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Luminescence of aniline blue in hydrophobic cavity of BSA

Yang Chang-Ying [a,b], Liu Yi [a,c,*], Zheng Dan [a], Zhu Jun-Cheng [a], Dai Jie [a]

[a] Department of Chemistry, College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, PR China
[b] College of Chemistry and Life Science, Three Gorges University, Yichang 443000, PR China
[c] State Key Laboratory of Virology, Wuhan University, Wuhan 430072, PR China

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Abstract

The fluorescence of aniline blue in the presence of bovine serum albumin (BSA) was investigated using total luminescence spectroscopy (TLS), fluorescence emission and synchronous fluorescence spectra. The fluorescence exhibits appreciable hypsochromic shift along with an enhancement in the fluorescence yield. It is suggested that the polarity-dependent twisted intramolecular charge transfer (TICT) process is responsible for the remarkable fluorescence sensitivity. The reduction in the rate of TICT in the hydrophobic interior of albumin leads to the increase of fluorescence yield. In BSA little rotation around N–C bond of aniline blue occurs during the excited state lifetime. Therefore, it could be concluded that aniline blue molecules have been included into the hydrophobic cavity of BSA and formed complex. The driving forces behind such association between the dye and biopolymer might include electrostatic attraction between ionic dye species and opposite charges on the biopolymer. In the synchronous fluorescence spectra of aniline blue when $\Delta\lambda = 170$ nm, dual fluorescence for aniline blue is observed, local excited state (LE) and TICT state, the relative fraction of the normal planar state increased quickly in low BSA concentration. It is indicated that hydrophobic pockets of BSA affected the LE-TICT equilibrium.

Keywords: Aniline blue; BSA; Total luminescence spectroscopy; Synchronous fluorescence; Twisted intramolecular charge transfer (TICT)

1. Introduction

BSA contains several hydrophobic binding sites, which can be expected to be involved in hydrophobic interaction with the aromatic regions of the dyes [1]. In addition, BSA has a high degree of α-helical content as a cylinder with an open channel and small molecules with suitable functional groups can occupy this channel [2,3].

Aniline blue water soluble ($C_{32}H_{25}N_3O_9S_3Na_2$), as shown in Fig. 1, is a kind of anionic triphenylmethane dyes derivate, which has application in histological and microbiological staining solutions. In histology, aniline dyes are most widely used as constituents of trichrome stains for demonstration of connective tissue elements. Triphenylmethane dyes derivate belong to a group of molecules called twisted intramolecular charge transfer (TICT) molecules. They are characterized by two excited singlet states, locally excited state (LE) and TICT state. Photoexcitation brings the molecule to the LE state from which TICT state forms at a rate competitive with other deactivation pathways from the LE state. The TICT state results from a twisting of the donor and the acceptor moieties, accompanied by a complete charge transfer from the donor to the acceptor moiety [4,5]. Some works showed that appreciable enhancement of fluorescence when TICT molecules bound to BSA because of the suppression of TICT state formation in the non-polar and restrictive environment of BSA binding site [6–10].

Aniline blue is lowly fluorescent in aqueous, the fluorescence of some of these dyes was greatly affected in a constrained medium such as glucose glass [11]. A study of response of their fluorescence parameters to binding with serum albumin can provide useful information regarding the nature of binding. In this paper, as a probe for the biomolecule, the binding of aniline blue to BSA was discussed using fluorescence spectroscopic technique.

* Corresponding author at: Department of Chemical Biology, College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, PR China. Tel.: +86 27 87218284/8304; fax: +86 27 68754067.
E-mail addresses: prof.liuyi@263.net, liuyi@public.wh.hb.cn (Y. Liu).

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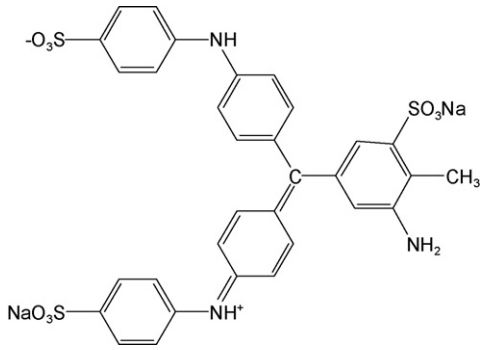

Fig. 1. Structure of aniline blue.

