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Quantification of the complexation of protein with neutral water borne polymer by fluorescence spectroscopy

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ABSTRACT

The complexation of bovine serum albumin (BSA) with poly(*N*-isopropylacrylamide) (PNIPAM) in an aqueous system (pH 7) has been investigated by means of fluorescence spectroscopy. Through analyzing the change of fluorescence intensity of protein chromophores caused by complexation, a quantitative mathematical equation has been established and the average number of bound proteins per neutral polymer (n_b) can be calculated accurately without destroying the dynamic equilibrium of aqueous complex system. At the same time, with the help of this equation, the extreme value of n_b can also be calculated when PNIPAM concentration is low enough. Compared with traditional calculation methods, this method has the advantages of rapid detectability, high sensitivity, accuracy and extensive applicability. Thus, it is a better way to study the complexation of protein with polymer.

1. Introduction

The interaction between proteins and polymers is of great importance in the field of biochemistry and has received much attention over the last few decades [1–9]. However, few studies have devoted to the researches of the complexation of proteins with neutral water borne polymers. This complexation is driven by hydrophobic effect and hydrogen bonding, besides there exists a dynamic equilibrium among free proteins, free polymers and complexes of proteins with polymers. It is very hard to isolate the intact complexes from aqueous system completely and to count the number of free proteins and free polymers accurately without destroying the dynamic equilibrium. Consequently, it is quite difficult to characterize this complicated complex system in quantities [1,2].

Up to now, most methods which have been adopted to measure the average complex ability of proteins with polyelectrolytes in aqueous system are inapplicable to the complexation of proteins with neutral polymers. For example, dialysis technique [1,2] is time-consuming, and with this technique, it is possible to break the dynamic equilibrium of complex system in the dialysis process and therefore the measurement results may not be correspondent with practice. Other methods, such as potentiometric titration [10] and conductometric titration [11], are also not suitable because for the complexation of protein with neutral polymer, potential and conductivity does not change obviously in the process of complex.

Today, fluorescence spectroscopy, as a simple and effective method to characterize the protein, has received wide attention [12–15]. Previous investigations have showed that fluorescence spectroscopy is sensitive to both the absorption and emission from either the intrinsic protein chromophores [13,14] or extrinsic fluorescence probe [12]. According to our investigation, the addition of neutral polymer into protein solution leads to the appearance of the fluorescent quenching of the intrinsic tryptophan chromophores, and ultimately results in the decrease of the fluorescence intensities at a given wavelength. The change of fluorescence intensity of protein chromophores was caused by the complexation of proteins with polymers. Therefore, fluorescence spectroscopy can be a kind of promising method to describe the complexation of proteins with neutral polymers in quantities. The aim of this study is to investigate the complexation of BSA with PNIPAM by fluorescence spectroscopy, and finally to establish a quantitative mathematical equation to calculate the average number of bound proteins per polymer in aqueous system.

2. Experimental part

2.1. Materials

BSA was commercially obtained from Sigma Chem. Co. It is a single polypeptide chain consisting of about 583 amino

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acid residues and no carbohydrates. At pH 5–7 it contains 17 intrachain disulfide bridges and 1 sulfhydryl group, $M_w = 66{,}430$ g/mol. Neutral water borne polymer (PNIPAM) was prepared via free radical polymerization of *N*-isopropylacrylamide (NIPAM) monomer in our laboratory and purified by dialysis method. The M_w value of PNIPAM is 45,000 g/mol.

2.2. Complex formation

Water, used as solvent, was deionized and distilled. Aqueous tris-buffer (0.02 mol/L) solution was adjusted to pH 7 by HCl and NaCl (0.01 M) as medium. The solutions of BSA or PNIPAM with different concentrations were prepared in the tris–HCl buffer solution. Both the BSA and PNIPAM solutions were filtered through 0.22 μm syringe filters before complexation. The concentration of BSA solutions were 5, 10 and 15 μmol/L and the concentration of PNIPAM solutions were 3, 5.25, 13.5, 18, 27, 40.5, 52.5, 75 μmol/L, respectively. The complexation was prepared via gently adding PNIPAM solutions with different concentrations into BSA solutions. Then the mixed solutions were standing for over 16 h for testing. The molar mixing ratio (r_{mixing}) is defined as:

$$r_{mixing} = \frac{[PNIPAM]_{total}}{[BSA]_{total}} \quad (1)$$

where $[PNIPAM]_{total}$ and $[BSA]_{total}$ represent the total molar concentration of PNIPAM and BSA in the mixed solution, respectively.

