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Conformational Dynamics of Poly(acrylic acid)-Bovine Serum Albumin Polycomplexes at Different pH Conditions

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Summary: This work presents the real time surface plasmon resonance (SPR) and fluorescence spectroscopy observation of cooperative interactions between aggregate-forming bovine serum albumin (BSA) and oppositely charged linear macromolecule poly(acrylic) acid (PAA). Interactions between protein and polymer result in formation of polyelectrolyte-protein polycomplexes, which exhibit expressed conformational transformations, especially at low pH. The rate constants of observed kinetic transformations were calculated and found to vary in the range from 0.8×10^{-3} to 6.7×10^{-3} for different pH values. The processes of aggregation, sedimentation and conformation of BSA-PAA complex are discussed.

Keywords: conformational analysis; drug delivery systems; sedimentation: protein-polymer complex; SPR spectroscopy

Introduction

Cooperative complexation between proteins and water-soluble polymers has been investigated because of its importance in colloid science as well as biological sciences.[1,2] The interaction between aggregate-forming proteins and oppositely charged linear macromolecules is known to result in formation of polyelectrolyte (PE)-protein complexes which exhibit a set of extremely interesting and practically important properties.[3,4] The conformational transitions of water soluble polymers were investigated in works[5,6] by using Raman spectroscopy, experimental titrations, fluorescence correlation spectroscopy[7] and other methods.[8,9] The weak polyelectrolytes such as poly(acrylic acid) are very sensitive to pH and chains are almost uncharged at low pH however are fully charged at high pH. Authors of work[10] reported that the conformation on PAA adsorbed on alumina estimated using a pyrene-labeled polymer changes appreciably with pH and affects the dispersion stability of polymer-protein complex solution. The pH-dependent complexation of PAA with phospholipid vesicles was characterized by[11] using fluorescence polarization, differential scanning calorimetry, and surface pressure measurements of phospholipid monolayers. The complexation was pronounced below pH 4, when the polymer carboxyl groups are protonated, as shown by the binding of PAA to vesicles and the decrease in polymer mobility. Although it is very important to investigate colloidal stability by adsorption of polymer-protein complexes in many applications, such as drug delivery, synthetic vaccines, very little work has been done to characterize the conformation of polymer-protein complexes adsorbed. Polyelectrolyte-protein polycomplexes (PCs) form as a result of the ion-exchange reaction between charged

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molecule of protein and ionized units of a macromolecule. There are two types of polymer-protein complexes: water-soluble nonstoichiometric and insoluble in water stoichiometric complexes which may form depending on the binding conditions, such as pH, ionic strength, concentration, chemical structure of the components and the reaction mixture composition.[12–14]

SPR is a surface sensitive technique providing easy route to perturb a molecule and register its relaxation in real time.[15] This technique is ideally suited for observation of conformational changes in gel-formed polymer materials due to possibility of easy detecting of the refractive index changes during real time measurements of studied object.

In the present study, *in situ* study of the real time conformational dynamics of polymer-protein polycomplexes under different pH of buffer solution, using fluorescence spectroscopy and SPR methods was under taken.

Materials and Methods

Materials

Polyacrylic acid (PAA) was prepared by radical polymerization of acrylic acid in toluene with benzoilperoxide as an initiator and fractioned from 3–4% solution in methanol by fractional precipitation by ethyl acetate as explained in the literature.[16] BSA was from Sigma Chem. Co.(USA), benzoylperoxide from Fluka, and were used as received. Acrylic acid was from Aldrich (Germany) and purified by vacuum distillation. Acetic acid was from Riedel (Germany). NaH_2PO_4, $Na_2HPO_4.7H_2O$, NaCl were from Fluka and NaN_3 was from Applichem. Ultra pure water was obtained from Millipore MilliQ Gradient system. Phosphate-buffered saline (PBS) was prepared with ultrapure water, and consisted of 50 mM phosphate and 150 mM sodium chloride for pH 6.0–8.0 studies. Acetate buffer with 0.01M CH_3COOH and 0.15M NaCl was prepared for pH 3.0-5.0 studies. Mobile phase solutions were filtered through a 0.45 μm cellulose nitrate filter and were degassed before use.

