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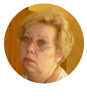

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Ternary Interpolyelectrolyte Complexes Insulin-Poly(methylaminophosphazene)-Dextran Sulfate for Oral Delivery of Insulin

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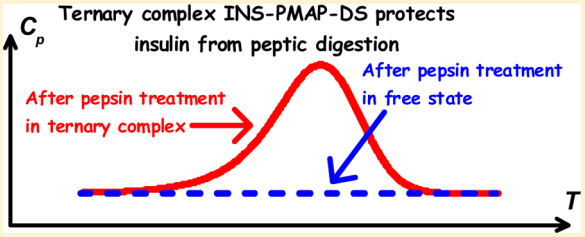
ABSTRACT: Ternary interpolyelectrolyte complexes of insulin with biodegradable synthetic cationic polymer, poly(methylaminophosphazene) hydrochloride (PMAP), and dextran sulfate (DS) were investigated by means of turbidimetry, dynamic light scattering, phase analysis, and high-sensitivity differential scanning calorimetry. Formation of ternary insoluble stoichiometric Insulin-PMAP-DS complexes was detected under conditions imitating the human gastric environment (pH 2, 0.15 M NaCl). A complete immobilization of insulin in the complexes was observed in a wide range of the reaction mixture compositions. The ternary complexes were shown to dissolve and dissociate under conditions imitating the human intestinal environment (pH 8.3, 0.15 M NaCl). The products of the complex dissociation were free insulin and soluble binary Insulin-PMAP complexes. The conformational stability of insulin in the soluble complexes of various compositions was investigated by high-sensitivity differential scanning calorimetry. The dependence of the excess denaturation free energy of insulin in these complexes on the PMAP content was obtained. The binding constants of the folded and unfolded forms of insulin to the PMAP polycation were estimated. Proteolysis of insulin involved in the insoluble ternary complexes by pepsin was investigated under physiological conditions. It was found that the complexes ensure an almost 100% protection of insulin against proteolytic degradation. The obtained results provide a perspective basis for development of oral insulin preparations.



INTRODUCTION

Design of insulin preparations for oral administration is the subject of a vast number of studies. Despite continuous searching in this field, no feasible solutions have been found up to now.[1−3] The key problems refer to protection of the protein from acid and/or enzymatic degradation in the gastrointestinal tract and to release of bioactive and muco-adhesive insulin in the intestine.[4] Among various strategies proposed for solving these problems,[1,5] the encapsulation approach seems to provide the most promising results.[6−9] Here, we will consider one of the encapsulation variants—incorporation of insulin into interpolyelectrolyte nanocomplexes formed by polycations and polyanions. The complexes should be stable in conditions of the stomach (pH 1.5−2.0) and capable of pH-sensitive dissociation in order to provide release of insulin under conditions reflecting the intestine (pH ∼ 8.0).

A key aspect of the complex formation is an appropriate choice of polycation. The list of potential polycations is rather poor, since most of the known synthetic polycations are cytotoxic.[10] A good candidate for encapsulation of oral insulin could be chitosan due to its biocompatibility.[11] Chitosan interpolyelectrolyte complexes as capsules for insulin immobilization were extensively investigated and demonstrated some promising results *in vitro*.[11−19] Nanocomplexes of chitosan with dextran sulfate were shown to immobilize up to 75% of a bioactive polypeptide[20] and about 85% of insulin.[21] Insulin was able to release from the complexes at pH 6.8 in a controllable way and retain its functionality. Interpolyelectrolyte complexes of insulin with some chitosan derivatives were shown to protect insulin from enzymatic degradation.[22] Despite numerous solutions concerning practical use of the delivery systems based on chitosan, this biopolycation is not approved by some health protecting organizations since its biodegradability and safety are still questioned.[23]

Water-soluble polyphosphazenes and their derivatives represent a real alternative to chitosan.[24,25] Poly(alkylaminophosphazenes) acquire positive charges in the backbone as a result of nitrogen protonation. Under physiological conditions, they are hydrolyzed in a controllable manner to the harmless ammonium salts and phosphates, which are easily excreted from the body.[26] Yet another important feature of polyphosphazenes, distinguishing them from chitosan, is the backbone flexibility. According to the model

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studies,[27,28] the flexibility provides a particular bond strength between components of the interpolyelectrolyte complex.

To date, only one study has been carried out on the polyphosphazene-based systems for insulin immobilization.[29] Polyphosphazene microspheres loaded with insulin were designed for subcutaneous administration and tested on diabetic mice with some positive results.

In this paper, we report investigation on the ternary interpolyelectrolyte complexes of insulin with biodegradable synthetic polycation and polyanion, poly(methylaminophosphazene) (PMAP), and dextran sulfate (DS), respectively. PMAP carries positive charges in the backbone. An important advantage of this polycation is its pH-dependent ionization. PMAP was shown to form stable stoichiometric and nonstoichiometric interpolyelectrolyte complexes with two natural polyanions, ι-carrageenan,[30] and DNA.[31] The conformation of both polyanions was sensitive to the complex formation with PMAP. DS is a branched sulfated polysaccharide containing three sulfate groups per disaccharide unit. It is capable of complex formation with proteins at pH both below and above their isoelectric points.[32] In this work, the ternary Insulin-PMAP-DS complexes were characterized by turbidimetry, phase analysis, dynamic light scattering, and high-sensitivity differential scanning calorimetry (HS-DSC) under conditions imitating the human gastric (pH ∼ 2) and intestine (pH ∼ 8) environment. Insulin immobilized in the ternary complex was shown to be protected from pepsin digestion and retain the tertiary structure upon pH-induced complex dissociation.

