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Investigation of Ternary Complex Formations of Polyacrylic Acid with Bovine Serum Albumin in the Presence of Metal Ions by Fluorescence and Dynamic Light Scattering Measurements

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Abstract In this paper, the complex formation of bovine serum albumin (BSA) and polyacrylic acid (PAA) in the presence metal ions at pH = 7 has been examined by using fluorescence and dynamic light scattering measurements. It has been observed that the most stable complexes of polyacrylic acid and bovine serum albumin have occurred in the presence of copper(II) ions. The other ions have the ability to form weak complexes between polyions and bovine serum albumin. To prior characterizing the interaction between bovine serum albumin and polyacrylic acid, the dynamic light scattering technique have been applied to determine the intensity-size distributions of the solutions of bovine serum albumin, polyacrylic acid, and ternary mixtures containing various molar ratios of bovine serum albumin to polyacrylic acid (the molar ratios of bovine serum albumin to polyacrylic acid has been taken equal to 0.5, 1.0, 1.5, 2.0 and 2.5) prepared at different molar ratios of copper(II) ions/acrylic acid unit. When the molar ratio of copper(II) ions to acrylic acid in the ternary mixtures has been lower than and equals to 0.3, two peaks have been observed in the curves of the intensity-size distributions due to contents of free bovine serum albumin and ternary complexes of polyacrylic acid-copper(II)-bovine serum albumin whereas when the molar ratio of copper(II) ions to acrylic acid equals to 0.4, the hydrodynamic diameter has pointed out only one peak. This result indicates that soluble and stable ternary complexes has occurred when the molar ratio of copper(II) ions to acrylic acid has been taken equal to 0.4.

Keywords Fluorescence · Dynamic light scattering · Ternary metal complexes · Bovine serum albumin · Polyacrylic acid

Abbreviation

BSA	Bovine serum albumin
PAA	Polyacrylic acid
PE	Polyelectrolyte
PMC	Polyelectrolyte-metal-protein polycomplexes
M	Metal
Cu^{2+}	Copper ions
AA	Acrylic acid
M_w	Molecular weight (weight-average)
pI	Isoelectric points
DLS	Dynamic light scattering

1 Introduction

The formation of water-soluble and water-insoluble ternary polycomplexes of proteins with synthetic polyelectrolytes in the presence of transient metal ions (Fe^{3+}, Ba^{2+}, Cu^{2+}, etc.) have been described previously [1–3, 8–10, 20–27, 29, 32–34, 36]. Such polyelectrolyte (PE)-metal-protein polycomplexes (PMC) have been obtained by simple mixing of solutions of proteins, metal (M) ions, and PE in water at

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different pH values. This method consists of the use of small concentrations of metal additives, which promote PE binding to protein molecule without causing any unexpected changes in the chemical structure of components. These studies revealed that in the presence of metal ions many nitrogen- and carboxyl-containing PE [poly(acrylic acid) (PAA), polyvinylpyridines, poly-*N*-vinylimidazole, polyconidine, poly(*N*-isopropylacrylamide) (poly(NIPAAm)) and their copolymers irrespective of their molecular mass] can form ternary PMC with different proteins (bovine serum albumin (BSA), human serum albumin (HSA), ovalbumin (OA), bovine γ-globulin (BGG), etc.). These complexes can be both soluble and insoluble in water. The contacts between proteins and PE are achieved via formation of a chelate unit in which the metal ions are located at the interface. The solubility of the polycomplexes depends on the nature of proteins and correlates with their isoelectric points (pI) [21]. In these systems, the metal ions promoted two effects: (1) the binding of PE to the protein molecules and (2) intermolecular aggregation of polycomplex particles. The solubility, composition, and stability of these polycomplexes depend on pH, metal/PE, and protein/PE ratios. Some of these polycomplexes reveal strong immunogenecity and provide a high level of immunological protection [9, 10, 20–22, 24, 26, 27, 32].

