High Performance Liquid Chromatography Study of Water-Soluble Complexes and Covalent Conjugates of Polyacrylic Acid with Bovine Serum Albumin

Namık Akkiliç, Zeynep Mustafaeva, Mamed Mustafaev

Department of Bioengineering, Faculty of Chemical and Metallurgical Engineering, Yıldız Technical University, 34210 Esenler-Istanbul, Turkey

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ABSTRACT: Polycomplex formation of bovine serum albumin (BSA) and polyacrylic acid (PAA) was studied by pH titration, fluorescence, and HPLC methods in water solutions. It was shown that the complex formation and the solubility (phase state) of the polycomplexes depended on pH of the solutions and ratio of the components. The stability of PAA-BSA complexes was negligibly weak when pH = 6.0–7.0 [pH > pI (isoelectric pH)]. Stable water-soluble polycomplexes formed at pH = pI (pH 5.0) in a wide range of n_{BSA}/n_{PAA} ratios (0.05–50) and coexisted with free protein molecules at the higher ratios of components. Existence of water-soluble and insoluble PAA-BSA complexes has been observed at pH $\leq$ 4.5. The soluble to insoluble state transition of polycomplexes has been confirmed and binding mode of polyelectrolytes to proteins was assumed dependent on the ratio of components as a result of formation of soluble polycomplexes, complex coacervation, or amorphous precipitates. Soluble complexes were formed as fully homogenized mixtures. Fraction composition of the mixtures and insoluble complexes depended on the protein/polymer ratio and over the critical protein/polymer ratio, the soluble polycomplexes coexisted with free BSA molecules. On the other hand, the comparision of covalent conjugate formation and complexation of PAA with BSA has been revealed. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 105: 3108–3120, 2007

Key words: HPLC; polyelectrolytes; phase separation; proteins

INTRODUCTION

Water-soluble and insoluble protein–polyelectrolyte complexes as functional biopolymer systems represent a specific class of polymer–protein compounds that have important applications in various areas.[1–4] Synthetic polyelectrolytes (PEs) have been widely used to modify proteins via covalent attachment, increasing (or reducing) the immunoreactivity and/or immunogenicity of original antigenic proteins, and improving *in vivo* stability with prolonged clearance times.[2,5–8] Besides, the PE conjugates (or stable complexes) with individual viral antigens reveal strong protective properties and can be considered as novel vaccines.[2,9] Proteins and PEs, in the presence of transient metal ions, form ternary complexes as recent studies have shown.[10–13] Some of these complexes reveal high immunogenicity and provide high levels of immunological protection.[2,12–21]

The complex formation between PEs and proteins has been as subjects of numerous researches. Soluble polymer–protein complexes were studied by a wide range of methods, which are well known in colloid and polymer chemistry.[13,22–39] Interaction of polyelectrolytes with proteins in aqueous solutions is found to be sensitive to pH and ionic strength, as well as be dependent on isoelectric points of proteins.[14,37,40] Polymer–protein complexes form as a result of the electrostatic interaction of polyion chains with the oppositely charged groups of the protein molecules. Binding mode of PEs to proteins is assumed to be dependent on the ratio of components and may result in the formation of soluble polycomplexes,[14–16,24] complex coacervation,[25–27] or amorphous precipitates.[28,29]

The use of a weak polyelectrolyte [polyacrylic acid (PAA), polymethacrylic acid (PMAA), etc.] as a matrix for the protein binding provides additional possibilities for influencing the complex or conjugate properties.[11,22] The precipitation of proteins by PAA and PMAA and transition of insoluble complexes to soluble state were studied as a function of pH, ionic strength, polymer/protein ratio, and the degree of polymerization of the PEs. The sedimentation and dynamic light scattering studies[14–16,31] have shown that soluble protein–polyion complexes are precursors of phase separation and phase transition to regulate by reaction composition. The phase separation is

Correspondence to: M. Mustafaev (mustafa@yildiz.edu.tr).
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partially or completely prevented depending on protein concentration of the mixtures. However, detailed information on the physicochemical parameters of the soluble protein–polymer complexes or conjugates in the presence of their insoluble complexes is missing. Recently, application of HPLC methods to polymer–protein mixtures gives an opportunity to compare the results to that of conventional physicochemical methods as well as supplying us with novel information on complex formation.[11–13,18–21]

We have been investigating the covalent conjugations of protein antigens with polyanions, such as PAA, and copolymers of acrylic acid with *N*-isopropylacrylamide and *N*-vinylpyrolidone.[37,38] It was shown for the first time that the formation of the electrostatic inter (polymer–protein) complexes in carbodiimide activated PE–protein mixtures occurs as a most rapidly proceeding process and then the condensation between carboxyl and amino groups of components (covalent conjugation) occurs. It can be assumed that protein–polymer complexes are precursors of covalent conjugation reactions and thus, to prepare conjugate molecules with desired physicochemical properties, detailed analysis of the interaction (complex formation) of components is necessary.

In this study, interactions of bovine serum albumin (BSA) with PAA in different molar ratios of components and pH were systematically investigated by turbidimetric, fluorescence, and HPLC methods. From the analysis of the water-soluble mixtures and matrix solutions of insoluble BSA-PAA complexes, it was possible to analyze the fraction composition of polymer–protein systems and polycomplexes, the mechanism of the polycomplex formation and structure of forming particles. In addition, the transition of soluble polycomplexes to insoluble state and on the contrary, solubilization of water-insoluble polycomplexes at different complexation conditions was investigated. Our study also focused on the comparative analysis of the covalent conjugation mechanism of PAA with BSA.

EXPERIMENTAL

Materials

PAA was prepared by radical polymerization of acrylic acid in toluene with benzoilperoxide as an initiator and fractioned from 3 to 4% solution in methanol by fractional precipitation by ethyl acetate as explained in the literature.[41] BSA and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were from the Sigma Chemical, St. Louis, MO; benzoilperoxide was from Fluka and used as received. Acrylic acid was purchased from Aldrich (Germany) and purified by vacuum distillation; acetic acid was purchased from Riedel (Germany). NaH_2PO_4, $Na_2HPO_4{\cdot}7H_2O$, NaCl were obtained from Fluka and NaN_3 was from Applichem. Ultra pure water was obtained from Millipore MilliQ Gradient system.

Polymer–protein complexes

To produce polymer–protein mixtures, various concentrations of the BSA solution dissolved in phosphate or acetate buffer were added to PAA at different temperatures. Some experiments have been prepared by the titration of solutions of BSA with different concentrations of PAA to analyze the mixture order effect of the compositions. In the both cases, we did not observe any difference between adding the PAA solution onto the BSA solution or the BSA solution onto the PAA solution. The pH values were adjusted to the studied pH by using 1*M* NaOH. Water-insoluble products of the complexes were investigated spectrophotometrically at 500 nm, using Jasco V-530 Model UV/vis Spectrophotometer. The amount (m) of pellets was obtained by weighing after drying in vacuum. BSA/PAA ratios (n_{BSA}/n_{PAA}) were calculated using the equation of $n = cN_A/M$, where n is the number of the molecules in 1 mL; M is the molecular weight of components; N_A is the Avogadro number; c is the concentration in g/100 mL.

Polymer–protein covalent conjugates

Activation of polymer acid groups was carried out in water (pH 5.0) in a molar ratio 1 : 2 (EDC : AA). For this, PAA was dissolved in water, stirred at 4°C and EDC was added. After 1 h, the pH value of the mixture was adjusted to 7.0 with 1*M* NaOH. Then BSA solution in different concentrations was added to the reaction mixture and stirred for 12 h. After removal of *O*-acylisourea intermediate by dialysis, the sample was lyophilized. The purified conjugates were dissolved in 50 mL phosphate-buffered saline (PBS) at 4°C for the further analysis.