MATERIALS AND METHODS

Human recombinant zinc insulin (INSURAN, Institute of Bioorganic Chemistry, Moscow, Russia) with a purity of 99% and an activity greater than 28 IU·mg^{-1} was used without additional purification. Poly(methylaminophosphazene) was prepared by complete aminolysis of linear poly(dichlorophosphazene) as described previously.[30,31,33] IR and ^{31}P NMR spectroscopy as well as the element analysis showed that an almost complete displacement of chlorine atoms from the P−Cl groups by methylamine was achieved. Poly(methylaminophosphazene) hydrochloride (PMAP) was prepared by dissolution of poly(methylaminophosphazene) in 0.01 M HCl with subsequent lyophilization. According to the element analysis, the chlorine content in the PMAP sample was 14.9%. An aqueous solution of PMAP of a concentration 1.0 mg·mL^{-1} had pH of 3.16 ± 0.01. These data allowed us to estimate the maximum ionization degree of PMAP. It was about 0.5, which agrees with the results of determination of the ionization degree of octamethylaminocyclotetraphosphazene.[34]

Dextran sulfate (sodium salt) was purchased from Sigma (Lot 099K15011). According to the certificate, it contained about 2% of phosphates which are nondesirable impurities for investigation of interpolyelectrolyte reactions. To remove phosphates, DS was dissolved in water at a concentration of 5%, dialyzed exhaustively against deionized water for 1 day, and then lyophilized. According to the element analysis, the purified DS preparation contained 15.1% of sulfur.

Ternary interpolyelectrolyte complexes insulin-poly(methylaminophosphazene)-dextran sulfate (Insulin-PMAP-DS) were prepared using the following protocol. Stock solutions of all polymer components of a desired concentration were prepared in 20 mM glycine buffer containing 0.15 M NaCl (pH 2.0). Concentration of insulin in the stock solution varied from 1.0 to 12 mg·mL^{-1}, depending on the method of analysis. It was spectrophotometrically ascertained using $E_{276}^{1\ \mathrm{mg\cdot mL^{-1}}}$ = 1.01.[35] Concentrations of PMAP and DS in the stock solutions were determined by weight. They varied from 1.0 to 20 mg·mL^{-1} for PMAP and from 1.0 to 10 mg·mL^{-1} for DS, depending on the method of analysis. First, a polycation reagent (CR), that is a mixture of insulin and PMAP, was prepared and incubated for 1 h, then an aliquot of the polyanion stock solution (DS) was added to the polycation reagent. The ternary mixtures were incubated for 2 h at room temperature before measurements.

In order to perform phase analysis, the ternary INS-PMAP-DS mixtures were subjected to centrifugation at 15 000 rpm for 1 h. As a result, the system was separated into two phases: a supernatant (transparent solution) and a precipitate (the complex coacervate phase). The weights of the supernatant and precipitate were determined. The coacervate phase was washed twice with deionized water and lyophilized. The concentration of insulin in the supernatant was spectrophotometrically measured. The content of PMAP and DS in the coacervate phase was determined by element analysis from the content of phosphorus and sulfur, respectively.

Calorimetric measurements were carried out with a differential scanning microcalorimeter DASM-4 (BIOPRIBOR, Russia) within the temperature range 10−130 °C at a heating rate of 2 K min^{-1} and an excess pressure of 0.25 MPa. The primary data processing and conversion of the partial heat capacity of insulin into the excess heat capacity function of the denaturation transition was performed using the *NAIRTA 2.0* software (Institute of Organoelement Compounds, Moscow, Russia). The baseline in the transition area was obtained by a spline interpolation. The maximum temperature of the excess heat capacity curve was taken as the denaturation temperature, T_d. The denaturation enthalpy, $\Delta_d h$, was determined by integration of the excess heat capacity function.

Experiments on dynamic light scattering were carried out with a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd.) at 25 °C under the angle of 173°. A helium−neon laser was used as a 632.8 nm light source. A sample solution was filtered through a Millipore filter of 22 μm into a 1 cm measuring cuvette, which was preliminary washed with ∼1 mL of the filtrate. The time of temperature equilibration of the cuvette inside the instrument was 10 min. The final hydrodynamic radius distribution of the sample was found by averaging five measurements, each of which was a result of 10−16 scans. Concentrations of insulin, DS, and PMAP in the individual and mixed solutions were kept constant and equal to 3.0, 3.3, and 2.0 mg·mL^{-1}, respectively.

Proteolysis of insulin was carried out with pig pepsin ("OlainFarm", Latvia) taken without additional purification. A stock solution of the enzyme was prepared in 0.01 M HCl and 0.15 M NaCl at a concentration of 0.5 mg·mL^{-1}. After filtration of the pepsin stock solution, its concentration was determined spectrophotometrically using $E_{278\mathrm{nm}}^{1\mathrm{mg\cdot mL^{-1}}}$ = 1.47. A ternary complex Insulin-PMAP-DS was prepared at pH 2.0 in 20 mM glycine, 0.15 M NaCl. 40 μL of the pepsin stock solution was added to 2 mL of the complex suspension. A control insulin−pepsin mixture was prepared without PMAP and DS. Both systems were incubated at 37 °C for 1 h. Then, the proteolysis was stopped by a quick shift of pH of the reaction mixtures to pH 8.3 by addition of an aliquot of 1 M NaOH. The final systems were analyzed by HS-DSC.

Spectrophotometric measurements were performed with a UV−vis spectrophotometer Genesys 2 (ThermoSpectronic).

