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Polysaccharide-protein Covalent Conjugates and Their Ternary Metal Complexes

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The conjugates based on polysaccharides (polyelectrolyte) and bovine serum albumin (BSA) were studied by high performance liquid chromatography, fluorescence technique. Dextran sulphate(DS)-metal-protein complexes were studied by fluorescence technique and UV-vis absorption spectroscopic analysis. BSA was coupled to polysaccharide by two methods : by covalent binding of linear macromolecules and by complex formation with metal ions. Aldehyde derivatives of dextran, methyl cellulose and hydroxyethyl cellulose were cross-linked with albumin by covalent bonds. Dextran sulphate (DS) can interact with albumin in the presence of metal ions (M). Various metals (Cu^{2+}, Zn^{2+}, Fe^{3+}, Fe^{2+}, Ni^{2+}, Cd^{2+}, Co^{2+} and Mn^{2+}) were used for formation of complexes. The character of interactions depends on the composition of [M]/[DS] and [M]/[BSA] ratio, the nature of metals and pH of medium. These studies revealed that in presence of different metal ions DS can form ternary polymer-metal-protein complexes with BSA. Fluorescence quenching by metal ions of tryptophan chromophores of BSA in structure of polycomplexes were used to reveal the structural features of soluble ternary polycomplexes. Two types of bioconjugate particles are formed depending on the method of binding and the conditions of the reaction : at covalent binding of components and at the complex formation *via* metal ions (Zn^{2+}, Ni^{2+}, Cd^{2+}, Co^{2+} and Mn^{2+}) the protein molecules in the structure of particles are densely covered by the shell of a polysaccharide coil and practically fenced of from the water environment; at the complex formation *via* metal ions (Cu^{2+}, Fe^{2+} and Fe^{3+}) the ternary polycomplex particles have more friable structures in which protein molecules are practically exposed to the solution.

Key Words: Polysaccharides, Polyelectrolytes, Proteins, Covalent conjugates, Ternary polycomplexes.

INTRODUCTION

The formation of water-soluble and water-insoluble ternary polycomplexes of proteins with synthetic polyelectrolytes (PE) in the presence of metal ions (Fe^{3+}, Ba^{2+}, Cu^{2+}, *etc.*) is previously reported[1-16]. Such PE-metal-protein polycomplexes were obtained by simple mixing of solutions of

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proteins, metal ions and PE in water at different pH values. This method consists of the use of small concentrations of metal additives, which promote the PE binding to protein molecule without causing any appreciable changes in the chemical structure of components. Some of these polycomplexes reveal strong immunogenecity and provide high level of immunological protection[1,4-9].

Some publications in the current literature are devoted to the construction of drugs based on such metal-mediated complexes and covalent bioconjugates of natural PE and antigens. Tumour targeting of recombinant human tumour necrosis factor alpha (TNF) and consequently an enhancement of anti-tumour effect were achieved through conjugation with dextran having metal (Cu^{2+}) chelating, diethylenetriamine pentaacetic acid residues based on metal coordination[17].

Dextran, methyl cellulose and hydroxyethyl cellulose polymers contain adjacent hydroxyl groups on each glucose monomer. These polymers may be oxidized with sodium periodate to create a polyaldehyde derivatives. This procedure results in two aldehyde groups, thus producing a highly reactive multifunctional polymer able to couple with numerous amine containing, molecules. Aldehyde polysaccharides can be used to couple many small molecules, such as antigens. Some of these polycomplexes provide a high level of immunological protection, which is important for immunization and vaccine production. It is known that the polysaccharide macromolecules in the structure of their protein conjugates should be characterized as protein carriers or as haptens depending on molecular weight of the carbohydrate and modes of coupling[18]. Dextran polymer particles (Sephadex G-200) mixed with BSA or sheep red blood cells (SR Bc) enhanced the protein and cell-specific primary immune response[19]. However, reduction of the antigenicity and immunogenicity of β-lactoglobulin was achieved by conjugation with CHS[20,21]. Protein conjugates of polysaccharides or of their breakdown products are being used as improved T-dependent vaccines. All conjugates induced stronger anti-dextran antibody responses than the polysaccharide, and there was observed a range of antigenicities[18].

