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Mucoadhesive Polyelectrolyte Microparticles Containing Recombinant Human Insulin and Its Analogs Aspart and Lispro

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Abstract—Microparticles containing recombinant human insulin and its analogs aspart and lispro were prepared using an alternate adsorption of chitosan and dextran sulfate from solutions onto microaggregates of protein–dextran sulfate insoluble complex. The following properties of polyelectrolyte hormone-containing microparticles were studied: pH stability, surface charge, mucoadhesive properties, Ca^{2+} binding, degradation under the influence of proteases (trypsin, chymotrypsin). The influence of the self-association ability of encapsulated insulins on the form of protein releasing from microparticles was studied. Insulins aspart and lispro released from the microparticles as monomers were more liable to proteolysis than human insulin released as a hexamer. The combined effect of properties of polyelectrolyte microparticles and of encapsulated recombinant proteins on the bioavailability of insulin under peroral administration is discussed.

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Key words: polyelectrolyte microparticles, human insulin, insulin lispro and aspart, mucoadhesive properties, Ca^{2+} binding, proteolysis

Progress in bioengineering has enlarged the application of recombinant proteins and peptides in therapy. The market for therapeutic agents prepared using recombinant DNA technologies is predicted to be above 52 billion dollars by 2010 [1]. Therefore, it is urgent to develop special systems to deliver the new class of drugs that are now being intensively elaborated.

Recombinant human insulins are widely used in the treatment of *diabetes mellitus*, which is revealed in about 3% of the Earth's human population. Patients with type I *diabetes mellitus* need to strictly control blood glucose level with several daily insulin injections. An ideal approach for insulin deliver would be to imitate its natural secretion in humans [2]. Normally, insulin secreted from the pancreas enters via blood vessels into the liver, which controls the insulin amount for other organs and tissues. Peroral delivery of insulin would be the most natural among the alternative delivery pathways. Taken *per os*, insulin from the small intestine would be also delivered into the liver, which controls the hormone secretion unlike via invasive and other pathways of hormone delivery.

However, the bioavailability of insulin is very low on administration *per os*. This is caused by protein denaturation at the low pH of gastric juice, protein cleavage by proteolytic enzymes of the stomach and small intestine, and by the low permeability of the intestinal cell membranes for the rather large hydrophilic insulin molecule [3, 4]. Modern studies for creation of perorally delivered proteins are directed to prevent or attenuate these influences [5-8].

The purpose of the present work was to develop a system of peroral delivery of recombinant insulin using a combined influence on the physicochemical processes decreasing the hormone bioavailability. In the work insulin was microencapsulated by consecutive adsorption of oppositely charged dextran sulfate and chitosan onto a nanostructured micromatrix, which presents an insoluble polyelectrolyte complex of the protein with the polyanion [9-11]. Chitosan was chosen due to its ability to promote fixation of particles on a mucus layer and to increase the time of their contact with the intestinal mucosa [12, 13].

In the present work the human recombinant insulin was used (M_r 5807, p*I* 5.35), molecules of which associate

Abbreviations: BAEE, N-benzoyl-L-arginine ethyl ester; BTEE, N-benzoyl-L-tyrosine ethyl ester; GIT, gastrointestinal tract; IPC, insoluble polyelectrolyte complexes.

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into hexamers consisting of three dimers bound with two Zn^{2+}, and its analogs with changes on the C-terminus of the B-chain. These analogs are aspart (M_r 5826, pI 5.10) with Pro^{B28} substituted by Asp and lispro (M_r 5807, pI 5.35) with two amino acid residues in the positions 28 and 29 inverted (Pro^{B28} by Lys and Lys^{B29} by Pro). These two rapidly acting insulin analogs possess about 300-times lower ability for self-association due to destruction of the dimer structure [2, 14]. Insulins aspart and lispro remain as monomers even at high concentrations.

MATERIALS AND METHODS

Materials. Sodium dextran sulfate (M_r 500,000), mucin type I-S, N-benzoyl-L-tyrosine ethyl ester (BTEE), aprotinin (M_r 6500) (Sigma, USA); α-chymotrypsin (61 U/mg), ovalbumin (M_r 45,000), chitosan (M_r 400,000) with 85% deacetylation (Fluka, Switzerland); trypsin (40 U/mg), N-benzoyl-L-arginine ethyl ester (BAEE) (Merck, Germany); Sephadex G-50 superfine (LKB-Pharmacia, Sweden); recombinant insulins: human aspart and lispro as zinc salts (produced by Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences). Other reagents were of chemical purity and special chemical purity qualification.

