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Conjugation of BSA Protein and VP/AA Copolymers

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Abstract:

Macromolecular complex and water-soluble covalent conjugate formation of poly(N-vinylpyrrolidone-2-co-acrylicacid) (VP/AA 2:1) with bovine serum albumin (BSA) was studied at different pH and investigated by spectrofluorometric method. Conjugates of VP/AA-BSA were synthesized by cross-linking the carboxyl groups of anionic VP/AA copolymer to the amino groups of bovine serum albumin by using 1-ethyl -3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) as a coupling reagent. Water-soluble complexes of VP/AA with BSA were formed by mixing copolymer and protein solutions at different molar ratio ($n_{VP/AA}/n_{BSA}$) and different pH. When the pH is higher than the isoelectric pH of BSA (pI) the stability of the VP/AA-BSA complexes was weak, however when pH is approximately equal to pI of BSA (pH 5.0), VP/AA-BSA complexes were stable. The fluorescence spectroscopy results of VP/AA-BSA complexes and water-soluble covalent conjugates showed decreased fluorescence intensities and a distinct blue shift on maximum wavelength (λmax.). This indicates that tryptophanyls of BSA were completely isolated from water when the complex or conjugates are formed between VP/AA and BSA.

Key words: Polyelectrolyte, polyelectrolyte-protein complex, covalent conjugation, fluorescence.

INTRODUCTION

Protein-polymer conjugates are important and widely employed for applications in biotechnology and medicine. Covalent conjugation of proteins with synthetic polymers improves protein stability, solubility, and biocompatibility [1]. Water-soluble and insoluble protein–polyelectrolyte complexes as functional biopolymer systems represent a specific class of polymer–protein compounds that have important applications in various areas [2-5].

The PE conjugates and stable complexes with viral antigens reveal high immunogenicity, increase protective properties and they can be estimated as novel vaccines [3-5, 6-22]. It is well known that foreign natural polyelectrolytes (proteins, polysaccharides, nucleic acids) and their structural analogs (polypeptides, polynucleotides) in the organism manifest the properties of antigens. In addition, natural polyelectrolytes (polysaccharides, native nucleic acids, doublestrand synthetic polynucleotides) are known to activate the immune system toward other antigens, that is, serve as immunostimulants. It was of interest to understand how the system reacts to unknown synthetic polyelectrolytes (SPEs), whose chemical structures do not resemble biopolymers [10].

Studies carried out by R. V. Petrov et al. showed that poly(acrylic acid), (PAA) is a strong stimulant of immunogenesis. However, in view of its high toxicity, the preparation did not gain wide application in immunological research. To lower the toxicity of PAA, a series of copolymers of N-vinylpyrrolidone with acrylic acid of different compositions and molecular weights were synthesized. It was found that the copolymers of N-vinylpyrrolidone with acrylic acid, just as PAA, give a pronounced immunostimulating effect. Based on this, these copolymers were considered as a prospective basis for a future medicinal form and decided to study the distribution and paths of elimination from the organism of copolymers of PAA with N-vinylpyrrolidone (1:1, mol. wt. 40,000 and 100,000). The information obtained in these investigations was very useful for producing and testing new physiologically active compounds [23].

The complexes of synthetic polyanions with globular proteins have great importance in medicine, bioengineering and wide areas on basic science. These systems can be considered as a model of antigen-antibody interactions, specific nucleoprotein complexes and nucleotide-protein complexes, in particular supplying us with novel information on immunogenic compounds for synthetic vaccine innovation.

Ability of anionic copolymers to form complexes with bovine serum albumin (BSA) depends on both the composition of the copolymers and the pH of the medium [24]. Polymer-Protein complexes are formed as a consequence of interaction of polyion chain and oppositely charged groups in proteins [21,25]. Binding of polyelectrolyte to protein depend on the molar ratio of components, as a result of binding polycomplex [26-27] complex coaservats or amorphous precipitate can be occur.

In recent years there has been much attention to the preparation of bioconjugates by the derivates of prepared synthetic polymers with biological macromolecules using simple activation chemistry. It is known that conventional methods of synthesis of protein-protein and protein-linear PE covalent conjugates are based on reactions between activated functional groups of macromolecules in aqueous solutions [28]. A whole series of well-known reactions can be used to activate functional groups of a polymer carrier or a protein molecule and link them together in solutions by using different cross linker agent such as 1-ethyl -3-(3-dimethylaminopropyl) carbodiimide hydrochloride.