Measurements

HPLC gel filtration

Proteins, polyelectrolytes, and water-soluble polymer–protein mixtures were separated using HPLC. The molecular masses of proteins and the fraction compositions of the polymer–protein mixtures or complexes were estimated by gel filtration chromatography, using column Shim-Pack Diol-300 (7.9 mm ID × 50 cm) with Shim-Pack Precolumn Diol (4.0 mm ID × 5 cm) at room temperature. A Shimadzu model LC-6AD pump was run in different buffers at a flow rate of 1.0 mL/min. All solutions were filtered with 0.45-μm Sartorius RC-membrane filters before injec-

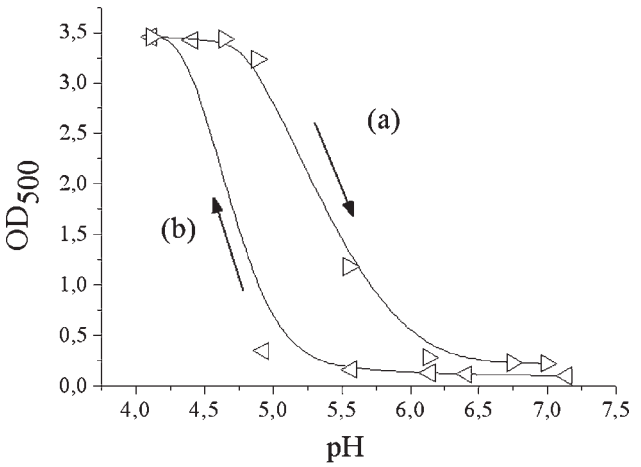

Figure 1 Dependence of optical density (OD_{500}) of the BSA-PAA mixture on the pH of the solutions at $n_{BSA}/n_{PAA} = 15$; (a) pH titration of BSA-PAA mixture solutions prepared at pH 4.0; (b) pH titration of BSA-PAA mixture solutions prepared at pH 7.0.

tion. A 20-μL sample volume was injected for analysis. The eluate was monitored at 280 nm with Shimadzu SPD-10AV VP Model UV–vis Dedector. PAA-BSA complexes were fractioned by using Shimadzu FRC-10A model fraction collector. The standards used to calibrate the column were thyroglobulin (670 kDa), immunoglobulin (155 kDa), BSA (66 kDa), ovalbumin (44 kDa), and myoglobin (16.9 kDa).

PBS was prepared from Millipore MilliQ Gradient system, and consisted of 50 mM phosphate and 150 mM sodium chloride for pH 6.0–7.0 studies. Acetate buffer with 0.01*M* CH_3COOH and 0.15*M* NaCl was prepared for pH 4.0–4.3–5.0 studies. Mobile phase solutions were filtered through a 0.45-μm cellulose nitrate filter and were degassed before use.

Fluorescence measurements

Fluorescence emission spectra were obtained using a QM-4/2003 Quanta Master Steady State Fluorescence Spectrometer (Photon Technology International, Canada) operating in quanta counting mode. The slits of excitation and emission monochromator are adjusted to 2 or 3 nm. The excitation was obtained at 280 nm. Interaction with other macromolecules appears as changing of protein fluorescence spectra, which was characterized by the wavelength at the maximum emission (λ_{max}) and maximum fluorescence intensity (I_{max}).

RESULTS AND DISCUSSION

Precipitation of PAA by BSA

Effect of pH

The effect of pH on the precipitation of PAA with BSA at constant temperature (25°C) is shown in Figure 1. Holding the n_{BSA}/n_{PAA} ratio constant and studying the precipitation as a function of pH was characterized by phase separation in soluble BSA-PAA mixture starting at pH = 5. The pH titrations of soluble PAA-BSA mixtures prepared at pH 7 showed that the mixtures of PAA-BSA solutions remained homogenous up to pH 5 whereas successive addition of acid leaded to phase separation. The maximum value of turbidity was reached at pH 4 and did not change practically below this value. At the same time, the titration of insoluble PAA-BSA mixture prepared at pH 4 was accompanied by shifting of pH solubility, which origins from dissolving of the precipitate at higher pH.

Effect of temperature and stirring

The effect of temperature was studied at 15–35°C at the ratio of $n_{BSA}/n_{PAA} = 1.0$ and pH 4.0. Figure 2 represents the optical densities (OD_{500}) of the PAA-BSA mixture depending on time at different temperatures. As it is shown in Figure 2, the OD_{500} values increased with time and absorbance values leveled off at about 5 min in all cases. The rate of the reaction (dA/dt) was obtained from the tangent of the curves in the early stages of the reaction, i.e., 0–3 min. The values of (dA/dt) 15°C, (dA/dt) 25°C, (dA/dt) 35°C, were 1.81×10^{-1}, 2.71×10^{-1}, and 3.75×10^{-1}, respectively. Evidently, the values indicated that the reaction at 35°C was faster than in the 15°C, i.e., the velocity of the reaction increased in temperature increase. The dependence of ln (dA/dt) values obtained from these curves on different temperatures was linear and fitted to the relation of $(dA/dt) = 9.3\, e^{-3172/T}$.

The formation of insoluble complexes also depended on the stirring of the complex mixture. The

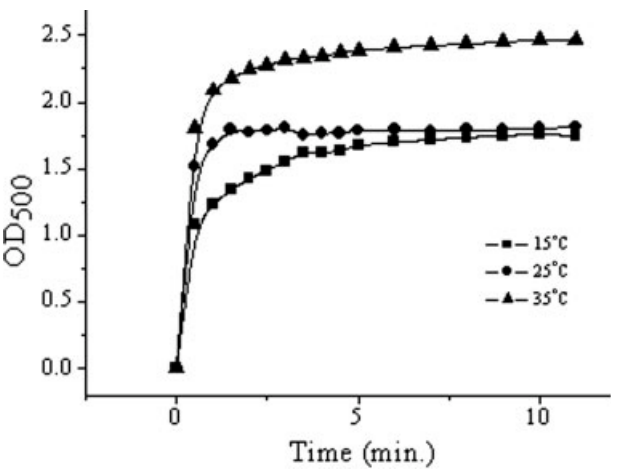

Figure 2 Dependence of optical density (OD_{500}) of the PAA-BSA mixture on time at different temperatures.