2.3. Fluorescence spectroscopy

Fluorescence spectra and fluorescence intensities were recorded on a Hitachi F-4500 fluorescence spectrophotometer. A high-quality quartz cuvette with 10 mm path length was employed for spectroscopic measurements. The excitation wavelength was set at 290 nm, and the emission was recorded from 310 to 450 nm. Before measurement, the cuvette was washed three times with blank solution (Tris–HCl buffer (0.02 mol/L), pH 7, NaCl (0.01 M)). All experiments were carried out at 25 °C.

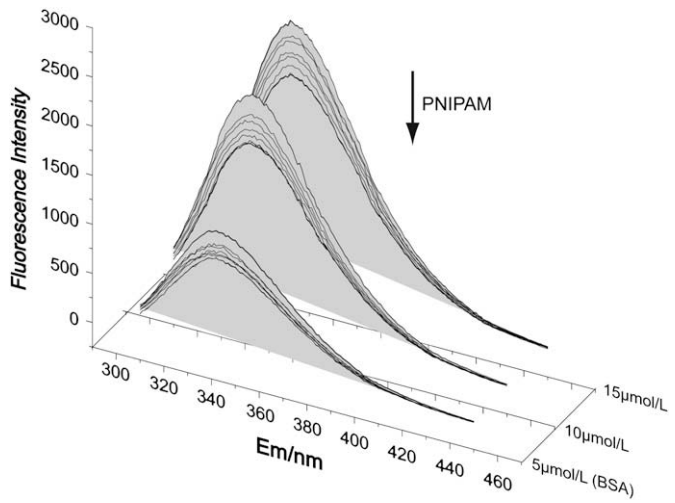

Fig. 1. The fluorescence spectra of the mixed solutions of BSA and PNIPAM with different molar mixing ratio. The concentration of BSA were 5, 10 and 15 μmol/L and the concentration of PNIPAM were 3, 5.25, 13.5, 18, 27, 40.5, 52.5, 75 μmol/L, respectively.

3. Results and discussion

In the aqueous complex system of protein and polymer, there exists a dynamic equilibrium among free proteins, free polymers and complexes of proteins with polymers. To describe the complexation accurately, the average number of bound proteins per polymer (n_b) in complexation is defined as follows:

$$n_b = \frac{[BSA]_{total} - [BSA]_{free}}{[PNIPAM]_{total} - [PNIPAM]_{free}} \quad (2)$$

In order to calculate the average number of bound proteins per polymer (n_b) in aqueous complex system, fluorescence spectrophotometer was used to measure the change of the fluorescence intensities of complexes. In the experiments, PNIPAM solutions with different concentrations were added into three groups of BSA solutions, respectively. The mixed solutions of each group were prepared under the same environmental condition (i.e. the same salt and protein concentration). Fig. 1 shows the fluorescence spectra of the mixed solutions of BSA and PNIPAM with different molar mixing ratio. Apparently, the addition of PNIPAM into BSA solutions produces a decrease of the tryptophan fluorescence intensity. The decrease of intensity of the tryptophan fluorescence