The first step of conjugation reactions, the carboxyl group is activated by the carbodiimide to form an O-acylisourea intermediate and which can react further with an amino group of protein molecules in the second step. Reaction with an amino group from a protein will lead to a cross-link between the polymer and protein components [5,13,21-22,29-33].

Mustafaev and his colleague investigated the covalent conjugations and complexes of protein antigens with polyanions, such as copolymer of acrylic acid with N-vinylpyrolidone [29,34]. It was shown that the formation of the electrostatic inter (polymer–protein) complexes in carbodiimide activated PE–protein mixtures occurs as a most rapidly proceeding process and then the condensation between carboxyl and amino groups of components (covalent conjugation) occurs [22].

In this study, the interaction between Bovine Serum Albumin (BSA) and Vinyl Pyrrolidone-Acrylic acid copolymer (2:1) will be discussed on the basis of experimental results obtained by a QM-4/2003 Quanta Master Steady State Spectrofluorimeter.

MATERIALS AND METHODS

Poly(N-vinylpyrrolidone-2-co-acrylic acid) (VP/AA = 2:1 ratio) was synthesized and fractioned as explained in the literature [35]. Glacial acrylic acid and N-vinylpyrrolidone-2 were polymerized in THF with benzoyl peroxide and cobalt naphthenate at 70 ^{o}C under nitrogen for 3 hr. The white solid was filtered and dried at 40 ^{o}C in vacuum.

Acrylic acid (Aldrich, Germany), vinylpyrrolidone-2 (Fluka), THF (Riedel-de Haen), benzoyl peroxide (Fluka) and cobalt naphthenate (Fluka) analytical grade reagents. Fractionally distilled solvents were used in the reaction. Bovine Serum Albumine (BSA) and 1-ethyl-3-(3-dimethylaminoprophyl) (EDC) were from Sigma Chemical Company, St. Louis, MO. NaH_2PO_4, Na_2H-$PO_4.7H_2O$, NaCl were obtained from Fluka and NaN_3 was from Applichem. Ultra pure water was obtained from Millipore MilliQ Gradient system.

In this study we aimed preparation of Protein-Copolymer complexes and conjugates of Bovine Serum Albumin (BSA) and copolymers of 1-Vinyl-2-pyrrolidone/acrylic acid (VP/AA 2:1).

BSA proteins - VP/AA Copolymer Complexes

BSA and VP/AA were dissolved in Phosphate Buffer (pH=7) and acetate buffer (pH=5) containing 0,15 M NaCl at 25 °C. To prepare BSA protein constant, VP/AA copolymer variable complexes (at pH 5 and pH 7); various concentrations of the protein solutions were added to BSA solutions (the weight concentrations of BSA was kept constant and equal to 1,0 mg/ml)

BSA proteins - VP/AA Copolymer Covalent Conjugates

In this study we synthesized of Bovine Serum Albumin (BSA) and copolymers of 1-Vinyl-2-pyrrolidone/acrylic acid (VP/AA) conjugates with carbodiimide method by using 1-ethyl -3-(3-dimethylaminopropyl) carbodiimide hydrochloride. The protein-copolymer conjugates were synthesized different molar ratio [for one protein molecule, different ratios of copolymer ($n_{protein}/n_{copolymer}$)] and investigated binding mechanism of polyelectrolyte to BSA protein. Activation of copolymer acid groups were carried out in water (pH 5.0) in a molar ratio 2 : 1 (EDC : AA). For this, VP/AA was dissolved in water, stirred at 12 h and EDC was added. After 1 h, the pH value of the mixture was adjusted to 7.0 with 1M NaOH. Then BSA solution in different concentrations was added to the reaction mixture and stirred for 12 h. After removal of O-acylisourea intermediate by dialysis, the sample was lyophilized. To purify the conjugates, the reaction mixture was passed through a column with TOSOH SP-550C (Toyopearl), equilibrated by 0.01 M phosphate buffer at pH 7.

The purified conjugates were dissolved in phosphate-buffered saline (PBS) at 25 °C for the further analysis. Complexes and purified conjugates were characterized and the mechanism of the binding process was investigated comparatively with BSA and VP/AA copolymer by Fluorescence Spectrophotometer.

