Fluorescence Study of Peptide and Protein Containing Interpolyelectrolyte Complexes

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Summary: Four polyelectrolyte (PE)-protein and PE-peptide systems were selected for fluorescence experiments. The mode of binding of proteins to PE and structure of forming polycomplexes depends upon the ratio of components and chemical composition of polymer macromolecules. Complex formation of proteins with oppositely charged polycations is realized by the self-assembly of non-stoichiometric polycomplex particles with characteristic composition. These polycomplex particles have a rigid rod-like carcass structures in which protein molecules are practically exposed to the solvent.
Two types of ternary polyanion-Cu^{2+}-protein polycomplex particles are formed depending on the monomer composition of the copolymer. At higher content of hydrophobic N-isopropylacrylamide monomer unites, the protein globules in the structure of ternary polycomplex particles are densely covered by the shell of a polymer coil and practically "fenced of" from the water environment. At higher content of acrylic acid unites, polycomplex particles have more friable structures in which protein molecules are practically exposed to the solution.
The PE-peptide conjugate species can be represented rather as a macromolecule of a segmented (block) copolymer in which the hydrophobic blocks, i.e. the sequences of copolymer and peptide unit pairs, which have formed the covalent and salt bonds alternate with hydrophilic ones, i.e. the sequences of the copolymer chain not participating in the formation of double strand blocks.

Keywords: bioengineering; covalent conjugates; fluorescence; peptides; polyelectrolytes; proteins

Introduction

Synthetic polyelectrolytes (PE) have been widely used to modify proteins via complex formation and covalent attachment, increasing (or reducing) the immunoreactivitiy and /or immunogenecity of originally antigenic proteins and improving their *in-vivo* stability with prolonged clearance times. Such conjugates seem to be of great importance for medicine and immunobiotechnology in particular with respect to drug delivery and vaccine innovation.[1-4]
The formation of polycomplexes (and/or conjugates) in the PE-protein mixtures and their structure were intensively studied by titration, high-performance liquid chromatography (HPLC), light scattering, electrophoretic, spectrophotometric and hydrodynamic (viscosity and sedimentation) methods.[5,6] The intrinsic tryptophan (Trp) fluorescence of proteins been

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used in some instances as a probe for the changes in conformation or microenvironment of a protein which can occur upon interaction with polymers.[7-9] Therefore, in this article the spectral changes in fluorescence, especially the emission maximum (λ_{max}), are used as parameters for the conformational changes of proteins indused by the formation of complexes (and conjugates) with synthetic PE. Much productive work has been done recently for creating highly efficient polymeric immunogens and synthetic vaccines based on polycomplexes of proteins and peptides.[4,10-13] We will discuss the mode of binding of serum proteins (bovine serum albumin-BSA, human serum albumin-HSA) and peptides (peptide epitops of VP1 protein of Foot-and-Mouth Disease Virus-FMDV) to PE and structure of forming polycomplexes depending on the ratio of components as determined by fluorescence measurements. These polycomplexes have been studied in detail and described in the literature.[14-17]

Complexation of Serum Albumins with Polycations

Typical fluorescence spectra of pure BSA and BSA in mixtures with poly(N-ethyl-4-vinylpyridine) (PEVP) at different molar ratios R_i ($R_i=n_{PE}/n_{BSA}$, where n_{PE} and n_{BSA} are molar concentration of the polymer and the protein, respectively), at pH 7 are shown in Figure 1.