Fluorescence techniques have recently been used to study protein-polyelectrolyte complexation[22]. From the fluorescent emission shift of tryptophan residues in proteins, it is possibble to localize the interaction between proteins and polyelectrolytes at certain protein domains. The Cu^{2+} -induced complex formation of bovine serum albumin (BSA) with synthetic anionic polyelectrolytes [polyacrylic acid [PAA], Poly(N-isopropylacrylamide) {PAA (NIPAAm)} and copolymers of N-isopropylacrylamide (NIPAAm) and acrylic acid in aqueous solution] was studied by fluorescence technique and high performance liquid chromatography[2].

EXPERIMENTAL

Dextran, dextran sulphate, methyl cellulose, hydroxyethyl cellulose and bovine serum albumin (BSA) were purchased from Sigma chemical Company (St. Louis, USA). Metal salts : $FeSO_4.7H_2O$, $MnSO_4.H_2O$, $CoCl_2.6H_2O$, $NiSO_4.7H_2O$, $CdSO_4.8/3H_2O$, $CuSO_4.5H_2O$, $ZnCl_2$, $FeCl_3.6H_2O$ were purchased from Merck (Darmstadt, Germany) and used without further purification.

Preparations of covalent conjugates: Sodium periodate ($NaIO_4$) (5×10^{-4} mol/g) was added to the solutions of dextran, methyl cellulose and hydroxyethyl cellulose in water (10 mL/g) (Fig. 1). The mixture was stirred at 20ºC for 5 h and then dialysed for 24 h. The aldehydic polymers thus obtained were recovered by freeze-drying. BSA and polysaccharides were mixed in phospate buffer (pH 7) in proportions 1:1 (w/w). Mixtures were stirred at 20ºC for 8 h.

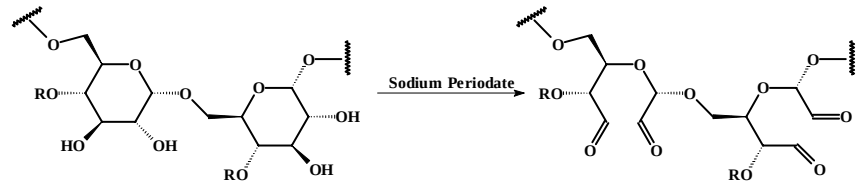

Fig. 1. Dextran polymers can be oxidized with sodium periodate to create a polyaldehyde derivative

Protein-metal-polymer complexes: BSA and DS solutions were mixed in required proportion (1:1) in phosphate buffer (pH 7). The values were adjusted with 1 M NaOH to give the desired pH. To produce a BSA-metal-DS complex, the same concentration (0.625×10^{-5} mM) of metals solutions were added to BSA-DS. n_M/n_{BSA}, $n_M/n_{Polymer}$ 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/100 mL.

Gel filtration HPLC: The heterogeneity of polymers and proteins and the fractional compositions of the mixtures were estimated by HPLC system : Gel filtration chromatography Shimpack Diol 300 column (7.9 mm i.d. × 500 mm) with Shimpack Precolumn Diol and Pump LC-10 Ai and SIL-10 Ai HPLC automatic sample injector. The eluant was monitored at 280 nm (SPD-10 Ai UV-Vis detector). The HPLC system consisted of a Waters model 501 pump, a Waters model U6K sample injector, a λ_{max} model 481 LC spectrophometer, and a Waters 746 data module integrator. A 7.8 × 300 mm stainless steel HPLC column was run in a phosphate buffer

containing 0.1 g/mol NaCl at a aflow rate of 1.0 mL/min at room temperature. The standard proteins with known molecular masses used to calibrate the column were thyroglobulin (670 kDa), immunoglobulin (155 kDa), ovalbumin (44 kDa), myoglobin (16.9 kDa) and vitamin B_{12} (1.35 kDa).

UV-vis light-absorption measurements: Spectrophotometric measurements for metal-polymer, metal-BSA and ternary complexes were made on UV-visible spectrophotometer Varian Bio-3. Absorption of metal-polymer complexes were measured on 400 nm (A_{400}), for metal-BSA and ternary complexes the measurements were taken at the maximum of absorption of BSA at 280 nm (A_{280}).

