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Fabrication and Characterization of Polyelectrolyte Microparticles with Protein

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Abstract—The incorporation of proteins into microparticles fabricated by layer-by-layer adsorption of oppositely charged polyelectrolytes (dextran sulfate and protamine) on protein microaggregates was studied. Microaggregates with insulin were prepared by two different techniques: 1) formation of insoluble polyelectrolyte complex consisting of insulin and dextran sulfate (aggregate size of 7-20 μm), or 2) salting out of insulin from solution by sodium chloride (aggregate size of 5-13 μm). Microparticles varying in the number of cycles (from 1 to 8) of polyelectrolyte adsorption on protein aggregates were examined and compared. Morphology of the microparticles was studied by scanning electron and optical microscopy. It was shown that polyelectrolyte microparticles retained the shape and dimensions of the initial protein aggregates used as a template. Ultrasonication of microparticles obtained using salted out protein aggregates resulted in the formation of stable nanoparticles (100-200 nm). Regulation of protein release from the microparticles of both types by varying the number of polyelectrolyte adsorption cycles and pH of the medium was demonstrated. Insulin not bound to polyelectrolytes was released from the microparticles at pH values between 6 and 8, which corresponds to the pH of the human small intestine and ileum.

Key words: proteins, polyelectrolytes, polyelectrolyte microparticles, dextran sulfate, protamine, insulin

As demonstrated in our earlier work [1, 2], the incorporation of proteins into polyelectrolyte microparticles fabricated by layer-by layer adsorption of oppositely charged polyelectrolytes is a promising protein immobilization technique. In this case, two major approaches to immobilization are possible: 1) protein entrapment within prefabricated polyelectrolyte microparticles [2-4], and 2) formation of microparticles on a protein-containing template [1, 5].

Fabrication of hollow microcapsules [2, 6] or microparticles which have weakly linked gel-like internal structure [3, 7] includes 8-12 cycles of polyelectrolytes sorption on a core of a certain size with following complete or partial dissolution of the core under strictly determined conditions.

A simpler and fewer-step process is polyelectrolyte adsorption on a microtemplate formed by protein crystals [8, 9] or protein aggregates obtained by salting out from aqueous solutions [1, 5, 10]. However, the crystallization is only possible for a limited number of highly purified proteins [11] and salting out requires the optimization of conditions for each protein individually [11], which in a number of cases occurs at very high salt concentrations [10] and thus complicates polyelectrolyte adsorption on the aggregates. A large number of proteins cannot be salted out at all [11]. Under these circumstances, the application of micron-sized insoluble protein–polyelectrolyte complexes as an initial template appears to be a promising solution.

The goal of this work was to study and compare the properties of the microparticles fabricated by layer-by-layer absorption of biocompatible polyelectrolytes on salted out protein aggregates and insoluble protein–polyelectrolyte complexes. Insulin (M_r 6500) was chosen as a model protein; the charge of its protein globule varies in the physiological pH range (p*I* 5.5). This protein is capable of aggregation and crystallization under different conditions [12]. Studies directed at the development of insulin microformulations, especially as peroral dosage forms, have been intensively carried out within the recent years [13].

The polyelectrolytes used in this work are natural biodegradable polymers: the polyanion dextran sulfate (DS) and the polycation the low molecular weight protein protamine (PR), containing up to 70% arginine (p*I* 10.5). This combination of polyelectrolytes (as illustrated by us in [3]) is suitable for the encapsulation of different proteins. Both biopolymers are well known and available. DS is used for the production of polyelectrolyte complexes with proteins [14] and other biologically active substances [15]. PR is applied in medicine for prolongation of insulin

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action and neutralization of heparin [16]. Being an excellent protein substrate for proteolytic enzymes [17], PR is easily excreted from the body.

MATERIALS AND METHODS

Materials. The following chemicals were used in this work: DS sodium salt (M_r 500,000), PR salmine (M_r 5000), and bovine insulin (Sigma, USA); Sephadex G-50f (LKB-Pharmacia, Sweden). Other substances were of chemical grade.

Microaggregates of insulin–dextran sulfate complex. To obtain an insoluble insulin–DS complex, equal volumes of insulin solution (20 mg/ml) in 1 mM HCl and DS (10 mg/ml) in 1 mM HCl containing 0.5 M NaCl were mixed. The mixture was stirred for 20 min and centrifuged (200*g*, 3 min). The microaggregate precipitate was twice resuspended in 1 mM HCl containing 0.5 M NaCl and centrifuged.

