the MF core of the microparticle was subjected to complete or partial dissolution. The MF resin which is obtained via polycondensation of melamine with formaldehyde is a thermoreactive oligomeric product with branched structure containing ether bonds, primary, secondary, and tertiary amino groups, methylene bridges, and hydroxyl groups [9]. When MF cores were dissolved in highly acidic solutions (pH 1.0) oligomers of low molecular weight were formed and eluted through the polyelectrolyte membrane (shell) of the microspheres. The result was microcapsules of a strictly determined size, which had thin polyelectrolyte membrane and hollow interior [11]. The proteins were adsorbed mainly in the membrane (90%) of the hollow microcapsules, and therefore the protein capacity of the microcapsule was not large (10^5-10^7 molecules per particle) [2].

During the slow hydrolysis of MF core under mild conditions (pH 1.7) [5, 7], the newly formed and positively charged amino groups interacted with polyanion of the first layer of the microcapsule shell [9]. This resulted in a redistribution of membrane polyelectrolytes and formation of homogeneous, weakly cross-linked, charged gelatinous matrix inside the microspheres [5, 7]. We obtained for the first time microspheres using MF particles and four bilayers of dextran sulfate (DS) and protamine (PR) [5]; we also demonstrated that the microspheres are permeable for negatively and positively charged species. Proteins with different molecular weights and isoelectric points are entrapped inside the microspheres and uniformly distributed across the entire volume of the particle [12]. The capacity was 10^8-10^9 protein molecules per microsphere and depended on the protein molecular weight [7, 12]. The proteins can be released from the microspheres, the rate of this process decreasing with the increase in their molecular weight [12].

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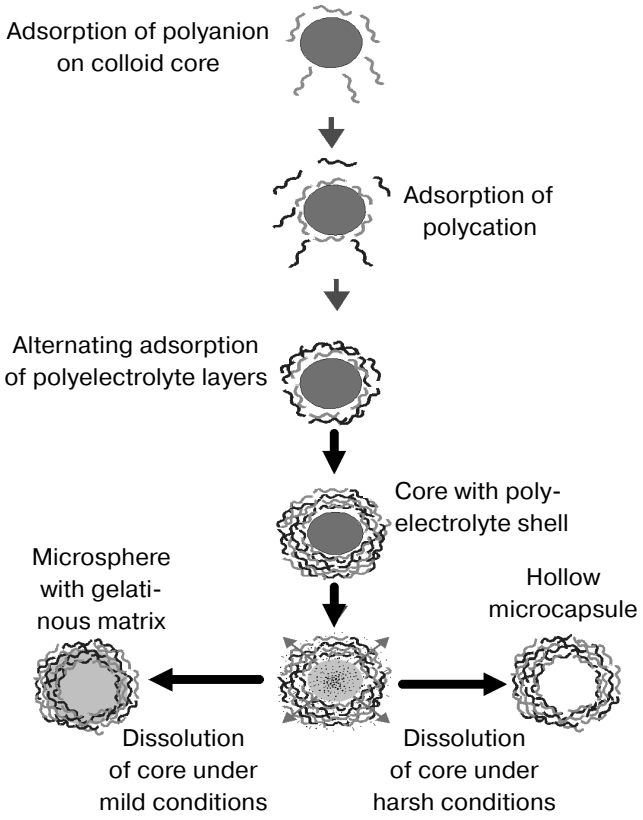

Fig. 1. Scheme of production of microspheres and hollow microcapsules using commercially available melamine formaldehyde particles and alternating adsorption of polyelectrolytes.

The aim of this work was to investigate the mechanism of encapsulation of high molecular weight proteins (using catalase (CAT) as model) in the polyelectrolyte microspheres obtained by alternating adsorption of DS and PR on MF particles, and also to clarify the nature of the forces responsible for this interaction.

MATERIALS AND METHODS

Materials. This work uses CAT from bovine liver (250 kD, p*I* 5.4), PR from salmon fish (5 kD, p*I* 10.5), DS sodium salt (500 kD), hydrogen peroxide, and fluorescein isothiocyanate (Sigma, USA); rhodamine-B-isothiocyanate (Aldrich, USA); melamine formaldehyde particles with diameter of 5.12 ± 0.15 μm, 20% suspension (Microparticles GmbH, Germany).