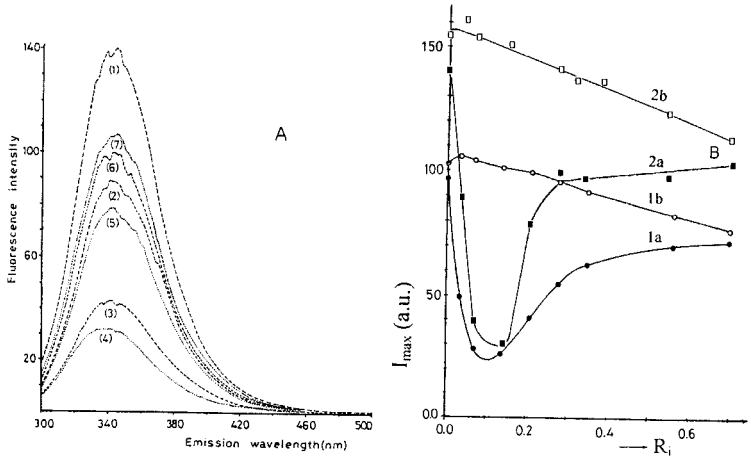

Figure 1. (A) Fluorescence spectra of BSA at 5.7μM (1) in the presence of increasing amounts of PEVP at pH 7.0. R_i = 0.035 (2), 0.07 (3), 0.14 (4), 0.21 (5), 0.35 (6) and 0.7(7); pH 7.0,20 °C. (B) Emission intensities (I_{max}) at 340 nm versus R_i for PEVP-BSA (1a, 1b) and PEVP-HSA (2a,2b) mixtures at pH 7.0 (a) and pH 4.3 (b).

Intensity of BSA fluorescence in maxima (I_{max}) is quenched in the presence of PEVP and depends on molar ratio of components. The emission maximum (λ_{max}) of protein solutions were not effected by the concentration of PEVP. As the polymer concentrations in the mixture were increased, I_{max} of the solution decreased and passed through a minimum at about R_i=0.1 and then increased beyond R_i=0.2 to a constant value (Figure 1b). For the mixture PEVP+HSA the dependence between I_{max} vs. R_i is practically the same as that for PEVP-BSA solution. Emission intensity quenching at the pH=7 is due to the binding of protein molecules to the polycation chain since the intensity of solution increases again beyond R_i=0.2. The maximum number of protein molecules are bound to the polymer at about R_i= 0.1,[5]; this is based on the maximum reduction in fluorescence intensity. The electrostatic repulsive forces between positively charged PEVP and proteins (BSA and HSA) prevent the stable complex formation in acidic solution, pH= 4,3. The emission intensities of proteins were not effected by the concentration of polymer (Figure 1b, curve 3). The obtained value of λ_{max} suggests that at neutral pH in the structure of polycomplex particles and protein molecules are practically exposed to the solvent. The composition and structure of them depends on mol ratio of components: electrostatic interaction between protein molecules and oppositely charged PEVP promote the formation of a non-stoichiometric polycomplexes at $R_i \geq 0.3$ (at higher concentration of polycations) by the cooperative self-assembly mechanism. Excessive ionised groups on PEVP forms an extensive hydrophilic area on the protein surface, which promotes the solubility of polycomplex particles, since the polycomplex has a characteristic composition and loop-like organization. By decreasing the mole ratio (R_i), the inorganic groups of the polycations within the polycomplex do not form salt bonds with the protein molecules (loops) are responsible for binding supplementary protein to the polycomplexes (secondary interactions), which leads to intense quenching of protein fluorescence.

Cu^{2+} - Induced Interaction between BSA and Anionic Polyelectrolytes

It is known that under conditions where both PE (poly(acrylic acid)-PAA, copolymers of acrylic acid with N-isopropylacrylamide-(poly(NIPAA$_m$-AA)) and BSA have negative charges and are incapable of binding to one another, divalent Cu^{2+} ions act as "fasteners", promoting the formation of a fairly stable water-soluble ternary complexes.[10] The solubility, composition and stability of these polycomplexes depend on composition of PE macromolecules and metal/PE (or protein) and protein/polymer ratios. In this study, BSA interactions with PAA and poly(NIPAA$_m$-AA) in different monomer compositions at the presence of Cu^{2+} ions were investigated by fluorescence methods. The idea was to use Cu^{2+}

