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The formation of polycomplexes of poly(methyl vinyl ether-*CO*-maleic anhydride) and bovine serum albumin in the presence of copper ions

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The binary and ternary complex formations of poly(methyl vinyl ether-*co*-maleic anhydride) (PMVEMA) with copper ions and with bovine serum albumin (BSA) in the presence of copper ions in phosphate buffer solution at pH = 7 were examined by the techniques of UV-visible, fluorescence, dynamic light scattering, atomic force microscopy measurements. In the formation of binary complexes of PMVEMA-Cu(II), the addition of copper ions to the solution of PMVEMA in phosphate buffer solution at pH = 7 forms homogeneous solutions when the molar ratio of Cu(II)/MVEMA is 0.5. Then the formations of ternary complexes of PMVEMA-Cu(II)-BSA were examined. Study analysis revealed that the toxicities of polymer-metal and polymer-metal-protein mixture solutions depend on the nature and ratio of components in mixtures.

Keywords: polyelectrolyte, bovine serum albumin, copper ions, metal complexes, biological processes, biomaterials.

INTRODUCTION

Investigation of water soluble and insoluble complexes of polymer with protein in the presence of metal ions has important applications in various areas[1–14]. In recent studies, it has been shown that metal (M) ions (Cu^{2+}, Zn^{2+}, Fe^{2+}) which are complexed with functional polymers have an important role in biological processes[2, 15–23].

Relevant studies revealed that nitrogen- and carboxyl-containing PE such as poly(acrylic acid) (PAA), poly(vinyl pyridine), poly(N-isopropyl acrylamide) and PMVEMA have been performed for the formation of ternary polymer metal complexes (PMC) with protein such as bovine serum albumin (BSA), human serum albumin, ovalbumin and bovine γ-globulin. The contact of proteins with polyelectrolyte (PE) is located at interface. Solubility of polycomplexes depends on the nature of proteins and correlates with their isoelectric point. In these systems, metal ions generally promote two effects: (1) the binding of polyelectrolite to protein molecules and (2) inter molecular 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[2, 24–27].

Synthetic PE applications have been found to increase immunoresponse to the immunizing agent and to produce an adjuvant effect[17, 18, 28, 29]. PE used as carrier in ternary complexes is firmly linked to microbial and viral antigens to form a stable complex (i.e., increased by several orders of magnitude the immune responsiveness of the organism but also afforded effective immune protection), which allows an avenue to manufacture artificial vaccines against yet uncontrolled infections[2]. 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 proteins coupling without prior activated support, through formation of chelate[30]. Recent findings showed the existence of a ternary complex between proteins, Cu(II) ions and amino acid[31].

Recently, fluorescence techniques were used to study protein-polyelectrolyte complexation[2, 16, 32, 33]. 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[25]. 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[34, 35]. Considering that Cu(II) ions are efficient fluorescence quenchers, one may expect that fluorescence study of PE binding to BSA in the presence of Cu(II) ions can provide valuable information regarding the structural features of soluble ternary polycomplexes[25, 34].

In present study, the interaction between PMVEMA and BSA in the presence of M ions in aqueous solution was analyzed by using UV-visible, dynamic light scattering (DLS), atomic force microscope (AFM) and fluorescence measurements. More stable water soluble binary complexes were obtained, when the ratio $n_{Cu(II)}/n_{MVEMA} \leq 0.5$. Water soluble and insoluble polycomplexes have been obtained depending on different condition of $n_{Cu(II)}/n_{MVEMA}$ with and without BSA concentration and the n_{BSA}/n_{PMVEMA} at different molar ratios of Cu(II)/MVEMA. Technique of atomic force microscopy was used to indicate the structure of ternary complexes at nano-scale form. The toxicities of solution of the polymer-metal and polymer-metal-protein mixtures were

determined, depending on nature of pure components and the ratio of components in the mixtures.

MATERIALS AND METHODS

Materials

(PMVEMA) (Gantiez AN 139 BF) was supplied from ISP Europe and used without further treatment. Bovine serum albumin (BSA) (Mw = 70 kDa, pI = 4.9) was purchased from Sigma chemical company (St. Louis, USA), other chemicals such as copper sulfate pentahydrate ($CuSO_4$, $5H_2O$, Merck), sodium dihydrogen phosphate (NaH_2PO_4, Reiadel de Haën), disodium hydrogen phosphate (Na_2HPO_4, Fluka), which were used without further treatment. Ultra pure water was obtained from Millipare Milli-Q gradient system. The solutions of PMVEMA (3 g/L) used in this study were prepared in phosphate buffer solutions of pH = 7 in cold room (5°C) with stirring over 12 hours. The molecular weight of PMVEMA in phosphate buffer solution at pH = 7 was found as $\overline{M}_w$ = 41 kDa by the measurement technique of gel permeation chromatography. Copper sulfate and BSA were dissolved in ultra pure water and pH of solutions was adjusted to pH = 7 by adding of 0.1 N NaOH solution as needed.

Preparation of polymer-Cu(II) and polymer-Cu(II)-BSA complexes

In order to produce polymer metal complexes, copper sulfate pentahydrate at different concentrations and PMVEMA at constant concentration (3 g/L) were prepared. The molar ratios of Cu(II)/MVEMA were taken as 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, and 2.0. The total volume of the obtained solution 10 mL and the pH of solution was adjusted to 7 by using 1 N NaOH solution prepared in ultra pure water.

To prepare the ternary mixtures of PMVEMA-Cu(II)--BSA, each of solutions containing molar ratios of Cu(II)/MVEMA = 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, and 2.0 was mixed with the required amount of BSA as weight (mg). The molar ratios of BSA to PMVEMA were taken as 0.5, 1.0, 1.5, 3.0, 2.5 and 3.0 and the total volume of each solution was kept constant as 10 mL. The pH of each solution was adjusted to 7 using 1 N NaOH solution.

