The fluorescence spectroscopic study on the interaction between 3-ethyl-benzothiazospironaphthopyran and bovine serum albumin

Jie Liu1,2,* and Jianghua Liu3

1School of Nuclear Technology and Chemistry & Biology, Hubei University of Science and Technology, Xianning, China
2Hubei Key Laboratory of Radiation Chemistry and Functional Materials, Hubei University of Science and Technology, Xianning, China
3School of Electronics and Information Engineering, Hubei University of Science and Technology, Xianning, China

*Corresponding author e-mail: chemrenliujie@163.com

Abstract. The interaction of 3-ethyl-benzothiazospironaphthopyran (BSNP) with bovine serum albumin (BSA) was analyzed by fluorescence spectroscopy. Firstly, the optimal experimental conditions, including medium, pH and ionic strength, were investigated. Secondly, the fluorescence titration experiments at 298 K, 306 K, and 313 K showed that the intrinsic fluorescence of BSA can be strongly quenched by the small organic molecule BSNP. The formation of a BSA-BSNP complex suggested that the fluorescence quenching mechanism may be a kind of static quenching procedure. Finally, the binding constants (K) and the ratio of fluorescence quantum yields of BSA-BSNP complex and free BSA (f) at three different temperatures were obtained. The values of the former were 7.91×10^4 L mol^-1, 6.60×10^4 L mol^-1, and 5.22×10^4 L mol^-1. The values of the latter is 0.88, 0.98, and 1.00.

1. Introduction
Benzothiazospironaphthopyran is a kind of photosensitizer with good properties [1]. This kind of compound can form opening rings similar to the homologues of merocyanine under lighting conditions. Some photosensitizers, such as merocyanine 540, hematoporphyrin derivatives, phthalocyanines, hypericin anthrone, phenothiazine compounds, can produce singlet oxygen and hydroxyl free radicals in the light by combining with lipid membrane, protein and nucleic acid, causing extensive damage to the virus [2]. These kinds of compounds are expected to be used to develop photochemical techniques for virus inactivation in blood products. Meanwhile, the study on the binding of small organic molecules to proteins has become a hot topic [3, 4]. So in this work, the interaction of 3-ethyl-benzothiazospironaphthopyran (BSNP, Figure 1) with bovine serum albumin (BSA) was studied by fluorescence spectroscopy technology. Some informations about the interaction were obtained including binding constants, interaction mechanism, thermodynamic parameters and main force type of binding process. The results may provide a reference for the mechanism of photochemical inactivation of bacteria and provide important information for the development and application of this kind of photosensitizer.
2. Materials and methods

2.1. Materials
BSA was purchased from Huamei Biotechnology Co. Ltd, Beijing, China. In order to get a BSA stock solution of 1.00 mg mL⁻¹, 0.2500 g BSA was dissolved in 250.0 mL secondary distilled water. The BSA stock solution was placed in the refrigerator at 4°C and diluted to the appropriate concentration before the experiment. BSNP was obtained from the Group of organic synthesis, school of chemical engineering, Hubei university. The BSNP stock solution is 1.0×10⁻³ mol L⁻¹. It was prepared with 5% ethanol-water solution, and the operating solution of BSNP was obtained by diluting the stock solution with secondary distilled water. The pH value was adjusted by tris (trimethylolmethyaminomethane, 0.1 mol L⁻¹)-HCl (0.1 mol L⁻¹) buffer solution. The ion strength was maintained by NaCl solution (0.1 mol L⁻¹). The other reagents used were analytically pure, and the water used in the experiment was secondary distilled water.

2.2. Apparatus
The fluorescence spectra were measured on a RF-540 spectrofluorophotometer (Shimadzu, Japan). The quartz cuvette with 1.0 cm was used. The temperatures of reaction system were controlled by constant temperature water bath. All pH measurements were performed with a pHB-4 digital pH-meter (Shanghai Leici Co., Ltd, Shanghai, China) with a glass composite electrode.

2.3. Experimental methods
Add appropriate amount of buffer solution (pH 7.40), NaCl solution (0.1 mol·L⁻¹), BSA solution and BSNP solution into a 12.5 mL colorimetric tube, fix the volume with secondary distilled water, mix thoroughly, keep at a certain temperature and keep away from light. After 5 minutes of reaction, the fluorescence spectrum of the system was determined immediately. The operation parameters of the spectrofluorimeter were set as follows: the wavelength for excitation was 288 nm, and the emission spectra were recorded in the range of 300-525 nm; both the excitation slit and emission slit were 5 nm. The temperature was maintained by circulating water device. The experimental temperatures were 298 K, 306 K and 310 K, respectively.