both as an important component for the stabilization of the complexes and as a quencher. Control experiments were made with a different quencher (succinimide, potassium iodide, and cesium chloride), which do not form complexes with the polymers used. The monomer compositions (r) of copolymers poly ($NIPAA_m$-AA) used in this study were r=$NIPAA_m$/AA=3:1(P25), 1:1(P50) an1: 3 (P75), PAA(P100) and poly ($NIPAA_m$)(P0). The heterogenecity of polymers and proteins and the fraction compositions of the mixtures were estimated by the HPLC system. Fluorescence emission spectra were obtained using a Quanta Master spectrafluorimeter, the fluorescence intensity was measured using 280 nm excitation and 340 nm emission wavelength.

Study of a BSA tryptophan fluorescence for PE-Cu^{2+}-BSA mixtures in homogenous systems at different component ratios and polymer compositions permits elucidation of some important features characterizing ternary polycomplex formation (Figure 2).

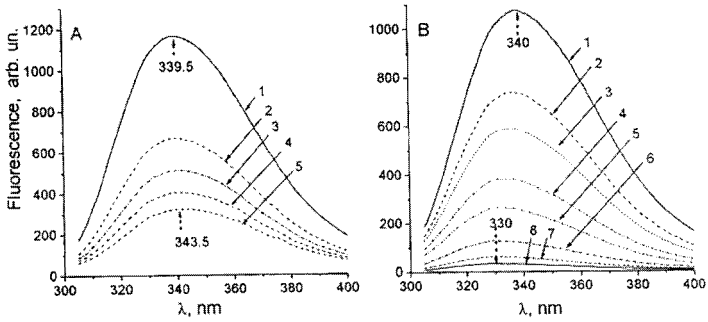

Figure 2. Fluorescence spectra of pure BSA (A) and BSA in mixtures of poly(NIPAAm-AA)-BSA (P50/BSA=3.52) (B) at different concentrations of [Cu^{2+}] ions in solution. BSA concentrations 0.71 mg/ml, phosphate buffer (pH7). CuSO4 concentrations (in mM): 0(1), 0.15(2), 0.3(3), 0.45(4), 0.6(5), 0.9(6), 1.2(7), 1.5(8).

The fluorescence intensity (I_{max}) of pure BSA at pH 7 decreases (quenching) and λ_{max} shows some red shift at Cu^{2+} concentration increase. Analysis of the dependence of λ_{max} and I_{max} vs. [Cu^{2+}] for BSA in the ternary mixture show that BSA fluorescence depends on the chemical composition of polymers and ratio of components. For the mixture P0+BSA the dependence of I_{max} and λ_{max} vs [Cu^{2+}] for all P0 concentrations is practically the same as that for pure BSA in solution, which witnesses for the absence of any interactions between BSA and P0 through Cu^{2+} ions. The pattern is quite different with the presence of polymer P50: as the fluorescence is quenched by increasing [Cu^{2+}], its maximum shifts toward the blue region . As the values of I_{max} of BSA in the presence of the same amount of PE were decreased by the increasing of

[Cu^{2+}] and practically do not depend on the polymer concentration in mixtures, the position of protein fluorescence was very sensible to the polymer/protein ratio (Figure 3).

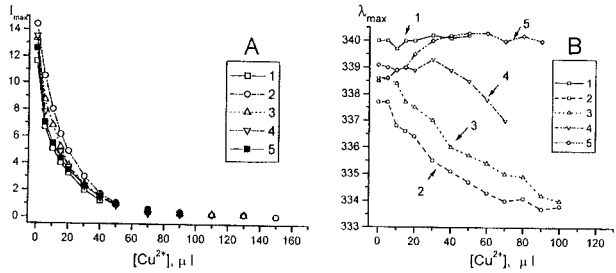

Figure 3. Intensity of BSA fluorescence in maxima (I_{max}) (A) and position of fluorescence maximum of BSA (λ_{max}) (B) for a mixture poly(NIPAAm-AA)-Cu^{2+}-BSA vs. Cu concentration in phosphate solution(pH 7) at different P50 concentrations(mg/ml): 0(1), 5(2), 2.5(3), 1.2(4), 0.83(5).