Fluorescence measurements: Fluorescence emission spectra were obtained by using the Quanta Master spectrofluorometer (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 behaviour depending on different influencing factors, which is due to the presence in almost all proteins of natural fluorophores tyrosine and tryptophan. The emission spectra of proteins are sensitive to the binding of substrates, association reactions, denaturation and interactions with other macromolecules. This results in the changes of fluorescence spectra. In present research we characterize them by the wavelength of maximum of fluorescence spectrum (λ_{max}), fluorescence intensity (I) and its width at half-maximum level ($\Delta\lambda$).

RESULTS AND DISCUSSION

Analysis of formation of covalent conjugates was carried out with HPLC-gel filtration method. Chromatogram of BSA is characterized by three peaks (Fig. 2A). On the chromatogram of BSA-aldehyde dextran (Fig. 2C) the peak of conjugate has larger area than the peak of BSA, and the peak that characterizes aldehyde dextran (Fig. 2B) disappears. Aldehyde hydroxyethyl cellulose bound with BSA (Fig. 2E) has two peaks that are different from pure BSA. BSA peaks are not seen in chromatogram. This phenomena are also observed in the case of aldehyde methyl cellulose, two peaks are observed in conjugate which are not characteristic for BSA and polymer (Fig. 2G).

HPLC analysis results indicate that protein globules bonded with aldehyde polymers form stable covalent conjugates. In equal concentrations the partial binding of BSA in aldehyde dextran is observed and the binding in the case of aldehyde hydroxyethyl and methyl cellulose is possibly complete.

Fluorescence spectra of pure BSA and protein connected aldehyde polysaccharides conjugate are presented in Fig. 3.