Polymer-Protein Complexes

To produce polymer-protein mixtures, various concentrations of the BSA solution in phosphate or acetate buffers were added to PAA. The experiments to analyze the mixture order effect of the compositions were carried out by the titration of solutions of BSA with different concentrations of PAA. In both cases, no differences were observed between adding the PAA solution onto the BSA solution or the BSA solution onto the PAA solution. The pH values were adjusted with 1M NaOH. 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.

Surface Plasmon Resonance (SPR) Spectroscopy

All experiments were performed with two-channel SPR device NanoSPR-321 (NanoSPR, USA). A monochromatic p-polarised 2mW laser light source at 670 nm wavelength in optical system with Kretchmann configurations was used. The carrier of plasmon oscillations, 50 nm Au layer and 5 nm Cr adhesion underlayer on the previously cleaned glass slides (refractive index $N = 1.61$) were evaporated. Au was employed as it provided stable measurements during the SPR-experiment. Use of a glass prism with $N = 1.61$ allowed the SPR curves to be recorded over the entire range of observed processes. After preparation the glass slides were placed on the glass prism of the SPR system. Optical contact between the prism and the glass slides was achieved using an immersion liquid ($N = 1.61$). For sample loading into SPR flow cell (volume 5 μL) and for measurements in flow regime the peristaltic pump was used. The flow rate was 15 μL/min for all measurements. During SPR experiments dependence of reflectance via angle

of laser beam incidence (SPR-curve) was recorded and position of SPR-curve minimum in real time regime was registered.

The SPR measurements were performed mainly with equal n_{BSA}/n_{PAA} ratio for most of experiments. In some measurements the ratio was changed and BSA/PAA mixture was centrifuged (promptness n = 5000, 10 min.) to remove the large particles. Samples of buffer solution with different pHs were added to PAA adsorbed layer which was previously formed on the surface of gold layer. Kinetic characteristic of SPR angle position for each of channels was registered and stored. The second channel was used as the reference. All measurements were performed at room temperature.

Fluorescence Spectroscopy

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 were adjusted to 2 or 3 nm. The excitation was obtained at 280 nm. Interaction with other macromolecules appeared as changes in the protein fluorescence spectra, which was characterized by the wavelength at the maximum emission (λ_{max}) and maximum fluorescence intensity (I_{max}).

Results and Discussion

Conformational Dynamics of Polycomplexes

SPR technique monitors the refractive index changes in close vicinity of the sensitive gold surface of the sensor. In studies of biomolecular interactions, the changes in the molecular mass at the surface of the sensor associated with these interactions are usually the ones responsible for the changes in the refractive index and therefore the changes in the SPR signal. Hence, SPR detects the amount of the adsorbed molecules on the gold surface and molecules in vicinity of the gold at the distance close to wavelength, in the present case it is 650 nm. However, effective detection can be performed at about 200 nm. This point is important for the colloid liquids, where dispersed molecules are stable in the solution and the adsorption process depends on the parameters of colloid stability. When colloidal solution is stable, SPR response depends mostly on effective refractive index of colloid, because only separated molecules are able adsorb on the surface of gold. However, when stability of colloidal solution is disrupted, adsorption processes become the main parameters effective on SPR response.

The BSA/PAA complex exhibited a stable colloidal behavior at pH > 5 even for different n_{BSA}/n_{PAA} values. For the pH 3.0–4.0, colloidal stability of complex strongly depended on the proportion of components of complex. In the 1.0–50 ratio range the solubility of the polycomplex was changed and processes of aggregation and sedimentation were observed.[15] Here, two state of BSA/PAA polycomplexes (PCs) with SPR, which exhibited insoluble state [$n_{BSA}/n_{PAA} = 1.0$; Figure 1(A)] and soluble [$n_{BSA}/n_{PAA} = 20$; Figure 1(B)] were studied. As was depicted on the figures, kinetics of SPR responses for PCs at the ratios of $n_{BSA}/n_{PAA} = 1.0$ and $n_{BSA}/n_{PAA} = 20$ exhibited the opposite behavior for purified (by centrifugation) and unpurified samples.