The values of λ_{max} were decreased by the increasing of both metal and polymer concentrations. 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. The blue shift indicates that the environment of the tryptophan becomes more hydrophobic and shielded from the aqueous solvent, which can be understood from the partially hydrophobic nature of the polymer backbone.

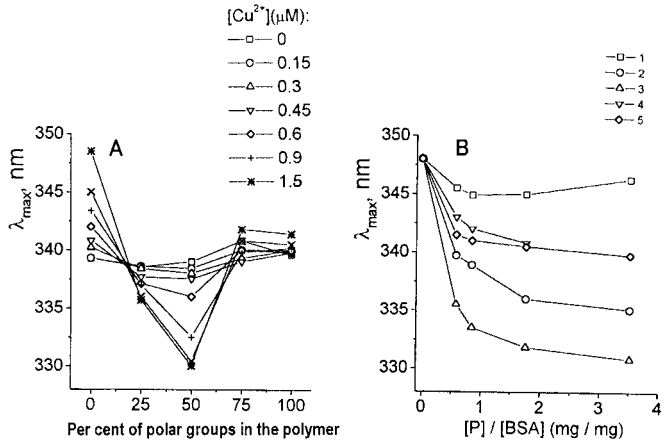

Figure 4. Position of fluorescence maximum (λ_{max}) for PE-Cu^{2+}–BSA mixtures vs. percent of –COOH groups in the PE (A).PE/BSA=1.76 and vs ratio PE/BSA (B) for different polymer compositions:1(P0), 2(P25), 3(P50), 4(P75), 5(P100). [Cu^{2+}]=1.2mM, pH7.

The fluorescence behavior of the systems is quite different for another composition of copolymers (Figure 4A). In the case of P25 the spectral shifts are less pronounced. So P25 covering of BSA in complex P25-Cu^{2+}-BSA looser and, as a result, BSA tryptophanyls are not completely isolated from water solution. P75 and P100, which contain 75 and 100 % of polar side chains, accordingly, must be strong binder of Cu^{2+} ions. However, at their presence in solution and [Cu^{2+}] increasing, the BSA spectrum shifts to longer wavelengths as for pure BSA but less effectively. A Cu^{2+}-dependent red shift suggests that at neutral pH they do not form a covering around BSA molecules. It is remarkable that the values of λ_{max} depend on the ratio of components and decreased with increasing of polymer concentration (Figure 4B).

Covalent Conjugates of BSA with Anionic Polyelectrolytes

The objective of the present study is to examine the covalent binding mechanism of BSA with poly(NIPAA$_m$-AA) copolymers depending upon the weight concentration ratio (C_{BSA}/C_{Pol}) of BSA to copolymer by intrinsic fluorescence measurements. The fraction composition of BSA-copolymer conjugates (NIPAA$_m$/AA= 1:1; P50) preparing by the activation of water soluble carbodiimide, and degree of binding were estimated by HPLC, electrophoretic and fluorescence measurements.[17] These conjugates, which had been prepared at different C_{BSA}/C_{Pol} were studied by fluorometric method. The characteristics of BSA-P50 conugates are given in Table 1.

Table 1.Characteristics of poly(NIPAAm-AA)-BSA covalent conjugates

C_{PE} / C_{BSA}	β	RT_n (min)	RT_{SDS} (min)	I_{fl}	λ_{max} (nm)
3.60	48	10.5	10	4.4	338.0
1.70	39	13	11	6.5	339.9
0.90	35	15	11	9.2	340.9
0.60	35	15	—	9.5	341.0
BSA	—	15	12.5	10	341.0

[a]RT_n and RT_{SDS}— retention times in native and SDS-electrophoresis;
I_{fl}— intensity of fluorescence in maxima, λ_{max} — position of fluorescence maximum;
β— average number of binding NH_2 – groups.