RESULTS AND DISCUSSION

Molecular and Electrochemical Characteristics of the Polyelectrolytes. The molecular weights and electrochemical characteristics of the studied polyelectrolytes are summarized in Table 1. The experimental conditions referred to two pH values, namely, pH 2.0 and pH 8.3, imitating the gastric and intestine medium, respectively.

Table 1. Molecular Weight (M_{sD}), Ionization Degree (f), and Equivalent Weight (M^*) of the Polyelectrolytes

		f		M^*, g·mol^{-1}	
polymer	M_{sD}, kDa	pH 2.0	pH 8.3	pH 2.0	pH 8.3
PMAP	320 ± 20	0.5	0.08	250	1390
DS	200 ± 40	1.0	1.0	212	212
Insulin	6.0[35]	1.0[a]	0.28[b]	935	2100

[a]The fraction of the positively charged groups of the total number of the insulin basic groups.[38] [b]The fraction of the negatively charged groups of the total number of the insulin acid groups.[38]

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PMAP of molecular weight M_{sD} = 320 kDa was used as a polycationic reagent. An important characteristic of a polyelectrolyte in interpolyelectrolyte reactions is its equivalent weight (M^*), that is, the polymer weight per one charge. This parameter for PMAP is a function of pH since its charge depends on pH. The charge of PMAP was derived from its ionization degree (f) which was calculated using the values of exponents of the ionization constants of octamethylaminocyclotetraphosphazene (OMACTP), p$K_{a,1}$7.95, and p$K_{a,2}$5.2.[34] The existence of two ionization constants for the cyclic phosphazene oligomer is a consequence of a so-called "effect of anticooperativity" when ionization of each subsequent link of a polymer chain becomes more and more hampered. This effect is typical of polyelectrolytes.[36] Based on the structural similarity between OMACTP and PMAP, we used the above ionization constants for estimation of the ionization degree of PMAP. The ionization degree of PMAP amounted to 0.5 and 0.08 at pH 2.0 and 8.3, respectively. Accordingly, the equivalent weight of PMAP (M^*_{PMAP}) increased notably from 250 to 1390 g·mol^{-1} when pH shifted from pH 2.0 to 8.3 (Table 1).

Dextran sulfate (DS) was used as a polyanion reagent. This is a biodegradable branched sulfated polysaccharide. According to the sulfur analysis, the DS preparation contained about 3 sulfate groups per one disaccharide unit. The ionization degree of DS is equal to 1 independently of pH. Accordingly, the equivalent weight of DS is M^*_{DS} = 212 g·mol^{-1} (Table 1).

Insulin is a protein hormone controlling the glucose level in the human blood.[35] This is a small globular protein composed of two subunits which are linked by two disulfide bonds into a monomeric form. Depending on pH and protein concentration, insulin exists in solution as monomers, dimers, hexamers, and their clusters. Insulin specifically binds zinc ions which affect its quaternary structure and conformational stability.[37] The molecular weight of the insulin monomer is about 6 kDa. Its equivalent weight (M^*_{INS}) depends on pH. As followed from the titration curve of insulin,[38] M^*_{INS} amounts to 935 and 2100 g·mol^{-1} at pH 2.0 and 8.3, respectively (Table 1).

Conformational Stability of Insulin. Conformational stability of insulin under conditions imitating the gastric (pH 2.0) and intestinal (pH 8.3) environment was characterized by HS-DSC. At pH 2.0 and low ionic strength, insulin exists in diluted solution predominantly as a monomer. Its thermal denaturation is a reversible transition between two states—native and unfolded. Insulin has the denaturation temperature T_d = 60.0 ± 0.4 °C and enthalpy $\Delta_d h$ = 11.2 ± 0.9 J·g^{-1} (Table 2). The addition of NaCl in physiological concentration significantly affects the denaturation behavior of insulin even in the diluted solution. A reproducible exothermic heat effect was observed on the thermogram assigned to formation of fibril-like aggregates of the unfolded protein. The aggregation results in a significant decrease in the denaturation enthalpy, although the denaturation temperature of insulin increases. A similar picture of the aggregation was reported for insulin solutions of high concentrations without salt.[39] Here, we should note that because of the significant aggregation heat effects it was not possible to apply HS-DSC for investigation of the insulin stability in ternary systems with PMAP and DS at pH 2.0 in 0.15 M NaCl. At pH 8.3, no aggregation heat effects were seen on the denaturation thermograms of insulin. Its denaturation temperature and enthalpy increase both with and without NaCl with respect to those at pH 2.0 indicating the stabilization of the insulin tertiary structure in the basic medium.

Turbidimetric Study of the Ternary Insulin-PMAP-DS Interpolyelectrolyte Complexes. Formation and properties of the ternary interpolyelectrolyte complexes of insulin with PMAP and DS were investigated under conditions imitating the human gastric environment (pH ~ 2). For this purpose, 20 mM glycine buffer of pH 2.0, containing 0.15 M NaCl, was used as a solvent. In these conditions, insulin and PMAP are polycations while DS is a polyanion. The composition of the reaction mixture Insulin-PMAP-DS was characterized by two parameters: the apparent weight fraction of the polycationic reagent CR (Insulin-PMAP) in the reaction mixture, w_{CR}, and the apparent weight fraction of insulin in the cationic reagent, w_{INS}.

Ternary complexes Insulin-PMAP-DS were obtained at various compositions of the reaction mixture. The yield of an insoluble complex was estimated from turbidity of the system at 436 nm, τ_{436}. Figure 1a shows the τ_{436} values as a function of the parameter w_{CR} for w_{INS} = 0.2. The turbidity passes through a maximum. According to the continuous variation method,[40] the mixture composition, corresponding to the turbidity maximum, provides a maximal yield of an insoluble stoichiometric complex.