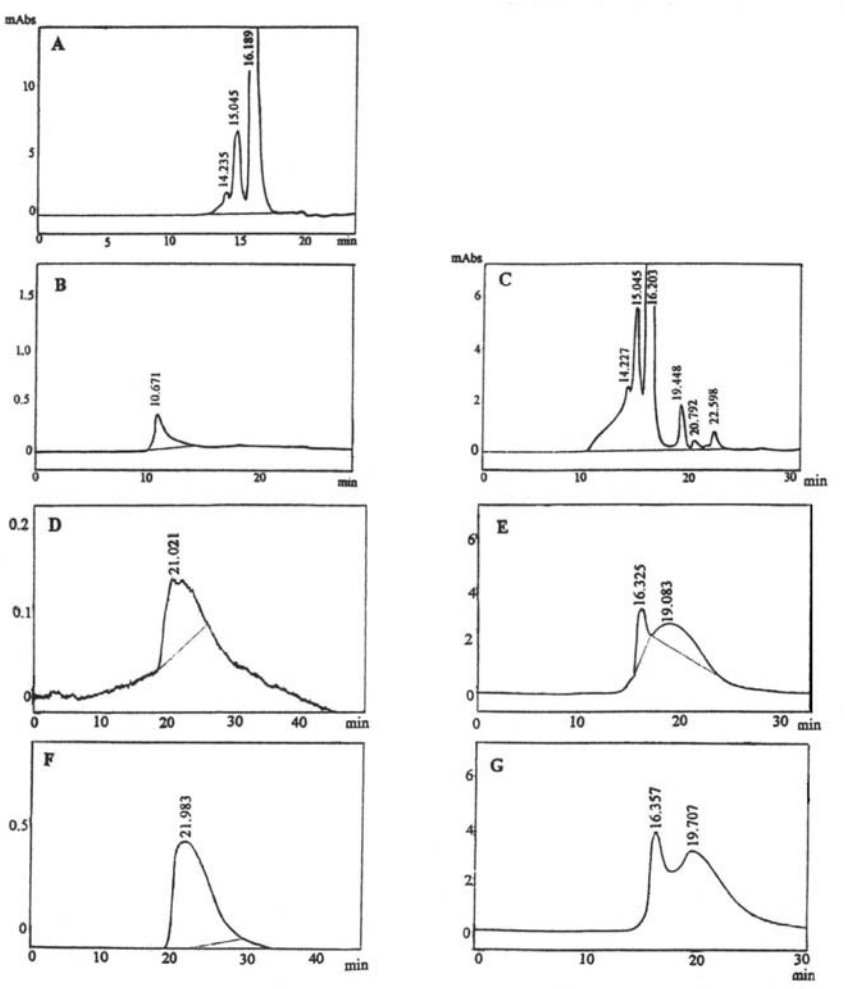

Fig. 2. HPLC results of BSA (A), aldehyde dextran (B), aldehyde dextran-BSA conjugate (C), aldehyde hydroxyethyl cellulose (D), aldehyde hydroxyethyl cellulose-BSA conjugate (E), aldehyde methyl cellulose (F), aldehyde methyl cellulose-BSA conjugate (G), concentrations of polymer and BSA 0.1 g/mL

In almost all proteins, there are natural fluorophores and the fluorescence is dominated by tryptophan residues. By conjugation reaction, the properties of protein may definitely change due to formation of new compounds polymer-protein. In all cases of aldehyde dextran, aldehyde hydroxyethyl cellulose and aldehyde methyl cellulose, spectrum of fluorescence was recorded for each of conjugate solutions at equal concentrations of BSA.

It was observed that in conjugate the fluorescence intensity is decreased substantially and the maximum is shifted to the shorter wavelenghts. In control experiments the effects of added dextran, methyl cellulose and

hydroxyethyl cellulose are not found. A small decrease of insensity is observed, possibly due to some screening of BSA tryptophanyls from water surrondings, but the shifts of BSA maximum are absent.

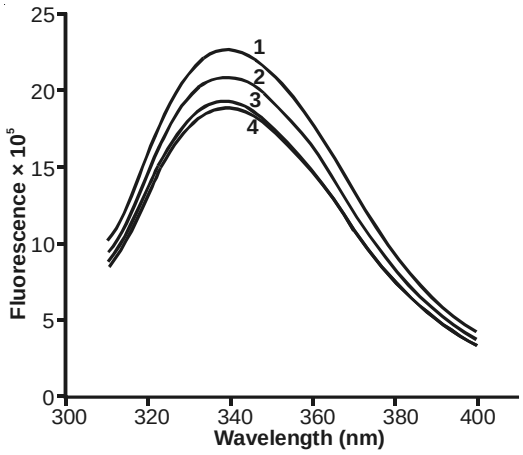

Fig. 3. Fluorescence spectra of pure BSA (1) and BSA connected with aldehyde methyl cellulose (2), aldehyde dextran (3) and aldehyde hydroxyethyl cellulose (4).

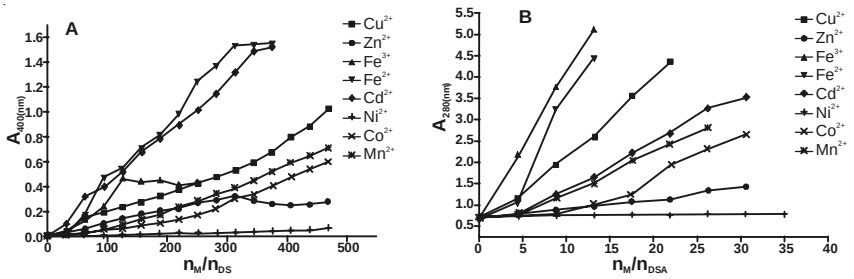

Fig. 4. Dependence of optical density for DS in the presence of metals, $A_{400\ (nm)}$ (A) and BSA, $A_{280\ (nm)}$ (B)

The results of measurement of optical density at selected wavelength 400 and 280 nm for system M-DS (Fig. 4A) and M-BSA (Fig. 4B). Our observations demonstrate a variety of effects. All the metals show different affinity to complex formation with DS and BSA. In the system DS-M, there is an increase of optical density $A_{400\ nm}$ with increasing the quantity of metal ions observed. In the system M-BSA, Fe^{2+} and Fe^{3+} increases the ultraviolet absorption already at small intial additions to solution. Regarding Cd^{2+}, the increase is observed, but to a smaller extent. For Zn^{2+} and Ni^{2+}, there is no detection of sediment, and the absorbance increases insignificantly. The ions Cu^{2+}, Co^{2+} and Mn^{2+} occupy intermediate position.