Polyelectrolytes of synthetic origin have been found to increase immunoresponse to the immunizing antigen and to produce an adjuvant effect [12, 13, 15]. The use of PE as a carrier, which is firmly linked to microbial and viral antigens to form a stable complex (or conjugate), not only increased by several orders of magnitude the immune responsiveness of the organism but also afforded effective immune protection. This has, in turn, opened the way to the construction of artificial vaccines against yet uncontrolled infections [15]. Such systems include complexes stabilized by cooperative electrostatic and hydrophobic interactions between the fragments of PE and antigen molecules and conjugates in which the functional groups of the components are linked by covalent bonds. In those cases, where PE macromolecules do not contain the corresponding electrostatic or hydrophobic groups for antigen binding, it is necessary to modify the carrier polymer, which can give rise to changes in its effect(s) upon biological systems.

A relatively good technique involves the use of M ions compounds as means of activating the support surface and allowing direct coupling of proteins without prior derivation of the activated support, through formation of chelate [16]. Evidence has recently been presented for the existence of a ternary complex between proteins, Cu^{2+} ions, and amino acid [35]. Some publications in the current literature are devoted to the construction of drugs based on such M-mediated complexes of natural PE and antigen [38–42].

The formation of polycomplexes in the mixtures PE-metal- protein and their structure were intensively studied by titration, high-performance liquid chromatography (HPLC), electrophoretic, spectrophotometric (FT-IR, UV–vis), and hydrodynamic (viscosity and sedimentation) methods. These methods provide general information about the structure of polycomplex particles, while the information about the structure of protein globules in these particles is practically absent.

Fluorescence techniques have recently been used to study protein-polyelectrolyte complexation [11, 19, 37, 43]. From the fluorescent emission shift of tryptophan residues in proteins, it is possible to localize the interaction between proteins and PE at certain protein domains [6]. The BSA molecule is known to contain two tryptophan (Trp) residues. One of them is located on the bottom of hydrophobic cleft between domains 1 and 3 whereas the other is on the surface of the molecule [4, 30, 31]. Considering that Cu^{2+} ions are efficient fluorescence quenchers, [4] one may expect that fluorescence study of PE binding to BSA in the presence of Cu^{2+} ions can provide valuable information regarding the structural features of soluble ternary polycomplexes [10].

In this study, the interaction between anionic PAA and BSA in the presence of M ions in aqueous solution was analyzed with fluorescence measurements and the formation of the polycomplexes in the mixture BSA-Cu^{2+}-PAA was examined by dynamic light scattering. It is shown that more stable water soluble ternary complex was obtained on the ratio copper(II)/acrylic acid = 0.4. Occurring the pattern of distribution of Cu^{2+} ions between PAA coil and of protein globules, they consist of ternary polymer-metal-protein complexes. These ternary complexes occur the self-assembly principle.

2 Materials and Methods

2.1 Materials

Polyacrylic acid (PAA) was synthesized and fractionated as explained in the literature [28]. Polymer was prepared by radical polymerization of acrylic acid in toluene with benzoilperoxide as initiator. PAA was fractionated from 3 to 4% solution in methanol by fractional precipitation with ethyl acetate. The weight-average molecular weight (Mw) of the fraction used in this study was 100 kDa. Bovine serum albumin (BSA) (M_w = 70000, pI = 4.9) were purchased from Sigma Chemical Company (St. Louis, USA). Ultra pure water was obtained from Millipore MilliQ Gradient System. Solutions of polymers used in the study were prepared on ultra pure water in a cold room (5 °C) with stirring over 12 h. After dissolution, the needed

quantities of BSA were weighed and solved in ultra pure water. pH was adjusted to pH 7 if needed by addition of 0.1 N NaOH.

Metal salts: $MnSO_4 \cdot H_2O$, $CoCl_2 \cdot 6H_2O$, $NiSO_4 \cdot 7H_20$, $CdSO_4 \cdot 8\backslash 3H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnCl_2$, were purchased from Merck (Darmstadt, Germany) and dissolved in ultra pure water.

2.2 Polymer-Metal-Protein Complexes

In order to produce polymer-metal complexes, various concentrations (metal ions/acrylic acid = 0.1, 0.2, 0.3 and 0.4) of the $CuS0_4 \cdot 5H_20$, $MnSO_4 \cdot H_2O$, $CoCl_2 \cdot 6H_2O$, $NiSO_4 \cdot 7H_20$, $CdSO_4 \cdot 8\backslash 3\ H_2O$, $ZnCl_2$, solutions were added to PAA dissolved in ultra pure water. The solution was stirred at 20 °C for 24 h. Then, pH was adjusted to pH 7 by adding 1 N NaOH solution.