For the experiments, VP/AA-BSA complexes and conjugates ratios ($n_{VP/AA}/n_{BSA}$= 1, 3, 5) were calculated using the equation, $n = C.N_A/M$ where n presents the number of the molecules in 1 ml, M is the molecular weight of the components, N_A is Avagadro's number and C represents concentration in mg/ml.

Fluorescence Measurment

Fluorescence emission spectra were obtained using a QM-4/2003 Quanta Master Steady State 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 was obtained at 280 nm. Binding of substrates, association reactions, denaturation and interaction with other macromolecules may result in the changes of protein fluorescence spectra. In the present study we characterized them by the wavelength at the maximum emission (λ_{max}) and maximum fluorescence intensity (I_{max}) [36].

RESULTS AND DISCUSSION

Covalent conjugation mechanism is investigated depending on changes in fluorescence properties of tryptophane amino acid which exists in protein molecule. Water soluble VP/AA-BSA complexes and conjugates occurrence and important changes in their 3D structure are determined according to components ratio and pH degree of media. Therefore, in this article the spectral changes in fluorescence, especially the emission maximum (λ_{max}) and fluorescence intensity (I_{max}) of BSA, are used as parameters for the conformational changes of proteins induced by the formation of complexes and conjugates with VP/AA copolymer.

Fluoresans Measurements Results

Typical fluorescence spectra of pure BSA and BSA in complexes with VP/AA copolymer at different molar ratio ($n_{VP/AA}/n_{BSA}$=1,3,5) at pH 7 and pH 5 are shown in Figure 1 and Figure 2.

As it shown in Figure 1 the emission maximum of VP/AA-BSA complexes at pH 7 is practically the same as pure BSA in solution (λ_{max}=337 nm). Thus at pH 7 Coulombic repulsive forces between the negatively charged

protein and the negatively charged polyelectrolyte prevent the formation of stable polycomplexes and the VP/AA and BSA molecules. There for protein and polymer formed weakly complexes at pH 7. λ_{max} of complexes which are around 336 nm proved this condition.

The complex formation between copolymer and protein depends on the value of pH. The results shown in Figure 2 that at pH=5, which approximately match to isoelectric point of BSA (pI=4.9 [37]) influences the binding of BSA protein with VP/AA copolymer and the formation of stable water-soluble VP/AA-BSA complexes. BSA molecules have positively charged groups at pH=5 because of this it formed complexes with negatively charged copolymer as it shown in Figure 2. The fluorescence intensity (I_{max}) of BSA at pH 5 decreases and shows a marked blue shift of the λ_{max} for free BSA and VP/AA-BSA complexes from 337 to 324 nm. This is point that when complexes were formed between VP/AA and BSA tryptophanyls of BSA were isolated from water.

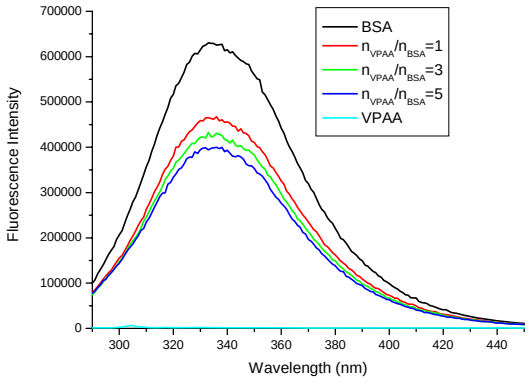

Figure 1: Fluorescence spectra of BSA at (1 mg/ml) in the presence of increasing amounts of VP/AA at pH 7 $n_{VP/AA}/n_{BSA}$ = 1, 3, 5 and VP/AA (1 mg/ml) pH=7, 25 °C

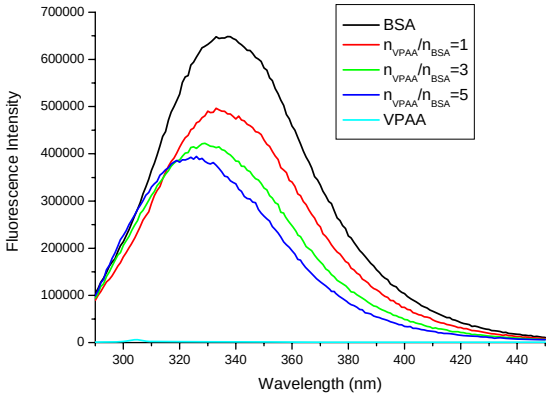

Figure 2: Fluorescence spectra of BSA at (1 mg/ml) in the presence of increasing amounts of VP/AA at pH 5 $n_{VP/AA}/n_{BSA}$ = 1, 3, 5 and VP/AA (1 mg/ml) pH=5, 25 °C

Typical fluorescence spectra of pure BSA and BSA in conjugates with VP/AA copolymer at different molar ratio ($n_{VP/AA}/n_{BSA}$=1,3,5) at pH 7 and pH 5 are shown in Figure 3 and Figure 4.