Figs. 5A-B show the positions of fluorescence maxima in systems BSA-M and ternary complexes BSA-M-DS. Intensity of fluorescence possesses similar character. In these experiments in the mixture BSA:DS of the ratio 1:1 and in simple BSA solution the concentration of BSA was equal. Solutions of metals were prepared in equal molar concentrations. Turbidimetric titration was carried out at pH 7, for the observation of precipitate formation. At lower concentrations of metal ions, the interaction can be only with macromolecules of DS due to sulphate groups in polymer chain. At higher the concentration, metal ion can act as a cross-linking agent in polymer-protein systems.

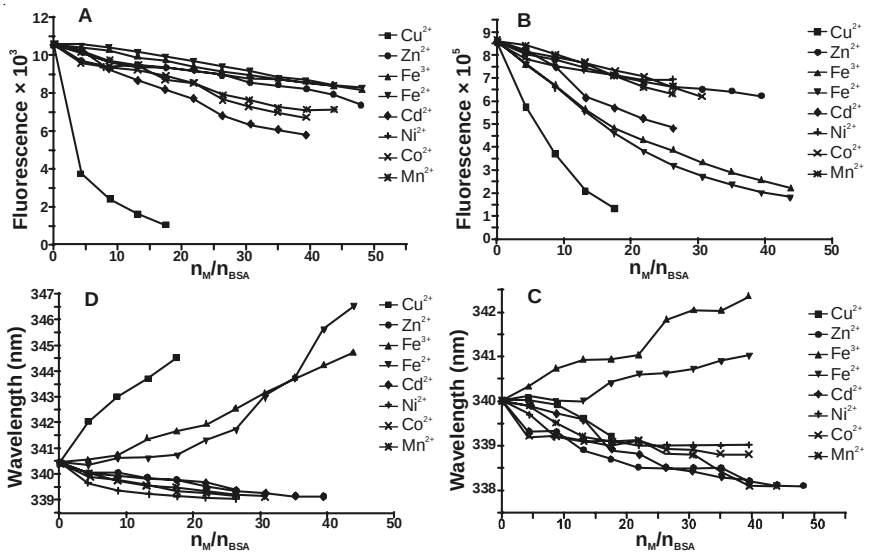

Fig. 5. Fluorescence intensity at emission maximum (A, B) and position of the maximum (C, D) for M-BSA system (A, C) and ternary complexes (B, D)

Thus, on addition of the metal ions the system remains homogeneous over a wide range of the concentration ratios [M]/[BSA]. Conformation changes in albumin molecule can be indicated as the changes in position of the maximum of ultraviolet fluorescence [Wavelength (λ_{max})]. As shown in Fig. 5C, the Fe^{3+}, Fe^{2+} has very high efficiency of Trp quenching, and as a result BSA emission spectrum shifts to longer wavelenght. For other metals the observed shift occurs towards short wavelenght, which indicates the formation of stable water-soluble complexes. Results obtained in the ternary mixture with diffirent metals at constant concentrations of protein and dextran sulphate are showed at Fig. 5D. These results demonstrate clearly that BSA fluorescence in mixtures depends on the metal ions. But the effects which they produced are different. With the exceptions of

Fe^{2+}, Fe^{3+} and Cu^{2+} all of metal ions in very low interaction shift λ_{max} to shorter wavelenghts. For Cu^{2+} it is known that this ion shifts BSA maximum in the spectra to longer wavelength[23] and this fact is supported by present experiments. For Fe^{2+} and Fe^{3+} in a ternary system the long-wavelength shift of spectra is observed.

Conclusion

On the basis of the results obtained we can suggest the following model. The two types of bioconjugate particles are formed depending on the method of binding and the conditions of the reaction. At covalent binding at the components and at the complex formation *via* metal ions (Zn^{2+}, Ni^{2+}, Cd^{2+}, Co^{2+} and Mn^{2+}) the protein molecules in the structure of particles are densely covered by a shell of a polysaccharide coil and practically fenced of from the water environment. In contrast, at the complex formation *via* metal ions (Cu^{2+}, Fe^{2+} and Fe^{3+}) the ternary polycomplex particles have more friable structures in which protein molecules are practically exposed to the solution.

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