This phenomenon can be explained by the process of aggregation of PCs with subsequent sedimentation and adsorption on the surface of gold. At low pH, the conformational processes in PCs with $n_{BSA}/n_{PAA} = 1.0$ were responsible for the existence of the insoluble aggregates with large size, which could not reach the sensitive surface of sensor due to steric hindrance [Figure 1(A), curve 2]. After centrifugation the aggregates with small size (supernatants) easily reached the surface [Figure 1(A), curve 1]. As seen in Figure 1(B), the opposite processes were registered for PCs with the component ratio of $n_{BSA}/n_{PAA} = 20$. Considered

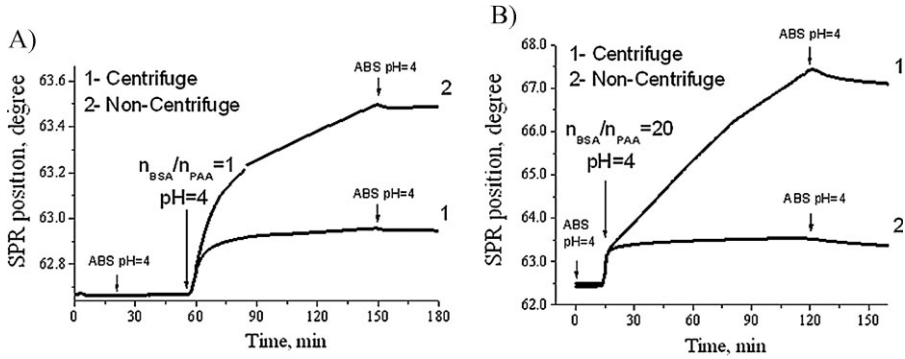

Figure 1.
Temporary dependence of SPR angle position on sedimentation of PCs, $n_{BSA}/n_{PAA} = 1.0$ (A) and $n_{BSA}/n_{PAA} = 20$ (B) at pH 4.0; 25 °C.

conformational dynamic of PCs was observed only at low pH and disappeared at pH ≥ 5. It can be seen also from Figure 1, the mass of the PAA-BSA complexes without centrifugation was about three times larger then that of the supernatants. Due to this, 70% of the aggregates remained in the pellet during centrifugation.

The sensitivity of SPR decays exponentially and increases at the surface of gold, so, it is optimal to study the conformational dynamic of PCs, placed directly on the surface of gold layer. In this case one can register real time changes in the structure of adsorbed BSA/PAA layer by simple changes of pH of solution in flow mode. The kinetic of changes in structure of adsorbed BSA/PAA layer ($n_{BSA}/n_{PAA} = 1.0$) at consequently changed pH is depicted in Figure 2(A). When pH increased from pH 4.0 to pH 7.0, the carboxylic acid groups on the polymer backbone deprotonated and the electrostatic repulsion caused an increase in thickness of the layer. As a result of this, the solvent molecules started reaching into the inner layers while the effective refractive index of BSA/PAA layer is decreased. One can assume that the increasing pH caused the structure of PAA opened up (*stretching*). The dependence of plasmon resonance angular shift

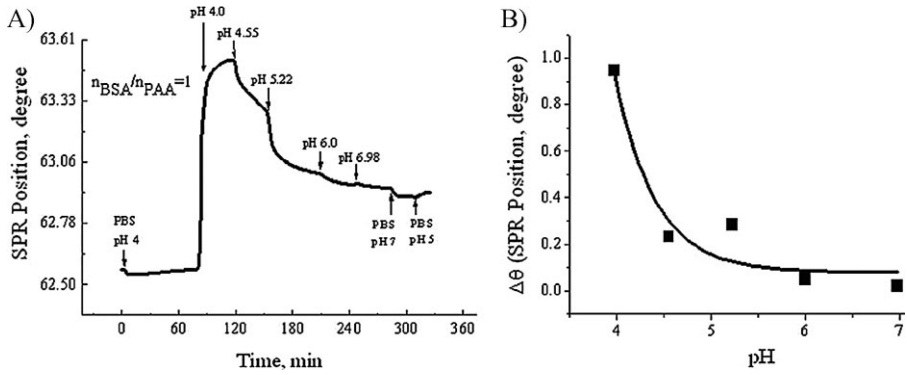

Figure 2.
(A) Kinetic dependence of SPR angular position, that reflects conformational changes in structures of PAA-BSA mixture at the ratio of $n_{BSA}/n_{PAA} = 1.0$ when the overlayer pH was changed from acidic to basic (pH 4.0 → pH 6.98). The horizontal part of graph- gold surface is without PAA/BSA layer. (B) Maximum of SPR responses depending on the wide range of pHs.