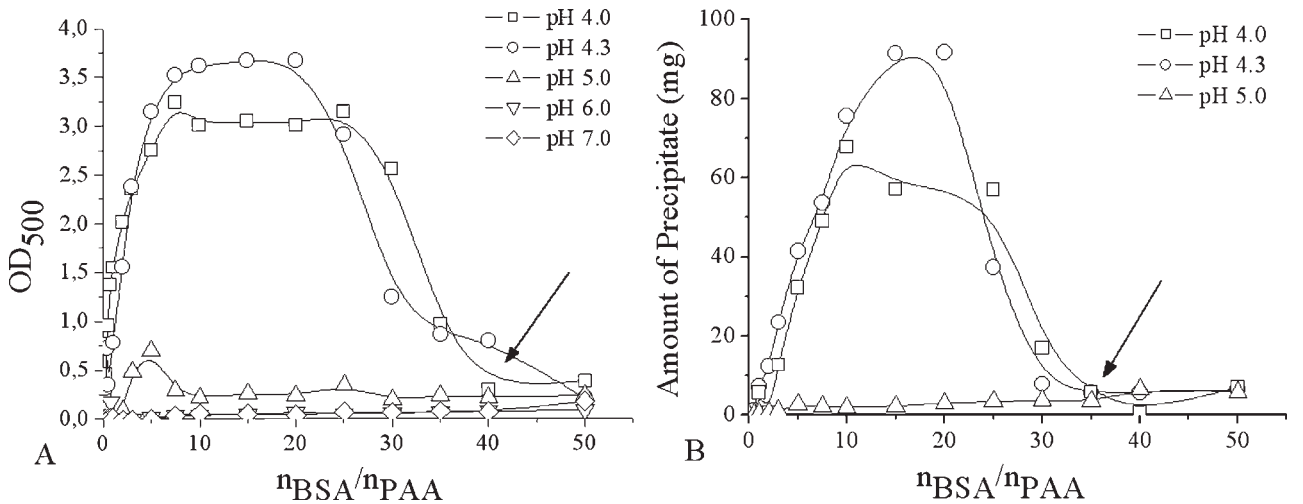

Figure 3 (A) Dependence of optical density (OD_{500} nm) of the solutions of BSA-PAA mixtures on the n_{BSA}/n_{PAA} at different pH values. (B) Dependence of the amount of precipitates obtained in the BSA-PAA mixtures on the ratio of components at different pH values.

stirring of the complex mixture caused an increase in the rate of insoluble complex formation.

Effect of n_{BSA}/n_{PAA} ratio

Figure 3(A) presents the dependence of the optical density (OD_{500} nm) of the PAA-BSA mixture on the amount of added protein at constant concentration of the PAA. It can be seen that the solubility of mixture depended on the n_{BSA}/n_{PAA} ratio and pH of the solutions. Starting with minimal values of n_{BSA}/n_{PAA} ratio of 0.5, a phase separation took place at pH: 4.0–4.3. OD_{500} values increased as protein concentration increase and reached a maximum value at $10 < n_{BSA}/n_{PAA} < 20$. Similar to OD_{500}, the amount (m) of the precipitate increased as the ratio of BSA in the components increased and then attained a limiting value as shown in Figure 3(B).

The pattern in both cases changed significantly with further increase in n_{BSA}/n_{PAA}. Depending on the protein concentration in the mixture, partial or complete prevention of phase separation took place. The minimal amount of the protein [indicated by the arrow in Fig. 3(A)], which must be introduced into the system to prevent the precipitation corresponded to the point N_i, where only the homogenous mixture existed in the system. Therefore, depending on the BSA concentration, transformation of insoluble protein-PAA mixture to soluble state at pH 4.0–4.3 took place. The solubility of BSA in supernatant, which was registered by UV–vis spectrophotometer at different molar ratio of components, was shown in Figure 4(A).

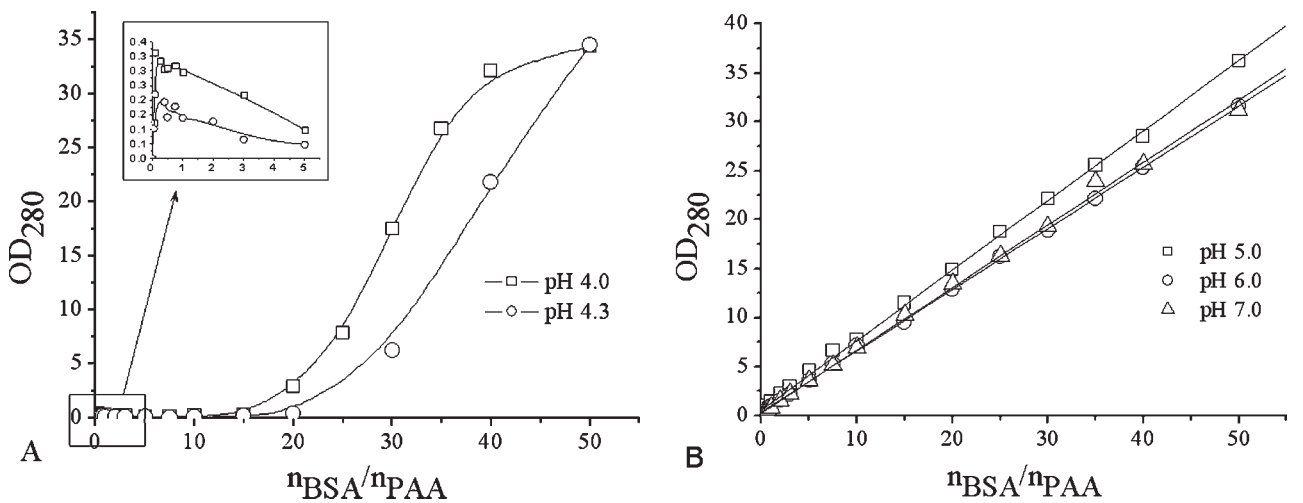

Figure 4 Dependence of optical density (OD_{280}) of supernatants of BSA-PAA mixtures on the n_{BSA}/n_{PAA} ratios obtained at different solution pHs: (A) 4.0–4.3, and (B) 5.0–7.0.

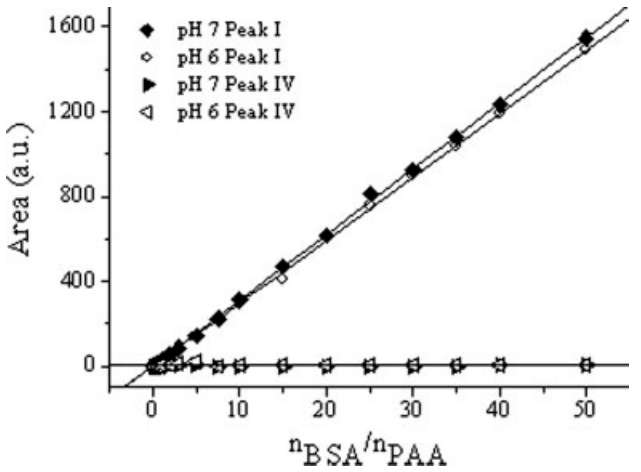

Figure 5 Dependence of areas of Peaks I and IV on the n_{BSA}/n_{PAA} ratios at pH 6.0–7.0.

Analysis of the BSA-PAA mixtures at pH 5.0–7.0 revealed that mixture of PAA/BSA appeared to remain soluble in a wide range of n_{BSA}/n_{PAA} contrary to pH < pI (isoelectric point of BSA, $pI_{BSA} = 4.9$)[42] at Figure 4(B). These results are consistent with results obtained for mixtures of PAA with human serum albumin, hemoglobin, globin, and transferrin.[11–22] The solubility of these mixtures is closely correlated with the pI values of protein molecules and also depended on the concentration of components in the mixture.

Analysis of the soluble systems

Complex formation at pH 5.0–7.0

The water-soluble mixtures of BSA-PAA were analyzed by spectrophotometer and HPLC under different experimental conditions, which allow us to study the fraction composition at relatively low concentrations of the mixture components.

As it was mentioned earlier, solutions did not affect the solubility of PAA within a certain range of n_{BSA}/n_{PAA} values at pH 6.0–7.0 with the addition of protein. The mixture of PAA-BSA was characterized in chromatograms by the peaks corresponding to free fraction of BSA (Peaks I, II, and III correspond to monomer, dimer and trimer fractions of BSA, respectively) and PAA (Peak IV). Dependence of the areas of these peaks on the ratio of components at pH 6.0 and pH 7.0 was given in Figure 5. As suggested by the slight change in areas of Peak IV, and the linear increase in Peaks I–III areas depending on the ratios showed that the interaction between BSA and PAA at the pH 7.0 was weak, if not negligible. The results are consistent with the results obtained by sedimentation analysis.[19] The preexisting electrostatic repulsive forces between PAA and protein molecule prevented the formation of the stable polyelectrolyte complexes of BSA.