2. Experimental

2.1. Materials

Aniline blue water soluble, was obtained from Sigma, 1.0×10^{-3} mol L^{-1} stock solution was firstly prepared. Fatty acid free BSA was obtained from Sigma and used without further purification, and its molecular weights were assumed to be 67,000. All chemicals were of analytical reagent grade and double distilled water was used for all the measurements. The 0.04 mol L^{-1} Britton–Robinson buffer solution of pH 7.24 was prepared.

2.2. Spectral measurements

All Fluorescence spectra were recorded on F-4500 spectrofluorimeter (Hitachi, Japan) equipped with 1.0 cm quartz cells and thermostat bath, the excitation and the emission slit widths were set at 5 nm.

Fluorometric experiments: 2.0 mL solution containing appropriate concentration of aniline blue was titrated by successive additions of stock solution of BSA. Titrations were done manually by using trace syringes, and the fluorescence intensity was measured (excitation at 302 nm).

Three-dimensional spectra were obtained by measuring the emission spectra, in the range 250–550 nm, repeatedly, at excitation wavelengths from 200 to 350 nm, spaced by 5 nm intervals in the excitation domain. Fully corrected spectra were then concatenated into an excitation–emission matrix. Three-dimensional plots and contour maps of total luminescence spectra were produced using origin program. All contour maps were plotted using the same scale range of fluorescence intensities.

The synchronous fluorescence spectra were collected by simultaneously scanning the excitation and emission monochromator in the 250–400 nm range, with constant wavelength differences, $\Delta\lambda$, between them. Fluorescence intensities were plotted as function of the excitation wavelength.

3. Result and discussion

3.1. Total luminescence spectroscopy

Total luminescence spectroscopy (TLS) is the simultaneous measurement of excitation, emission, and intensity wavelengths of compound fluorophores [12–14]. This technique permits specific fluorescence signatures to be represented in a three-dimensional matrix or excitation–emission matrix (EEM). EEMs serve as unique "finger prints" for single compounds or a mixture of fluorescent components [15–18].

The EEM fluorescence and the respective three-dimensional projections of aniline blue before and after addition of BSA can be seen in Fig. 2. The relatively intense band, observed for aniline blue, with excitation in the range of about 200–350 nm and emission in the range of about 320–620 nm, and the most intense region is centred at $\lambda_{ex}/\lambda_{em}$ = 302/470 nm. After the addition of BSA, the location of fluorescence maxima markedly shifted the $\lambda_{ex}/\lambda_{em}$ maxima towards shorter wavelength ($\lambda_{ex}/\lambda_{em}$ = 289/437 nm), and the fluorescence intensity increase dramatically. Aniline blue is lowly fluorescent in aqueous, it has been suggested that the poor photoactivity is due to the fast relaxation processes that occur by the rotational motion of their aromatic rings, which is called twisted intramolecular charge transfer (TICT). The intense region shift and enhancement of the fluorescence intensity can be rationalized in terms of binding of this probe to BSA and the relaxation was restricted.

3.2. Fluorescence emission of aniline blue in presence of BSA

The more comprehensive data concerning change of aniline blue in BSA were achieved from the emission spectra. The solutions were excited at 302 nm. As Fig. 3 shows, the emission of aniline blue centered at 450 nm in pH 7.24 B-R buffer is strongly hypsochromic shifted with greater enhancement in the intensity on adding the protein. The fluorescence shift is leveled off above 0.6 μmol L^{-1} BSA, where a broad band appears at 400 nm.

One goal of the present work was to show an efficient switch from hydrophilic to hydrophobic interactions, so the emission spectra of aniline blue were recorded in β-CD and surfactant cetyltrimethylammonium bromide (CTMAB). As Fig. 4 shows, the fluorescence band maximum observed at 450 nm in water is blue shifted on adding β-CD and CTMAB (27 and 55 nm, respectively), with greater enhancement in the intensity.

Cyclodextrins (CD), a family of torus-shape cyclic oligosaccharides capable of forming inclusion complexes with a variety of hydrophobic species from aqueous solutions, have been widely utilized as the host moiety [19]. The high fluorescence intensity of aniline blue in the complexes with β-CD was explained in terms of hindrance of the formation of the TICT states by the interaction and confinement in hydrophobic cavity [20,21]. In micelles, the hydrophobic core is formed by flexible alkyl chains, the spectral shift and enhancement of the fluorescence intensity can also be rationalized in terms of binding of this hydrophobic probe to a less polar site in micelles.