Fabrication of microspheres. DS (5 mg/ml) and PR (5 mg/ml) were sequentially adsorbed on MF particles in the presence of 0.2 M NaCl at pH 5.0 and 20°C [5]. Application of every polyelectrolyte layer was carried out for 10 min, then the particles were centrifuged (3 min, 1000*g*) and washed twice in 0.2 M NaCl solution, pH 5.0. Four bilayers of DS/PR ($(DS/PR)_4$) were applied. The resulting microparticles were placed into HCl solution at pH 1.7 for partial hydrolysis of the MF spheres, and after that rinsed in 1 mM HCl. The suspension of microspheres (denoted as $MF(DS/PR)_4$) with concentration $(7 \pm 3)\cdot 10^7$ particles/ml was stored at 5°C in 1 mM HCl solution.

An additional DS layer was applied on the microparticles with undissolved MF core as described above.

Characterization of microparticles. The concentration of microparticles was determined by counting the number of particles in a Goryaev chamber. The morphology of the microspheres was investigated using a Zeiss DSM 40 scanning electron microscope (Zeiss, Germany, 3 kV). Permeability of MF spheres coated with polyelectrolyte layers and microspheres $MF(DS/PR)_4$ was measured using a TSC Leica confocal laser microscope (Leica, Germany).

To study the interaction with fluorescein isothiocyanate, 50 μl of microparticle suspension was incubated for 2 h with 100 μl of fluorescein isothiocyanate solution (0.5 mg/ml) and 850 μl of 50 mM borate buffer, pH 9.0, containing 150 mM NaCl. After that, the particles were rinsed three times with buffer and three times with water.

To investigate localization of protein inside the microspheres, 50 μl of microparticle suspension in universal buffer (pH 4-8) was incubated for 5 min with 5 μl of CAT solution (1 mg/ml), labeled with rhodamine-B-isothiocyanate (1 : 3). After that, the microparticles were washed with the buffer three times.

Investigation of interaction of catalase with microspheres. For the encapsulation of protein, 50-200 μl of microsphere suspension was mixed with 550-700 μl of universal buffer solution (0.02 M H_3PO_4, 0.02 M CH_3COOH, 0.02 M H_3BO_3 + 0.1 M NaOH, pH 4-8) and 250 μl of 0.08-8.0 mg/ml CAT solution. When needed, 1-2 M NaCl or 30% isopropanol was added to the buffer. The mixtures were incubated for 1 h at 20 or 50°C, centrifuged for 4 min at 1000*g*, and supernatants were separated. The microspheres with CAT were rinsed three times and resuspended in 1 ml of the corresponding buffer solution.

To release the protein, microsphere suspension with CAT ($1.4\cdot 10^7$ particles/ml, enzyme entrapped at pH 7.0, enzyme concentration in microcapsule volume: 152 mg/ml) was incubated in buffer with pH 7.0 at 20°C; suspension aliquots were withdrawn after 10 and 30 min, 1, 3, and 7 h, and 14 days. The aliquots were centrifuged for 4 min at 5000*g*, and protein content in supernatants was determined.

Determination of protein concentration. Protein concentration in solutions and microparticle suspensions was determined according to Lowry et al. [13]. Number of protein molecules in a single microparticle was calculated according to the following formula: $L = 10^3[c]N_A/M_r[N]$, where [c] is protein concentration in suspension of microparticles, mg/ml; N_A, Avogadro's number; M_r,

molecular weight of CAT, g/mol; [*N*], microparticle concentration in suspension, particles/ml. CAT concentration in the internal volume of the microparticle (C_{CAT}, mg/ml) was determined according to: $C_{CAT} = [c]/[N]V$, where V (cm^3) is the volume of one microparticle at pH 7.0.

Measurement of catalase activity. CAT activity was determined from the depletion of hydrogen peroxide concentration as a result of enzymatic reaction [14]. Mixture of 30-80 μl of enzyme solution or microparticle suspension containing 0.005-0.010 mg/ml CAT, of 920-970 μl of 0.1 M phosphate buffer, pH 7.0, and of 100 μl of 0.196 M hydrogen peroxide was prepared. Decrease in optical density was registered spectrophotometrically at 240 nm.