A starting point for the interpretation of the turbidimetry data can be an assumption on the equality of the equivalents of the anionic (AR) and cationic (CR) reagents at the maximal yield of the insoluble complex:

$$n_{AR} = n_{CR} \quad (1)$$

Further, one can suggest that the total equivalent of the cationic reagent, composed of insulin and PMAP, is equal to the sum of the equivalents of insulin (INS) and poly(methylamino-phosphazene) (PMAP):

$$n_{CR} = n_{INS} + n_{PMAP} \quad (2)$$

It follows from this equality that the dependence of the apparent equivalent fraction of the cationic reagent, corresponding to the maximal yield of the insoluble stoichiometric complex (w^*_{CR}), on the composition of the cationic reagent has a form

$$w^*_{CR} = \frac{M^*_{CR}/M^*_{AR}}{1 + M^*_{CR}/M^*_{AR}} \quad (3)$$

where M^*_{CR} and M^*_{AR} are the equivalent weights of the cationic and anionic reagents, respectively. The equivalent weight of the

Table 2. Temperature (T_d) and Enthalpy ($\Delta_d h$) of Denaturation of Human Insulin at pH Values, Imitating the Human Gastrointestinal Conditions

system	NaCl, M	PMAP, mg·mL^{-1}	DS, mg·mL^{-1}	pH 2.0[b] T_d, °C	pH 2.0[b] $\Delta_d h$, J·g^{-1}	pH 8.3[c] T_d, °C	pH 8.3[c] $\Delta_d h$, J·g^{-1}
Native insulin	–	–	–	60.0 ± 0.4	11.2 ± 0.9	74.5 ± 0.6	15.4 ± 1.7
Native insulin	0.15	–	–	64.7 ± 0.8	6.6 ± 1.0	81.7 ± 0.7	17.0 ± 0.7
Insulin-PMAP-DS after dissolution of the coacervate phase	0.15	2.0	3.3	nd	nd	70.5 ± 0.5	22.0 ± 0.9
Insulin-PMAP-DS (control)	0.15	2.0	3.3	–	–	71.4 ± 0.9	26.5 ± 0.8

[a]Protein concentration 3.0 mg·mL^{-1}; heating rate 2 K min^{-1}. [b]Buffer solution: 20 mM glycine–HCl. [c]Buffer solution: 20 mM glycine–NaOH.

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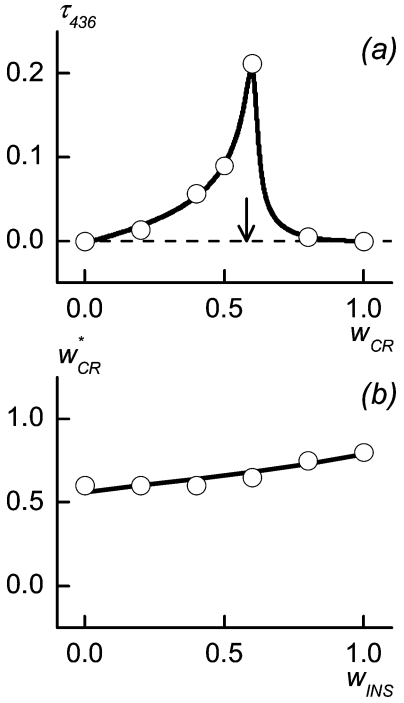

Figure 1. (a) Turbidity of the Insulin-PMAP-DS system vs the apparent weight fraction of the polycationic reagent (Insulin-PMAP) at pH 2.0 (20 mM glycine, 0.15 M NaCl). The apparent weight fraction of insulin w_{INS} = 0.2; the total polymer concentration 0.05 mg·mL^{-1}; the equivalent mixture composition is marked by an arrow. (b) Equivalent apparent weight fraction of the polycationic reagent vs the insulin content in this reagent at pH 2.0 (20 mM glycine, 0.15 M NaCl): (○) experimental data; the solid line is calculated by eqs 3 and 4 at M^*_{INS} = 763 g mol^{-1}, M^*_{PMAP} = 261 g mol^{-1}, M^*_{DS} = 202 g mol^{-1}.

cationic reagent may be expressed via its composition and the equivalent weights of insulin and PMAP (M^*_{INS} and M^*_{PMAP}, respectively):

$$\frac{1}{M^*_{CR}} = \frac{1}{M^*_{INS}} w_{INS} + \frac{1}{M^*_{PMAP}}(1 - w_{INS}) \quad (4)$$

A combination of eqs 3 and 4 allows one to calculate the equivalent value of the apparent weight fraction of the cationic reagent, corresponding to the maximal yield of the insoluble stoichiometric complex, as a function of the insulin content in the cationic reagent.

Figure 1b shows the experimental and calculated results as a dependence of the apparent weight fraction of the polycationic reagent, corresponding to the maximal yield of the stoichiometric complex, w^*_{CR}, on the apparent weight fraction of insulin in the cationic reagent. The calculated line was obtained as the best fit of eqs 3 and 4 to the experimental data with the parameters M^*_{INS} = 763 g mol^{-1}, M^*_{PMAP} = 261 g mol^{-1}, and M^*_{DS} = 202 g mol^{-1}. It agrees well with the experimental data. The fit parameters are close to the values of the equivalent weights of the constituting polyelectrolytes (Table 1). Consequently, the insoluble complexes Insulin-PMAP-DS, formed under conditions of the human gastric environment, represent the ternary stoichiometric (equivalent) complexes of these polyelectrolytes.