METHODS

Viscosity

The viscosity measurements were performed at a constant temperature of 25°C with an Ubbelohde automatic viscometer (Schott Gerate, Berlin, Germany).

Fluorescence measurements

Quanta Master spectrofluorometer (Photon Technology International, Canada) operating in quanta counting mode was used to obtain the fluorescence emission spectra. The slits for the excitation and emission monochromators were set to 2 or 3 nm. The excitation wavelength was 280 nm. We characterized them through measurements of the maximum wavelength of the fluorescence emission spectrum (λ_{max}), and the fluorescence intensity (I).

Dynamic light scattering method

Photon correlation spectroscopy with a Zetasizer Nano ZS instrument equipped with 4.0 mV He-Ne laser at a wavelength 633 nm at a temperature 25°C and manufactured by Malvern Instruments, UK was used to examine the properties of the average size and size distribution of protein, polymer and complexes of protein-metal-polymer. Before DLS measurement, each solution was filtered with 0.2 μm RC-membrane Sartorius filters to remove the impurities from the solutions.

Measurements by atomic force microscopy

Atomic force microscopy (Shimadzu Scanning Probe Microscope SPM-9600) is a very high-resolution type of scanning probe microscopy with demonstrated resolution on the order of fractions of a nanometer more than 1000 times better than the optical diffraction limit. The AFM is one of the foremost tools for imagining, measuring and manipulating matter at the nano-scale. The information is gathered by "feeling" the surface with a mechanical probe. Piezoelectric elements that facilitate tiny but accurate and precise movements on commend enable very precise scanning[36, 37].

Technical applications appear to be abundant because the AFM can make three dimensional quantitative measurements with high resolution and on a wide variety of samples than virtually any other technique[37–41]. All samples were prepared in ultrapure water [PMVEMA (0.15 μg/1ml), BSA (0.15 μg/1ml) and PMVEMA-Cu(II)--BSA (BSA 0.15 μg/1ml)].

Preparation of Thiazolyl Blue Tetrazolium Bromide Solution (MTT)

Solution prepared by dissolving 50 mg Thiazolyl blue tetrazolium bromide powder (Sigma M5655) in 5 mL phosphate buffered saline was filtered through filter with respective pore sizes, 0.45 μL and 0.22 μL (Sartorius MiniSart RC filter).

Investigation of Toxic Effects of Polyelectrolyte, Polyelectrolyte-Copper and Polyelectrolyte-Copper-BSA complexes on L929 cells with MTT Method

L929 cells (with 10^4 cell/mL concentration) were seeded on ninety-six-well polystyrene plates. After cell adhesion on the wells, 10 μL of polyelectrolyte, polyelectrolyte--copper, polyelectrolyte-copper-protein solution were added on the cells adhered to the wells. Ultra-pure water was used as the control group. Cells were incubated at 37°C with humid air for 48 hours. And then 10 mg/mL of MTT solution was added to each well and incubated for 4 hours. 100 μL of MTT stop solution was added and incubated at room temperature for 30 min. The same procedure was used (i.e., without cells) to evaluate interaction of the polymer conjugates with medium. Optical density was measured at 570 nm wavelength with micro plate photometer measuring device[15].

RESULTS AND DISCUSSION

Formation of binary polymer-metal complexes

The addition of different amount of copper ions to PMVEMA solutions gives homogeneous solution at pH = 7. With increasing of the molar ratios of Cu(II)/

MVEMA, the solubility of binary complexes decreased and insoluble complexes took place. This was confirmed by UV-visible, gravimetric, viscometric and DLS measurements.

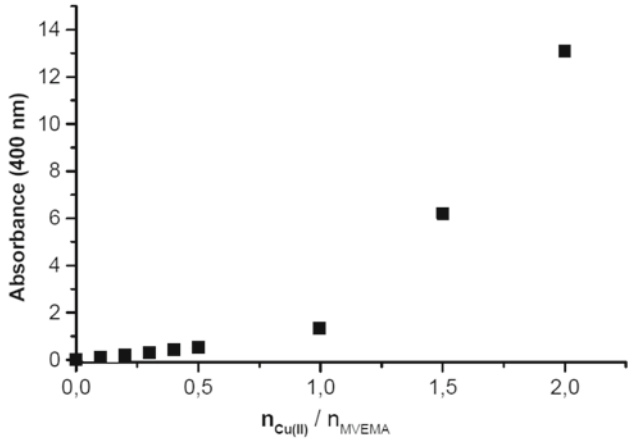


Figure 1. The dependence of optical density at 400 nm (A_{400}) on the molar ratio of Cu(II)/MVEMA for the formation of binary complexes of PMVEMA-Cu(II) prepared at phosphate buffer solution at pH = 7. The molar ratio of Cu(II)/MVEMA = 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5 and 2.0. The total volume of solution is 10 mL. C_{PMVEMA} = 3 g/L

The dependence of optical density at 400 nm is illustrated in Figure 1. As can be seen from Figure 1, the absorbance values at 400 nm increased slowly with an increase in copper concentration up to molar ratio of Cu(II)/MVEMA = 1 then increased sharply after Cu(II)/MVEMA>1. These results were attributed to increasing copper concentration caused by the augmentation of formation of insoluble intermolecular complexes between copper ions and PMVEMA.