After the cross-linking of carboxyl groups of anionic VP/AA copolymer with the amino groups of bovine serum albumin, it was observed that fluorescence intensity (I_{max}) of conjugates decreased and λ_{max} slide toward blue region from 337 nm to 327 nm (Figure 3-4).

Mustafaev and his colleague considered that protein–polymer complexes are precursors of covalent conjugation reactions and thus, to prepare conjugate molecules with desired physicochemical properties, detailed analysis of the interaction (complex formation) of components is necessary [22].

It was shown in recent studies that the formation of conjugates can involve several sequential steps. The first and most important step is primary complex formation between the polymer activated by carbodiimide and the protein by electrostatic interactions and strengthening of the structure by hydrophobic interactions. Covalent cross-linking reaction is limited by structure formation and occurs on a slower time scale [5,13,21-22,29-33].

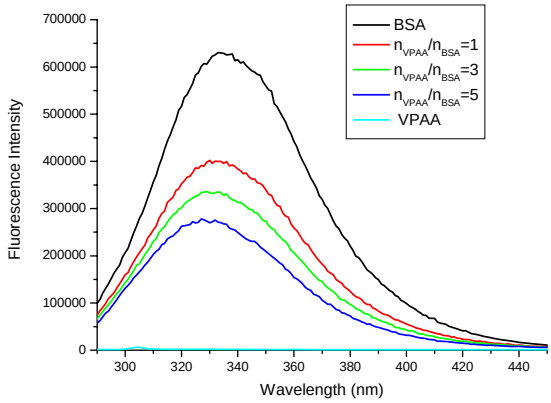

Figure 3: Fluorescence spectra of BSA at (1 mg/ml) and VP/AA-BSA conjugates at pH 7 $n_{VP/AA}/n_{BSA}$ = 1, 3, 5 and VP/AA (1 mg/ml) pH=7, 25 °C

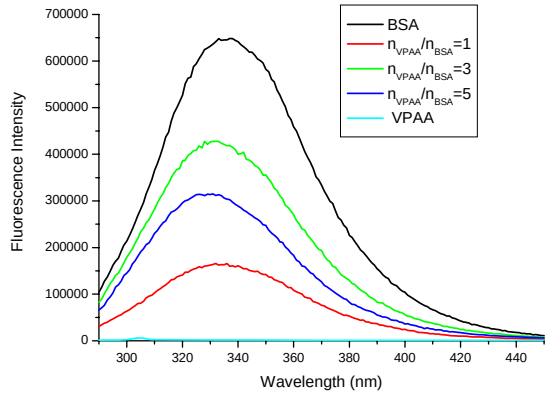

Figure 4: Fluorescence spectra of BSA at (1 mg/ml) and VP/AA-BSA conjugates at pH 5 $n_{VP/AA}/n_{BSA}$ = 1, 3, 5 and VP/AA (1 mg/ml) pH=5, 25 °C

In this study we prepared complexes and covalent conjugates of VP/AA with BSA and investigated changes of BSA 3D structure by applying Tyrptophanes fluorescence properties. It is well known that tryptophan (Trp) fluoresence of proteins varies with their conformational changes resulting in changes in the fluorescence parameters, such as the emission maximum (λmax), quantum yield and lifetime [38]. We showed that tryptophanyls were completely isolated from

water when the VP/AA-BSA conjugates and complexes were formed.

CONCLUSIONS

The present study we examined complex formation and covalent conjugation between VP/AA copolymer and BSA protein by using Fluorescence spectroscopy method. BSA contains two Tryptophans (Trp) [29]. One Trp that located on the bottom of the BSA hydrophobic cleft, the second Trp which has a low quantum yield is located exteriorly and completely accessible to aqueous solvent [39]. We finally conclude that the results in Figure 1-4 indicate that when the complex or conjugate formed between VP/AA and BSA tryptophanyls of BSA were completely isolated from water which seems to cover the entire BSA surface.

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