Phase Analysis of the Ternary Insulin-PMAP-DS Complex Systems. For the quantitative estimation of the insulin encapsulation in the ternary complexes, a phase analysis of mixtures Insulin-PMAP-DS was carried out. The insoluble complexes (coacervate phases) were isolated by centrifugation and the insulin content in the supernatant was determined spectrophotometrically. A content of insulin in the coacervate phase was derived from a mass balance of the separated phases. Insulin yield in the coacervate phase at various compositions of the reaction mixture is shown in Figure 2. At $w_{CR} \leq 0.6$, 100% of

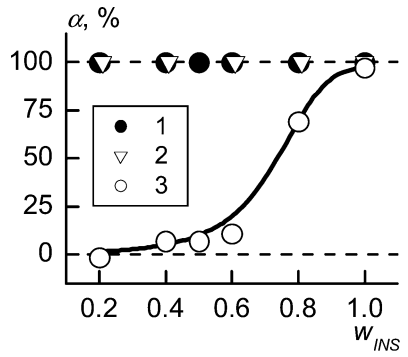

Figure 2. Insulin yield in the stoichiometric ternary Insulin-PMAP-DS interpolyelectrolyte complexes vs the apparent weight fraction of insulin in the polycationic reagent (Insulin-PMAP) at pH 2.0 (20 mM glycine, 0.15 M NaCl). The apparent weight fraction of the polycationic reagent w_{CR} = 0.4 (1), 0.6 (2), and 0.8 (3); the total polyelectrolyte concentration is 1.0 mg·mL^{-1}.

insulin precipitates as the complexes independently of the protein content in the polycationic reagent. At w_{CR} = 0.8, a dependence of the insulin yield on its fraction in the polycationic reagent arises. The higher the PMAP content in the polycationic reagent, the lower the insulin yield in the complex coacervate phase. It seems to reveal a more successful competition of PMAP with insulin for binding to the polysaccharide matrix. This suggestion agrees with data by Izumrudov et al. on the substitution of a polyelectrolyte from the interpolyelectrolyte complex by its more charged and long-chain analogue.[41−43]

The content of PMAP and DS in the insoluble complexes was determined from the element analysis of the coacervate phase according to the phosphorus and sulfur content, respectively. It was of interest to compare the content of each polymer in the coacervate phase with its content in the initial reaction mixture. Figure 3 shows data on the yield of insulin, PMAP, and DS in the coacervate phase, and the corresponding composition of the reaction mixture at w_{CR} = 0.6. It is seen that within the limits of experimental errors all points lie on the bisector of the quadrant. This result suggests an approximately complete precipitation of the polyelectrolytes in all systems investigated. Consequently, one can conclude that the coacervate phase formed at pH 2.0 is composed of the ternary Insulin-PMAP-DS interpolyelectrolyte complexes.

In view of the pharmacological relevance of the Insulin-PMAP-DS complexes, their dissociation under conditions simulating the human intestine; environment (pH ~ 8) is of particular importance. The dissociation of the complexes should provide release of bioactive insulin. To verify these assumptions, we investigated the behavior of the insoluble ternary Insulin-PMAP-DS complexes, prepared at pH 2.0, after their redispersion in a weakly basic medium. Most of the ternary complexes dissolved completely at pH 8.3. This allowed one to determine the insulin content spectrophotometrically with a small correction for light scattering.[44] Data on insulin release from the ternary Insulin-PMAP-DS complexes after their dissociation in the basic medium (pH 8.3) are shown in Figure 4. The release was characterized by the fraction of soluble insulin formed after the complex dissociation on the insulin content in the complexes at pH 2.0. The results show that the

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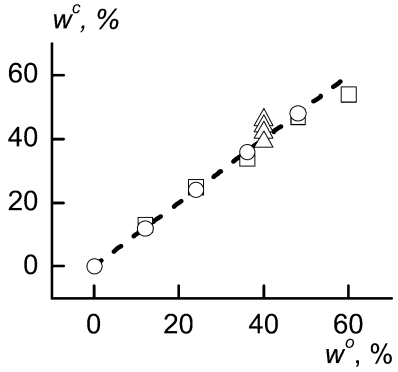

Figure 3. Composition of the stoichiometric ternary Insulin-PMAP-DS interpolyelectrolyte complexes (w^c) formed at pH 2.0 (20 mM glycine, 0.15 M NaCl, w_{CR} = 0.6) vs the composition of the Insulin-PMAP-DS reaction mixtures (w^o): insulin content (○), PMAP content (□), DS content (△); the dashed line is the bisector of the quadrant.

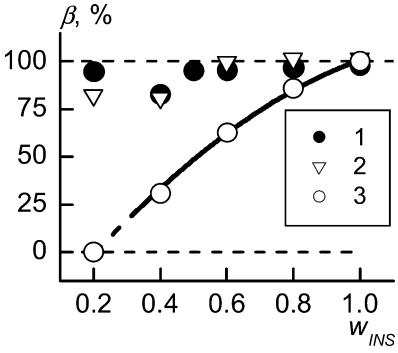

Figure 4. Release of soluble insulin from the stoichiometric ternary Insulin-PMAP-DS interpolyelectrolyte complexes as a result of their dissociation at pH 8.3 (20 mM glycine, 0.15 M NaCl) vs the apparent weight fraction of insulin in the polycationic reagent (Insulin-PMAP). The apparent weight fraction of the polycationic reagent, w_{CR} = 0.4 (1), 0.6 (2), and 0.8 (3); the total polyelectrolyte concentration is 1.0 mg·mL^{-1}.

release of insulin amounts to almost 100% over a sufficiently wide range of the complex compositions.