The noticeable structural features of aniline blue are: (i) possibility of rotation around N–C bond; (ii) presence of secondary amino group. The observations correlate well with the generally accepted mechanism of TICT formation. Lowering the polarity of the medium destabilizes the excited state more than the ground state. As a consequence, the energy gap between the emitting

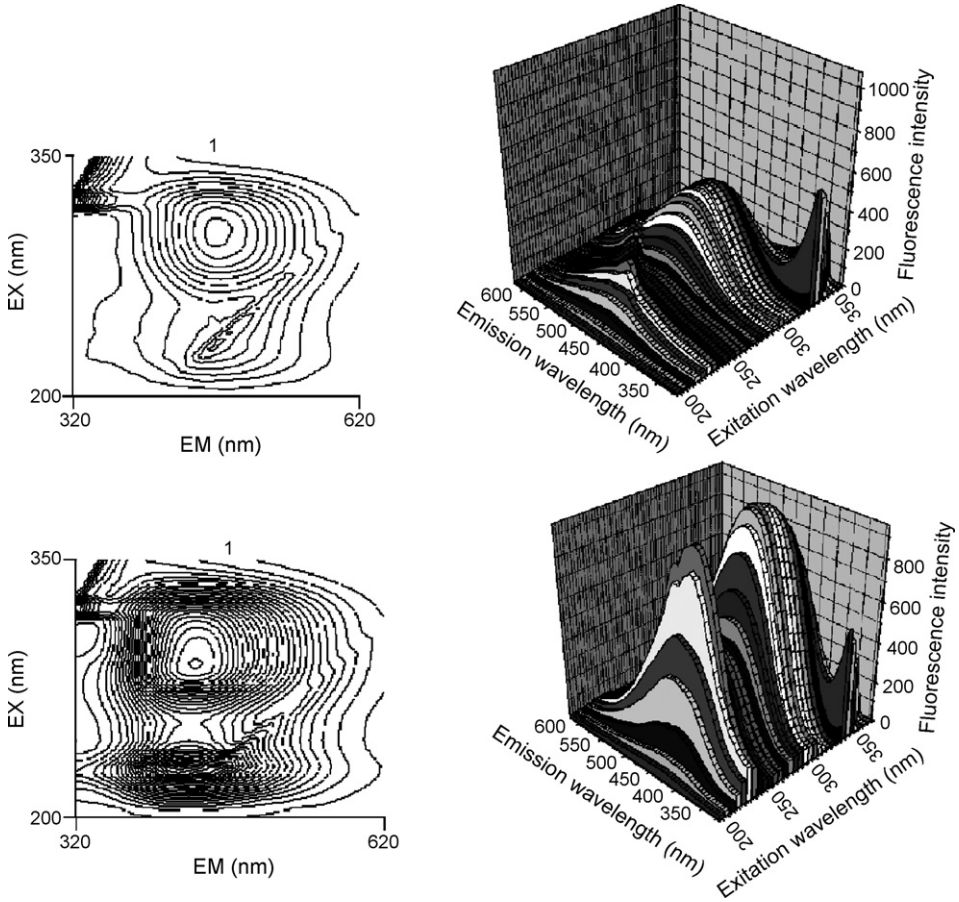

Fig. 2. EEM fluorescence diagrams and the corresponding three-dimensional projections of 1.0×10^{-5} mol L^{-1} aniline blue before (up) and after 4.0×10^{-7} mol L^{-1} BSA addition (down).

and the ground state increases, thus, the emission maximum is shifted to the blue side.

This behavior observed in BSA is similar to that in the β-CD, but the emission shift is more hypsochromic shifted in BSA solutions. In BSA little rotation around N–C bond of aniline blue occurs during the excited state lifetime. Therefore, it could be concluded that aniline blue molecules have been included into the hydrophobic pockets of BSA and formed complex. The driving forces behind such association between a dye and a biopolymer might include electrostatic attraction between ionic dye species and opposite charges on the biopolymer, hydrophobic interactions between the dye and the non-polar regions of the protein and hydrogen bonding interactions between the dye and the protein [22,23]. The polarity-dependent TICT process is responsible for the remarkable fluorescence sensitivity of these biological fluorophores to the environment. The reduction in the rate of TICT in the hydrophobic interior of albumin leads to the increase of fluorescence yield. The blue shifted emission and the fluorescence yield of aniline blue provides information about the size and flexibility of the hydrophobic cavity.