Salted out insulin microaggregates. To obtain salted out protein microaggregates, NaCl was added to insulin solution (20 mg/ml) in 1 mM HCl up to the concentration of 0.5 M. The mixture was stirred for 1 h and centrifuged (200*g*, 3 min).

Fabrication of polyelectrolyte microparticles was performed by the layer-by-layer adsorption of PR or/and DS onto insulin-containing microaggregates. For this procedure, a polyelectrolyte solution (10 mg/ml) in 1 mM HCl containing 0.5 M NaCl was added to microaggregate precipitate. The mixture was stirred for 20 min and centrifuged (200*g*, 3 min). The precipitate was twice suspended in 1 mM HCl solution containing 0.5 M NaCl and centrifuged. After achieving a required number of polyelectrolytes sorption cycles, the microparticles were suspended in 1 mM HCl containing 0.5 M NaCl, stored, or rinsed three times by 1 mM HCl and lyophilized. The lyophilized preparations were stored at 4°C. An aliquot of the microparticle suspension was ultrasonicated for 10 min at 10°C (Sonorex digital 10P, 100%).

Characterization of microparticles. The morphology of microparticles was determined using optical microscopy (Opton III, Carl Zeiss, Germany) and scanning electron microscopy (Zeiss DSM 40, Germany, 3 kV). The average size of microparticles was determined by measuring the size of 100 particles using optical microscopy.

Determination of protein content. Protein concentration in the solutions was determined according to Lowry et al. [18], insulin concentration by absorbance at 280 nm [12] (PR does not absorb light under these conditions), and PR concentration based on the difference in total protein concentration and insulin concentration. To determine protein content in the microparticles the lyophilized preparations were suspended in 0.1 M NaOH, where the complete dissolution due to the disruption of polyelectrolyte complex as a result of the change in total charges of insulin and PR was observed. The resulting solution was analyzed as described above. Protein content in the preparations corresponded to the ratio between the mass of the protein and lyophilized preparation. DS content was determined as the ratio of mass difference of lyophilized preparation (taking into account an average humidity of 5-6%) and mass of the two proteins in it to the mass of lyophilized preparation.

Study of insulin release from microparticles. Microparticle suspension was mixed with a universal buffer (0.02 M H_3PO_4, 0.02 M CH_3COOH, 0.02 M H_3BO_3 + 0.1 M NaOH, pH 3-8) up to final protein concentration of 0.2-0.3 mg/ml and incubated under stirring (100 rpm) at room temperature. Suspension aliquots were collected after 10 and 30 min and 1, 2, 4, and 24 h, the samples were centrifuged (10,000*g*, 5 min), and protein concentration in the supernatant was determined. Protein release from the microparticles was characterized by the ratio between protein content in the supernatant and microparticle suspension.

To study the form of insulin released from the microparticles, the supernatants obtained after the particle incubation in universal buffer (pH 7.0) for 1 h were separated by chromatography on a column with Sephadex G-50 fine (19.5 × 2 cm) in the same buffer.

RESULTS AND DISCUSSION

Fabrication of polyelectrolyte microparticles and their morphology. The encapsulation of insulin involved formation of insoluble microaggregates containing the protein and used as a template and subsequent layer-by-layer adsorption of the charged polyelectrolytes on them (Fig. 1). The original microaggregates, which contained insulin, were obtained at pH 3.0 by formation of an insoluble polyelectrolyte protein–DS complex or by salting out the native protein. The difference in efficiency of protein encapsulation in the microaggregates was insignificant, 75-80% and 80-85% for the two mentioned techniques, respectively.

The microscopic investigation revealed that microaggregates obtained from protein–DS complex are irregularly shaped closed particles with the size of 7-20 μm (average size is 13.3 μm). Microaggregates obtained by salting out of the protein had the same shape but smaller size: 5-13 μm (average size is 7.5 μm).

Subsequent layer-by-layer application of PS and DS (oppositely charged compared to the original charge of the surface) on the microaggregates was performed in the presence of 0.5 M NaCl at pH 3.0 until the required number of polyelectrolytes sorption cycles (N), $N = 8$ has been reached. The loss of protein in the course of polyelectrolyte sorption due to the loss of microparticles during centrifugation was insignificant (5-30%) at N from 2 to 5.