In the fluorescence measurements of the ternary complexes, PAA-M-BSA was prepared as follows: BSA was dissolved in ultra pure water at pH 7, and then added to the PAA-M complex solution. The pH of solution was also adjusted to 7. To produce a PAA-M-BSA complexes, the same concentration of metals solution were added to PAA. In the measurements of UV–Vis and dynamic light scattering, the mole ratio of Cu^{2+} ions to the PAA was kept constant at copper(II)/acrylic acid = 0.1, 0.2, 0.3 and 0.4. The metal ions/protein and metal ions/acrylic acid ratios were calculated using the equation $n = CN_A/M$, where n is the number of the molecules in 1 ml, M is the molecular weight of components and N_A is the Avagadro number and C represent concentration in g/mL.

2.3 Fluorescence Measurements

Fluorescence emission spectra were obtained by using the Quanta Master spectrofluorimeter (Photon Technology International, Canada) operating in quanta counting mode. The slits of excitation and emission monochromators were adjusted to 2 or 3 nm. The excitation wavelength was 280 nm. The fluorescence of proteins is widely used to study their behaviors depending on different influencing factors, which is due to the presence in almost all proteins of natural fluorophores tyrosine and tryptophan. In present research we have characterized them by the measurements of wavelength of maximum of emission fluorescence spectrum (λ_{max}), fluorescence intensity (I).

2.4 Dynamic Light Scattering Method

The average size and size distribution of protein, polymer and protein-metal-polymer mixtures (complexes) were investigated by using photon correlation spectroscopy with a Zetasizer Nano ZS instrument (Malvern Instruments, UK) equipped with 4.0 mV He–Ne laser at a wavelength 633 nm at a temperature 25 °C. Scattered light was detected at an angle 173° using non-invasive back scattering (NIBS) technique. All solutions were filtered with 0.2 μm RC-membrane filters (Sartorius) before DLS measurement.

3 Results and Discussion

BSA contains two Trp [4, 28, 30, 31]. One of them (spectral class 2 by Burstein [4] with λ_{max}) 340-342 nm and half width $\Delta\lambda$ = 53–55 nm) is located on the bottom of BSA hydrophobic cleft. The second Trp of class 3 (λ_{max} = 350–352 nm, half width $\Delta\lambda$ = 59–61 nm) with low quantum yield (1/5 of the total BSA fluorescence [4]) is superficial and completely accessible to aqueous solvent. 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 (I_{max}) and position of maximum (λ_{max}), quantum yield, lifetime, and others [5, 7, 17]. Figure 1 shows the emission maximum (I_{max}) of fluorescence intensity of BSA with PAA at pH = 7 in the presence of metal ions with increase in the concentration of metal ions. As can be seen from Fig. 1, with augmentation in concentration of metal ions, the fluorescence intensity (I_{max}) of BSA in the presence of PAA at pH 7 decreases due to quenching of fluorescence intensity (I_{max}) of BSA The results in Fig. 1 suggest the following model. The two types of bioconjugate particles form depending on the method of binding and the conditions of the reaction. The complex formation via M (Zn^{2+}, Ni^{2+}, Cd^{2+}, Co^{2+} and Mn^{2+}) with the protein molecules in the presence of polymer particles are densely

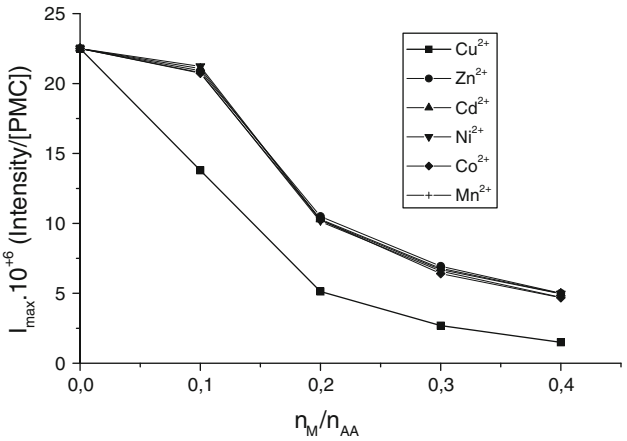
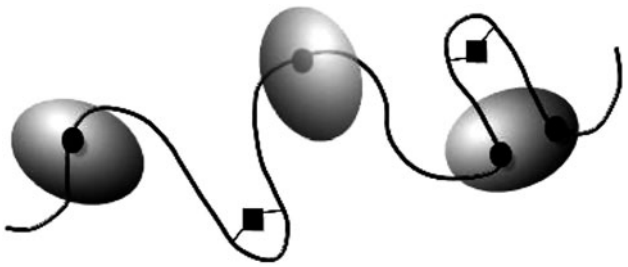