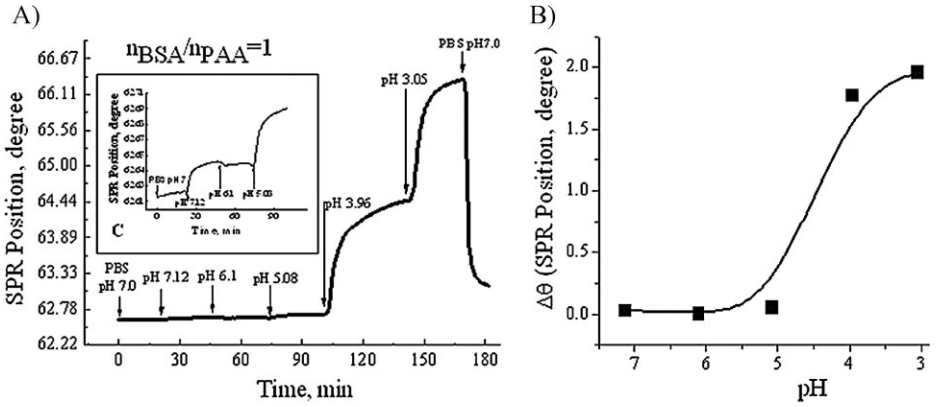

Figure 3.
(A) Kinetic dependence of SPR angular position of PAA-BSA mixture at the ratio of $n_{BSA}/n_{PAA} = 1.0$ when the overlayer pH was changed from basic to acidic (pH 7.0 → pH 3.05) conditions. Here, BSA/PAA layer exists on the surface of gold before start of measurements. (B) Respective maximum of SPR responses depending on the wide range of pHs. (C) Inset: zoomed part of sensogram for basic pH.

on value of pH of buffer solution was presented in Figure 2(B) for the case of acidic-basic transformation.

Figure 3 shows the transformation of PAA-BSA mixture from soluble to insoluble phase at the ratio of $n_{BSA}/n_{PAA} = 1.0$. As it follows from Figure 3(A), PAA protonated and the overall thickness of the layer decreased while the pH is decreased; as a result, the refractive index after such transformation became higher. One can assume that, decreasing pH caused PAA to assume a *coiled* conformation due to the ensuing intramolecular hydrogen bond formation and the layer became more compact. Thus, the increasing SPR response showed the increasing mass of adsorbed molecules at the surface.

The rate of structural transformation of PCs during pH variation changed dramatically between pH 4.0 and pH 5.0 that implied an existing of threshold charge

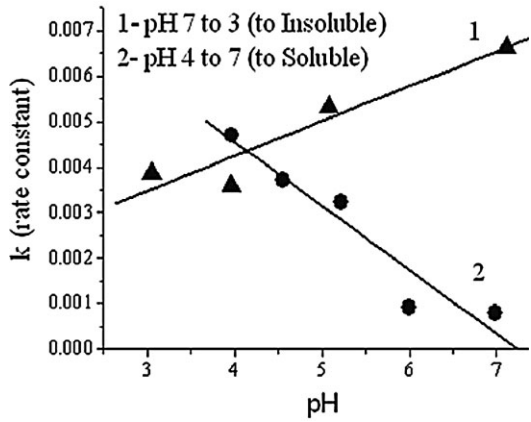

Figure 4.
The calculated rate constants of the kinetic of structure transformation for BSA/PAA complex at the surface of gold due to change of pH of buffer solution from 3.0 to 7.0. Change of pH from 7.0 to 3.0 (1); from 4.0 to 7.0 (2).