RESULTS AND DISCUSSION

Properties of the microspheres. Polyelectrolyte microspheres $MF(DS/PR)_4$ were obtained from MF particles with a diameter of 5.12 ± 0.15 μm by alternating adsorption of four DS and PR bilayers with subsequent dissolution of the MF core under mild conditions (pH 1.7) (Fig. 1). The microspheres were homogeneous in size with a diameter of 8.0 ± 0.2 μm at pH 3-5 and 9-10 μm at pH 7-8, which is more than the diameter of the original MF particles. The increase in microsphere size compared to the MF particles was caused by the significant increase in osmotic pressure during the hydrolysis of MF resin [11]. Scanning electron microscopy revealed (Fig. 2) that the microspheres have a spongy shell and a bulging "core" in the middle. This confirmed the presence of a gelatinous matrix inside the microspheres.

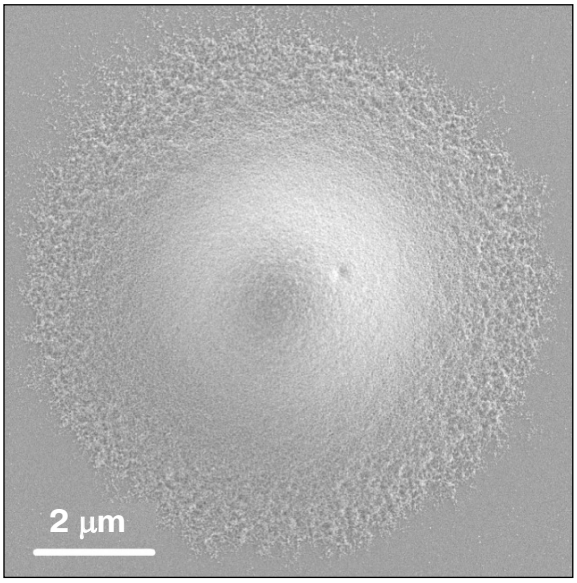

Fig. 2. Scanning electron microscopy of $MF(DS/PR)_4$ microspheres.

According to the data of confocal laser microscopy, the fluorescein isothiocyanate formed covalent bonds both with the $(DS/PR)_4$ shell coating the undissolved MF core (Fig. 3a) as well as with the shell and microsphere matrix of $MF(DS/PR)_4$ (Fig. 3b). This provides