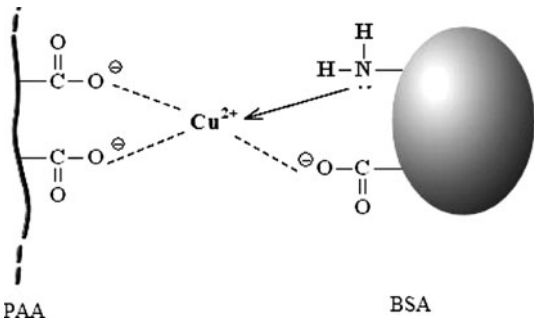
Fig. 1 The dependence of the emission maximum (I_{max}) of fluorescence intensity/[PMC] on the ratios of n_M/n_{AA} = 0.1; 0.2; 0.3; 0.4 and n_{BSA}/n_{PAA} = 2 (the excitation wavelength was 280 nm and emission wavelength was 339.3 nm)

covered by a shell of a polymer coil and practically "fenced off" from the water environment. In contrast, at the complex formation via Cu^{2+} ions and BSA in the presence of PAA, the ternary polycomplex particles of BSA-Cu^{2+}-PAA have more friable structures in which protein molecules are practically exposed to the solution as given in Schemes 1 and 2 and described in previous studies [10, 14, 18, 19, 21, 26].

Figure 2 shows that the position at maximum emission (λ_{max}) of ternary complexes of BSA-Cu^{2+}-PAA in fluorescence measurements is also effected from the augmentation in metal ions concentration. The maximum emission wavelength (λ_{max}) of ternary mixture changes shift to red (longer wavelength) region with increasing the concentration of Cu^{2+} ions in the solution of ternary mixtures. Whereas the increase in other metal ions (Zn^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+} and Mn^{2+}) in the solutions of ternary mixtures changes shift to blue (shorter wavelength) region. These results are indicative of changes in the local conformation around the Trp residues resulting from interaction of polymer with the protein via copper ions. Blue shift indicates that the environment of Trp becomes more hydrophobic and shielded from aqueous solvent which can be understood from partially hydrophobic nature of polycomplex [10, 18]. The Scheme 2 shows the hypothetical structure of ternary water soluble complex of PAA-Cu^{2+}-BSA.



Scheme 1 *Filled circle* binding of polymer-protein functional groups in Cu^{2+} presence. *Filled square* binding between COO^- groups in Cu^{2+} presence



Scheme 2 The hypothetical structure of ternary water soluble complex of PAA-Cu^{2+}-BSA

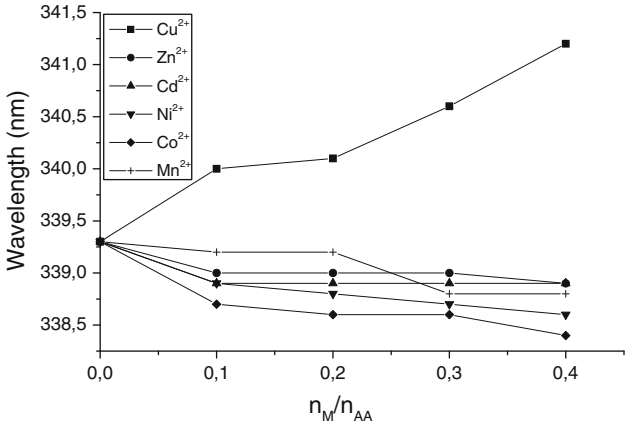


Fig. 2 The effect of the ratios of n_M/n_{AA} on the position of maximum emission(λ_{max}) ($n_M/n_{AA} = 0.1$; 0.2; 0.3; 0.4 $n_{BSA}/n_{PAA} = 2$)