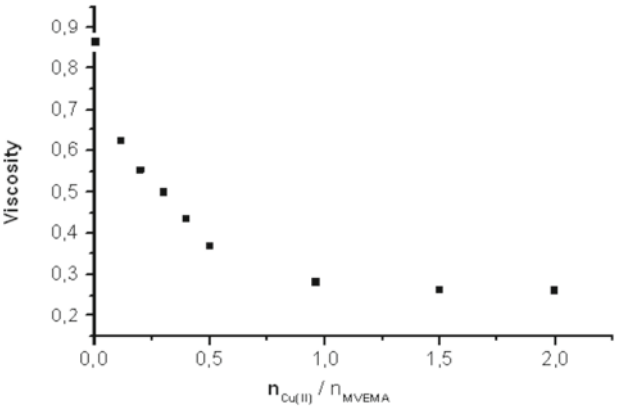


Figure 2. Viscosity values of the binary mixtures of PMVEMA prepared at different copper concentrations (intrinsic). The molar ratio of Cu(II)/MVEMA = 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5 and 2.0. The total volume of solution is 10 mL. The pH of each solution equals to 7. C_{PMVEMA} = 3 g/L

Figure 2 indicates the relationship between the viscosity of binary solution of copper ion and the ratio of $n_{Cu(II)}/n_{MVEMA}$ at pH = 7. The viscosity of solution decreased with an increase in the molar ratio of copper ions to monomer unit (MVEMA) in phosphate buffer solution at pH = 7 (Fig. 2). The decrease in the viscosity of binary solution of Cu(II) and PMVEMA with increase in copper concentration is attributed to increasing copper concentration acting to the increase of the formation of insoluble complexes and after separation of these insoluble particles, the viscosity of solution decreased.

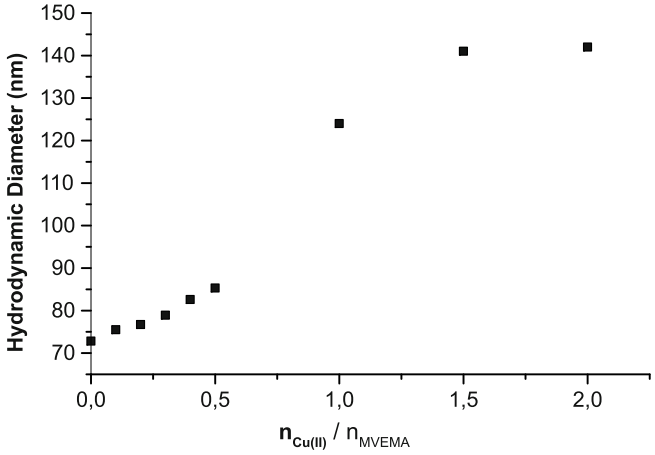


Figure 3. The dependence of hydrodynamic diameter of binary mixture of PMVEMA-Cu(II) prepared at different copper concentration (The molar ratio of Cu(II)/MVEMA = 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5 and 2.0). The pH of each solution equals to 7. The total volume of solution is 10 mL. C_{PMVEMA} = 3 g/L

The average particle size of soluble and insoluble complexes of binary mixtures of Cu(II)-PMVEMA was determined by using DLS measurements depending on the molar ratios of copper ions to polymer (PMVEMA) (Fig. 3). Figure 3 illustrated, at the beginning of adding of copper ions to the binary mixture, average particle sizes of soluble and insoluble complexes started to be increased and reached at the ratio of $n_{Cu(II)}/n_{MVEMA}$ = 1 with an increase in the copper concentration. This result indicates that insoluble complexes caused the augmentation of hydrodynamic diameters of particles due to formation of intermolecular complexation.

The dependence of zeta potential of binary mixture of Cu(II)-PMVEMA on the ratio of $n_{Cu(II)}/n_{MVEMA}$ is given in Figure 4. Zeta potential of binary mixture of

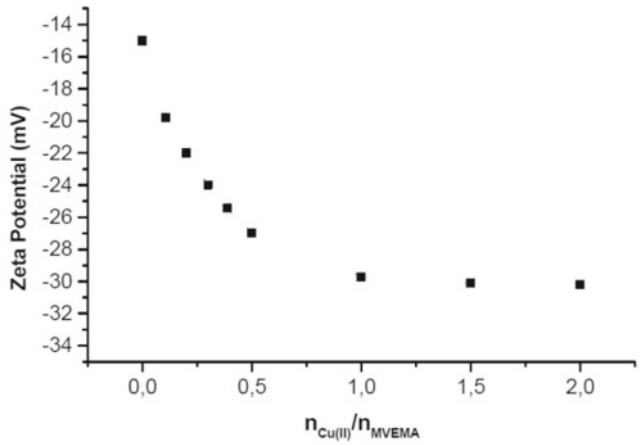
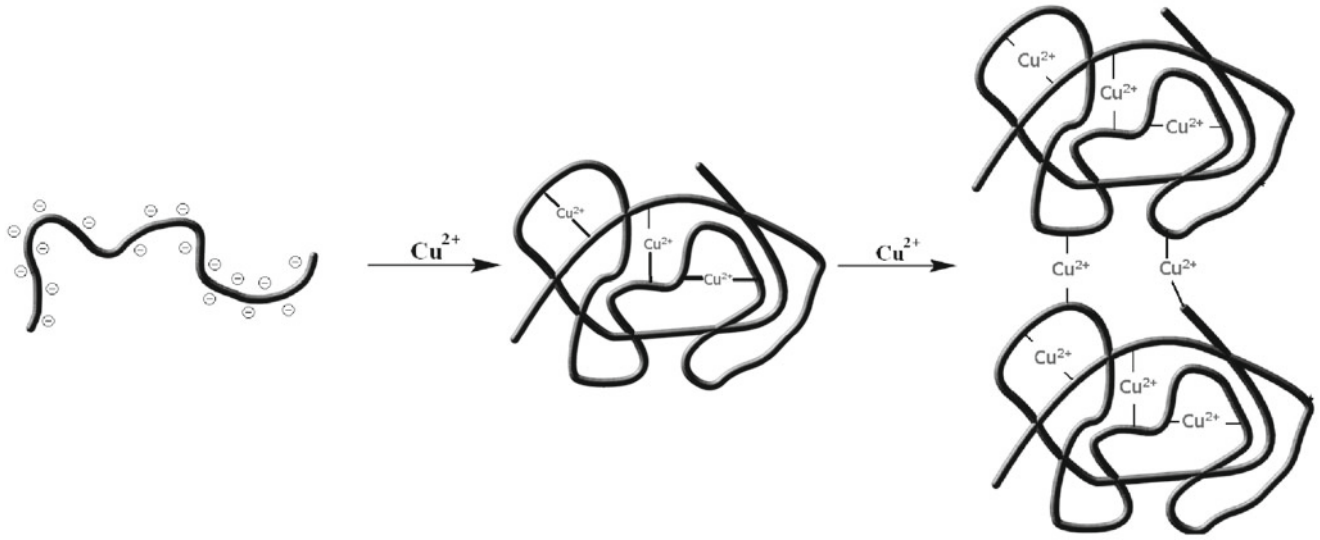