However, the optical density (OD_{280}) of the Peak IV corresponding to PAA fraction was increased by increase in concentration of the protein molecules in mixture and may reflect the involvement of part of PAA and BSA molecules in polymer–protein complexes at pH 6.0 (We can not analyze the distribution of protein molecules between polymer coils in soluble mixture because free PAA does not separate from polymer–protein complex in HPLC at reaction conditions). Therefore, the presented results showed that the forming of weakly polyelectrolyte complexes and free components coexisted in solution in a wide range of ratios at pH 6.0.

However, stable complexation took place upon decrease of pH values of solution of PAA-BSA mixture. The extent of complex formation, which depends on the value of pH, is nearly quantitative under the experimental conditions at pH 5.0. Figures 6–8 show the HPLC results of the titration of PAA solutions with different concentration of BSA molecules at pH 5.0. One can distinguish three regions corresponding to reaction mixture composition. In Region I ($n_{BSA}/n_{PAA} = 0.05–1.0$), the BSA-PAA solutions were characterized on chromatograms only by one peak. The RT corresponding to this peak did not change and remained equal to that free PAA. At the same time, the presence of BSA gave rise to an increase in the optical density (areas of the peaks) of the mixture. The free BSA molecules were hereby absent in the PAA-BSA mixture as indicated the HPLC results in the Figure 6. An increase in the

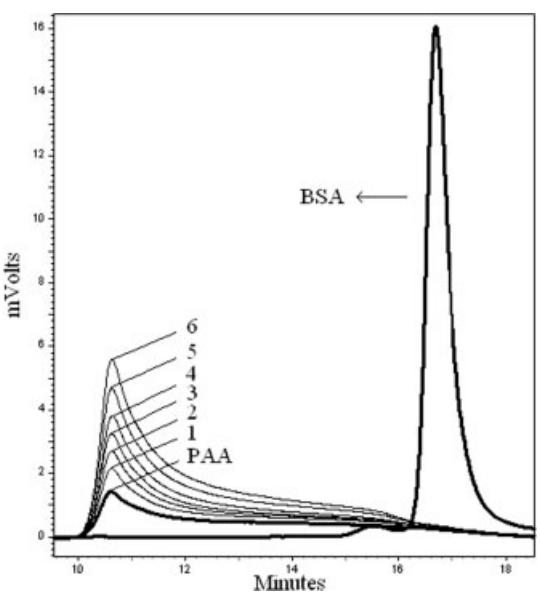

Figure 6 HPLC chromatograms of BSA, PAA, and BSA-PAA mixtures prepared at the ratios of $n_{BSA}/n_{PAA} = 0.1$ (1); 0.25 (2); 0.4 (3); 0.5 (4); 0.75 (5); 1.0 (6), at pH 5.0.

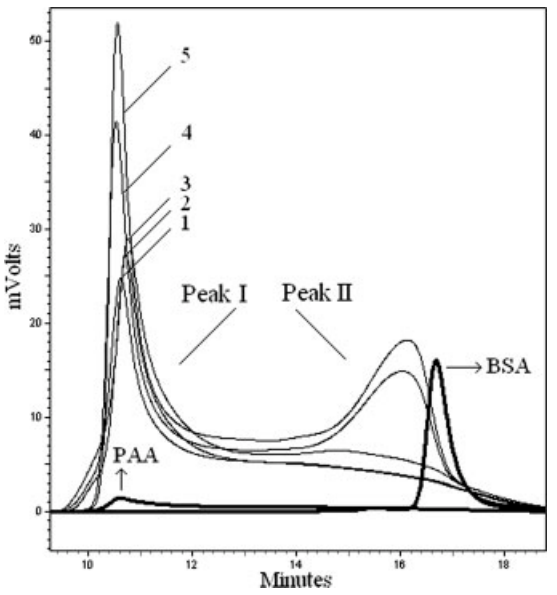

Figure 7 HPLC chromatograms of BSA, PAA, and BSA-PAA mixtures prepared at the ratios of $n_{BSA}/n_{PAA} = 2$ (1); 3 (2); 5 (3); 7.5 (4); 10 (5), at pH 5.0.

areas of the peaks might reflect the involvement of BSA in polyelectrolyte complexes.

As it follows from Figures 7 and 8, two peaks were observed on the chromatograms of the solutions obtained at $n_{BSA}/n_{PAA} = 1.0–7.5$ (Region II). These results meant that the BSA-PAA complexes at $n_{BSA}/n_{PAA} = 5.0$ coexisted with the free BSA molecules. Successive addition of BSA ($n_{BSA}/n_{PAA} = 7.5–50$ (Region III) did not lead to noticeable change of the ''fast'' peak area, whereas the rate of growth of ''slow'' peak area increased. The retention times (RT) of these ''slow'' peaks corresponded to RT of the free BSA. Accordingly, regarding this region the soluble BSA-PAA complexes coexisted with free BSA molecules.

Complex formation at pH 4.0–4.3

Figure 3(A) shows the curves of turbidimetric titration of PAA solution with BSA solution at pH 4.0 and 4.3. One can distinguish three regions correspond to the reaction mixture composition. Analysis of the supernatant of these insoluble BSA-PAA mixtures was carried out with HPLC-gel filtration and spectrophotometric methods. In the Region I ($n_{BSA}/n_{PAA} = 0.05–1.0$) the matrix solutions remained homogenous and two peaks with RT = 10.5 min. and 15.5 min. were thereby seen in the chromatograms (Fig. 9). The OD_{280} of the Peak I was depended essentially on protein concentration and decreased with the increase in the n_{BSA}/n_{PAA} ratio. The turbidity also increased in proportion with the increase in protein/polymer ratio. One can assume that the Peak I corresponds to the fraction of free PAA. The peak with RT corresponding to retention time of free BSA in the chromatograms of the supernatants was absent. Moreover, spectrophotometric UV-analysis of supernatant revealed the protein fractions also [Fig. 4(A)]. Therefore, the ''slow'' Peak II may correspond to soluble protein–polymer complexes.

As it follows from the chromatograms of the mixture at $5 < n_{BSA}/n_{PAA} < 20$ (Region II), there were no polymeric compounds (the protein, the polyanion or water-soluble BSA-PAA complexes) in the solution after removing of the precipitate (Fig. 10). In

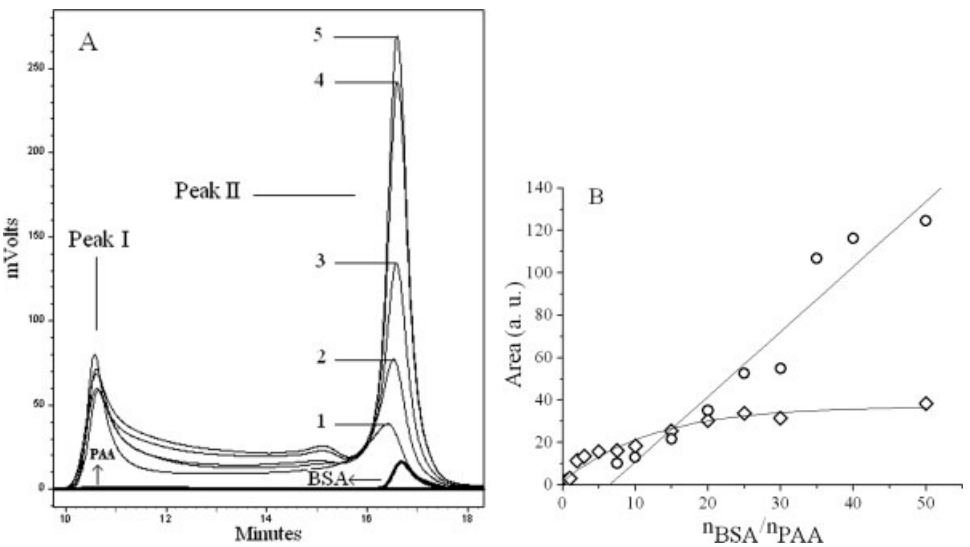

Figure 8 (A) HPLC chromatograms of BSA, PAA, and BSA-PAA mixtures prepared at the ratios of $n_{BSA}/n_{PAA} = 15$ (1); 20 (2); 25 (3); 35 (4); 40 (5), at pH 5.0. (B) Dependence of areas of the Peak I (◇) and Peak II (○) on the n_{BSA}/n_{PAA} ratios at pH 5.0.