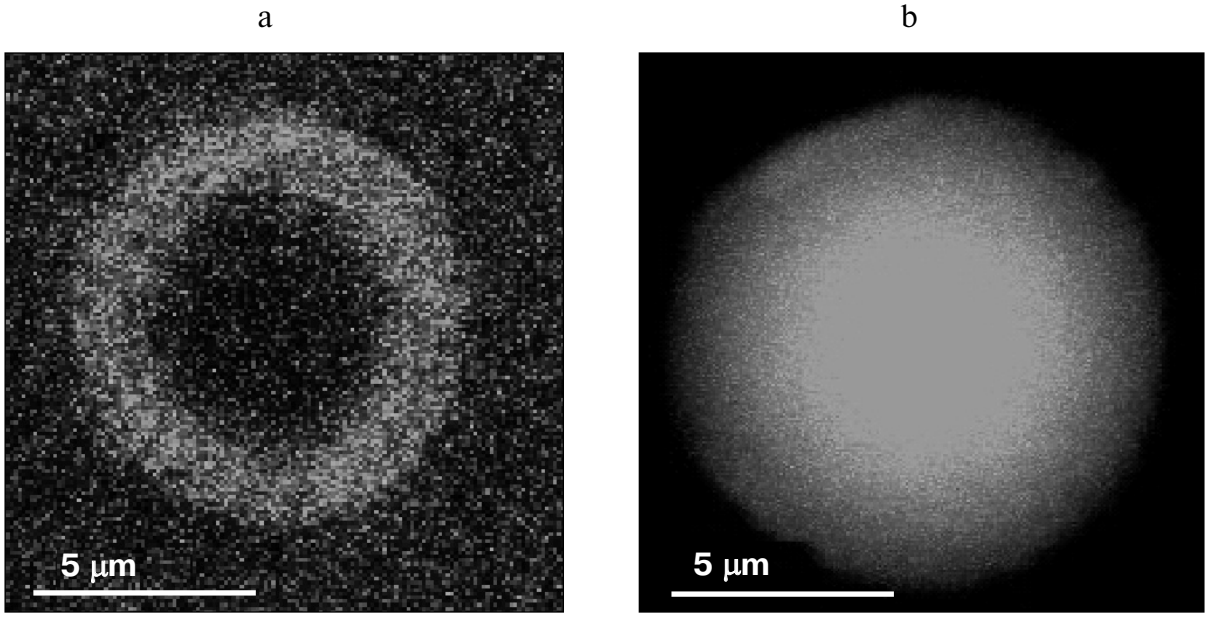

Fig. 3. Confocal laser scanning microscopy: binding of fluorescein isothiocyanate to melamine formaldehyde (MF) core coated with polyelectrolyte shell (a) and $MF(DS/PR)_4$ microsphere (b).

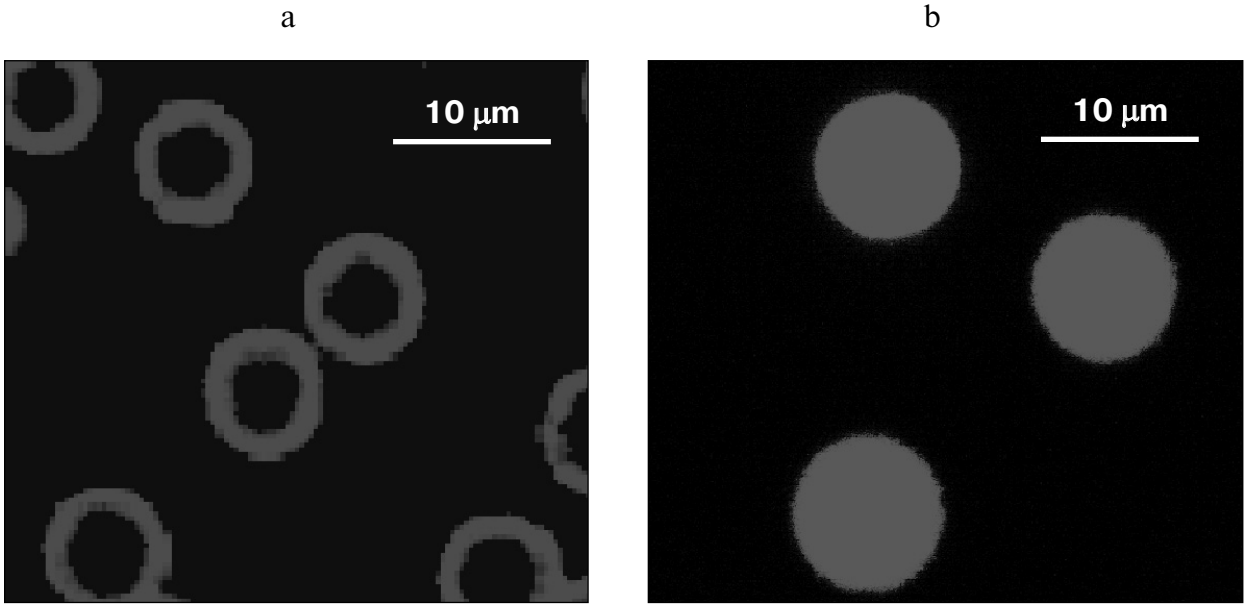

Fig. 4. Confocal laser scanning microscopy: binding of rhodamine-B-isothiocyanate-labeled catalase (pH 7.0) to melamine formaldehyde (MF) core coated with polyelectrolyte shell (a) and $MF(DS/PR)_4$ microsphere (b).

evidence of the presence of free amino groups in the polyelectrolyte shell and interior matrix of the microspheres. Analogously to low molecular weight dye, CAT (enzyme with molecular weight 250 kD) permeated and concentrated at pH 4-8 in the polyelectrolyte shell of $(DS/PR)_4$ microparticles with undissolved MF core spheres (Fig. 4a) or inside $MF(DS/PR)_4$ microspheres (Fig. 4b). Moreover (similarly to the experiments with lower molecular weight proteins [5, 12]), the enzyme was uniformly distributed throughout the microsphere volume.