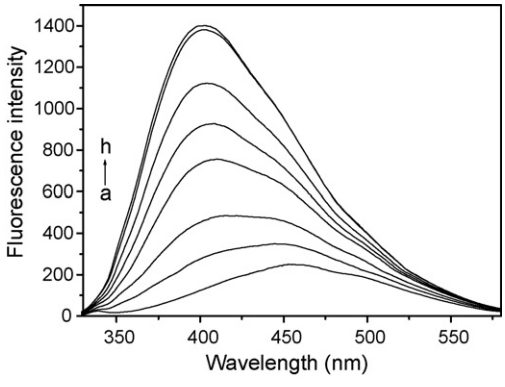

Fig. 3. The fluorescence spectra of Aniline blue with increasing concentrations of BSA. The concentration of the dye was kept constant at 1.0×10^{-5} mol L^{-1}; c(BSA) ($\times 10^{-7}$ mol L^{-1}): (a–h) 0–7.0. $T = 303$ K, pH 7.24.

3.3. Synchronous fluorescence spectra

The synchronous scan collects fluorescent emission only from the waveband where the absorption and emission bands of a species overlap by the specified wavelength interval. This reduces the complexity of fluorescence spectra of a species into a trace, where usually one main fluorescent component is presented in the form of one peak [24]. The synchronous fluorescence spectra (SFS) give information about the molecular environment in the vicinity of the chromosphere molecules. In the SFS, the sensitivity associated with fluorescence is main-

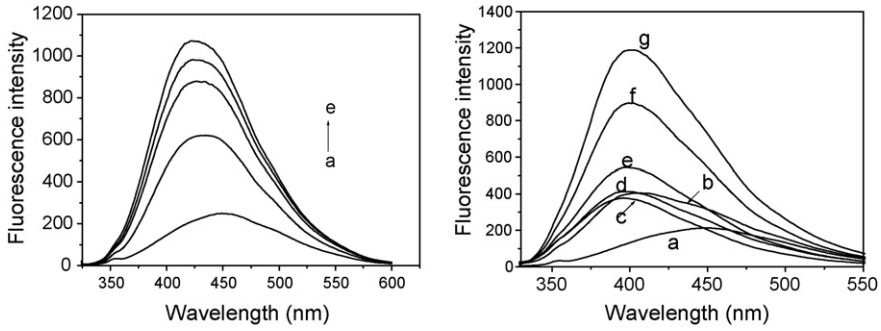

Fig. 4. The fluorescence spectra of 1.0×10^{-5} mol L^{-1} aniline blue with increasing concentrations of β-CD (left) and CTMAB (right); c(β-CD) ($\times 10^{-4}$ mol L^{-1}): (a–e) 0, 1.0, 2.0, 3.0, 5.0; c(CTMAB) ($\times 10^{-4}$ mol L^{-1}): (a–g) 0, 1.0, 3.0, 5.0, 7.0, 9.0, 11. T = 303 K, pH 7.24.

tained while offering several advantages: spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects.

A TICT-based fluorophore emits, in general, two bands of fluorescence, of which the band at the shorter wavelength (B band) is an emission assigned to LE state (emission from the planar geometry), and that at the longer wavelength (A band) the emission originates from TICT state. In Fig. 5, the synchronous fluorescence spectra of the aqueous buffer containing 1.0×10^{-5} mol L^{-1} aniline blue and different concentrations of BSA are shown. In the SFS of aniline blue aqueous buffer, when $\Delta\lambda = 170$ nm (stokes shift of aniline blue attained from EEM spectrum), aniline blue emits only TICT fluorescence (A band). With the addition of BSA, dual fluorescence for aniline blue is observed. Increasing of BSA concentration leads to both bands enhancement, but the local fluorescence band is more sensitive at low BSA concentration than the TICT band. In addition, the blue shift observed in band A is larger than that observed in B band. It is concluded that the non-polar environment in the hydrophobic cavity reduced the formation of the TICT state of the aniline blue.