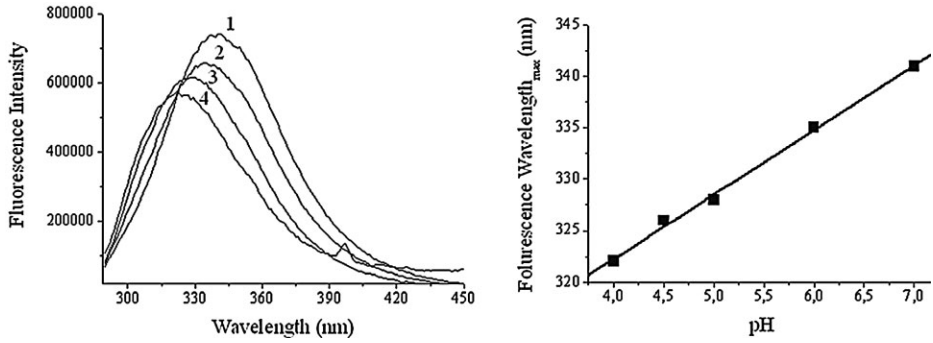

Figure 5.
(A) Fluorescence measurements of PAA-BSA mixture at the ratio of $n_{BSA}/n_{PAA} = 1.0$ at 280 nm wavelength: pH 7.0 (1); pH 6.0 (2); pH 5.0 (3); pH 4.0 (4). (B) Its maximum fluorescence wavelengths depending on the pH.

state. As depicted on Figures 2 and 3, the swelling of PAA-BSA complexes exhibited the lowest rate constants K_d for process of dissociation then reverse associative process of shrinking of PCs layer. The higher rate constant K_a values in the interaction of PAA-BSA complexes without centrifugation compared with centrifuged complexes can be explained in terms of an increase in binding capacity due to multivalent interaction, since SPR detects the mass of the molecules interacts with the surface. The rate constants of observed kinetic transformations were calculated using standard fitting procedure and found to be changed in the range from 0.8×10^{-3} to 6.7×10^{-3} for different pH values and direction of change (Figure 4).

Fluorescence spectroscopy was used to study the interactions and dynamics of protein-polymer complexes. Figure 5(A) shows the spectrofluoremetric titration of PAA-BSA mixture at the ratio of $n_{BSA}/n_{PAA} = 1.0$ and in a wide range of pH values (from pH 4.0 to pH 7.0) with 1M NaOH. Maxiumum fluorescence wavelength (nm) of this mixture depending on the pH was depicted in Figure 5(B). It is well-known that tryptophan (Trp) fluorescence of proteins varies with their conformational changes resulting in changes of fluorescence parameters, such as the emission maximum (λ_{max}), quantum yield, lifetime, and others.[17,18] BSA (isoelectric point of BSA, $pI_{BSA} = 4.9$) contains two Trp.[19] One of them (spectral class 2 by Burstein[17] 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 to aqueous solvent.

Fluorescence maximum shifted toward the red region ($\lambda_{max} = 342$ nm) at pH 7.0 as can be seen from Figure 5A. The titration of $n_{BSA}/n_{PAA} = 1.0$ complex from pH 4.0 to pH 7.0 resulted in a 20 nm red shift (Figure 5B). The emission maximum of BSA-PAA mixture at pH 7.0 is practically the same as that for pure BSA in solution ($\lambda_{max} = 340$nm). The preexisting electrostatic repulsive forces between PAA and protein molecule prevented the formation of the stable polyelectrolyte complexes of BSA at pH 7.0. One can assume that the large red shift obtained in the samples upon changing the pH of solution up to pH 4.0 indicated that the BSA trypthophanyls became more accessible to aqueous solution, which might be due to the binding of the polymer with the protein.

Conclusion

The structural changes of BSA/PAA complex can be easily monitored by Surface Plasmon Resonance spectroscopy in real time mode. The dynamic of conformational

changes of protein-polymer complex expressed the strong dependence on pH of liquid environment and proportion of components of PCs. Soluble PCs corresponded with high pH and a swelled structure, whereas insoluble PCs were responsible on more compact, shrunk complexes, which showed that at low pH the ability of aggregation and sedimentation depended on proportion of components. The rate constants of formation of insoluble PCs at high pH appeared higher than the ones for the transformation into soluble transparent structure at low pH. The fluorescence spectroscopy should be considered as a useful additional method fort he verification of results of SPR experiments. The combination of these two methods appeared to be a promising approach for the investigation of protein-polymer complexes and even for the development of synthetic vaccines.

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