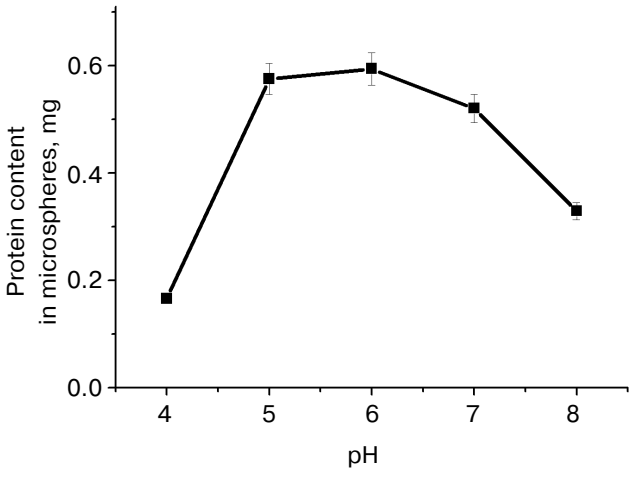

Fig. 5. Influence of pH on the encapsulation of catalase in $MF(DS/PR)_4$ microspheres. The conditions were as follows: $3.5 \cdot 10^6$ particles, catalase concentration 2.0 mg/ml, universal buffer (0.02 M H_3PO_4, 0.02 M CH_3COOH, 0.02 M H_3BO_3 + 0.1 M NaOH).

Encapsulation of catalase into the microspheres. The pH value of the medium affected the inclusion of CAT into $MF(DS/PR)_4$ microspheres (Fig. 5). It should be mentioned that the enzyme concentration inside a single microsphere is significantly (by 1.5-2 orders) exceeded the concentration of CAT in the solution (2 mg/ml) used for protein immobilization. The maximum amount of CAT ($4 \cdot 10^8$ molecules per microsphere) was entrapped at pH close to its isoelectric point (p*I* 5.4). At pH higher or lower than the isoelectric point, a decrease in the amount of the entrapped protein was observed. The possible reason for this is that pH value affects both the charge of the enzyme molecule and the structure of the microspheres. At acidic pH values, the decrease in CAT amount occurs due to the decrease in microsphere volume, which was described earlier. At the alkaline pH values the structure of the microsphere could change due to either alteration of polyelectrolyte shell structure caused by decrease in charge at PR as it approaches the isoelectric point (p*I* 10.5), or by the alteration in the total charge of the internal matrix caused by partial deprotonation of its amino groups.

The influence of CAT concentration on the efficiency of microencapsulation and specific activity of the enzyme at pH 7.0 (corresponding to the optimum of enzyme action) is presented in the table. CAT was entrapped in microparticles with high efficiency (70-100% depending on its original concentration). The observed specific activity of CAT was dependent on the amount of protein entrapped in the microspheres.

Inclusion of catalase in $MF(DS/PR)_4$ microparticles at pH 7.0 ($1.4 \cdot 10^7$ particles/ml)

Initial catalase concentration, mg/ml	Efficiency of encapsulation, %	Catalase specific activity, % of initial	Protein content per microsphere	
			mg/ml	molecules
0.89	68 ± 3	13 ± 1	83 ± 5	$(1.12 \pm 0.07) \cdot 10^8$
0.17	99 ± 1	29 ± 3	24 ± 1	$(0.29 \pm 0.01) \cdot 10^8$
0.08	100 ± 6	72 ± 4	15 ± 1	$(0.14 \pm 0.01) \cdot 10^8$

Enzymatic reaction inside the microparticles requires that substrate diffuses inside through the polyelectrolyte membrane while the reaction product is released into the surrounding medium. The increase in enzyme concentration in the microcapsule increases the role of diffusional factors. At the same time, the actual rate of the enzymatic reaction may become lower than the theoretically possible rate, because of the diffusional resistance created by the microsphere. This resistance results in that almost all substrate will be cleaved by the surface layers of enzyme, while the interior of the microspheres will be depleted in the substrate [15]. Apparently, the increase in specific activity of the enzyme with the decrease in CAT content inside microcapsules depends on the transition of the reaction from the diffusion to the kinetic regime.