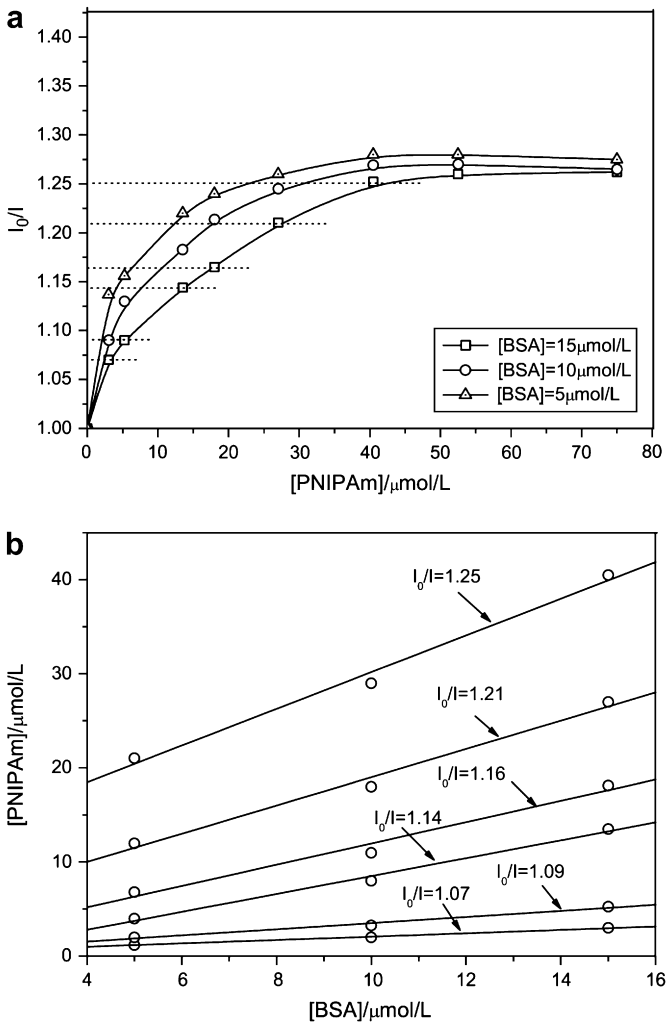

Fig. 2. (a) The variation of I_0/I value, evaluated from the fluorescence spectra of Fig. 1, as a function of $[PNIPAM]_{total}$. (b) The linear relationship between $[PNIPAM]_{total}$ and $[BSA]_{total}$ obtained from sets of data taken at different I_0/I values. The intercept of these straight lines corresponds to $[PNIPAM]_{free}$ value, while the slope of these straight lines presents *n* value.

can be quantified in terms of the ratio I_0/I (I_0 and I being the fluorescence intensities, registered at a given wavelength, in the absence and presence of the PNIPAM considered, respectively).

$$[\text{PNIPAM}]_{\text{total}} = [\text{PNIPAM}]_{\text{free}} + n[\text{BSA}]_{\text{total}} \tag{3}$$

$$n = \frac{[\text{PNIPAM}]_{\text{total}} - [\text{PNIPAM}]_{\text{free}}}{[\text{BSA}]_{\text{total}}} = \frac{[\text{PNIPAM}]_{\text{complex}}}{[\text{BSA}]_{\text{total}}} \tag{4}$$

where $[\text{PNIPAM}]_{\text{free}}$ represents the molar concentration of PNIPAM which are not complex with BSA, $[\text{PNIPAM}]_{\text{complex}}$ represents the molar concentration of PNIPAM which are complex with BSA and n represents the average number of bound PNIPAM molecules per BSA molecule, including free BSA and complex BSA.

It should be noticed that, in Eq. (3), n value cannot be defined as the reciprocal value of n_b. To quantify the amount of $[\text{PNIPAM}]_{\text{free}}$ or $[\text{PNIPAM}]_{\text{complex}}$, it is necessary to establish the linear relationship between $[\text{PNIPAM}]_{\text{total}}$ and $[\text{BSA}]_{\text{total}}$,. In Fig. 2(a), for an arbitrary I_0/I value, three data sets of $[\text{PNIPAM}]_{\text{total}}$ and $[\text{BSA}]_{\text{total}}$, can be obtained by drawing a horizontal line parallel to abscissa. With the help of these data sets, the linear relationship between $[\text{PNIPAM}]_{\text{total}}$ and $[\text{BSA}]_{\text{total}}$ can be established, as it is demonstrated in Fig. 2(b). According to Eq. (3), the intercept of these straight lines in Fig. 2(b) corresponds to $[\text{PNIPAM}]_{\text{free}}$ value, while the slope of these straight lines presents n value.