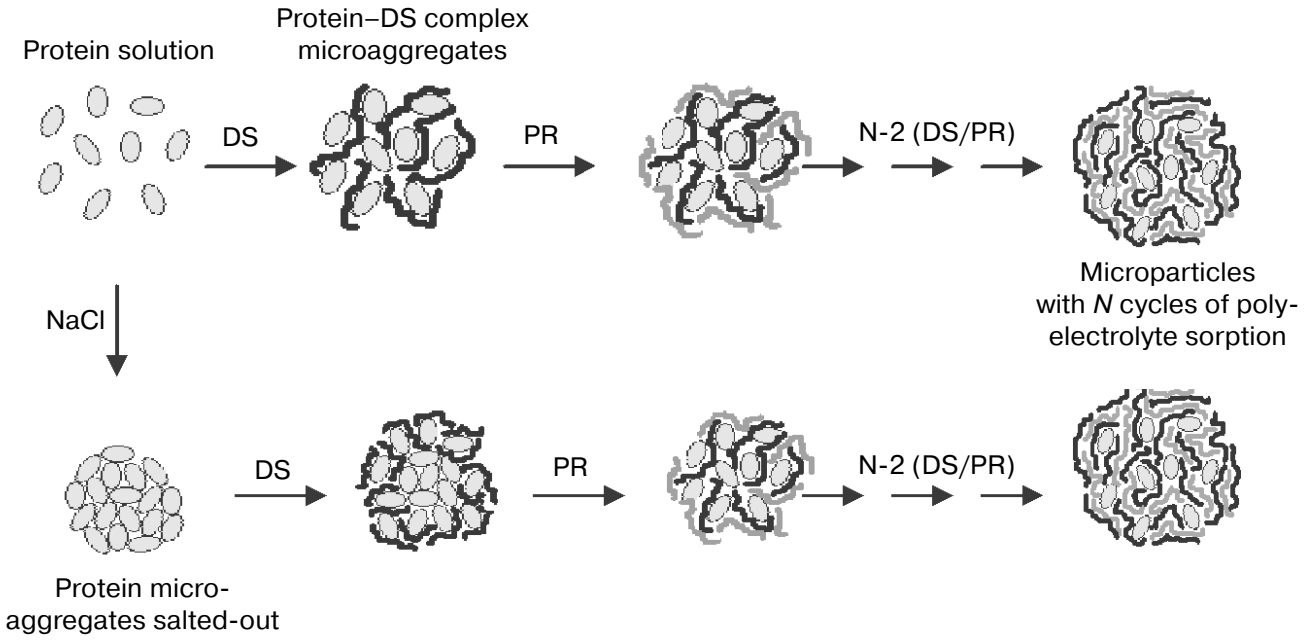

Fig. 1. Principal scheme of fabrication of the polyelectrolyte microparticles with insulin using protein–polyelectrolyte complex and salted out protein aggregates.

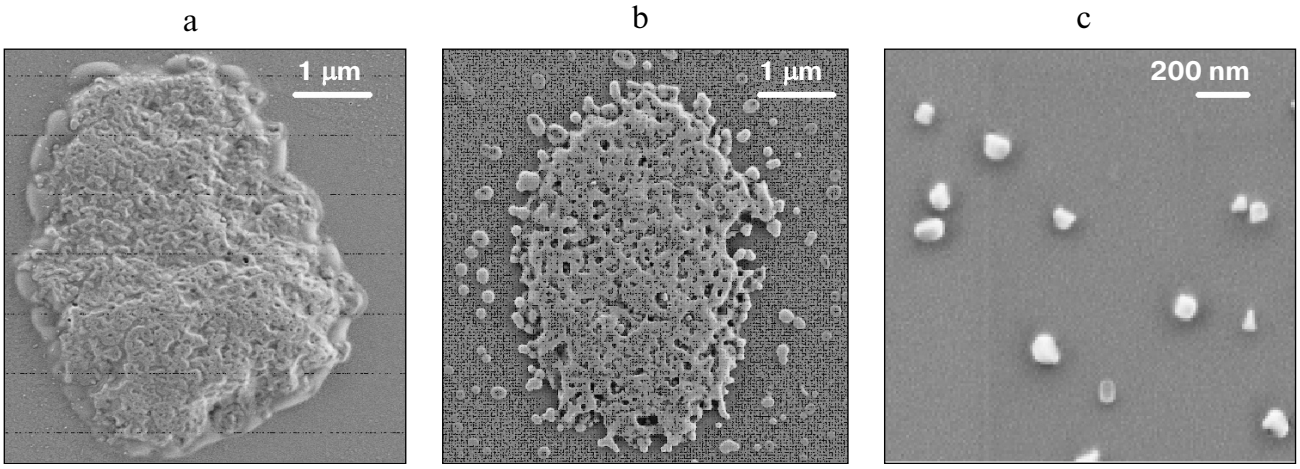

Fig. 2. Scanning electron microphotographs of microparticles with protein after three polyelectrolyte sorption cycles obtained using insulin–DS complex (a) and salted out insulin aggregates before (b) and after ultrasound treatment (c).