The results, obtained by the analysis of the electrophoretic measurement data are conformed by the data of fluorescence analysis. The character of binding depends on the initial ratio of reaction components. The emission maximum of BSA at 340 nm was seen to shift to shorter wavelengths and became reduced in intensity with increasing polymer concentration. This indicates that in the conjugate particules, BSA tryptophanyls are partially isolated from water solution by the polymer, which covers apparently the protein surface. By increasing the amount of protein, the position of the maximum of the spectrum approaches λ_{max} of free BSA. Thus at $C_{BSA}/C_{Pol} > 1$ the polymer chains do not form covering around particles possess more friable structures in which protein molecules are practically open for the solution.

Covalent Conjugates of FMDV Peptide with Anionic Polyelectrolytes

Triptophan residues containing peptide [Trp+(135-160)] (P) serotype of FMDV was synthesized by Merrifield solid-phase technique using α-NH_2 terminus of polypeptide (135-160) fragments an automatic synthesizer (Millipore's Automated Peptide Synthesizer, USA).

P: Trp-Lys-Tyr-Ser-Ala-Thr-Gly-Glu-Arg-Thr-Arg-Gly-Asp-Leu-Gly-Ala-Leu-Ala-Ala-Arg-Val-Ala-Thr-Gln-Leu-Pro-Ala

Poly(NIPAA$_m$-AA) (P50)-Peptide conjugate was prepared by WSC activation method in surfactant stabilized micro emulsion of water in organic solvents (Hydrated Reversed Micelle Systems-HRM).[12] The fraction composition of reaction products was characterized by gel filtration and ion exchange HPLC. The yield of conjugation (α-NH_2 contents) studied by fluorescamine method. The results in Figure 5 indicate that peptide P solution shows discrete (structured) emission spectra at λ_{max}~315 and 325 nm. Such Trp residues were attributed to class S in the hypothesis of discrete states.[18] Therefore, P tryptophanyls exist in the hydrophobic environment of polypeptide chain and completely isolated from water solution.

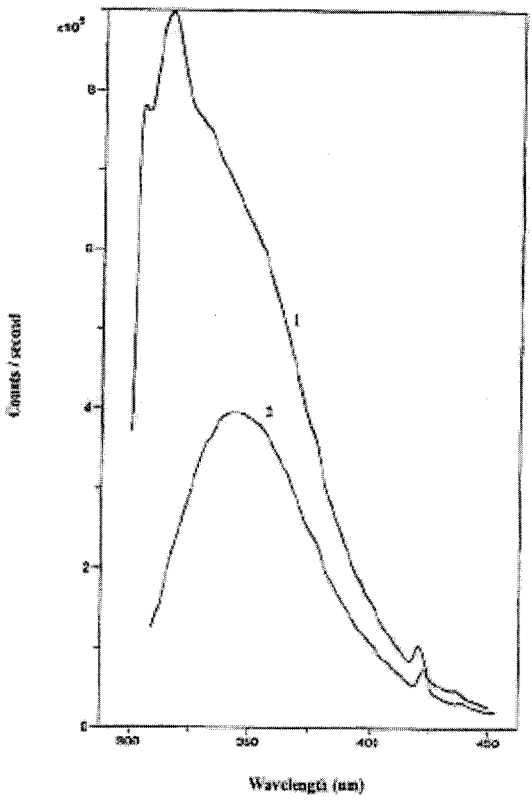

Figure 5. Fluorescence spectra of pure FMDV peptide and P50-peptide conjugate in water solutions. Peptide concentrations 0.07 mg/ml; phosphate buffer (pH 7.0), 25°C. Quanta Master spectrofluorimeter (Photon Technology International, Canada)The excitation wavelength 280 nm.