2.4. Optimization of experimental conditions
When measuring the fluorescence intensity, the change of experimental conditions will have a great impact, so it is usually necessary to control the experimental conditions strictly. Different experimental factors including media, pH and ion strength of the solution were studied in order to choose the optimum conditions. The BSNP concentration was maintained at 1.6×10⁻⁵ mol L⁻¹ in all these experiments.

2.4.1 Effect of media. Under the same conditions, different buffer solutions, including Tris-HCl medium, NaAc-HAc medium and Na₂HPO₄-KH₂PO₄ medium were compared in a series of experiments. It was found that the reaction in Tris-HCl had the highest fluorescence sensitivity, so Tris-HCl was selected to control the pH of the solution.
2.4.2 Effect of pH. The effect of pH on the intensity ratio $F_0/F$ for the BSA and BSA-BSNP system studied in the range of 5.5-8.0. At concentrations of BSA and BSNP of 32 μg mL$^{-1}$ and 1.6×10$^{-5}$ mol L$^{-1}$, the emission wavelength of BSA and BSA-BSNP did not changed, but the fluorescence intensity of both were altered substantially. The maximum of $F_0/F$ occurred at pH 7.46. Consequently, the pH of the reaction solutions was maintained at 7.46 in the subsequent experiments.

2.4.3 Effect of ion strength. The ion strength of the solutions affected obviously the stability and intensity of the fluorescence in the BSA and BSA-BSNP system. So it is important to control the ion strength with the addition of 0.1 mol L$^{-1}$ NaCl. The influence of ion strength on the intensity ratio $F_0/F$ was studied. When 1.4 mL 0.1 mol L$^{-1}$ NaCl was added, the intensity ratio $F_0/F$ got the maximum value. So 1.4 mL 0.1 mol L$^{-1}$ NaCl was taken to maintain the ion strength of the system in this study.

3. Results and discussion

3.1 Fluorescence spectra

Under the above experimental conditions, the fluorescence spectrum of BSA-BSNP system were investigated (Figure 2). It can be seen from Fig. 2 that when excited by 288 nm, BSA showed a strong emission peak at the wavelength of 348 nm. When BSNP was added gradually, the intrinsic fluorescence intensity of BSA decreased significantly, and a new fluorescence peak appeared at about 427 nm. When the concentration of BSNP increased, the fluorescence intensity of BSA continued to decrease, but the peak position of BSA remained unchanged. The intensity of the new fluorescence peak continued to increase and red shifted to 432 nm. There is an equal emission point at 412 nm. These results indicate that BSNP can quench the fluorescence of BSA and form new compound [5], but it has no effect on the conformation of BSA. It can also be proved when the UV absorption spectrum of the system had not changed significantly (not listed).

![Figure 2](image_url)

**Figure 2.** The fluorescence emission spectra of BSNP-BSA system at 298 K

pH=7.46; c (BSA)= 64 μg mL$^{-1}$; c (BSNP) (10$^{-5}$ mol L$^{-1}$). a→f: 0, 1.6, 3.2, 4.8, 6.4 and 9.0, respectively.

3.2 Quenching mechanism

It is generally believed that the endogenous fluorescence of biological macromolecules (such as protein) is reduced by small organic molecule through two mechanisms. One is static quenching caused by complex formation, and the other is dynamic quenching caused by intermolecular collision. It can be differentiated by variable temperature experiments. In static quenching process, the BSNP-BSA complex is formed between BSNP and the ground state molecules of BSA. If the complex has no fluorescence and its stability decreases with the increase of temperature, the quenching degree of
fluorescence intensity of BSA decreases with the increase of temperature. In dynamic quenching process, the interaction occurs between BSNP and the excited state molecules of fluorescent substances BSA, which results in the decrease of fluorescence intensity. This process mainly depends on diffusion, so the quenching usually increases with the increase of temperature.

The \( F_0/F-1-[Q] \) curves of BSA with BSNP at three different temperatures (298 K, 306 K, 310 K) by the fluorescence spectra of the BSA-BSNP system are shown in Figure 3. It can help us to infer which is the main fluorescence quenching process.

![Figure 3. Stern-Volmer plots of BSNP-BSA](image)

Figure 3. Stern-Volmer plots of BSNP-BSA

\[ \text{pH}=7.46; \ c(\text{BSA})=64 \ \mu\text{g mL}^{-1}; \ c(\text{BSNP}) (10^{-6} \ \text{mol L}^{-1}): \ 0, 1.6, 6.4, 12.8, 16.4, 32, 48 \text{ and } 64, \] respectively.