For neutral water (pH = 7) the addition of Cu^{2+} ions to PAA starts to give homogeneous solution at given value of copper(II)/acrylic acid = 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5 and 2.0. This has been confirmed by UV–visible spectrophotometric measurements. The dependence of optical density at 400 nm on the different ratio of metal ions to acrylic acid monomers is illustrated in Fig. 3. The absorbance increases with an increase in Cu^{2+} ions concentration. The relation suggests that the Cu^{2+} ions are bounded to PAA molecules, which in turn generates a water soluble (copper(II)/acrylic acid $\leq$ 0.4) PAA-Cu^{2+}.

Dynamic light scattering measurements are used characterize the complex systems between protein, metal and polymer. Figure 4 shows the relation between the scattering intensities and hydrodynamic diameters of PAA, BSA and the ternary mixtures of BSA-Cu^{2+}-PAA. As seen from Fig. 4, the hydrodynamic diameter of BSA shows a peak at 9.3 nm while PAA gives a broad distribution where there

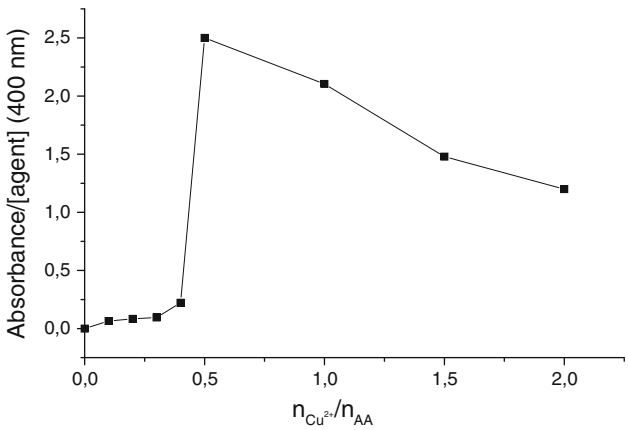


Fig. 3 The dependence of optical density of 400 nm (OD_{400}) on the ratio of metal ions to acrylic acid monomers ($n^{2+}_{Cu}/n_{AA} = 0.1$; 0.2; 0.3; 0.4; 0.5; 1; 1.5; 2)

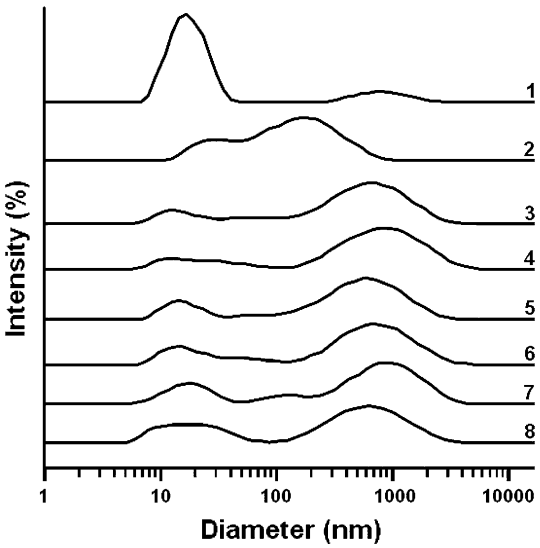


Fig. 4 Intensity-size distributions of BSA (1); PAA (2); the different ratios: $n_{BSA}/n_{PAA} = 0.5$ (3), $n_{BSA}/n_{PAA} = 1.0$ (4), $n_{BSA}/n_{PAA} = 1.5$ (5), $n_{BSA}/n_{PAA} = 2.0$ (6), $n_{BSA}/n_{PAA} = 2.5$ (7), $n_{BSA}/n_{PAA} = 3.0$ (8); at pH 7.0 at the constant ratio: $n_{Cu}^{2+}/n_{AA} = 0.1$