Preparation of polyelectrolyte microparticles. To prepare microaggregates of insoluble polyelectrolyte complex, equal volumes of protein (20 mg/ml) and dextran sulfate (5 mg/ml) were flowed together at pH 3.0, mixed for 20 min, and centrifuged for 3 min at 200*g*. Microparticles were prepared in 0.15 M NaCl (pH 3.0) using step-by-step adsorption on microaggregates of chitosan, dextran sulfate, and again chitosan, as described in [8]. Then the microparticles were suspended in 0.15 M NaCl (pH 3.0) and stored at 4°C or washed thrice with 1 mM HCl and lyophilized.

Characterization of microparticles. Morphology of the microparticles was determined using a confocal laser scanning microscopy (FV300; Olympus, Japan). The average size of the microparticles was determined by optical microscopy (Opton III; Carl Zeiss, Germany) from the results of measurement of 100 microparticles.

Composition of microparticles. Preparations of dried microparticles were suspended in 0.1 M NaOH, the protein content was determined by the method of Lowry et al. [15], and the dextran sulfate was determined by the method of Dubois et al. [16]. The chitosan content was determined by the ratio of the difference of weights of lyophilized preparation (considering its average humidity of 5-6%) and weights of the protein and dextran sulfate to the weight of the lyophilized preparation.

Protein release from microparticles. Suspension of microparticles containing 1 mg protein was supplemented with HCl (pH 1.1) to the volume of 4 ml. The suspension was incubated with mixing (100 rpm) at room temperature for 8 h. Two and 4 h after beginning the experiment, the suspension was centrifuged at 160*g*, the supernatant was separated, and the precipitate was supplemented with the same volume of universal buffer (pH 6.0 and 7.4, respectively). One to eight hours after the starting the process, aliquots of the suspension were taken and centrifuged for 5 min at 1000*g*. The protein concentration was determined in the supernatants.

The protein released from the microparticles was studied using chromatography on Sephadex G-50 (1 × 13 cm column). Specimens were prepared by mixing the suspension of microparticles and 0.05 M Na-phosphate buffer (pH 7.4) to the protein concentration of 2 mg/ml, then they were incubated for 2 h, centrifuged, and 0.5 ml of the supernatant was placed onto the chromatographic column.

Proteolytic degradation of the protein. Suspension of microparticles (0.5 mg/ml protein) was incubated for 1 h at 37°C in 0.05 M Tris buffer (pH 7.1) supplemented with 700 U/ml trypsin and 4 U/ml chymotrypsin [17]. Activities of chymotrypsin and trypsin were determined using BTEE [18] and BAEE [19], respectively, as substrates. Proteolysis was stopped by addition of trifluoroacetic acid to the concentration of 0.1%, the specimens were centrifuged, and the supernatants were analyzed by rapid liquid chromatography on a Superose 12 column (1 × 30 cm) using as eluent 0.02 M Tris acetate (pH 7.4), 0.2 M NaCl, 0.01% NaN_3. The protein degradation was evaluated by the ratio of areas of peaks corresponding to the intact protein before and after treatment with the proteases.

Mucin adsorption on microparticles. To dried microparticles (2 mg), 1.6 ml of mucin solution (0.25 mg/ml) was added. The suspension was incubated for 1 h at 37°C on a shaker at 100 rpm. The supernatant was separated by centrifugation (5 min, 1500*g*), and the mucin content was determined using the Schiff reagent [20]. The mucin adsorption was calculated by the difference between the added amount of mucin and its remains in the supernatant.

Binding of Ca^{2+} by microparticles. To 0.2 ml of the microparticle suspension (12.7 mg/ml), 1 ml of 0.01 M solution of $CaCl_2$ was added. The mixture was incubated for 30 min (100 rpm) and centrifuged for 5 min at 1200*g*. In the supernatant the Ca^{2+} content was determined by titration with EDTA and using Eriochrome Black T [21].

RESULTS AND DISCUSSION

Microencapsulation was performed in 0.15 M NaCl at pH 3.0 in two steps [4]. In the first step human insulin, lispro, and aspart (which were positively charged under these conditions) and dextran sulfate produced insoluble polyelectrolyte complexes (IPC) that included about 99% of each protein. In the second step chitosan, dextran sul-

Properties of polyelectrolyte microparticles containing insulin and its analogs

Insulin	Inclusion efficiency, %	Content, wt. %			ζ-Potential, mV	Average particle size, μm
		protein	dextran sulfate	chitosan		
Human	67 ± 6	55 ± 2	25 ± 4	15 ± 3	32 ± 2	6 ± 3
Aspart	68 ± 8	56 ± 2	27 ± 1	16 ± 3	33 ± 3	5 ± 2
Lispro	64 ± 3	57 ± 1	26 ± 2	17 ± 3	30 ± 2	5 ± 2

fate, and again chitosan on IPC microaggregates were absorbed step-by-step. The losses of all proteins were most pronounced during the chitosan sorption on IPC (22-29%). These losses were partly due to the displacement of insulin from IPC by chitosan. During the following stages of the sorption losses of the proteins were insignificant.