Figure 4. The effect of the ratios of $n_{CU(II)}/n_{MVEMA}$ on zeta potential of the solutions of binary mixtures of PMVEMA-Cu(II) prepared at phosphate buffer solution in pH = 7. The molar ratio of Cu(II)/MVEMA = 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5 and 2.0. The total volume of solution is 10 mL. C_{PMVEMA} = 3 g/L



Scheme 1. Chemical structural model of binary complexes of Cu(II)-PMVEMA

Cu(II)-PMVEMA decreased with increasing copper concentration, reaching approximate minimum value at the molar ratio of Cu(II)/MVEMA = 1. The mechanism of formation of insoluble complexes of Cu(II)-PMVEMA is illustrated in the Scheme 1.

Formation of ternary polymer-metal-protein complexes

The solutions of polymer-metal complexes of Cu(II)--PMVEMA were used to prepare the ternary complexes of PMVEMA-Cu(II)-BSA in phosphate buffer solution at pH = 7. These complexes were researched by spectrophotometric measurements.

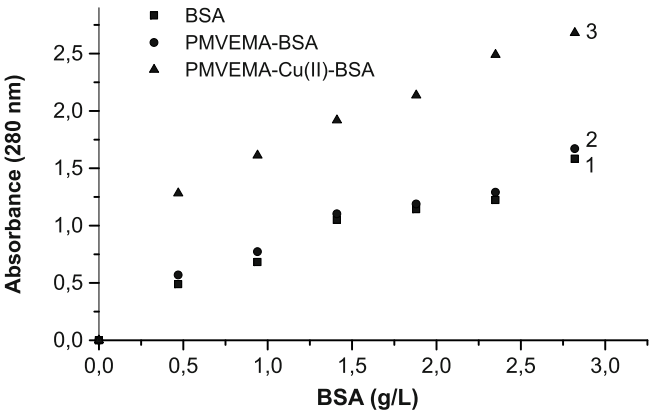
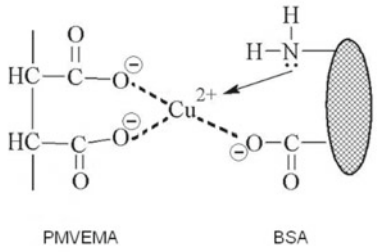


Figure 5. The dependence of optical density (A_{280}) on the BSA concentration for BSA solutions, the binary mixtures of PMVEMA-BSA and the ternary mixtures of PMVEMA-Cu(II)-BSA prepared at phosphate buffer solution at pH = 7. C_{PMVEMA} = 3 g/L, the molar ratio of Cu(II)/MVEMA = 0.5 and n_{BSA}/n_{PMVEMA} = 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0

The optical density (A_{280}) of the solutions of BSA, BSA-PMVEMA and BSA-Cu(II)-PMVEMA (n_{BSA}/n_{PMVEMA} = 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0) were examined. Figure 5 shows the dependence of optical density (A_{280}) for the solution of BSA, PMVEMA-BSA and PMVEMA-Cu(II)-BSA on BSA concentration. Figure 5 indicates that the absorbance values at 280 nm shows an augmentation with increasing protein concentration in the system depending on free protein concentration and complex formation between polymer and protein in presence of copper ions. The value of A_{280} of the solutions of BSA and PMVEMA-BSA increased with increasing of BSA concentration (Fig. 5 curve 1 and 2), respectively. However, the slopes of these curves are almost of the same value, so there isn't any interaction between BSA and PMVEMA. But, when protein solutions (n_{BSA}/n_{PMVEMA} = 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0) in phosphate buffer solution at pH = 7 were mixed with the solution of PMVEMA-Cu(II) (PMVEMA = 3 g/L, $n_{Cu(II)}/n_{MVEMA}$ = 0.5) at pH = 7, the absorbance value of A_{280} changed more with increasing of the concentration of BSA (Fig. 5, curve 3). These results indicate that protein can reacts with copper ions to form polymer and protein complexes (see scheme 2 for hypothetical chemical structure of ternary PMVEMA-Cu(II)-BSA complex).



Scheme 2. The hypothetical chemical structure of ternary water soluble complex of PMVEMA-Cu(II)-BSA

The velocity of the formation of soluble and insoluble ternary complexes of PMVEMA-Cu(II)-BSA depends on what sequence BSA to PMVEMA-Cu(II) (PMC) or PMC to BSA is added.

In both cases, ternary complexes of PMVEMA with BSA in the presence of copper ions are formed depending on time. The effect of the method of preparation was given in Figure 6. The variation of absorbance at 280 nm versus time for two earlier method preparations, *i.e.*, the BSA solutions were added to the PMC solutions and PMC solutions were added to the BSA solutions (Fig. 6) respectively. As can be seen from Figure 6, in both cases the A_{280} values increased with time and absorbance values reached an equilibrium at 300–350 min. Results reveal that when PMC solutions were added to the BSA solutions, the formation of ternary complexes between PMC and BSA was faster than the formation of ternary complexes between BSA and PMC when BSA solutions were added to PMC solutions.