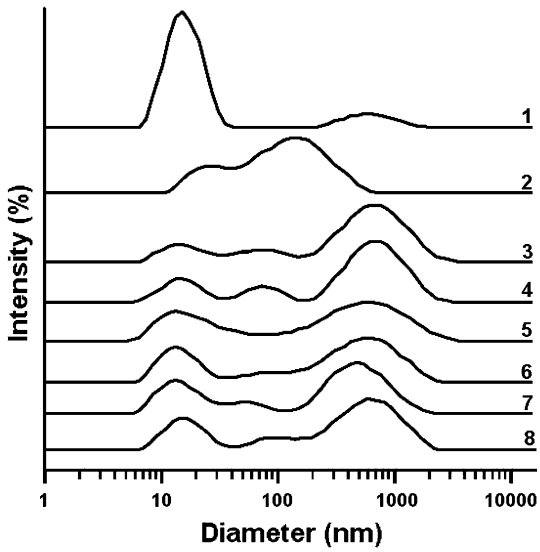


Fig. 5 Intensity-size distributions of BSA (1); PAA (2); the different ratios: $n_{BSA}/n_{PAA} = 0.5$ (3), $n_{BSA}/n_{PAA} = 1.0$ (4), $n_{BSA}/n_{PAA} = 1.5$ (5), $n_{BSA}/n_{PAA} = 2.0$ (6), $n_{BSA}/n_{PAA} = 2.5$ (7), $n_{BSA}/n_{PAA} = 3.0$ (8); at pH 7.0 at the constant ratio: $n_{Cu}^{2+}/n_{AA} = 0.2$

can be seen two peaks at 14.3 and 96.0 nm. The second peak has 80% part of the distribution. This bimodal character of PAA is thought to be because of intermolecular aggregation of PAA at pH = 7. The solubility composition and stability of polyelectrolyte-protein complexes depend on metal/PE and protein/PE ratios. It has been investigated the effect of the protein/polymer ratios of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 for each of metal/polymer ratios of 0.1, 0.2, 0.3 and 0.4 individually. The dynamic light scattering measurements has showed that soluble complexes of BSA, Cu^{2+} and PAA are formed at average 379 nm at copper(II)/acrylic acid = 0.1(Fig. 4, curves 3–8). However, there are also peaks at around 9 nm which are associated with free (unbounded) BSA in the solutions. The amount of free BSA increases from 13 to 32% as BSA amount increases in ternary mixtures of PAA-Cu^{2+}-BSA. Therefore, it can be said that the excess of added BSA to the ternary mixtures of BSA-Cu^{2+}-PAA does not enter into the complex molecules and the initially formed complexes of BSA-Cu^{2+}-PAA do not alter.

With increase in BSA content in the ternary mixtures of PAA-Cu^{2+}-BSA, the soluble complexes at ratio copper(II)/acrylic acid = 0.2 have also formed (Fig. 5). The hydrodynamic diameter of the first formed complex is 483 nm at protein/polyacrylic acid = 0.5 and this size decreases to 386 nm while protein/polymer ratio increases to 3.0. There are also peaks around 9 nm which are thought to be free BSA and this free BSA amount increases from 15 to 25% as BSA amount increases. Besides, a third peak appears in this metal/polymer ratio at about 44 nm with a 10% in the intensity-size distribution.

At the ratio copper(II)/acrylic acid = 0.3, the hydrodynamic diameter of the soluble complex is found to be 379 nm for the ratio protein/polyacrylic acid = 1.0 and rises to 408 nm at protein/polyacrylic acid = 3.0 (Fig. 6). This suggests that there is still free BSA indicating a peak at around 9 nm. The free BSA content in ternary mixture increases from 15 to 25% as protein/polymer ratio increases. The third peaks, clearer in this ratio, are about 50 nm with a 15% in the intensity-size distribution.