Release of catalase from microspheres. The efficiency of CAT encapsulation (entrapped at pH 7.0) inside the microspheres was investigated (Fig. 6). When enzyme solution used for encapsulation was changed for a buffer with the same pH value, 7% of the CAT was released from the microspheres during 7 h. Seemingly, the protein molecules released first are those which are weakly bound in the surface layers of the microspheres. It should be mentioned that CAT release was occurring with lower rate than for all other proteins with lower molecular weights investigated in our previous work. Within 7 h and under the mentioned conditions, 43% of insulin (6.5 kD), 44% aprotinin (6.5 kD), 25% peroxidase (44 kD), and 10% glucose oxidase (160 kD) were released from the microspheres [5, 12]. It was possible to increase the rate of CAT release from the microspheres by increasing the ionic strength of the solution to 1 M by adding NaCl (Fig. 7).

Nature of interaction of catalase with microspheres. Initially, at pH 7.0, the interaction of CAT (whose protein globule is negatively charged under these conditions) with MF core covered with polyelectrolyte shell was studied. The polyelectrolyte $(DS/PR)_4$ shell of a single microparticle having positive surface charge adsorbed $1.4 \cdot 10^7$ CAT molecules. When an additional DS layer was adsorbed in a polyelectrolyte shell of the $(DS/PR)_4DS$ microparticles (whose surface charge thus became negative), $3.7 \cdot 10^7$ of similarly charged enzyme molecules were entrapped.

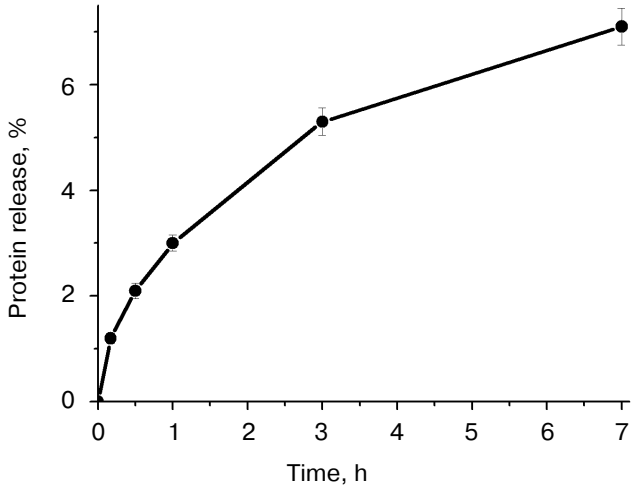

Fig. 6. Kinetic curve for the release of catalase from $MF(DS/PR)_4$ microspheres at pH 7.0 (universal buffer).

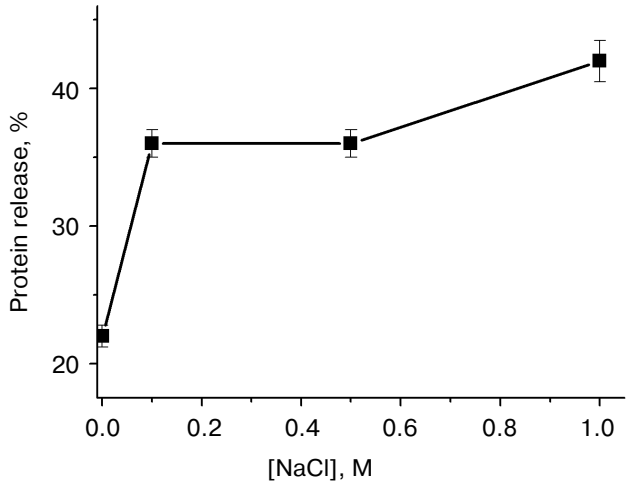

Fig. 7. Influence of ionic strength on the release of catalase from $MF(DS/PR)_4$ microspheres at pH 7.0 (universal buffer) during 14 days.