Properties of polyelectrolyte microparticles are presented in the table. Microparticles containing insulin and its analogs were characterized by a high content of protein (53-59%), and the contents of dextran sulfate and chitosan were, respectively, 21-29 and 12-20%. Microparticles were enclosed and had irregular shape with the average size 3-9 μm. Upon lyophilization and resuspension, the microparticles did not aggregate but retained their initial shape and size.

Due to the use of chitosan in the last stage of the preparation, the surface of microparticles carried a positive charge (28-36 mV). Chitosan is capable of producing electrostatic and hydrogen bonds with mucin, which is the most important component of the mucosa [11]. At physiological pH values this glycoprotein has a negative charge due to the presence of fucose and sialic acid on the ends of carbohydrate chains [22]. The binding of mucin with the polyelectrolyte microparticles was measured. This feature is used for predicting mucoadhesive properties of particles [23-25]. Mucin adsorption for human insulin, aspart, and lispro-containing microparticles was, respectively 50 ± 6, 36 ± 6, and 46 ± 5 μg/mg dried particles, and their ζ-potential was changed to the opposite and became –(42-44) mV. For comparison, microparticles with the size of 3-12 μm prepared by disperse drying and consisting only of chitosan [23] bound mucin in the amount of ~50 μg/mg.

The kinetics of protein release from the microparticles (Fig. 1) were determined under conditions which modeled changes in the environmental pH during the passage of microparticles through the human gastrointestinal tract (GIT) [26]. During the first 2 h the microparticles were incubated in medium at pH 1.1 imitating the stomach environment. Then the microparticles were transferred into medium with pH 6.0 imitating the upper parts of the small intestine and incubated there for 2 h. Then the microparticles were placed into medium with pH 7.4 imitating the lower parts of the human small intestine. Human insulin and its analogs were not released from the microparticles in the acidic medium. Human insulin and its analogs were released only at pH 7.4. Within 1 h about 90% of the hormones were released, and during the following 3 h the remaining proteins were stepwise released from the microparticles.

Concurrently with studies on the kinetics of protein release, changes in the surface charge of the polyelectrolyte particles and binding of Ca^{2+} were studied because this can promote an increase or decrease in their interaction with the small intestine mucosa and opening of tight intercellular contacts of the intestinal epithelium, respectively [27]. At pH 1.1 the ζ-potential of the microparticles was unchanged (30-33 mV). At pH 6.0 the microparticles became negatively charged (–(27-36) mV). Under these conditions, chitosan (pK_a ~ 6.5) has virtually no charge. Insulin and its analogs acquire a weak negative charge, and consequently their IPC with dextran sulfate was decomposed. However, all recombinant insulins have a poor solubility at pH close to p*I* and therefore remain inside the particles. At pH 7.4 the proteins still possessing a high negative charge are released into the solution. The

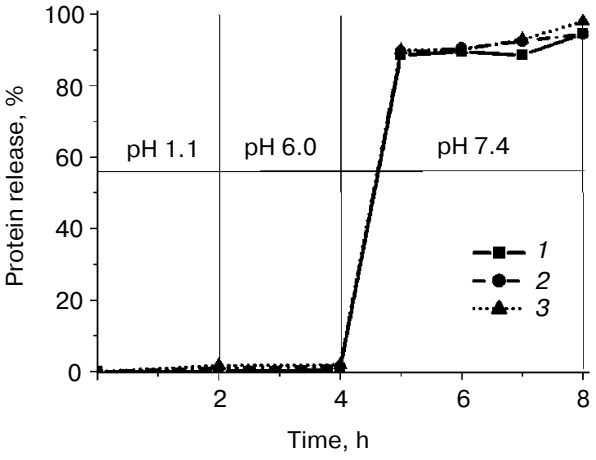

Fig. 1. Kinetics of protein release from polyelectrolyte microparticles under conditions modeling the passage through the human GIT: *1*) human insulin; *2*) aspart; *3*) lispro.