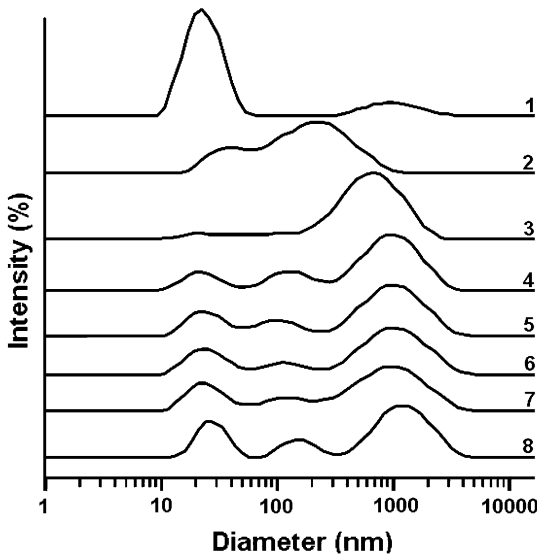


Fig. 6 Intensity-size distributions of BSA (1); PAA (2); the different ratios: $n_{BSA}/n_{PAA} = 0.5$ (3), $n_{BSA}/n_{PAA} = 1.0$ (4), $n_{BSA}/n_{PAA} = 1.5$ (5), $n_{BSA}/n_{PAA} = 2.0$ (6), $n_{BSA}/n_{PAA} = 2.5$ (7), $n_{BSA}/n_{PAA} = 3.0$ (8); at pH 7.0 at the constant ratio: $n_{Cu}^{2+}/n_{AA} = 0.3$

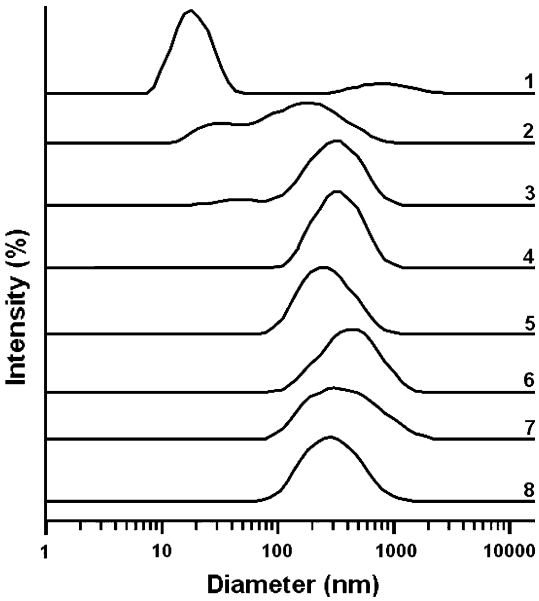


Fig. 7 Intensity-size distributions of BSA (1); PAA (2); the different ratios: $n_{BSA}/n_{PAA} = 0.5$ (3), $n_{BSA}/n_{PAA} = 1.0$ (4), $n_{BSA}/n_{PAA} = 1.5$ (5), $n_{BSA}/n_{PAA} = 2.0$ (6), $n_{BSA}/n_{PAA} = 2.5$ (7), $n_{BSA}/n_{PAA} = 3.0$ (8); at pH 7.0 at the constant ratio: $n^{2+}_{Cu}/n_{AA} = 0.4$

When the ratio of copper(II)/acrylic acid has been taken equal to 0.4, only one peak in the intensity-size distribution of the ternary mixtures of PAA-Cu^{2+}-BSA has been observed at around 174 nm and the soluble complexes have formed (Fig. 7). There is not any free BSA in the solution although metal amount is enough to bind all added BSA to PAA. This indicates that with increasing BSA in the ternary mixtures of PAA-Cu^{2+}-BSA, stable and water soluble ternary complexes are obtained at the ratio of copper(II)/acrylic acid = 0.4.

4 Conclusion

The present study demonstrates the formation of the ternary complexes of PAA with BSA in the presence of metal ions at pH = 7. Fluorescence and dynamic light scattering techniques have been applied. The results of fluorescence measurements show that the most stable complexes between PAA and BSA in the presence of Cu^{2+} have occur. The dynamic light scattering measurements show that the solubility composition and stability of polyelectrolyte complexes depend on metal/protein and protein/polymer ratios. At the ratios of copper(II)/acrylic acid = 0.1, 0.2 and 0.3, with increasing BSA in the ternary mixture of PAA-Cu^{2+}-BSA, two peaks have been observed in the results of the intensity-size distributions of ternary mixtures. When the copper(II)/acrylic acid equals to 0.4, only one peak has been observed in the curve of intensity-size distribution of ternary mixture. This indicates that stable and soluble ternary complexes have been obtained at this ratio. At the complex formation via metal ions, the ternary polycomplex particles have more friable structures in which protein molecules are practically exposed to solution. These results are important because they can be used for developing new type of synthetic immunogens.

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