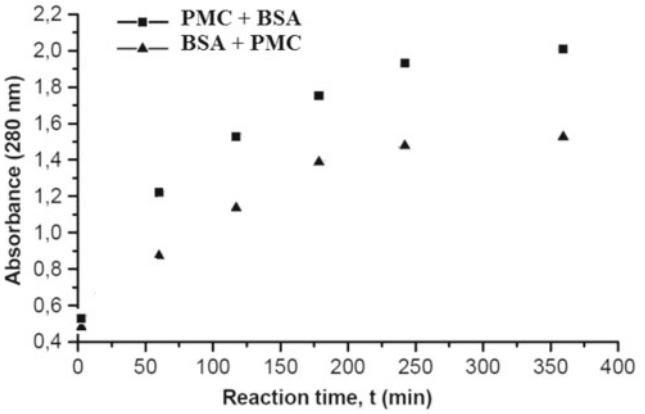


Figure 6. The velocity of the formation of soluble and insoluble ternary complexes of PMVEMA-Cu(II)-BSA depending on the order of addition (BSA to PMC (PMVEMA-Cu(II)) (▲) or PMC to BSA (■). The pH of each solution containing phosphate buffer solution is 7. The total volume of solution is 10 mL. C_{PMVEMA} = 3 g/L

BSA contains two Trp residues. Trp has a wavelength of maximum absorption at 280 nm and emission peak that is solvatochromic ranging from 300 to 350 nm depending on the polarity of local environment. Hence, protein fluorescence could be used to examine the properties of the mixtures of protein-metal and protein-metal--polymer by measuring the values of the fluorescence parameters such as I_{max}, λ_{max}, quantum yield and lifetime and others[34, 35, 42].

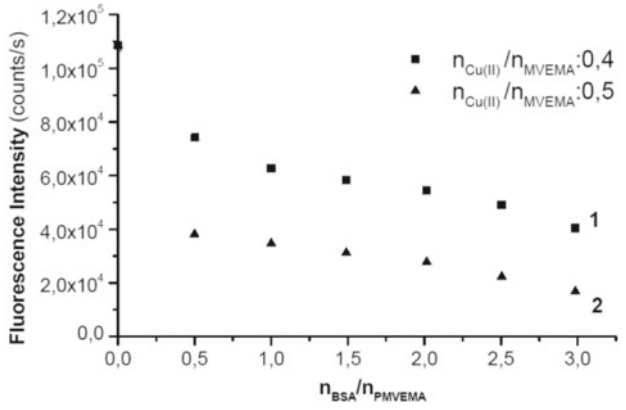


Figure 7. The relation between I_{max} of ternary mixtures of PMVEMA-Cu(II)-BSA and molar ratios of BSA/PMVEMA (n_{BSA}/n_{PMVEMA} = 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0) prepared at the molar ratios of CU(II)/MVEMA = 0.4 (■) and 0.5(▲). C_{PMVEMA} = 3 g/L

The dependence of I_{max} of fluorescence intensity of BSA with PMVEMA in the presence of different Cu(II) concentrations ($n_{Cu(II)}/n_{MVEMA}$ = 0.4 and 0.5) in phosphate buffer solution at pH=7 on the increase in BSA concentration was shown in Figure 7. It was observed that with augmentation in Cu(II) concentration, the intensity of emission maximum (I_{max}) decreased due to quenching of Trp in BSA. Moreover, the increase in copper concentration leds to the formation of conformational changes and quenching effect. For this reason, in the case of the complex formation of PMVEMA with BSA in the presence of the $n_{CU(II)}/n_{MVEMA}$ = 0.4 (Fig. 7, curve 1), the fluorescence intensity of ternary mixture of PMVEMA-Cu(II)-BSA is higher than that occurred in complex formation of PMVEMA with BSA in the presence of the $n_{CU(II)}/n_{MVEMA}$ = 0.5 (Fig. 7, curve 2).

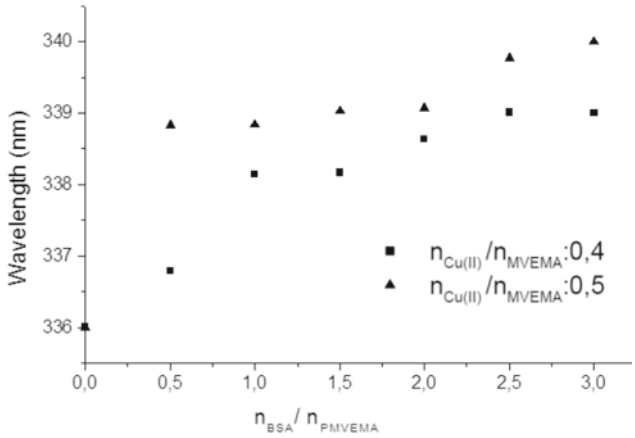


Figure 8. The effect of molar ratios of BSA/PMVEMA on the position of λ_{max} of ternary mixtures of PMVEMA-Cu(II)-BSA prepared at different copper concentrations ($n_{Cu(II)}/n_{MVEMA}$ = 0.4 (▲) and 0.5 (■)). n_{BSA}/n_{PMVEMA} = 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0. The pH of each solution containing phosphate buffer solution is 7. The total volume of solution is 10 mL. C_{PMVEMA} = 3 g/L

Interaction between the values of λ_{max} in the fluorescence spectra of the ternary complexes of PMVEMA--Cu(II)-BSA and the ratios of n_{BSA}/n_{PMVEMA} was given in Figure 8, indicating an increase in λ_{max} values as BSA concentration increased; on the other hand, decreased by augmentation of copper concentration. This phenomenon was described as red shift indication in previous studies[2, 3, 25, 42]. Experimental results indicate that tryptophan of BSA is influenced from local environment resulting from interaction PMVEMA, BSA with copper ions. Therefore, tryptophan fluorescencing can be very sensitive in determining the conformational state of BSA because of the tryptophan residues. For this reason, the red shift indicates that the environment of Trp becomes more hydrophilic feature; and the interaction between Trp molecules of BSA and water increases with increasing maximum wavelength emission of ternary mixture of PMVEMA-Cu(II)-BSA as describe in previous studies[25].