Conformational Stability of Insulin in the Insulin-PMAP-DS System. Incorporation of insulin into the ternary interpolyelectrolyte complexes formed at pH 2 implies a placement of the protein in conditions extreme for its native conformation. Along with the risk of acid hydrolysis, an additional protein destabilization arises because of preferential binding of the unfolded protein form to the charged matrix.[45−49] In this view, a necessary condition for release of the functionally active insulin from the interpolyelectrolyte complex after its dissociation is renaturation of the protein. The conformational state of insulin after dissociation of the ternary Insulin-PMAP-DS complexes was investigated by HS-DSC. A ternary Insulin-PMAP-DS complex (w_{CR} = 0.6, w_{INS} = 0.6) was formed at pH 2, then separated by centrifugation and redispersed in a buffer solution of pH 8.3 (20 mM glycine, 0.15 M NaCl). As a result, a transparent solution was formed that implied either a complete dissociation of the stoichiometric ternary complex or its transformation into a nonstoichiometric soluble complex. The denaturation thermogram of insulin in this solution is shown in Figure 5, and the denaturation parameters are given in Table 2 in comparison with the parameters of native insulin in a reference solution. Additionally, data for the control ternary system, prepared by direct mixing of insulin, PMAP, and DS at pH 8.3, are given. The denaturation thermograms show that insulin retains its cooperative tertiary structure upon transfer of the coacervate phase from pH 2.0 to 8.3 and its dissolution (Figure 5),

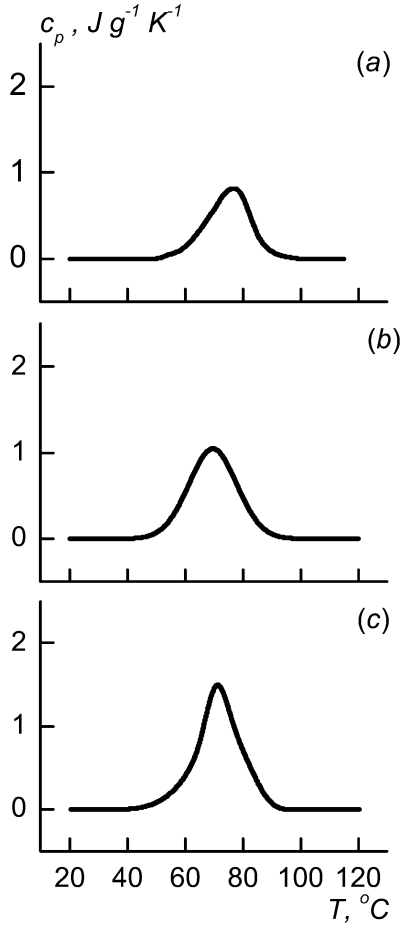

Figure 5. Denaturation thermograms of native insulin (a) and insulin in the Insulin-PMAP-DS mixtures (b,c) at pH 8.3 (20 mM glycine, 0.15 M NaCl). The ternary mixtures were obtained by the dissociation of the stoichiometric Insulin-PMAP-DS complex, prepared at pH 2.0 (b), and by direct mixing of the polyelectrolytes at pH 8.3 (c). The concentrations of the polyelectrolytes in all systems are C_{INS} = 3 mg·mL^{-1}; C_{PMAP} = 2 mg·mL^{-1}; C_{DS} = 3.3 mg·mL^{-1}.

although it slightly changes the conformational stability (Table 2). The denaturation temperatures of insulin in both ternary systems are lower than that of native insulin. In contrast, the denaturation enthalpies in the ternary systems are larger than the enthalpy of native insulin. The reason for such differences will be explained below. Here, it is important that the denaturation enthalpy of insulin released from the ternary complex amounts to 80% of the enthalpy of native insulin in the control ternary system. This fact implies preservation of the functionality of insulin immobilized in the ternary complex upon its transfer from the acid medium to the basic one.

Structure of Ternary Insulin-PMAP-DS Systems after Dissociation of the Ternary Stoichiometric Complexes at pH 8.3 (0.15 M NaCl). A composition of the products of dissociation of the ternary stoichiometric Insulin-PMAP-DS complexes was qualitatively determined by dynamic light scattering using average hydrodynamic radii of insulin, PMAP, and dextran sulfate in the corresponding reference solutions as assignment

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Table 3. Average Hydrodynamic Radius (R_h, nm) of Particles in Solutions of Individual Polyelectrolytes and of Their Mixtures at pH 8.3 (20 mM glycine, 0.15 M NaCl)

system	insulin	PMAP	DS	complex
Individual polyelectrolyte	3.0 ± 0.2	26 ± 1	14 ± 3	–
Insulin-PMAP-DS	2.5 ± 0.6	nd	12 ± 6	50 ± 14
Insulin-PMAP	2.8 ± 0.3	16 ± 3	–	70 ± 30
Insulin-DS	3.1 ± 0.2	–	16 ± 2	–

criteria (Table 3). According to these data, the dissociation products involve free insulin ($R_h \simeq 3$ nm) and dextran sulfate ($R_h \simeq 12$ nm), as well as some very large particles ($R_h \simeq 50$ nm). Similar particles were apparently observed in the Insulin-PMAP mixed solutions. One can suggest that insulin and PMAP are able to form soluble interpolyelectrolyte complexes in the weakly alkaline medium because they are still oppositely charged under these conditions.

Thus, the light scattering data show that the stoichiometric Insulin-PMAP-DS complex being dissociated in the basic medium partially liberates the protein and partially transforms into a soluble binary complex involving insulin and PMAP. The existence of soluble Insulin-PMAP complexes is of particular interest since it can provide a prolonged kinetics of the insulin release in the intestine environment that is an attractive property of insulin preparation for oral delivery.