As mentioned above, the value of I_0/I increases with the increase of PNIPAM concentration and reaches a plateau ($I_0/I_{\min}$) when PNIPAM concentration exceeds a certain value. Fig. 3 is the schematic diagram of fluorescent quenching. As Fig. 3 shows, $I_0/I_{\min}$ represents the maximum fluorescent quenching ability of the complexation of PNIPAM and BSA. The value of $1-I_{\min}/I_0$ can be defined as the maximum fluorescent quenching ratio of complex, and the value of $1-I/I_0$ as the fluorescent quenching ratio of complex at any r_{mixing} value. The concentration of free BSA in the mixed solutions can be calculated according to Eq. (5):

$$\frac{[\text{BSA}]_{\text{total}} - [\text{BSA}]_{\text{free}}}{[\text{BSA}]_{\text{total}}} = \frac{I_0 - I}{I_0 - I_{\min}} = \frac{1 - I/I_0}{1 - I_{\min}/I_0} \tag{5}$$

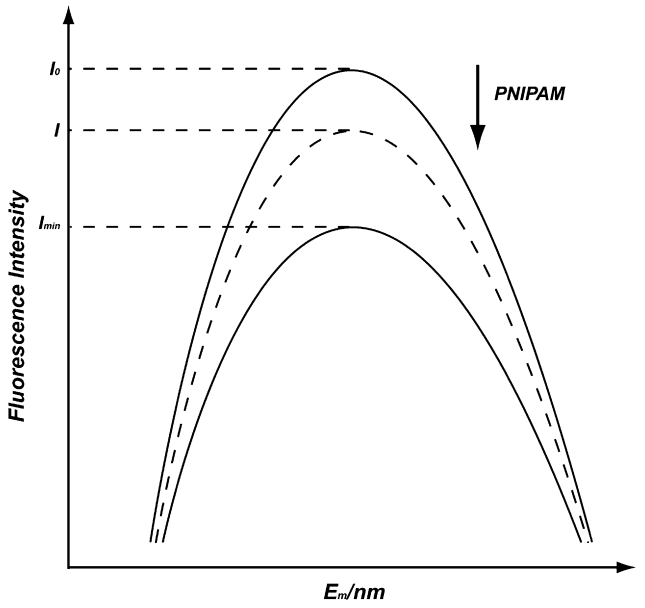

Fig. 3. The schematic diagram of fluorescent quenching. The value of $1-I_{\min}/I_0$ can be defined as the maximum fluorescent quenching ratio of complex, and the value of $1-I/I_0$ as the fluorescent quenching ratio of complex at any r_{mixing} value.

Combined with Eqs. (1), (2) and (5), the n_b value can be calculated as follows:

$$n_b = \frac{[\text{BSA}]_{\text{total}} \times \frac{1 - I/I_0}{1 - I_{\min}/I_0}}{r_{\text{mixing}} \times [\text{BSA}]_{\text{total}} - [\text{PNIPAM}]_{\text{free}}} \tag{6}$$

Here, the values of I/I_0, $I_0/I_{\min}$ and $[\text{PNIPAM}]_{\text{free}}$ can be obtained from Fig. 2(a) and (b), and the average number of bound proteins per polymer (n_b) in complexation can be calculated accurately by Eq. (6). At the same time, with the help of this formula, the extreme value of n_b can also be calculated when PNIPAM concentration is low enough ($r_{\text{mixing}} < 0.01$). This extreme value can hardly be obtained by other methods.

Fig. 4(a) shows the changing patterns of n_b value with r_{mixing} under different BSA concentrations. As mentioned above, the $I_0/I_{\min}$ values of the three groups of mixed solutions are different, hence, with the same r_{mixing} value, their n_b values are also different. It is obvious that n_b value of the three group mixed solutions all decrease with th7e increase of r_{mixing}. In addition to this, n_b value of the complex solution with lower BSA concentration is higher than that of the complex solution with higher BSA concentration. This changing pattern of n_b with r_{mixing} is similar to that of previous literatures [1,2] which measured the n_b value with dialysis technique.