Previously, the large hydrophobicity and amphipatic α-helical structure of 135-160 fragments was demonstrated by Pffaf and co-workers.[19] The fluorescence intensity (I_{max}) of peptide after conjugation with P50 decreases (quenching) which testify conjugate formation. On the other hand, conjugation of P peptide with copolymer macromolecules induces a marked red shift of λ_{max}. This indicates that in the P50- peptide conjugates, peptide Trp as compared with pure peptide molecules are essentially exposed to the solution.

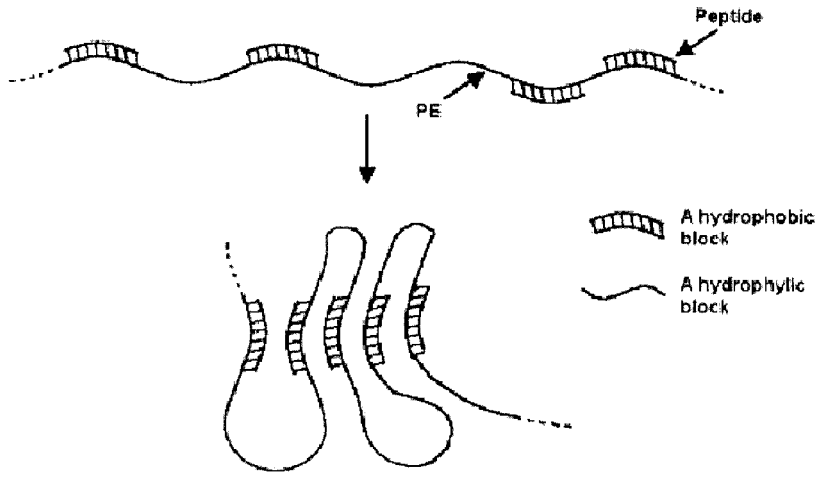

Figure 6.Schematic representation of polyelectrolyte-peptide conjugate species.

We can assume that the conjugate species can be represented rather as a macromolecule of a segmented (block) copolymer in which the hydrophobic blocks, i.e. the sequences of copolymer and peptide unit pairs which have formed the covalent and salt bonds alternate with hydrophilic ones, i.e. the sequences of the copolymer chain not participating in the formation of double strand blocks (Figure 6). Such mechanism proposed "frozen" of peptide molecules in the structure of conjugate at the unfolding state, which Trp environment are exposed to the solution.

In a later study we have shown that these peptide containing polymeric conjugates characterize the higher immunogenecity. This immunogens were used in vaccinating guinea pigs for estimation of the potency against FMDV and dose dependent high protection was achieved. Such a modulated system is attractive for application as a novel immunogenic system in vaccine technology.

Conclusions

The present study demonstrates that the mode of binding of proteins to PE and structure of forming polycomplexes depends upon the ratio of components and chemical composition of polymer macromolecules. Complex formation of proteins with oppositely charged polycations at low protein concentrations ($R \geq 0.3$) is realized by the self-assembly of non-stoichiometric polycomplex particles with characteristic composition (nonuniform distribution of protein globules among PE (hosts) in electrostatic complexes). After $R < 0.3$ the character of the

reaction changes, polycomplex particles possess ability for binding supplementary protein molecules via free polymer sections (secondary interactions). These polycomplex particles have a rigid rod-like carcass structures in which protein molecules are practically exposed to the solvent.

When polymer-metal complexes solution is titrated with protein solution, the protein globules are cross linked with a linear polyion via copper ions .One can assume that the protein molecules were uniformly distributed between polymer coils and the number of protein globules binding with one polymer chain increased by the increasing of the protein/polymer ratios. Two types of ternary polycomplex particles are formed depending on the monomer composition of the copolymer. At higher content of hydrophobic N-isopropylacrylamide monomer unites, the protein globules in the structure of ternary polycomplex particles are densely covered by the shell of a polymer coil and practically "fenced of" from the water environment. At higher content of acrylic acid unites, polycomplex particles have more friable structures in which protein molecules are practically exposed to the solution.

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