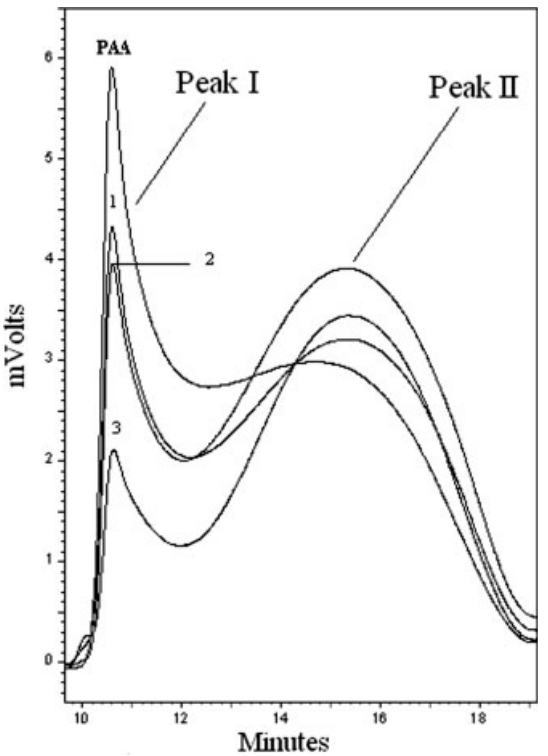

Figure 9 HPLC chromatograms of free PAA and BSA-PAA mixtures prepared at the ratios of $n_{BSA}/n_{PAA} = 0.5$ (1); 0.75 (2); 1.0 (3), at pH 4.0.

this region, practically all protein and polymer molecules were trapped in the fraction of insoluble complexes.

Addition of protein above these ratios is accompanied by a decrease in the turbidity of the solution, and at $n_{BSA}/n_{PAA} = 20$ the system again becomes homogeneous (Region III). On the chromatograms of the solutions (supernatants) obtained at $20 < n_{BSA}/n_{PAA} < 50$, two peaks are observed (Fig. 11). The retention time RT = 10.5 min. of quickly eluting particles virtually coincided with that of free PAA whereas slowly eluting particles were characterized by RT = 17.5 min. corresponding to RT of the free BSA. The most probably free BSA molecules coexist only with soluble BSA-PAA complexes. Dependence of the relative areas of ''slow'' and ''fast'' peaks on the mixture composition n_{BSA}/n_{PAA}, it can be seen that addition of BSA till $n_{BSA}/n_{PAA} = 35$ is accompanied by a concurrent increase in the areas of both peaks, and the area of the ''slow'' peak is much bigger than one of the ''fast'' peak. This means that starting at some critical ratios of components the free protein coexists with the water-soluble (Peak I) and insoluble BSA-PAA complex particles. Successive addition of BSA after complete solution of the precipitate does not lead to noticeable change of the ''fast'' peak area whereas the rate of growth of ''slow'' peak area increases.

The Peaks I and II on chromatograms for the ratio of $n_{BSA}/n_{PAA} = 40$ were separated [Fig. 12(A)] and the solutions corresponding to these peaks were analyzed by the fluorescence measurements. The fluorescence spectra of BSA-PAA mixture and of the fraction I (Peak I) and II (Peak II) of this mixture solutions are given in Figure 12(B). It is well-known that tryptophan (Trp) fluorescence of proteins varies with their conformational changes resulting in

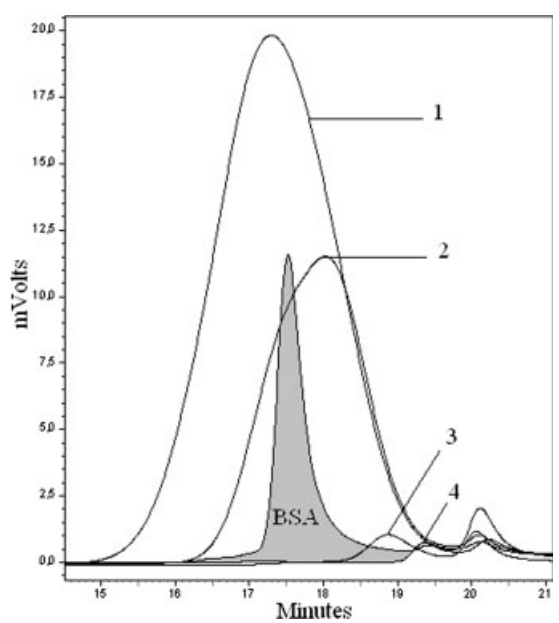

Figure 10 HPLC chromatograms of free BSA and BSA-PAA mixtures prepared at the ratios of $n_{BSA}/n_{PAA} = 2$ (1); 3 (2); 5 (3); 7.5 (4), at pH 4.0.

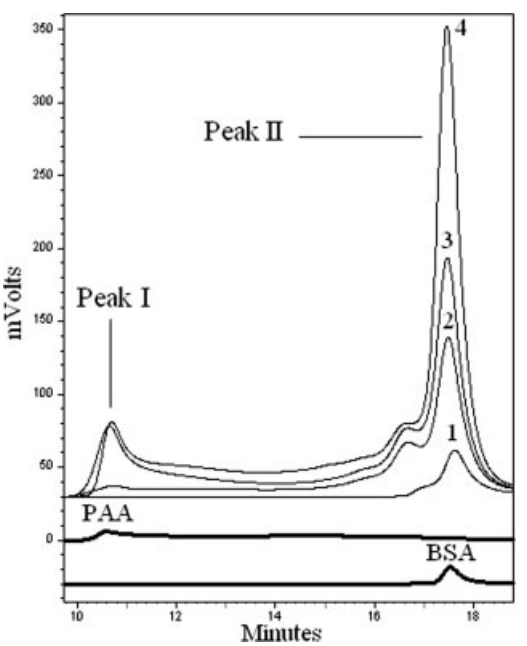

Figure 11 HPLC chromatograms of free BSA, PAA molecules, and BSA-PAA mixtures prepared at the ratios of $n_{BSA}/n_{PAA} = 25$ (1); 30 (2); 35 (3); 50 (4), at pH 4.0.