Conformational Stability of Insulin in the Soluble Insulin-PMAP Complexes. The Insulin-PMAP mixtures of different PMAP/Insulin weight ratios, q, were studied by HS-DSC. The thermograms of the mixtures are shown in Figure 6. With

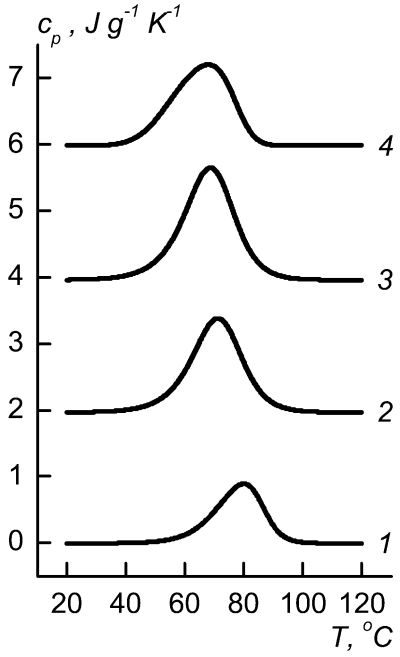

Figure 6. Denaturation thermograms of insulin in soluble complexes with PMAP at pH 8.3 (20 mM glycine, 0.15 M NaCl). The PMAP/Insulin weight ratio, q: 0 (1); 0.33 (2); 0.67 (3); and 1.67 (4). The insulin concentration in all systems is 3.0 mg·mL^{-1}. The curves are shifted arbitrarily along the y-axis for better presentation.

increasing content of PMAP, a gradual shift of the denaturation peak of insulin to the left is observed, that is accompanied by an increase in the peak area. The denaturation parameters of insulin in the mixtures are presented as a function of q in Figure 7. With the

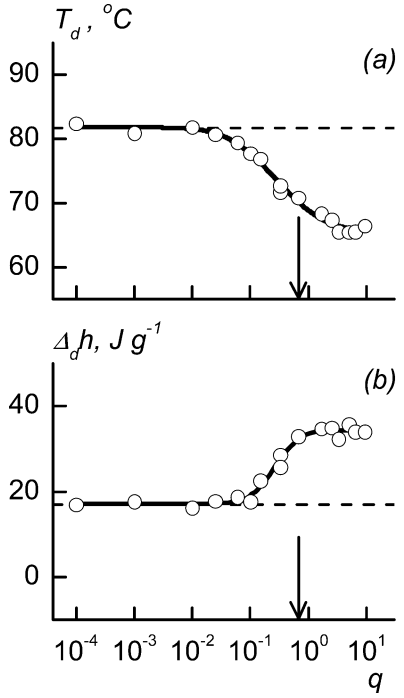

Figure 7. Denaturation temperature (a) and enthalpy (b) of insulin in complexes with PMAP vs the PMAP/Insulin weight ratio, q, at pH 8.3 (20 mM glycine, 0.15 M NaCl). The denaturation parameters of insulin in the absence of PMAP are shown by dashed lines. Arrows mark the PMAP/Insulin equivalent ratio, $q^* = 0.67$.

increase in the PMAP content, the denaturation temperature decreases, while the denaturation enthalpy increases. These dependences can be regarded as the sigmoid transition curves passing from the stability level of free insulin to some new level corresponding to the conformational stability of the protein in bound state. Both curves have inflection points in the vicinity of the equivalent PMAP/Insulin ratio ($q^* = 0.67$).

The dependences $T_d(q)$ and $\Delta_d h(q)$ were used for calculation of the excess denaturation free energy of insulin, $\Delta_d G^E$, normalized per equivalent weight of the protein estimated for pH 8.3 ($M^*_{INS} = 2100$ g·mol^{-1}), as a function of the parameter q (Figure 8). This dependence can be expressed in terms of the binding constants of PMAP to the native and denatured forms of the protein (K_N and K_D, respectively)[31]

$$\Delta_d G^E = -RT_d^0 \ln(1 + K_D L) + RT_d^0 \ln(1 + K_N L) \quad (5)$$

where R is the gas constant; T_d^0 is the denaturation temperature of insulin at $q = 0$; L is the equivalent concentration of PMAP ($M^*_{PMAP} = 925$ g·mol^{-1}).

Equation 5 was fitted to the experimental dependence $\Delta_d G^E(q)$. This procedure was performed using both binding constants as adjusted parameters. A good fit was reached at $K_N = 325 \pm 30$ M^{-1} and $K_D = 1100 \pm 81$ M^{-1}. This means that PMAP has a higher affinity to the denatured protein form than to the native one ($K_D \gg K_N$). The result can probably be explained by lower entropic costs of the formation of complementary ionic pairs between the polycation and the protein when the latter adopts an unfolded flexible conformation.

Proteolysis of Insulin in the Ternary Insulin-PMAP-DS Interpolyelectrolyte Complex. An essential problem in design of the insulin preparations for oral administration is maintenance of intactness of the protein structure during its passage through the gastric tract, i.e., protection of the protein

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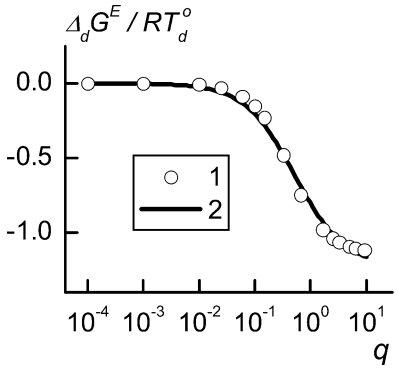