Within 6-8 sorption cycles, an increased aggregation of the particles as well as their adherence to the container walls was observed, which complicated the centrifugation and resulted in more significant loss of the microparticles with insulin.

The prepared particles were rinsed and stored in solution at pH 3.0. Under these conditions, the microparticles of both types were stable during the observation period (1.5 years).

The optical microscopy data revealed that microparticles at $N = 1$-5 imitate the shape and size of the original templates (protein aggregates) on which they were formed. Hence, the size of microparticles (5-13 μm) fabricated by salting out of insulin aggregates was approximately twice smaller than the size of microparticles (7-20 μm) fabricated from insulin–DS complex. At $N = 6$-8 and due to the adherence an average size of the microparticles formed on the aggregates of both types increased up to 10-100 μm.

The results of scanning electron microscopy presented in Fig. 2 (a and b) demonstrated the structural similarity of microparticles ($N = 1$-5) with their 1.5-2-fold dif-

ference in size. For microparticles fabricated on salted out protein aggregates, the presence of repeating structural motifs (microformations) with dimensions of 100-200 nm was more noticeable. The difference was especially explicitly exhibited when the microparticles were treated with ultrasound. During this process, a fragmentation of microparticles (N = 1-5) was observed. However, for microparticles fabricated from insulin–DS complex, along with the small number of microformations (less than 1 μm in size), original unfragmented microparticles with average size approximately 10 μm were also observed. For microparticles fabricated from salted out protein aggregates, the fragmentation process was more explicit and fragmented microparticles were more homogeneous by size. As Fig. 2c illustrates, the most striking results of ultrasound treatment were observed for microparticles with N = 3 fabricated from salted out insulin aggregates, when it was possible to obtain a suspension of nanoparticles with a long-term stability of 1.5 years and particle size of 100-200 nm. Evidently, three polyelectrolyte sorption cycles stabilized the original smallest salted out insulin aggregates; therefore, they did not undergo further aggregation after the ultrasound treatment.

Properties of polyelectrolyte microparticles with insulin. The table illustrates the microparticle composition corresponding to different number of polyelectrolyte adsorption cycles (N). Microparticles obtained from the salted out protein aggregates were characterized by higher insulin content for equal N. As N increased and regardless the initial aggregates with insulin the following was observed: 1) decrease in insulin content (as in the case of the microencapsulation of other proteins by this method [1, 5], especially at N = 7-8); 2) increase in PR content, and therefore; 3) increase in the other polyelectrolyte (DS) content. During 1.5 years of microparticle storage none ($N > 3$) or insignificant ($N \leq 3$) release of insulin was observed.

Properties of polyelectrolyte particles with insulin

Number of polyelectrolyte sorption cycles (N)	Insulin content, %	Protamine content, %	Protein release after 1.5 years in 1 mM HCl, %
Obtained using insulin–DS complex			
1	91 ± 3	0	3 ± 0.1
2	87 ± 3	2 ± 0.1	2 ± 0.1
3	80 ± 3	1 ± 0.1	1 ± 0.1
4	70 ± 2	6 ± 0.1	0
5	58 ± 2	8 ± 0.2	0
6	44 ± 2	9 ± 0.2	0
7	28 ± 1	16 ± 0.2	0
8	23 ± 1	17 ± 0.3	0
Obtained using salted out insulin aggregates			
1	95 ± 3	0	5 ± 0.2
2	94 ± 3	1 ± 0.1	2 ± 0.1
3	88 ± 3	2 ± 0.1	2 ± 0.1
4	75 ± 2	8 ± 0.2	0
5	62 ± 2	9 ± 0.2	0
6	55 ± 2	10 ± 0.2	0
7	43 ± 2	15 ± 0.2	0
8	35 ± 1	14 ± 0.2	0