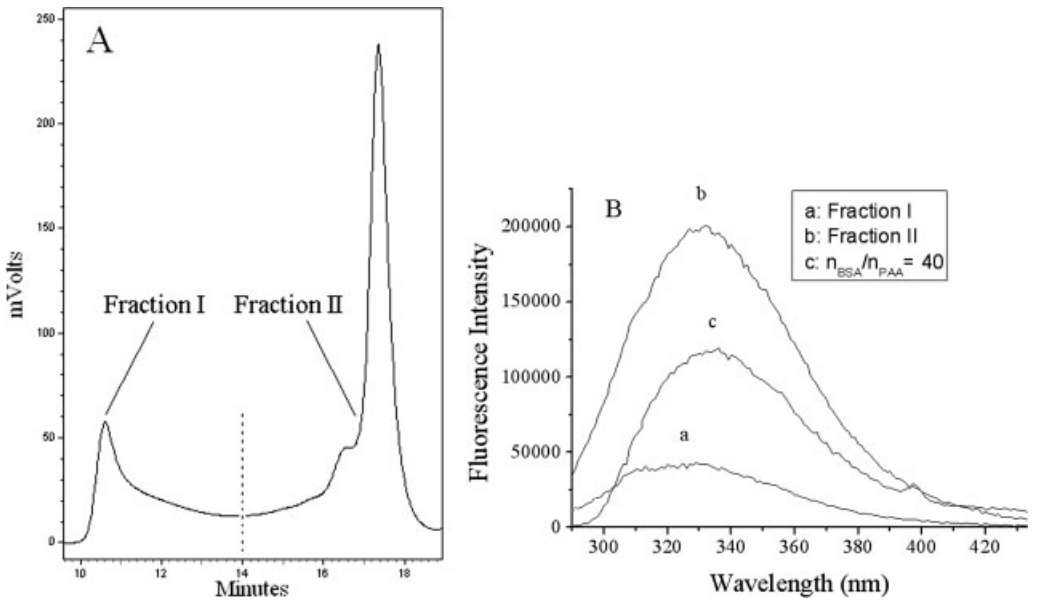

Figure 12 (A) HPLC chromatograms of the fractions and supernatant of complex $n_{BSA}/n_{PAA} = 40$ at pH 4.0. (B) Its fluorescence measurements at 280 nm wavelength; (a) fraction I, (b) fraction II, and (c) supernatant of complex $n_{BSA}/n_{PAA} = 40$ at pH 4.0.

changes of fluorescence parameters, such as the emission maximum (λ_{max}), quantum yield, lifetime, and others.[43,44] As it is shown in Figure 12(B), the emission maximum of BSA-PAA mixture at pH 4.0 is practically the same as that for pure BSA in solution ($\lambda_{max} = 340$ nm), which witness for the large surplus of the free BSA in mixtures. The pattern is quite different with the solutions of fraction I: fluorescence maximum shifts toward the blue region ($\lambda_{max} = 330$ nm). BSA contains two Trp.[42] One of them (spectral class 2 by Burstein[45] with $\lambda_{max} = 340$–342 nm) is located on the bottom of BSA hydrophobic cleft. The second Trp of class 3 ($\lambda_{max} = 350$–352 nm) with low quantum yield is superficial and completely accessible

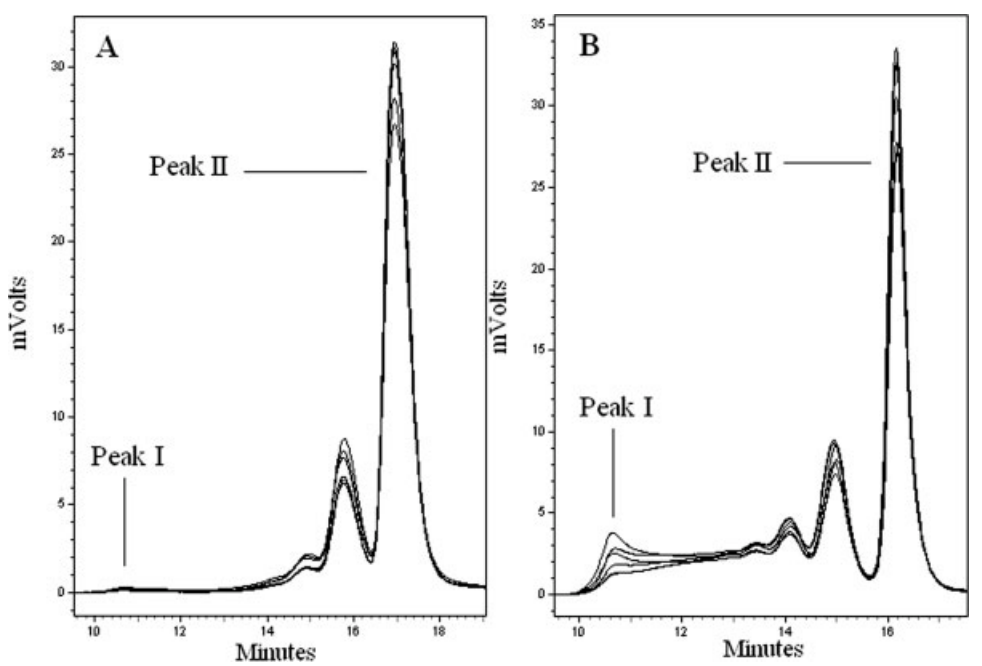

Figure 13 HPLC chromatograms of the BSA-PAA pellets: (A) The pellets were dissolved at pH 7.0 immediately after removing at pH = 4.0. (B) The pellets were dissolved at pH 7.0 after vacuum drying of the precipitate at pH 4.0; the ratios of $n_{BSA}/n_{PAA} = 7.5$–25.

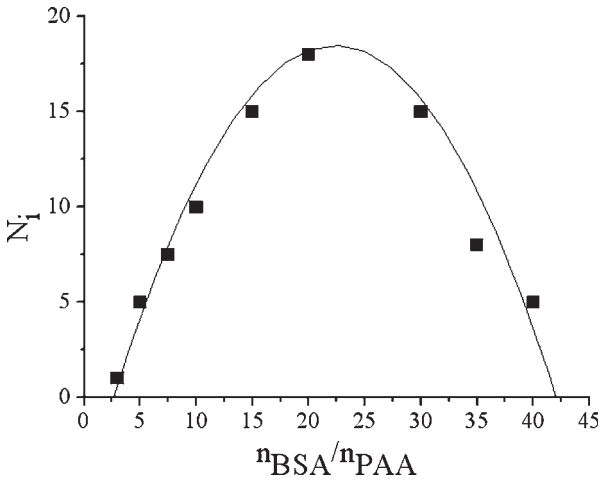

Figure 14 The number of BSA molecules in the pellets, $n_{BSA}/n_{PAA} = 1.0–50$, pH 4.0.

to aqueous solvent. One can assume that the obtained in our samples large blue shift (ca. 10 nm) indicates that the BSA trypthophanils become less accessible for water solution, which might origin from increasingly tighter binding of the polymer with the protein, that is the Peak I corresponds to protein–polymer complexes, which coexist with free protein molecules.

Fraction composition of insoluble polycomplexes

For the analysis of the behavior of the insoluble polycomplex particles, the precipitates of PAA-BSA mixtures after removing at pH 4.0–4.3 were dissolved by titration into pH 7. Figure 13 presents the HPLC results for soluble mixtures of BSA-PAA, which were obtained by dissolving of insoluble complexes prepared by two methods: (1) the precipitate was dissolved immediately after removing [Fig. 13(A)], and (2) the precipitate was firstly dried about 20 h in vacuum at room temperature and then was dissolved [Fig. 13(B)]. As it follows from the chromatograms for the soluble mixtures prepared by the method 1, practically all insoluble complexes were fully dissolved into free components at pH 7. The negative charges on both the protein globules and polyanion coils prevent the complex formation. At the same time as it is seen from the Figure 13(B), the measured BSA-PAA solutions were characterized by two peaks over a wide range of protein concentrations $n_{BSA}/n_{PAA} = 7.5–25$. The RT of the Peaks I and II equal to 10.5 min. (Peak I) and 17 min. (Peak II) corresponding to soluble PAA-BSA complexes (free PAA fractions) and free BSA molecules, respectively. Therefore, pH-stabilization of insoluble polycomplex particles was increased by the vacuum drying (probably by conformational changing).