If the fluorescence quenching process is dynamic quenching, it can be described by Stern-Volmer equation [6]:

\[ F_0/F=1+K_{qs} [Q]=1+K_{sv}[Q] \]  

(1)

Where \( F_0 \) is the fluorescence intensity of BSA and \( F \) denotes the fluorescence intensity of BSA-BSNP system; \([Q]\) is the equilibrium concentration of free quenching agent (BSNP). Under the experimental conditions, \( c(\text{BSA}) \) is about \( 10^{-7} \ \text{mol L}^{-1} \), \( c(\text{BSNP}) \) is about \( 10^{-5} \ \text{mol L}^{-1} \), which means that the quencher is far excessive, so the total concentration of BSNP can be used to replace the equilibrium concentration. The Stern-Volmer quenching constant, \( K_{sv} \), is also the slope of linear regressions of Fig. 3.

It can be seem from Fig.3 that the Stern Volmer plots have good linear relationship and the slopes decrease with the increase of temperature. This means that the previous assumption does not hold. That is to say, the fluorescence quenching mechanism between BSNP and BSA is not dynamic quenching, but static quenching.

In addition, \( \tau_0 \) in Formula 1 is the average lifetime of molecules which have fluorescence in the absence of quenching agent, and it is about \( 10^{-8} \) seconds for proteins. \( K_q \) is the quenching rate constant of the biomacromolecule (BSA). Obviously,

\[ K_q \tau_0=K_{sv} \]  

(2)

The quenching constant \( (K_q) \) can be calculated according to Equation 2. The results are exhibited in Table 1. If there is dynamic quenching between small molecule fluorescence quenching agent and biological macromolecule, the collision quenching constant \( (K_q) \) controlled by scatter is not greater than \( 2 \times 10^{10} \ \text{L mol}^{-1} \ \text{s}^{-1} \). Obviously, the fluorescence quenching rate constant of BSA caused by BSNP is far greater than which occurred in the collision procedure. This means that the fluorescence quenching is not caused by the collision process, but by the formation of BSA-BSNP complex.

3.3 Binding constants

The static quenching is presented by the Stern Volmer equation modified [7]:

\[ F_0/F=1+K_{qs} [Q]=1+K_{sv}[Q] \]  

(1)
Where $F_0$ is the fluorescence intensity of BSA and $F$ denotes the fluorescence intensity of BSA-BSNP system, $[Q]$ is the concentration of quencher (BSNP), $K$ is the binding constant for BSA-BSNP complex, and $f$ is the ratio of the quantum yield for BSA-BSNP complex and free BSA. This equation can be transformed into a linear form:

$$
F_0/F = (1+K[Q])/(1+fK[Q])
$$

(3)

The linear curves between $F_0/ (F_0-F)$ and $1/[Q]$ were plotted and shown in Figure 4. Some parameters can be calculated by using the slope of the straight line and the results are also list in table 1. The sizes of values of binding constants ($K$) mean that there was a strong interaction between BSNP and BSA. The changes of values mean the interaction was weakened when the temperature rose. This further verifies the previous inference, that is, BSNP quenched the fluorescence of BSA through the static process of complex formation.

\[\text{Figure 4. Stern-Volmer equation modified plots of BSNP-BSA}\]

\[\text{pH}=7.46; \ c(\text{BSA})=64 \ \mu\text{g mL}^{-1}; \ c(\text{BSNP})\ (10^{-6} \ \text{mol L}^{-1}): 1.6, 6.4, 12.8, 16.4, 32, 48 \text{ and } 64, \ \text{respectively.}\]

**Table 1.** The Stern-Volmer quenching constants ($K_{sv}$), the dynamic quenching rate constants ($K_q$), binding constants ($K$), the ratio of fluorescence quantum yields of BSA-BSNP and BSA ($f$)

| $T$ (K) | $K_{sv}$ ($\times 10^4$ L mol$^{-1}$) | $K_q$ ($\times 10^{12}$ L mol$^{-1}$ s$^{-1}$) | $K$ ($\times 10^4$ L mol$^{-1}$) | $f$ |
|--------|-----------------------------------|-----------------------------------|-----------------------------------|-----|
| 298    | 5.89                              | 5.89                              | 7.91                              | 0.88|
| 306    | 5.58                              | 5.58                              | 6.60                              | 0.98|
| 313    | 5.37                              | 5.37                              | 5.22                              | 1.00|

4. Conclusion

The interactions between BSNP and BSA were investigated by fluorescence spectroscopy. The results showed that a BSNP-BSA complex was formed in the static quenching procedure. The binding constants ($K$), the ratio of fluorescence quantum yields of BSA-BSNP and BSA ($f$) were obtained according to modified Stern Volmer equation.
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