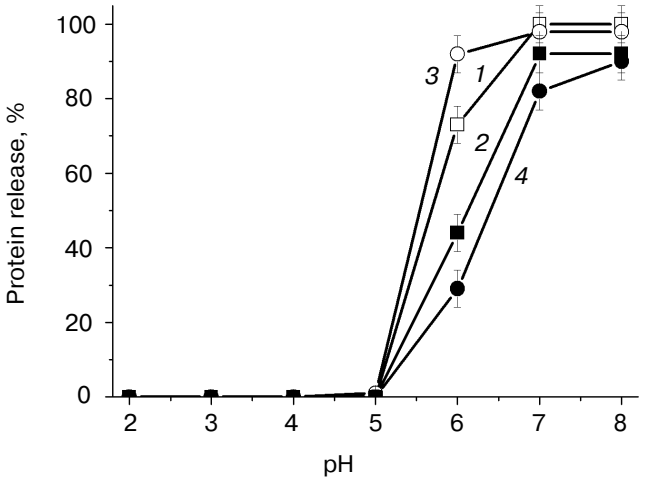

Fig. 3. Influence of pH on the release of protein from microparticles with insulin after one (*1*, *3*) and three polyelectrolyte (*2*, *4*) sorption cycles. Microparticles were obtained using insulin–DS complex (*1*, *2*) or salted out insulin aggregates (*3*, *4*). Incubation time, 1 h.

Figure 3 demonstrates the influence of pH on protein release from the microparticles. Microparticles with insulin appeared to be pH-sensitive, which correlates with our previous observations where microencapsulation was performed using salted out aggregates of other proteins (trypsin, chymotrypsin) and synthetic polyelectrolytes (polystyrene sulfonate, polyallylamine) [1, 5, 10]. Microparticles with insulin fabricated from both types of aggregates were stable at pH 1.7-5 and the release of protein was observed at pH higher than 5. With the increase of pH up to 8, the rate of protein release also increased. This is due to the fact, that at pH higher than the isoelectric point (pI 5.5) insulin acquired a cumulative negative charge and its complex with polyelectrolyte of the first DS layer was disrupted. The intensity of protein release decreased with N growing from 1 to 3 due to diffusion limitations caused by the polyelectrolyte net. In the course of protein release, the microparticles were changing their shape, structure, decreased in size, and at the moment of complete protein release, became transparent.

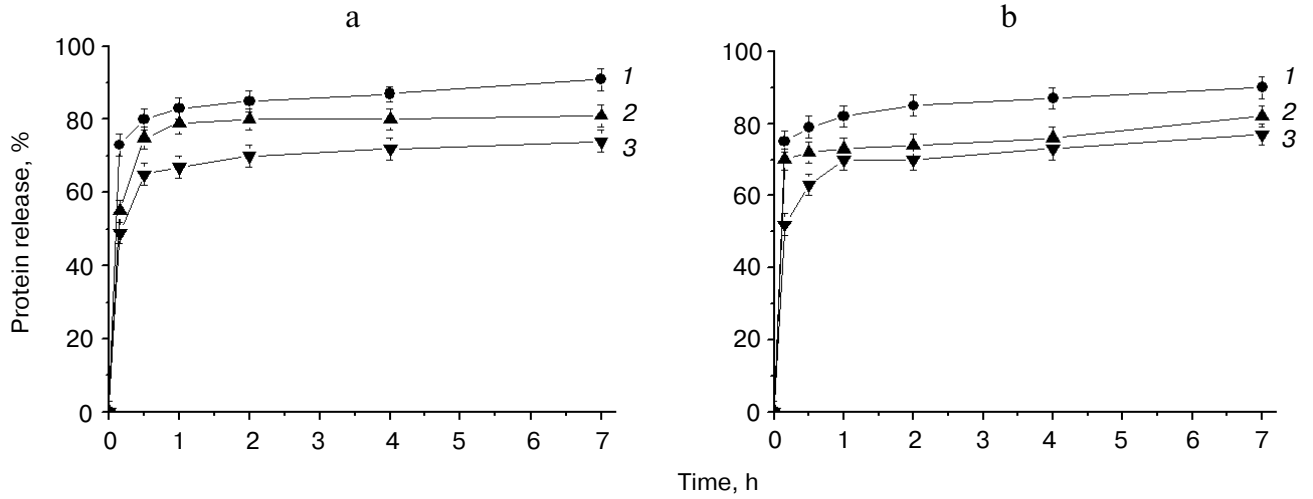

Fig. 4. Kinetics of the release of protein from microparticles at pH 7.0 with three (*1*), five (*2*), and seven (*3*) polyelectrolyte sorption cycles obtained using insulin–DS complex (a) or salted out insulin aggregates (b).