The dependence of the number of the protein molecules (N_i) in the composition of the precipitates on the initial ratios of the components was obtained by the HPLC analysis using their soluble mixtures and depicted in Figure 14. N_i values were increased by adding of protein molecules, and then decreased after reaching to maximal values. This type of dependence is correlated with the dependence of the amount of insoluble complexes on initial protein/polymer ratios and with the results obtained for soluble mixtures. These results have an importance in the production of the insoluble polycomplex particles with different composition by titration of soluble PAA-BSA mixtures.

Covalent conjugation

In this part of the study, PAA and BSA covalently conjugated by carbodiimide reaction. Here, in the presence of an amine, carbodiimides promote the formation of an amide bond in two steps.[46] In the initial reaction, the carboxyl group adds to the carbodiimide to form an *O*-acylisourea intermediate (Fig. 15, step 3) with an amine for the polymeric carboxylic acids, yields the corresponding amide. Covalent bioconjugates were synthesized in different n_{BSA}/n_{PAA} ratios and HPLC chromatograms of related samples were given in Figure 16.

As seen from this figure, the system is characterized by one peak of the elution components on the chromatograms up to $n_{BSA}/n_{PAA} = 1.0$. The RT corresponding to the peaks were practically coincidenced with the RT of the free PAA solutions. The results obtained at the $n_{BSA}/n_{PAA} < 1.0$ convinced the absence of pure BSA in the solutions of the reaction products. Therefore, only one peak in HPLC chromatograms corresponded to covalent bioconjugates of PAA-BSA as it was mentioned earlier.

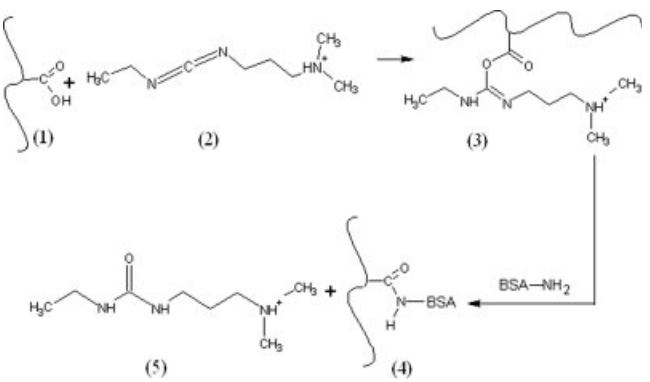

Figure 15 Covalent conjugation reaction scheme of PAA-BSA: Carboxylate Compound (1); EDC (2); *O*-Acylisourea Intermediate (3); PAA-BSA conjugate (4); Isourea by-product (5).

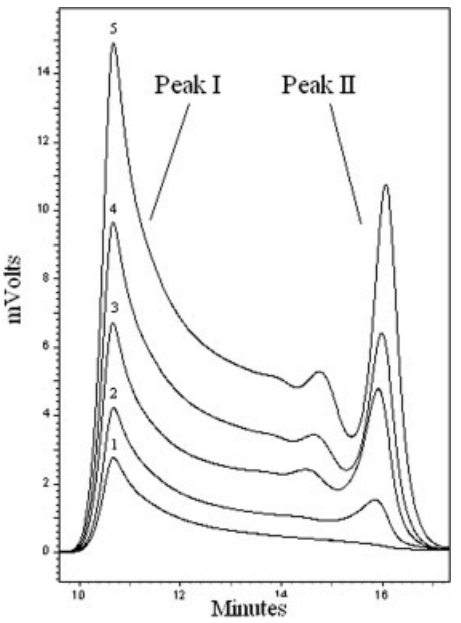

Figure 16 HPLC chromatograms of BSA-PAA covalent conjugates prepared at the different molar ratios of components, n_{BSA}/n_{PAA} = 0.5 (1), 1.0 (2), 2.0 (3), 3.0 (4), 5.0 (5); pH 7.0; 25°C.

At $n_{BSA}/n_{PAA} \geq 1.0$ the reaction products of BSA-EDC-PAA were characterized by a bimodal distribution of elution components. A comparision of the values of the RTs corresponding to the Peaks I and II of the studied mixtures and the individual components showed that the slowly eluting substance (Peak II) corresponded to the free BSA and, as before, Peak I corresponded to the polymer–protein conjugate. As seen from Figure 17, the areas of these peaks were increased with further increase of n_{BSA}/n_{PAA} molecular ratios.

From the HPLC results of soluble complexes and covalent conjugates, we have calculated the amount of BSA molecules involved in the composition of the complex and conjugate particles (N_i, the characteristic of composition of conjugate and complex molecules, which corresponds to the number of BSA molecules bound to one polymer chains). Figure 18 shows the dependence of the average N_i on the initial BSA-PAA mixture composition (n_{BSA}/n_{PAA}). It is seen that an increase in BSA content in the initial protein–polymer mixtures results in linear increase for the number of protein molecules, bounded with one polymer macromolecule for the both complex formation and covalent conjugation. The average values of N_i were in a good agreement up to the n_{BSA}/n_{PAA} ratio of 1.0. At the same time at $n_{BSA}/n_{PAA} >$ 1.0, some divergence in relationships was observed (ca. 5–10% depending on the ratios). Based on the results, one can assume that the formation of electrostatic (and hydrophobic) interpolymer–protein complexes in PAA-carbodiimide-BSA mixture is very important for the organization of the structure of bioconjugate molecules as a most rapidly proceeding process. It is reasonable to conclude that the compactness of the complex particles is increased after the condensation reaction and the bioconjugate molecules lost part of the protein molecules at certain ratios.

Mechanism of complex formation

Polyelectrolytes may be classified by their behavior against pH variations. Charged polymers are usually termed as strong polyelectrolytes when the charge amount and distribution along the polyelectrolyte are not sensible to large pH variations and depend only on the initial chemistry. On the other hand, when the amount of charged sites varies as a function of pH, and therefore ionization sites are able to

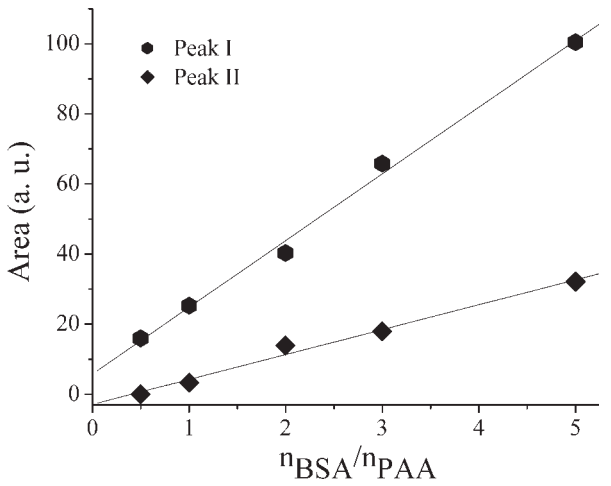

Figure 17 Dependence of areas of the Peaks I and II on the ratios of n_{BSA}/n_{PAA}; pH 7.0; 25°C.