Figure 8. Excess denaturation free energy of insulin in complexes with PMAP at pH 8.3 vs the PMAP/Insulin weight ratio, q: 1, experimental; 2, calculated by eq 5 at $K_N = 325 \pm 30$ M^{-1} and $K_D = 1100 \pm 81$ M^{-1}. The excess free energy is normalized per the equivalent weight of insulin in the interpolyelectrolyte reactions ($M^*_{INS} = 2100$ g·mol^{-1}). $T^\circ_d = 354.9$ K is the denaturation temperature of insulin in the absence of PMAP.

from attack of gastric proteolytic enzymes. We have investigated proteolysis of insulin incorporated into the ternary Insulin-PMAP-DS interpolyelectrolyte complex by a major enzyme of the human gastric juice, pepsin. The complex Insulin-PMAP-DS ($C_{INS} = 3$ mg·mL^{-1}; $w_{CR} = 0.6$; $w_{INS} = 0.6$) was prepared at pH 2.0. The complex was treated by pepsin at the enzyme/substrate ratio 1/1280 at 37 °C for 1 h (this was comparable to the time of normal food presence in the stomach). Then, the proteolysis was stopped by a quick shift of pH to pH 8.3, and the system was analyzed by HS-DSC. In the control, the proteolysis of insulin in a free state (i.e., in the absence of PMAP and DS) was carried out.

Figure 9 shows the denaturation thermograms of the native insulin at pH 8.3, as well as of two insulin samples subjected to proteolysis with pepsin at pH 2.0: free insulin and insulin in the ternary Insulin-PMAP-DS complex. The native insulin has the denaturation temperature and enthalpy $T_d = 81.7$ °C and $\Delta_d h = 17.0$ J·g^{-1}, respectively (Table 2). No heat capacity peak is observed in the thermogram of the control insulin sample subjected to the pepsin treatment in the free state (Figure 9b). It means that there is no native protein among the proteolysis products. On the other hand, the denaturation peak of insulin is clearly seen on the thermogram of the Insulin-PMAP-DS system treated by pepsin (Figure 9c). It corresponds to the denaturation temperature $T_d = 67.6$ °C and enthalpy $\Delta_d h = 20.6$ J·g^{-1}. These parameters differ slightly from those of the native protein. As shown above, the differences are caused by the complexation of insulin with PMAP in the weakly basic solutions.

Characteristics of the protein stability measured by HS-DSC were shown to be relevant for primary estimates of the protein functionality.[50−52] In fact, this method directly detects a global cooperativity of the protein tertiary structure, thus indicating its folding degree (nativity). Maintenance of the global cooperativity is a necessary condition for a protein to perform its biological function. In this view, HS-DSC can be considered a promising, easily accessible, and high-throughput tool of the protein drug express analysis.[51,53,54]

Thus, using HS-DSC we have shown that insulin retains its native tertiary structure in the ternary Insulin-PMAP-DS interpolyelectrolyte complex being attacked by the gastric protease. It is expected that the intactness of tertiary structure of insulin in the complex will ensure preservation of its functional activity upon oral administration of the complex preparation.

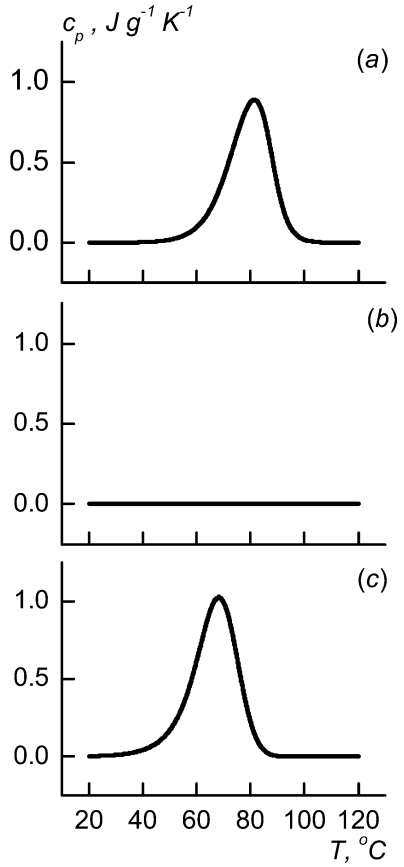

Figure 9. Denaturation thermograms of native insulin (a) and insulin treated by pepsin at pH 2.0 (20 mM glycine, 0.15 M NaCl) in the free state (b) or in the stoichiometric ternary Insulin-PMAP-DS interpolyelectrolyte complexes at $w_{CR} = 0.6$ and $w_{INS} = 0.6$ (c). The thermograms were obtained under the conditions simulating the human intestinal environment (pH 8.3, 20 mM glycine, 0.15 M NaCl). The insulin concentration is 3.0 mg·mL^{-1}.

CONCLUSION

Insulin has been immobilized in stoichiometric ternary interpolyelectrolyte complexes with biodegradable cationic (poly(methylaminophosphazene)) and anionic (dextran sulfate) polyelectrolytes in conditions imitating the human gastric environment (pH ∼ 2, 0.15 M NaCl). The efficiency of the insulin immobilization in the complexes reached 100% in a wide range of mixture compositions. Insulin incorporated in the ternary complex was inaccessible for the gastric proteolytic enzyme. The complexes dissociated completely under conditions imitating the human intestine (pH ∼ 8.5, 0.15 M NaCl) providing freely soluble insulin and its nonstoichiometric complex with the polycation. The tertiary structure of insulin released from the ternary complexes as well as of insulin bound to the polycation seems to be close to that of the native protein. The key properties of the Insulin-Poly(methylaminophosphazene)-Dextran sulfate system are of potential significance for design of oral insulin preparations.

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Notes

The authors declare no competing financial interest.

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