The relative fraction of normal planar state can be expressed by the fluorescence intensity of band B to band A, I_B/I_A. The relationship between I_B/I_A and BSA concentration are shown in Fig. 6. It is observed the value is strongly dependent of BSA especially in low concentration which indicated an appreciable influence of the BSA on the ratio of fluorescence intensities from both states. Probe molecules, both free and included into the hydrophobic pockets of BSA, exhibit dual fluorescence:

$$\xrightarrow{h\nu} \text{LE(B)} \rightleftarrows \text{TICT(A)}$$

The presence of an influence of BSA on I_B/I_A indicates that hydrophobic pockets of BSA changes the microscopic viscosity in the range affecting measurable changing of the LE-TICT equilibrium. As the TICT state involves a complete charge transfer, it has a high dipole and a strong dependence of its fluorescence emission on the polarity of the microenviroment. The increase of polarity would lead to the equilibrium shifts in the direction

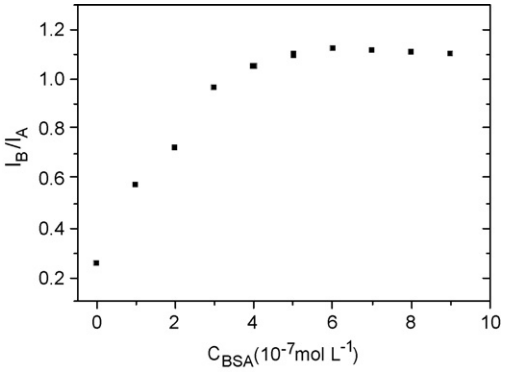

Fig. 6. Values of I_B/I_A for Aniline blue with increasing concentrations of BSA when $\Delta\lambda = 170$ nm. T = 303 K, pH 7.24.

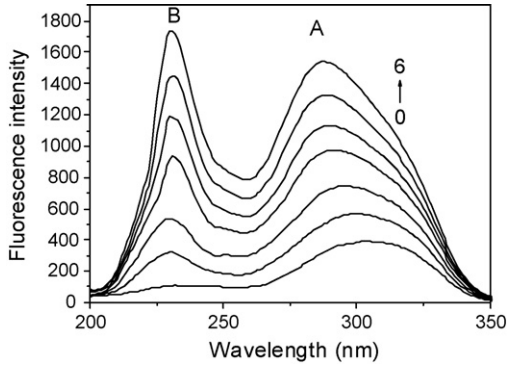

Fig. 5. SFS of Aniline blue with increasing concentrations of BSA when $\Delta\lambda = 170$ nm. The concentration of the dye was kept constant at 1.0×10^{-5} mol L^{-1}; c(BSA) ($\times 10^{-7}$ mol L^{-1}): (0–6) 0–6.0. T = 303 K, pH 7.24.

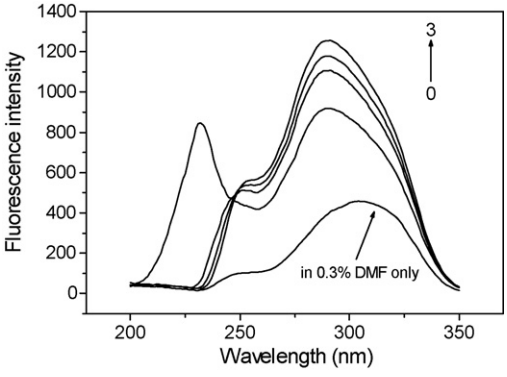

Fig. 7. The effect DMF on SFS of aniline blue BSA system when $\Delta\lambda = 170$ nm. The concentration of the dye was kept constant at 1.0×10^{-5} mol L^{-1}; c(BSA) = 2.5×10^{-7} mol L^{-1}; c(DMF): (0–3) 0–0.9% (V%).

that tends to TICT state. Fig. 7 shows the effect of strong polarity solvent *N*,*N*-dimethyl formamide (DMF) on the equilibrium, the addition of DMF led to the disappearance of peak A and strongly enhancement of peak B, which indicated the shifts of LE-TICT equilibrium.

4. Conclusion

Total luminescence spectroscopy and fluorescence emission spectroscopy evidence that the photophysical behavior of aniline blue water soluble is modified remarkably in hydrophobic cavity of BSA compared to in aqueous phase. This behavior is similar to that observed in the β-CD. The high fluorescence intensity of aniline blue in the complexes with BSA was explained in terms of hindrance of the formation of the non-fluorescent TICT states by the interaction and confinement in cavity. Dual fluorescence was clearly observed in synchronous fluorescence spectra. The addition of BSA would lead to the equilibrium shifts in the direction that tends to locally excited state, while the increase of polarity would lead to the equilibrium shifts in the direction that tends to TICT state.

Acknowledgments

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