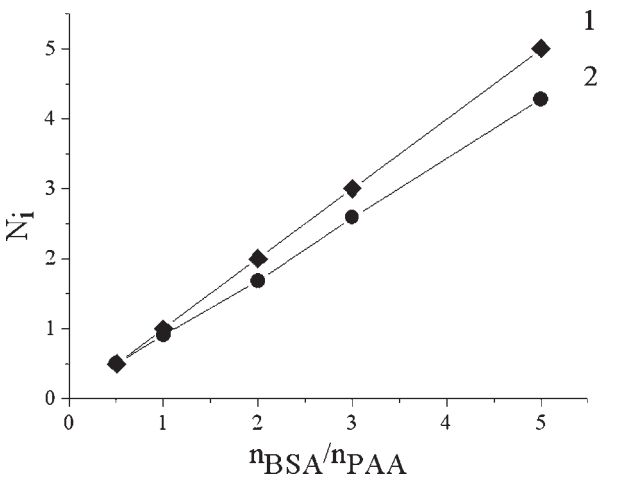

Figure 18 The number of BSA molecules bound to each PAA chain (1) in the complex at pH 5.0, and (2) covalent conjugate at pH 7.0 on the ratios of n_{BSA}/n_{PAA}; 25°C.

move along the chain, the polymer is defined as a weak polyelectrolyte such as PAA. The degree of ionization (α) of PAA is increased with increasing pH of solutions (pH = 4.0–8.0; α = 5–77%).[47] Obviously, the electrostatic interactions play a major role in the solution behavior of PAA solution, the presence of vicinal charges making further ionization of the PAA chain more difficult.[47] At low pH (pH 4), PAA exhibits a low degree of ionization: 0.1. In this region, the electrostatic interactions are not dominant, van der Waals interactions and hydrogen bonding are expected to play a nonnegligible role by promoting compact conformations.

BSA is characterized by a high content of cystine (35) and the charged amino acids, aspartic (41) and glutamic (58) acids, lysine (60) and arginine (26). Based on hydrodynamic[48–50] and low-angle X-ray scattering[51] serum albumin was postulated to be an oblate ellipsoid with dimensions of 140 × 40 Å^2. The BSA molecule is not uniformly charged within the primary structure. At neutral pH, Peters (1985)[52] calculated a net charge of −10, −8, and 0 for Domains I, II, and III for BSA. It is known that the protein globules undergo reversible conformational isomerization with changes in pH. The N (normal)–F (fast) transition involves the unfolding of Domain III. The N form is characterized by a higher helical content (55%), common negative charge and globular structure. At pH values lower than isoelectric points (pI = 4.9), albumin molecules are characterized by positive charge because of protonization of lysine and arginine amino acid residues, by an increase in viscosity and a significant loss in helical content (45%).

The pH 6–7 of the BSA-PAA mixtures corresponds to the condition of pH > pI. In this case, the globules of BSA and polyions of PAA negatively charged and preexisting electrostatic repulsive forces between them prevent the formation of stable polycomplex particles. As suggested by the slight increase in Peak I, the interaction between BSA and PAA at the pH 6.0 was weak, if not negligible.

The results obtained at the pH = 5.0 which is practically correspond to isoelectric point of BSA (pI = 4.9) convince the protein binding by PAA and formation of water-soluble polymer–protein complexes at a wide range of reaction components. The character of the dependence of the studied parameters of the reaction products on the n_{BSA}/n_{PAA} ratios shows that the mechanism of the binding and the composition (and structure) of the polycomplexes depend on the initial ratios of components. At this pH, BSA globules are weakly and negatively charged and binding of protein molecule with the negatively charged polyanions (α = 18%) takes place despite of electrostatic repulsion. Under this, the efficient of the negative charge of every sorbing protein globules may only increase at the expence of the ''stuck'' units of polyanion. Then the ''docking'' of globules in the polycomplex particles are not profitable (or less profitable) and they settle down separate each other by forming the polycomplex structure. In this model, one polyelectrolyte molecule forms a complex with many of the protein molecules until all of the polyion functional groups are stoichiometrically interacted with the corresponding groups of the protein (formation of hydrogen bonds, electrostatic and hydrophobic interaction, etc.). Most charged groups of the polyanion in the polycomplex remain free. These groups are situated in loops on the surface of the particles and provide its solubility in water.

As it was mentioned earlier, the areas of free BSA chromatographic peaks are plotted in Figure 8(B) versus the ratio of the number of protein molecules to that of PAA chains in the system. The intersection points obtained at the extrapolation of these plots to the zero area of the free BSA peak correspond to n_{BSA}/n_{PAA} when all protein molecules are bound to complex with PAA. One can assume that this limit [lim $(n_{BSA}/n_{PAA}) = N_i$, when the peak area was equal to zero] equals to the number of the protein molecules bound by a polyanion of a given degree of polymerization under given conditions. It follows from the results that about 5 protein molecules are bound by one polymer chain with M_w = 100 kDa at pH 5.0 in the composition of water-soluble protein/polymer complexes.

The character of the binding of polyanion and protein molecules at pH < pI is essentially different. BSA globules are positively charged (N→F transition)[42] at pH = 4.0–4.3 and their binding with polyanion coils take place by electrostatic interactions of opposite charging functional groups. The formation of electrostatic (salt) bonds between negatively charged monomer units of PAA and positively charged groups on the surface of protein globules constitute the primary driving force at the adsorption of the proteins by polyanions. As a result, the positive charges of protein molecules are neutralized (screening) and do not prevent manifestation of nonpolar interaction (aggregation, etc.). At the same time, it is achieved the partial neutralization and screening of the charge of polyanions which promote draw together separate part of polyanions at the self-organization of the polycomplex structure. Depending on the ratio of components, different self-organization level and correspondingly different phase state (soluble complexes → insoluble complexes → soluble complexes) were resulted in PAA-BSA mixtures. Noticeable, that the character of the dependence of the amount and N_i of insoluble complexes were correlated with the ratios of components. Schematic representation of the mechanism of complex formation at pH 4.0 and pH 5.0 is shown in Figure 19.

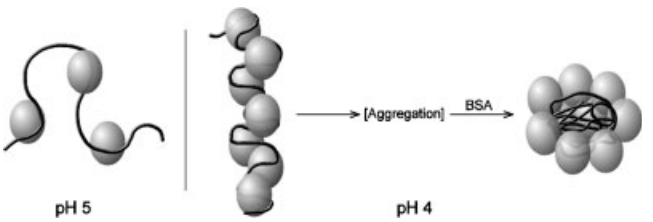

Figure 19 Schematic representation of the mechanism of complex formation at pH 4.0 and pH 5.0.

CONCLUSIONS

In this study, the complex formation and covalent conjugation between PAA and BSA were examined using the turbidimetry, HPLC, UV–vis spectroscopy, and spectrofluorometer methods. Depending on the charge density of the protein and polyanion, the different pH values revealed several types of polycomplex formations. We have observed the stable coils at pH 5.0 in the nature of the complexation and the peaks relative to PAA-BSA complexes increased depending on the protein concentration up to $n_{BSA}/n_{PAA} = 10$ and did not show the noticeable change above this ratio.

Secondly, at low n_{BSA}/n_{PAA} ratios and pH 4.0, two types of polycomplex molecules were observed on the chromatograms and the UV–vis spectroscopy demonstrated the presence of protein molecules. As we expected, all the protein and polymer molecules were trapped into the fraction of insoluble complexes by increase in n_{BSA}/n_{PAA} ratios. Nevertheless, transition of insoluble polycomplexes to soluble state was occurred at $n_{BSA}/n_{PAA} = 20$. Above this ratio, we have observed two types of peaks on the chromatograms by the increase in protein concentration. Most probably, one of them may be the formed PAA-BSA complex that was also proved by fluorescence measurements and the other one belonged to the free protein molecules.

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