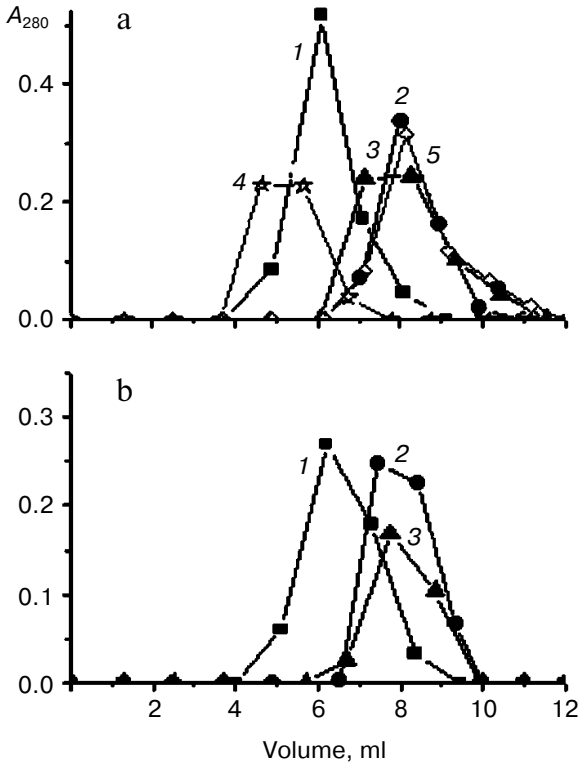

Fig. 2. Gel filtration on Sephadex G-50 (pH 7.4) of native proteins (a) and proteins released from the polyelectrolyte microparticles (b): *1*) human insulin; *2*) aspart; *3*) lispro; *4*) ovalbumin (M_r 45,000); *5*) aprotinin (M_r 6500).

ζ-potential of residues of polyelectrolyte microparticles containing only chitosan and negatively charged dextran sulfate still increased its negative charge (–(40-42) mV). The initial binding of Ca^{2+} by the insulin-containing microparticles was 4.4 ± 0.2 μg/mg, and upon the complete release of the insulin it increased to 11 ± 1 μg/mg.

The form of protein released from the polyelectrolyte microparticles into the medium with pH 7.4 imitating lower parts of the small intestine was determined by gel filtration. The column was precalibrated using proteins with M_r equal to 6500 and 45,000. According to the results of chromatography on Sephadex G-50, human insulin was released mainly as a hexamer, and lispro and aspart as monomers (Fig. 2). The space between the intestinal epithelium cells is estimated to be from 1 to 3-5 nm [28]. Therefore, the paracellular transporting of insulin analog monomers with their average size of 2.3 nm was easier than the transport of insulin hexamer with its average size of 5 nm.

Trypsin and chymotrypsin are the main enzymes of the pancreatic juice capable of degrading insulin in the small intestine and which act on, respectively, two and five main sites of proteolysis in the hormone molecule. Proteolysis of microencapsulated insulin and its analogs was observed during 1 h in medium imitating the human pancreatic juice [17] containing trypsin and chymotrypsin (the weight ratio insulin/protease is 5 : 1 for each protease, pH 7.1). All insulins in the native states were completely degraded under these conditions. The polyelectrolyte microparticles significantly protected human insulin (40% non-degraded protein), which was released as a hexamer. The amount of non-degraded aspart released from the microparticles as a monomer was 23%. The lispro monomer was not protected by microparticles under these conditions.

Thus, by adsorption of dextran sulfate and chitosan, microparticles were prepared which contained recombinant human insulin, aspart, and lispro and were characterized by a high content of protein (no less than 55%). The polyelectrolyte microparticles protect the recombinant insulins against the aggressive gastric medium by preventing the protein release. Positive charge and mucoadhesive properties of the particles caused by the use of chitosan in the last stage of their preparing can promote the attachment of the microparticles on the intestinal epithelium mucosa. Due to specific interactions of chitosan with proteins of tight intercellular contacts (occludin, actin, and ZO-1), chitosan can reversibly increase the intercellular permeability of the epithelium at the site of the contact with it [11, 29, 30]. At pH > 6, i.e. under conditions corresponding to the middle and lower parts of the intestine, insulin is released from the microparticles as a hexamer, whereas the insulin analogs aspart and lispro are released as monomers. It seems that the monomer shape of the insulin analogs can be favorable for increasing their penetration across the intestinal epithelium as compared to human insulin. The binding of calcium ions by the microparticles and the increase in this binding on the protein release can promote the opening of the tight contacts of the intestinal epithelium and the paracellular penetration of the hormone into the blood flow. Change in the positive surface charge of microparticles to negative upon the protein release should promote elimination of the remaining chitosan and dextran sulfate from the intestine. The protective effect of polyelectrolyte microparticles against proteolytic enzymes decreases in the following series: human insulin > aspart > lispro. Thus, incorporation of insulin and its analogs into polyelectrolyte microparticles is shown to increase the bioavailability of insulin on administration *per os*. The hypoglycemic effects of polyelectrolyte microparticles containing human recombinant insulin are now under study.

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