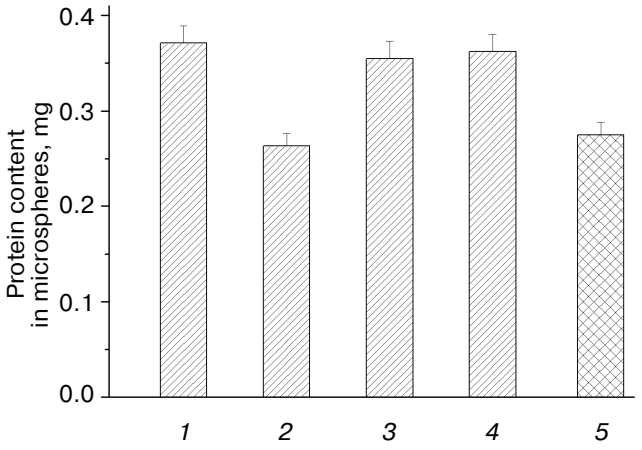

Fig. 8. Influence of temperature (20°C (*1-4*), 50°C (*5*)), ionic strength, and polarity of the medium on the encapsulation of catalase in $MF(DS/PR)_4$ microspheres: *1*, *5*) no additives; *2*) 1 M NaCl; *3*) 2 M NaCl; *4*) 30% isopropanol. Conditions: $3.5 \cdot 10^6$ particles, catalase concentration 0.9 mg/ml, universal buffer, pH 7.0.

Hence, this experiment illustrates that interaction between CAT and polyelectrolyte shell is realized not only due to electrostatic interactions with upper polyelectrolyte microparticles layer.

To establish the nature of the forces responsible for protein interaction with microspheres at pH 7.0, the influence of temperature, hydrophobicity, and ionic strength of the medium on CAT encapsulation in microspheres was studied (Fig. 8). It should be noted that the microscopic investigation did not reveal any changes in size and shape of the microspheres or in protein distribution inside the particles when the chosen parameters vary within the ranges mentioned below.

Increasing the temperature from 20 to 50°C resulted in 30% lower content of CAT in the microspheres. The increased temperature leads to a weakening of all kinds of intermolecular interactions (especially electrostatic) except for hydrophobic interactions [16]. When temperature increases the formation of hydrophobic bonds is more thermodynamically favorable since the greatest contribution in these interactions is provided by the entropy component, which is linearly decreasing with growing temperature [16]. Therefore, it can be assumed that the electrostatic interactions played a significant role in CAT encapsulation in the microspheres.

The increase in ionic strength of the medium weakens electrostatic interactions and strengthens hydrophobic ones [16, 17]. The increase in NaCl concentration to 1 M resulted in 30% decrease in entrapped CAT amount, compared to the buffer solution. However, small additions of salts up to 1 M less affected the hydrophobic interactions in disperse systems [17], and the decrease of CAT entrapped in $MF(DS/PR)_4$ microspheres in solution with the mentioned ionic strength can be caused by decrease in electrostatic interactions. During further increase in NaCl concentration to 2 M, the microspheres strongly aggregated, while the amount of CAT in the microspheres increased by 24% compared to the analogous experiment with 1 M NaCl (only 6% less than the inclusion into microspheres in buffer solution). These data indicate the presence of hydrophobic interactions (both during incorporation of the protein and aggregation of microspheres), whose contribution exceeded the decrease in electrostatic interactions between CAT and the microspheres.

Decrease in medium polarity leads to a strengthening of electrostatic and a weakening of hydrophobic interactions due to disruption of water structure [16]. Addition of 30% isopropanol into the medium had virtually no affect on the inclusion of CAT into the microspheres, which points to the contribution from both electrostatic and hydrophobic interactions to CAT encapsulation into the microspheres, their increase and decrease being apparently compensated.

Hence, the microspheres from melamine formaldehyde, dextran sulfate, and protamine, due to the presence of an internal gelatinous matrix, are capable of entrapping CAT with high efficiency and preserving its activity. The increase in the specific activity during the decrease in catalase amount inside the microspheres was a result of the switch of enzymatic reaction from diffusion to kinetic mode. With CAT as example, it has been shown that electrostatic and hydrophobic interactions are responsible for the interaction of the protein with the microspheres. Therefore, encapsulation and release of proteins from $MF(DS/PR)_4$ microspheres can be controlled via adjustment of pH, ionic strength, and temperature. Microspheres $MF(DS/PR)_4$ studied in this work have unique properties which might find application in the concentration of high molecular weight proteins.

This work was supported by the Ministry of Industry, Science, and Technology of Russian Federation. The authors express their gratitude to D. V. Volodkin (PhD student, Faculty of Chemistry, Lomonosov Moscow State University) for providing the data on confocal laser scanning microscopy.

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