DLS measurements solutions, of pure BSA, PMVEMA and the mixtures of BSA with PMC at varied molar ratios of BSA/PMVEMA = 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 in phosphate buffer solution at pH = 7 are shown in Figure 9. As made mention of Figure 9, hydrodynamic diameter of BSA and PMVEMA show peaks at 9.75 nm and 40.02 nm, respectively. When BSA solution was added to PMC solution at the molar ratio of Cu(II)/MVEMA = 0.5, a new peak having higher dynamic diameter was observed at 455.60 nm attributed to the formation of ternary complexes ($n_{Cu(II)}/n_{MVEMA}$ = 0.5), supporting the formation of ternary complexes.

Zeta potentials of the solutions of the ternary mixtures BSA and PMC ($n_{Cu(II)}/n_{MVEMA}$ = 0.5) in phosphate buffer solution at pH = 7 were measured (Fig. 10). The zeta potentials of the solutions of BSA and PMVEMA have negative charges. This phenomenon prevents the formation of bond between BSA and PMVEMA.

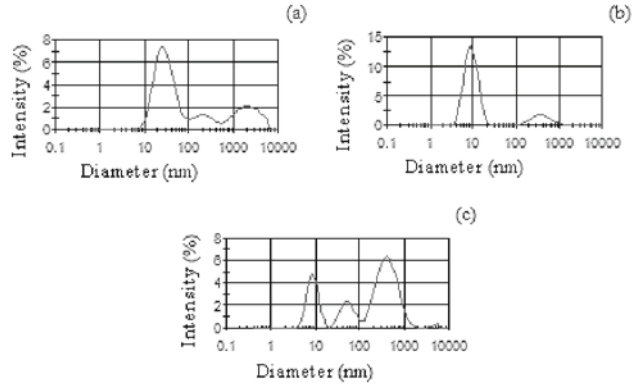


Figure 9. The relation between the intensities of solutions of PMVEMA (a), BSA (b) and ternary mixture of PMVEMA-Cu(II)-BSA ($n_{Cu(II)}/n_{MVEMA}$ = 0.5 and n_{BSA}/n_{PMVEMA} = 3.0) (c) prepared at phosphate buffer solution at pH = 7. C_{PMVEMA} = 3 g/L

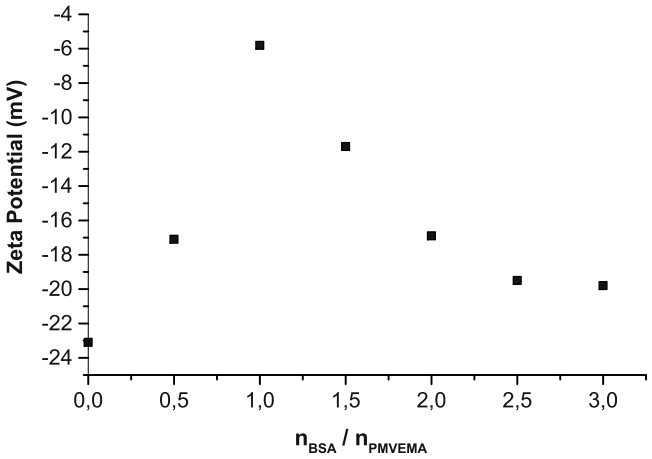


Figure 10. Zeta potentials of ternary mixtures of PMVEMA-Cu-(II)-BSA containing various BSA concentrations (n_{BSA}/n_{PMVEMA} = 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0) prepared at phosphate buffer solution at pH = 7. C_{PMVEMA} = 3 g/L

Divalent Cu(II) ions act as "fasteners" promoting the formation of ternary complexes. When BSA solution started to mix with PMC solutions, the zeta potential of solutions increased and reached a maximum value at the molar ratio of BSA/PMVEMA = 1.0 in the presence of copper ions ($n_{Cu(II)}/n_{MVEMA}$ = 0.5). Increase in the n_{BSA}/n_{PMVEMA} ratio caused a decrease in, the zeta potential of ternary BSA-Cu(II)-PMVEMA mixture. This indicates that solubility and stability of polycomplexes depend on the composition of PMVEMA, the molar ratio of BSA/PMVEMA and Cu(II)/MVEMA[3].

The solutions of PMVEMA (0.15 g/L), BSA (0.15 g/L) and the ternary mixture of PMVEMA-Cu(II)-BSA were prepared in phosphate buffer solution at pH = 7. Each of prepared solutions was placed on the mica surface. The surface of mica coated with the solution of PMVEMA, BSA or ternary mixture of PMVEMA-Cu(II)-BSA was dried at room temperature.

Figure 11 shows the scanning images of PMVEMA (Fig. 11, a), BSA (Fig. 11, b) and ternary mixture of PMVEMA-Cu(II)-BSA (Fig. 11, c). As seen from figure 11, when the structures of particle distributions in the scanning imagines of the solutions of PMVEMA (Fig. 11, a), BSA (Fig. 11, b) and ternary mixture of PMVEMA-Cu(II)-BSA (Fig. 11, c) prepared in phosphate buffer solution at pH = 7 have been compared, it has been observed that more uniform structure of particles distribution has appeared in the scanning imagine of the solution of ternary mixture of PMVEMA-Cu(II)--BSA (Fig. 11, c).