Kinetics of insulin release from the microparticles at pH 7.0 corresponding to the human lower intestinal tract is presented in Fig. 4. Rapid release of the protein was observed; however, as *N* increased from 3 to 7 the initial rate of insulin release (in first 10 min) decreased. This means that regardless of the aggregates used, the microparticles are stabilized with the increase in the number of polyelectrolyte adsorption cycles. It should be noted that the influence of the number of sorption cycles of natural DS and PR polyelectrolytes on insulin release is less profound than for earlier studied synthetic polyelectrolytes: polystyrene sulfonate (M_r 70,000) and polyallylamine (M_r 60,000), and a protein with higher molecular weight, chymotrypsin (M_r 24,000, p*I* 8.8), when the enzyme was released from the microparticles as soluble complex with polyanion [5]. Insulin released from the microparticles fabricated from the aggregates of both types at pH 7.0 is practically unbound with polyelectrolytes, as proved by gel chromatography (Fig. 5).

Hence, in this work protein immobilization by subsequent adsorption of dextran sulfate and protamine on insoluble microtemplate obtained during the formation of an insoluble insulin–polyelectrolyte complex or by salting out of insulin was performed. High efficiency of insulin entrapment was observed regardless of the type of protein microaggregate used. The polyelectrolyte microparticles are stable under storage conditions and pH values corresponding to that of human gastric juice (pH 1.7). The final size of the polyelectrolyte microparticles with insulin, structure and possibility of their fragmentation by ultrasound were dependent on the size and structure of the original protein microtemplates. Microparticles fabricated from salted out protein aggregates had twice smaller size and a structure which allowed obtaining stable nanoparticles by ultrasound treatment.

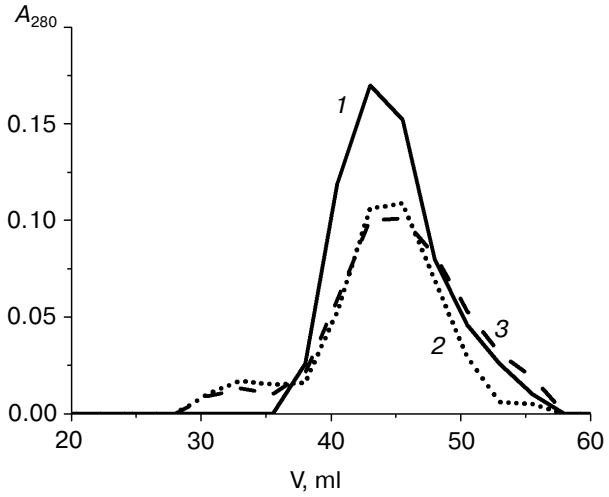

Fig. 5. Gel-permeation chromatography on Sephadex G-50 (pH 7.0) of insulin (*1*) and proteins released during 1 h incubation at pH 7.0 from the microparticles with three polyelectrolyte sorption cycles. Microparticles were obtained using insulin–DS complex (*2*) or salted out insulin aggregates (*3*).

However, use of insoluble protein–polyelectrolyte complex as a template appears to be an easier process. This technique is suitable for all proteins, even for those, which cannot be precipitated by salting out, if the oppositely charged polyelectrolyte is optimized.

Gradual protein release from the microparticles occurs at pH higher than 6 and regardless of the type of initial aggregates.

Similarity in the content and properties of the microparticles fabricated from the aggregates of both types indicates that a uniform protein–polyelectrolyte structure is formed on protein aggregates during the layer-

by-layer adsorption of oppositely charged polyelectrolytes (Fig. 1). The pH dependence of protein release from the microparticles and kinetics of this process depend on the properties of the encapsulated protein (M_r, p*I*) as well as properties of the polyelectrolytes (M_r, pK_a, pK_b, or p*I*), and therefore are largely determined by behavior of protein complex with polyelectrolytes, in particular with the first polyelectrolyte layer. These processes are controlled by the number of polyelectrolyte sorption cycles, as was also illustrated in our previous works [1, 5, 10].

These properties of the polyelectrolyte microparticles with insulin show the prospects for their use for peroral and intranasal application.

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