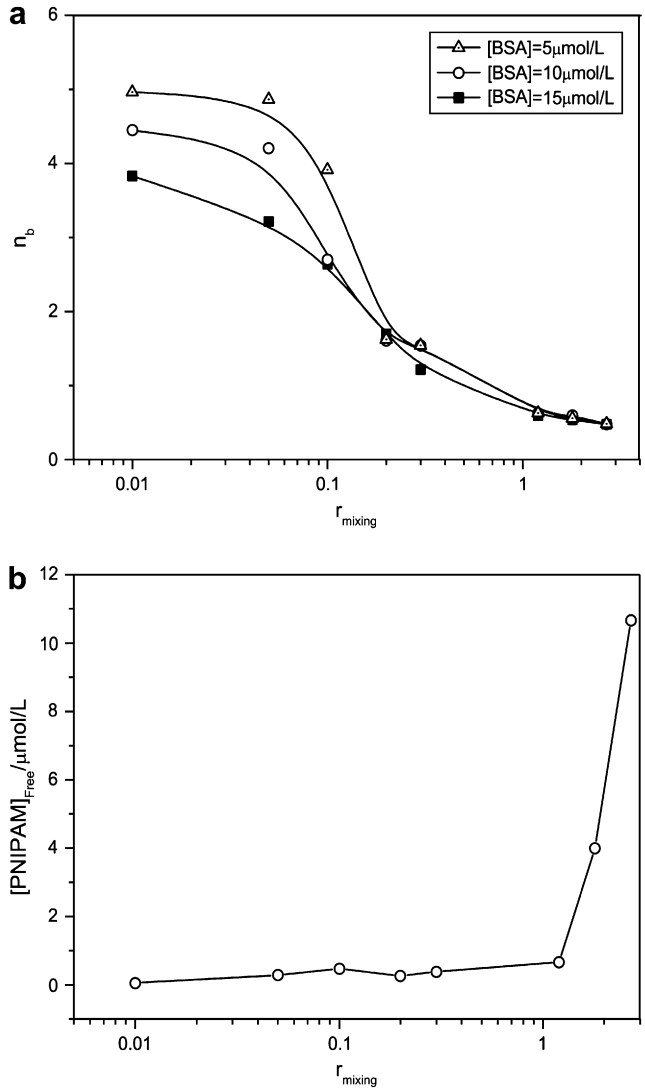

Fig. 4. (a) The changing pattern of n_b value with r_{mixing} at different BSA concentrations. (b) The changing pattern of $[\text{PNIPAM}]_{\text{free}}$ with r_{mixing}.

As it is known that, in the complex system of protein with neutral water borne polymer, light scattering intensity of free neutral polymers is lower than that of proteins and complexes. Therefore, the free neutral polymers cannot be detected precisely by dynamic light scattering (DLS) [1,2]. Previous literatures have studied this problem by investigating the complexation of HSA–PNIPAM with electrophoretic light scattering (ELS) [1]. It was found that, using ELS method, the signal of free PNIPAM molecules in the complex solutions can only be detected when the PNIPAM concentration exceeds a certain value ($r_{mixing} = 2.0$). On the contrary, with the help of our method, both $[PNIPAM]_{free}$ and the transition point at which large amount of free PNIPAM appears can be calculated accurately. Fig. 4(b) shows the changing pattern of $[PNIPAM]_{free}$ with r_{mixing}. It can be observed that, with the rise of r_{mixing} value, $[PNIPAM]_{free}$ first increase slowly, but when $r_{mixing} > 1.8$, the $[PNIPAM]_{free}$ value presents a rapid growth. Thus, the transition point ($r_{mixing} = 1.8$) obtained by this method is in accordance with the one measured by ELS ($r_{mixing} = 2.0$). In this way, the accuracy of this method has been further proved.

4. Conclusion

(1) Based on analysing the change of fluorescence intensity of protein chromophores caused by complexation, a quantitative mathematical equation has been established in this paper to calculate the average number of bound proteins per polymer without destroying the dynamic equilibrium of aqueous complex system.
(2) With the help of this method, the extreme value of n_b can also be calculated when PNIPAM concentration is low enough ($r_{mixing} < 0.01$). This extreme value can hardly be obtained by other methods.
(3) At the same time, both $[PNIPAM]_{free}$ values in the complex solutions and the transition point at which large amount of free PNIPAM appears can also be calculated accurately by this method.

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