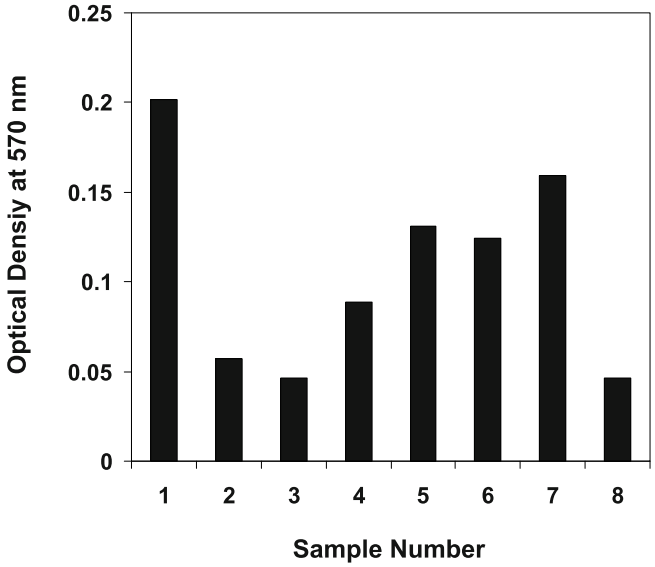


Figure 11. Scanning imagines of PMVEMA (a), BSA (b) and ternary mixture of PMVEMA-Cu(II)-BSA (c). C_{PMVEMA} = 0.15 g/L, C_{BSA} = 0.15 g/L and $n_{Cu(II)}/n_{MVEMA}$ = 0.5

The optical density of reference sample was given as Figure 12, sample 1. As can be seen from Figure 12, it was observed that the toxicity of the binary mixture of PMVEMA-Cu(II) ($n_{Cu(II)}/n_{MVEMA}$ = 0.5) (Fig. 12, sample 3) was higher than those of the solution PMVEMA (Fig. 12 sample 2) and PMVEMA-Cu(II)-BSA ($n_{Cu(II)}/n_{MVEMA}$ = 0.5 and n_{BSA}/n_{PMVEMA} = 2.0) (Fig. 12 sample 4). The efficiency of toxicities of these compounds has been in the order of PMVEMA-Cu(II)>PMVEMA->PMVEMA-Cu(II)-BSA. When the toxicities of the solutions and mixtures of PMVEMA and PAA with/without BSA in the presence of copper ions were compared, the toxicities of the solution PMVEMA and PMVEMA-Cu(II) and PMVEMA-Cu(II)-BSA (Fig. 12, samples 2-4) were higher than those of the solution of PAA and PAA-Cu(II) ($n_{Cu(II)}/n_{AA}$ = 0.4) and PAA-Cu-(II)-BSA ($n_{Cu(II)}/n_{AA}$ = 0.4 and n_{BSA}/n_{PAA} = 1.0) (Fig. 12, samples 5–7). When the toxicities of solutions of the ternary mixture of PAA-Cu(II)-BSA, containing two different BSA concentrations were examined, the toxicity of the ternary mixture of PAA-Cu(II)-BSA prepared at the molar ratio of protein/polymer = 1.0 (Figure 12 sample 7) is lower than that of the ternary mixture of PAA-Cu(II)-BSA (Figure 12 sample 8) prepared at the molar ratio of protein/polymer = 2.0[43]. These results indicate that the toxicities of solutions of polymer and mixtures of polymer-metal and polymer-metal-protein depend on the nature of pure components and the ratio of components in mixtures.

CONCLUSIONS

The binary and ternary complex formations of PMVE-MA with copper ions and PMVEMA with BSA in the presence of copper ions in phosphate buffer solution at pH = 7 have been investigated by using UV-visible,

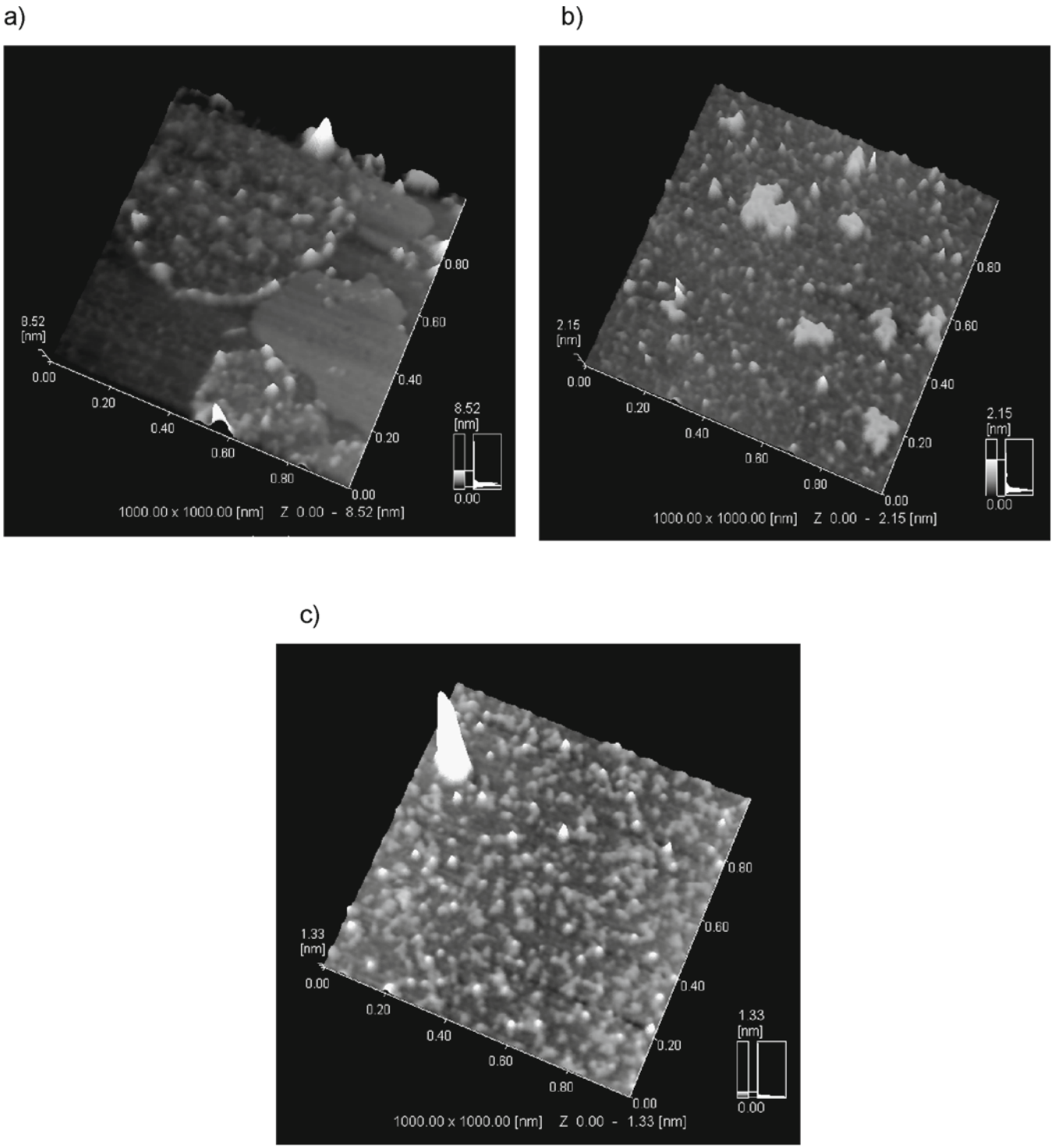


Figure 12. The toxicities of pure water (1) and the solutions of PMVEMA (2), binary mixture of PMVEMA-Cu(II) (nCu(II)/nMVEMA = 0.5) (3), ternary mixtures of PMVEMA-Cu(II)-BSA ($n_{Cu(II)}/n_{MVEMA}$ = 0.5 and n_{BSA}/n_{PMVEMA} = 2.0) (4), PAA (5), binary mixture of PAA-Cu(II) ($n_{Cu(II)}/n_{PAA}$ = 0.4) (6) and ternary mixtures of PAA-Cu(II)-BSA prepared at different BSA concentrations ($n_{Cu(II)}/n_{PAA}$ = 0.4, n_{BSA}/n_{PMVEMA} = 1.0 (7) and 2.0 (8)). C_{PMVEMA} = 3 g/L

fluorescence, DLS and AFM measurements. UV-visible, gravimetric and DLS measurements indicated that the addition of different amount of copper ions to PMVEMA gives homogeneous solutions at phosphate buffer solution at pH = 7. It was observed that insoluble particles started to occur after the molar ratio of copper ions to methyl vinyl ether maleic anhydride equals to 0.5, reaching a maximum value at the molar ratio of Cu(II)/MVEMA = 1. When the formations of ternary complexes of PMVEMA-Cu(II)-BSA were examined by UV-visible measurements, the values of A_{280} of the solution of BSA and PMVEMA-BSA increased with BSA concentration. However, the slope of the curves had almost the same value. Results suggest no interaction between BSA and PMVEMA; however, for BSA solutions mixed with the solutions of PMVEMA-Cu(II), the absorbance (A_{280}) increased more with increasing BSA concentration. When fluorescence measurements were applied to examine the formation of ternary complexes of PMVEMA-Cu(II)-BSA, the intensity of I_{max} decreased with augmentation in Cu(II) concentration due to quenching of Trp in BSA. The λ_{max} in the fluorescence measurements of ternary complexes of PMVEMA-Cu(II)-BSA increased with increasing BSA concentration

but decreased by augmentation of copper concentration. This finding showed as red shift indication in literature[2, 3, 25, 42] indicates that Trp becomes more hydrophilic feature and the interaction between Trp molecules of BSA and water increases with increasing maximum wavelength emission of PMVEMA-Cu(II)-BSA. The scanning image of the ternary mixture of PMVEMA--Cu(II)-BSA measured by AFM technique showed a uniform structure at nano-scale. The toxicity of binary mixture of PMVEMA-Cu(II) ($n_{Cu(II)}/n_{MVEMA}$ = 0.5) was higher than those of the solution of PMVEMA and ternary mixture of PMVEMA-Cu(II)-BSA($n_{Cu(II)}/n_{MVEMA}$ = 0.5 and n_{BSA}/n_{PMVEMA} = 2.0). Toxicity efficiencies of these compounds have been ordered as PMVEMA--Cu(II)>PMVEMA>PMVEMA-Cu(II)-BSA. Not only ternary complexes were lower than binary complexes but also lower than pure polymer. When solution toxicities of mixtures of PMVEMA and PAA with/without BSA in the presence of copper ions were compared, the toxicities of the solution of PMVEMA, PMVEMA-Cu(II) and PMVEMA-Cu(II)-BSA increased in comparison to solutions of PAA, PAA-Cu(II) ($n_{Cu(II)}/n_{AA}$ = 0.4) and PAA-Cu(II)-BSA ($n_{Cu(II)}/n_{AA}$ = 0.4 and n_{BSA}/n_{PAA}= 1.0). When the toxicity of the ternary mixtures, containing two different BSA concentrations were compared, the toxicity of the ternary mixture of PAA-Cu(II)-BSA prepared at the molar ratio of protein/polymer = 1.0 (Fig. 12, sample 7) was lower than the ternary mixture of PAA-Cu(II)--BSA (Fig. 12, sample 8) prepared at the molar ratio of protein/polymer = 2.0, revealing the contrast between PMVEMA with PAA polymers. Additionally the toxicities of solutions of polymer, polymer-metal and polymer--metal-protein mixtures depend on the nature of pure components and the ratio of components in mixtures. Most significant finding was its use in biotechnological applications, such as antitumor agent and new type of synthetic immunogens.

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ABBREVIATION

BSA	– Bovine serum albumin
PMVEMA	– Poly(methyl vinyl ether-*co*-maleic anhydride)
PE	– Polyelectrolyte
PMC	– Polymer-metal complexes
M	– Metal
MVEMA	– Methyl vinyl ether maleic anhydride
pI	– Isoelectric points
DLS	– Dynamic light scattering
AFM	– Atomic force microscope
PAA	– Poly(acrylic acid)
MTT	–3-(4,5-Dimethylthiazol-2-yl)-2,5-diphen– yltetrazolium bromide, a yellow tet– razole

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